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PURIFICATION OF RABBIT LIVER ALDEHYDE OXIDASE BY AFFINITY CHROMATOGRAPHY ON BENZAMIDINE SEPHAROSE 6B

J. G. P. STELL*, A. J. WARNE and C. LEE-WOOLLEY
The School of Pharmacy (Pharmaceutical Chemistry), University of Bradford, Bradford BD7 1DP (U.K.)
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SUMMARY

Highly purified rabbit liver aldehyde oxidase was prepared in high yield using affinity chromatography on Benzamidine Sepharose 6B. Rabbit liver was homogenised, heat treated and ammonium sulphate was added to the supernatant to give a crude preparation of the enzyme. Aliquots of the crude preparation were chromatographed on a Benzamidine Sepharose 6B column at pH 9 and the aldehyde oxidase was eluted by a benzamidine containing buffer. This single affinity step resulted in a 38-fold increase in purity over the crude preparation with an 84% recovery of enzyme activity. Further purification on a Mono Q ion-exchange column gave an additional 1.7-fold increase in specific activity to yield a highly purified preparation of the enzyme. The new method described is considerably simpler and faster than ones hitherto employed and gives a much better yield of the highly purified enzyme.

INTRODUCTION

Rabbit liver is a rich source of the molybdenum containing enzyme aldehyde oxidase (EC 1.2.3.1). Methods usually employed for its isolation and purification are based on that originally described by Rajagopalan *et al.*[1] and subsequently modified by Felsted *et al.*[2]. In the initial steps of this process, the tissue homogenate is subjected to a brief heat treatment, followed by centrifugation and ammonium sulphate precipitation to yield a crude preparation of the enzyme. Up to this stage there is little loss in enzyme activity and an approximately six-fold gain in specific activity. Further purification requires a number of stages, involving acetone precipitation, adsorption onto calcium phosphate gel, ion-exchange and gel permeation chromatography. Application of these techniques yields a product with a further 40–50 fold increase in specific activity and which is regarded as the pure enzyme[2]. However during this long involved procedure approximately 80% of the original enzyme activity present is lost. Recently Yoshihara and Tatsumi[3] improved the yield slightly by including an FMN Sepharose 4B affinity chromatography step in their isolation of the enzyme from guinea pig liver.

The present article describes a rapid affinity chromatography method for the

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purification of rabbit liver aldehyde oxidase in high yield using Benzamidine Sepharose 6B.

EXPERIMENTAL

Materials

The following materials were obtained from the suppliers as listed below. *p*-Dimethylaminocinnamaldehyde (DMAC), benzamidine, *p*-aminobenzamidine, xanthine, xanthine oxidase (grade 1 from buttermilk) from Sigma (Poole, U.K.). Benzamidine Sepharose 6B, Sephadex G-25M, PD-10 columns, Mono Q™ HR 5/5 anion-exchange column, from Pharmacia (Milton Keynes, U.K.). Bio-Rad Protein Assay Kit from Bio-Rad Labs. (Watford, U.K.). Millex-GV 0.22-μm filters, from Millipore (Harrow, U.K.). All buffer solutions used throughout this study were prepared from standard reagent grade chemicals and all contained EDTA at a concentration of 10^{-4} *M*.

Enzyme purification

Preliminary purification steps. Partially purified aldehyde oxidase was prepared from the livers of New Zealand White female rabbits. The livers were weighed then liquidised in an Atomix liquidiser in three volumes of ice-cold 0.1 *M* pH 7 phosphate buffer solution to give a 25% homogenate. The suspension was rapidly heated to 55–60°C on a water bath, maintained at this temperature for 10 min then rapidly cooled in an ice bath and centrifuged (15 000 *g*, 45 min, 4°C). To the clear supernatant, sufficient ammonium sulphate was added to give 50% saturation. The solution was stirred slowly on an ice bath for 20 min and the precipitate obtained collected by centrifugation (6000 *g*, 20 min, 4°C). The precipitate was dissolved in 0.01 *M* pH 7, phosphate buffer using 1 ml of buffer solution for every 10 g of original liver wet weight. The partially purified enzyme prepared in this manner was divided into small portions and frozen in liquid nitrogen until required.

Affinity chromatography on Benzamidine Sepharose 6B. All chromatographic operations were carried out in a refrigerated cabinet maintained at 4–6°C and using a Pharmacia dual pump fast protein liquid chromatography (FPLC) system equipped with a Pharmacia UV-M monitor and FRAC-100 fraction collector. The eluent was monitored at 436 nm.

Portions (1–2 ml) of the partially purified aldehyde oxidase preparation were passed down a small column of G-25M Sephadex (Pharmacia PD-10). Prior equilibration and elution being carried out with a 0.1 *M* pH 9 glycine–sodium hydroxide buffer solution containing 0.1 *M* sodium chloride. This removes contaminating ammonium sulphate and adjusts the sample to the starting conditions for the next operation. The protein containing eluent from the G-25M column was then applied to a column containing Benzamidine Sepharose 6B (60 mm × 15 mm I.D.) which had been equilibrated with the pH 9 glycine buffer–sodium chloride solution. Elution was carried out with the same buffer–sodium chloride solution until a large non-active protein peak had been clearly eluted from the column. The eluting solvent was then changed to one containing 6 m*M* benzamidine in the above pH 9 glycine buffer–sodium chloride solution to elute the aldehyde oxidase. The flow-rate was 1 ml/min throughout and 1-ml fractions were collected. Fractions containing aldehyde

oxidase activity were pooled and treated as below or passed down a PD-10, G-25M Sephadex column to adjust to pH 7 (using 0.067 *M* phosphate buffer) and remove the benzamidine present in the eluent from the affinity column. The purified enzyme preparation was used for further studies immediately or frozen and stored in liquid nitrogen until required.

Ion-exchange chromatography on the Mono Q column. An 11-ml volume of the ammonium sulphate fraction was chromatographed on the benzamidine sepharose column as described above using three separate applications of *ca.* 3.7 ml and the fractions containing aldehyde oxidase activity collected in each case. These were combined and ammonium sulphate added to 50% saturation. The resulting precipitate was collected by centrifugation (7000 *g*, 20 min, 4°C) and dissolved in 1 ml of pH 7 0.067 *M* phosphate buffer. Aliquots of this solution (0.5 ml) were passed down a Sephadex G-25M column (PD-10) to remove excess ammonium sulphate and equilibrate to the starting buffer to be used on the Mono Q column. After filtration (Millex-GV, 0.22 μm) the sample was applied to the Mono Q anion-exchange column via a superloop and eluted as follows. The start buffer (A) consisted of 20 m*M* Bis–Tris propane, pH 6.85. The gradient buffer (B) consisted of 20 m*M* Bis–Tris propane, pH 6.7 containing 1.0 *M* sodium chloride. The flow-rate was 2 ml/min, the temperature was 4°C and detection was performed at 280 nm. Elution profile: 0.0 to 10.5 ml, 0% B, 100% A; 10.5 to 30.5 ml, 0% B to 35% B (linear gradient); 30.5 to 34.5 ml, 35% B to 100% B (linear gradient). Fractions were collected and assayed for enzyme activity and protein content. The UV–VIS absorption spectrum was recorded of fractions containing the major portion of enzyme activity and the A_{280} to A_{450} ratio determined.

Enzyme assay

Aldehyde oxidase activity was determined by adding a suitable volume of the enzyme solution (usually 20 or 50 μl) to DMAC as substrate (25 μ*M*) in pH 7 phosphate buffer (0.067 *M*) and monitoring the decrease in absorbance at 398 nm due to disappearance of the substrate[4]. All determinations were carried out using a Pye Unicam SP8-200 spectrophotometer with cuvettes of 1-cm light path and thermostated at 30°C. Protein estimation was by the method of Bradford[5] using a Bio-Rad Protein Assay Kit in accordance with the manufacturers instructions and bovine serum albumin as standard. Specific activity was calculated as the number of enzyme units per mg of protein, one unit of activity being defined as the amount of enzyme which oxidised 1.0 μmol of *p*-dimethylaminocinnamaldehyde per min at 30°C. A molar extinction coefficient of 30 500 at 398 nm[4] being used to convert absorbance units to moles of substrate oxidised.

Inhibitor studies

The rate of reaction at pH 7 and pH 9 was observed using two different concentrations of DMAC (20 and 10 μ*M*) and several different concentrations of the amidines (0.0625–1.0 m*M*) to ascertain the degree of inhibition produced by the latter compounds. Rates of reaction were also measured over a range of DMAC concentrations (2–20 μ*M*) using a suitable concentration of the amidine in order to determine the type of inhibition occurring at pH 7 and pH 9. The rate of oxidation of xanthine (7, 14 and 28 μ*M*) by xanthine oxidase in the presence and absence of benzamidine (1.0 m*M*) at pH 7, 8 and 9 was determined by following the change in absorbance at 298 nm which accompanies the oxidation of xanthine to uric acid[6].

Polyacrylamide gradient gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out on Pharmacia "PhastGel" Gradient 8–25 ultrathin microelectrophoresis gels using a Pharmacia "Phastsystem" in accordance with the manufacturer's instructions. Samples of the purified material obtained from the benzamidine Sepharose column and the final preparation from the Mono Q column were run on both native and sodium dodecyl sulphate (SDS)-PAGE along with a sample of the crude ammonium sulphate preparation (native PAGE) and molecular weight marker proteins (SDS-PAGE). The samples to be run on SDS-PAGE were first diluted with an equal volume of a solution containing 5% SDS and 10% β-mercaptoethanol and then placed on a boiling water bath for 5 min. The amount of material applied to the gels was in the range 165–480 ng of protein for the purified preparations and 5 μg for the crude ammonium sulphate preparation. On completion of electrophoresis the gels were stained with Pharmacia PhastGel Blue R (Coomassie Brilliant Blue R) to locate the proteins.

RESULTS AND DISCUSSION

Benzamidine Sepharose 6B has been used as an affinity chromatography medium for the purification of trypsin[7] and a number of aromatic amidine compounds have been shown to be potent inhbitors of this enzyme[8,9]. Benzamidine bears some structural resemblance to certain α-aminoazaheterocyclic compounds which we have found to be competitive inhibitors of aldehyde oxidase, *e.g.*, 2-aminoquinoline[10,11], 1-aminoisoquinoline and 6-aminophenanthridine[11]. A study was therefore under-

TABLE I

INHIBITION OF ALDEHYDE OXIDASE CATALYSED OXIDATION OF DMAC BY BENZAMIDINE (BA) AND *p*-AMINOBENZAMIDINE (PABA) AT pH 7 AND pH 9

pH	*DMAC* (μM)	*Percentage inhibition*					K_i^a (M)
		[BA] (mM)					
		0.0625	*0.125*	*0.25*	*0.5*	*1.0*	
9	20	–	22	35	52	68	$1.65 \cdot 10^{-4}$
	10	–	28	43	61	76	
7	20	–	5	10	19	32	$1.04 \cdot 10^{-3}$
	10	–	7	13	23	38	
		[PABA] (mM)					
		0.0625	*0.125*	*0.25*	*0.5*	*1.0*	
9	20	22	37	54	70	–	$8.0 \cdot 10^{-5}$
	10	30	46	63	77	–	
7	20	4	10	18	31	–	$5.75 \cdot 10^{-4}$
	10	6	13	23	37	–	

[a] Inhibitor (I) constant (K_i), calculated from Dixon ($1/v$:[I]) plots. v is the rate of oxidation of DMAC in μmol l^{-1} min^{-1}.

taken to see if benzamidine possessed any inhibitory properties towards aldehyde oxidase and to investigate the possible application of Benzamidine Sepharose 6B as an affinity chromatography material for the purification of this enzyme. The less readily

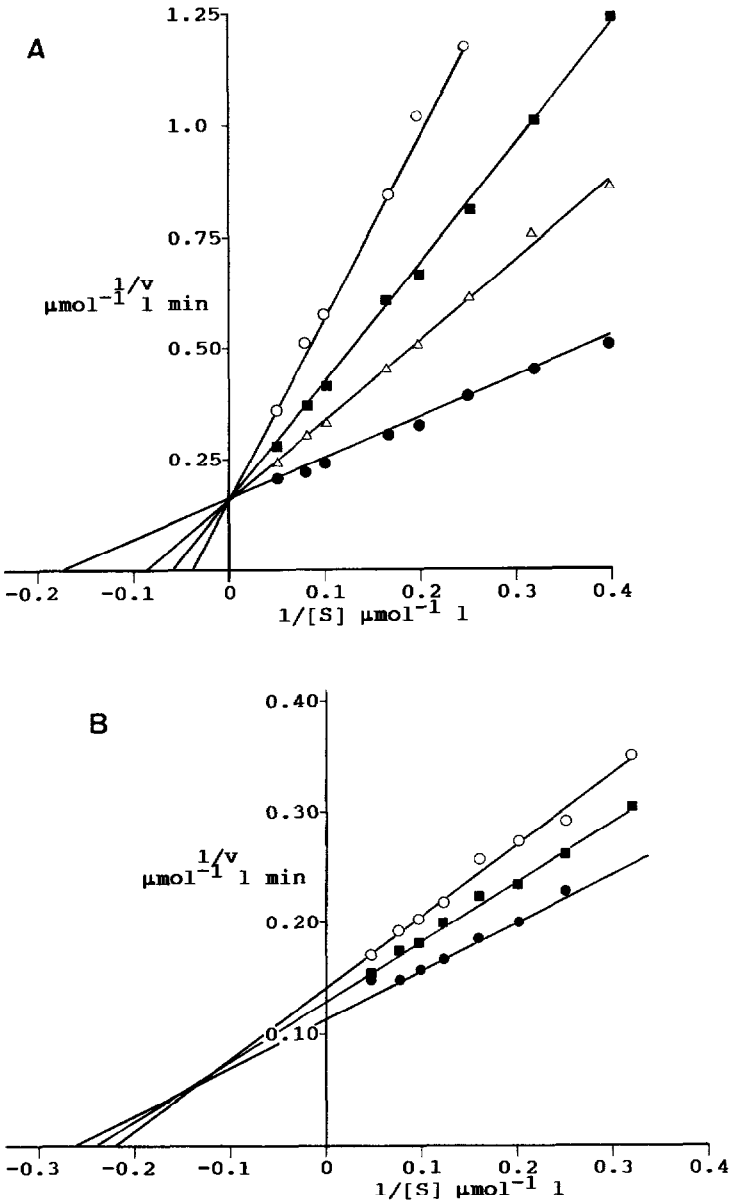

Fig. 1. Double reciprocal plots showing the effect of different concentrations of benzamidine on the rate of oxidation of DMAC by aldehyde oxidase. (A) Reaction carried out in pH 9, 0.1 *M* glycine–sodium hydroxide buffer in the presence of 0.00 (●); 0.125 (△); 0.25 (■); and 0.5 m*M* (○) benzamidine. (B) Reaction carried out in pH 7, 0.067 *M* phosphate buffer in the presence of 0.00 (●); 0.50 (■); and 1.00 m*M* (○) benzamidine.

available and considerably more expensive *p*-aminobenzamidine, which is the actual ligand on the Sepharose 6B, was also tested but to a more limited extent.

Preliminary experiments were carried out using the partially purified aldehyde oxidase from the ammonium sulphate precipitation stage to ascertain the effect different concentrations of the two benzamidines had on the rate of oxidation of DMAC by the enzyme between pH 7 and pH 9.

The enzyme is reasonably stable over this pH range and DMAC which is an excellent substrate with high extinction coefficient can also be used at these pH values. The results presented in Table I, show benzamidine to be an effective inhibitor of aldehyde oxidase with the inhibitory properties being much more pronounced at the higher pH values.

Kinetic determinations showed that at pH 9 the inhibition was purely competitive in nature (Fig. 1A) whereas at pH 7 a mixture of competitive and non-competitive inhibition appeared to be occuring (Fig. 1B).

The behaviour of *p*-aminobenzamidine was similar in all respects to that of benzamidine but was approximately twice as effective with respect to its inhibitory properties (Table I). Benzamidine was also tested to see if it had any inhibitory properties towards the closely related xanthine oxidase but no inhibition was observed over the pH range 7 to 9 in the presence of 1 m*M* benzamidine. These results suggested that Benzamidine Sepharose 6B ought to be an eminently suitable affinity chromato-

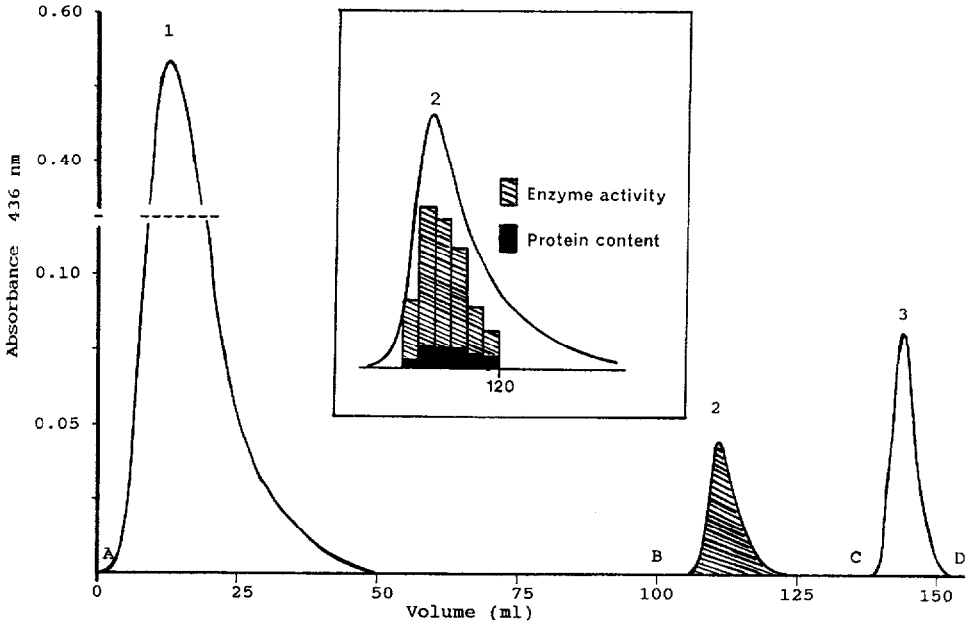

Fig. 2. Elution profile of crude ammonium sulphate fraction from rabbit liver on Benzamidine Sepharose 6B. Shaded area (peak 2) indicates enzyme activity. Eluting buffers: A to B, pH 9, 0.1 *M* glycine buffer + 0.1 *M* sodium chloride. B to C, as A to B + 6 m*M* benzamidine. C to D, pH 4, 0.067 *M* phosphate buffer + 0.5 *M* sodium chloride. Inset box: peak 2 (scale enlarged) showing levels of enzyme activity and protein content of fractions, conditions as in text.

graphy material for the purification of aldehyde oxidase. The affinity of material for the enzyme would be expected to be reasonably high at pH 9 but the binding not being so tight as to make displacement from the affinity ligand difficult, *e.g.*, either by lowering the pH or incorporating benzamidine in the eluting buffer. To test this hypothesis trial experiments were performed whereby small samples of the partially purified aldehyde oxidase in pH 9 buffer were applied to a short column containing a little Benzamidine Sepharose 6B (*ca.* 1 ml volume of the gel). On carrying out elution with a pH 9 glycine buffer–sodium chloride solution a considerable amount of non-active protein material passed rapidly through the column while all the aldehyde oxidase activity was retained on the affinity gel. It was found that the enzyme could be displaced from the gel by lowering the pH to 6.5 with 0.1 *M* phosphate buffer or more efficiently by incorporating benzamidine (6 m*M*) in the pH 9 eluting solution. A larger column of the Benzamidine Sepharose 6B was therefore prepared and samples of the partially purified aldehyde oxidase preparation chromatographed as described in the experimental section. The elution profile for a typical separation is shown in Fig. 2. On assaying the fractions it was found that virtually all the enzyme activity resided in the second smaller peak that was eluted by the benzamidine containing buffer. Owing to the strong UV absorption of benzamidine at 280 nm it was necessary to monitor the eluent at 436 nm where the enzyme still has a reasonable absorption and the benzamidine does not interfere. The contaminating benzamidine was easily removed from fractions containing enzyme activity by passage through a short column of G-25M sephadex. Specific activity measurements on these active fractions indicated a 38-fold increase in purification over the ammonium sulphate preparation (Table II). This high degree of purification had not only been achieved in essentially one relatively simple chromatographic operation but with a striking 84% recovery of enzyme activity. However despite the high specific activity, spectroscopic measurements indicated that some contaminating protein material was still present as the A_{280} to A_{450} ratio was somewhat higher at approximately 8.5 than the value of 5.2–5.3 quoted by Felsted *et al.*[2] for the exhaustively purified enzyme. Further purification was therefore attempted using a Mono Q anion-exchange column as described in the Experimental section. The elution profile is shown in Fig. 3. All the aldehyde oxidase activity was present in the large peak eluting between 24 and 33% concentration of the gradient eluting buffer B. The recovery of enzyme activity was 83% and the specific activity had increased to 2.83 units per mg of protein, *i.e.*, by a factor of 1.7 over the product from the affinity column, and consequently raised the purification factor to

TABLE II

PURIFICATION OF ALDEHYDE OXIDASE FROM RABBIT LIVER ON BENZAMIDINE SEPHAROSE 6B

Fraction	*Volume (ml)*	*Total protein (mg)*	*Protein concentration (mg/ml)*	*Total activity (units)*[a]	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
Crude ammonium sulphate	4.65	165	36	7.28	0.044	100	1
Benzamidine Sepharose purified	22	3.36	0.15	6.10	1.68	84	38

[a] One unit of activity = amount of enzyme that converts one μmol of DMAC per min at pH 7 at 30°C.

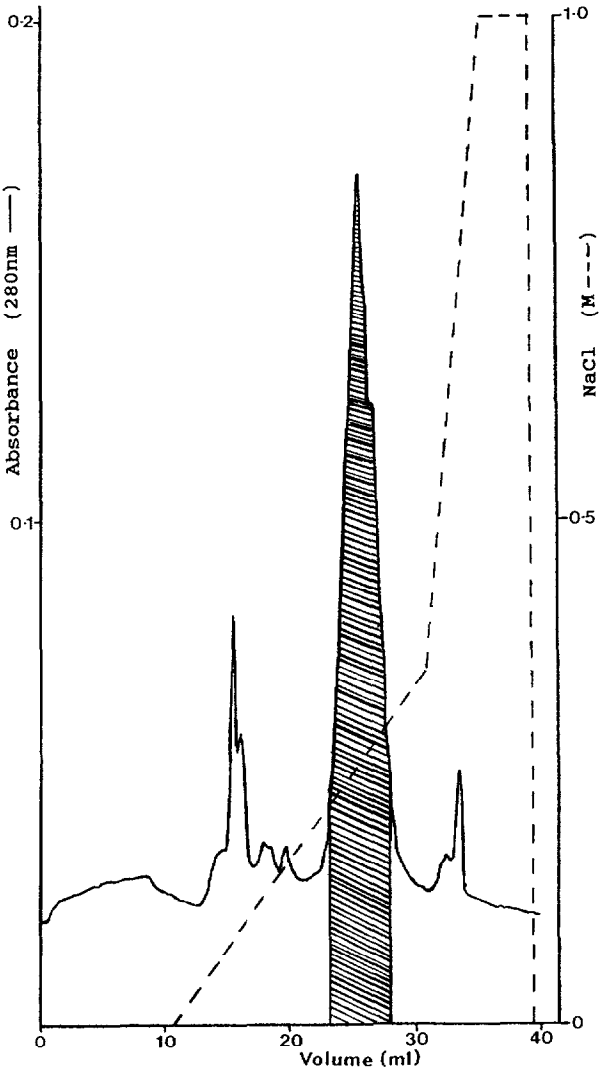

Fig. 3. FPLC on the Mono Q HR 5/5 anion-exchange column of active material from Benzamidine Sepharose 6B affinity column. Shaded area indicates enzyme activity.

a value of 64 when compared to the crude ammonium sulphate preparation. As the latter shows an approximately 6-fold increase in specific activity with respect to the initial homogenate[1,2] then the overall purification in the present study is of the order of 350–400 fold.

Native polyacrylamide gel electrophoresis (Fig. 4a) of the Mono Q purified material showed only a single band even at high sample loading (480 ng), corresponding in position to a band present in the crude ammonium sulphate preparation. The material from the benzamidine sepharose column showed a similar

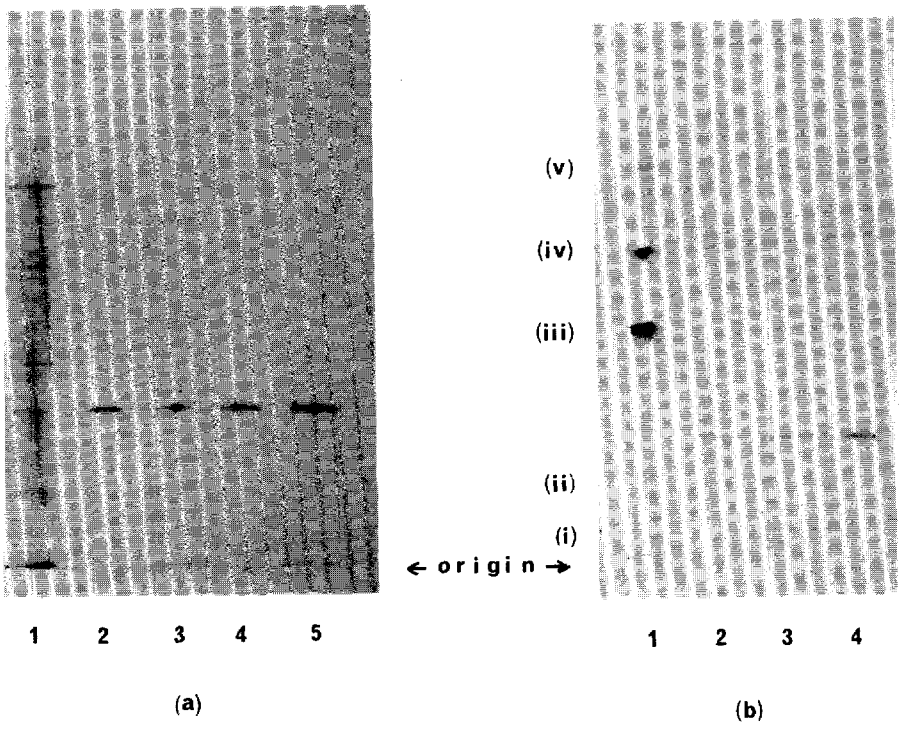

Fig. 4. (a) Native PAGE. Lane 1, crude ammonium sulphate preparation (5 μg). Lanes 2 and 3, material eluted from Benzamidine Sepharose 6B column (2 and 3 = 330 ng). Lanes 4 and 5, material eluted from Mono Q column (4 = 240 ng; 5 = 480 ng). (b) SDS-PAGE. Lane 1, marker proteins; (i) thyroglobulin (330 000), (ii) ferritin (220 000), (iii) albumin (67 000), (iv) lactate dehydrogenase (36 000), (v) ferritin (18 500). Subunit molecular weight in parentheses. Lanes 2 and 3, material eluted from the Benzamidine Sepharose 6B column (2 and 3 = 165 ng). Lane 4, material eluted from Mono Q column (240 ng).

intense band in the same position, but in this sample an additional faint band at $R_F \approx 0.7$ was also present.

On electrophoresis in the presence of SDS (Fig. 4b) both of the purified preparations showed a major band corresponding to a subunit molecular weight of approximately 144 000 and a minor band at approximately 130 000 (estimated from log MW to R_F plot of the marker proteins). These results are very similar to those obtained by Yoshihara and Tatsumi[3] for purified guinea pig aldehyde oxidase. No other bands were present in the Mono Q preparation but the material from the benzamidine sepharose column showed an additional four very faint bands of lower molecular weight material. Further confirmation of the high degree of purity of the aldehyde oxidase prepared in the above manner is afforded by the absorption spectrum (Fig. 5) and a value of 5.4 for the A_{280}/A_{450} ratio, both of which are comparable to those reported by Felsted *et al.*[2] for the pure enzyme.

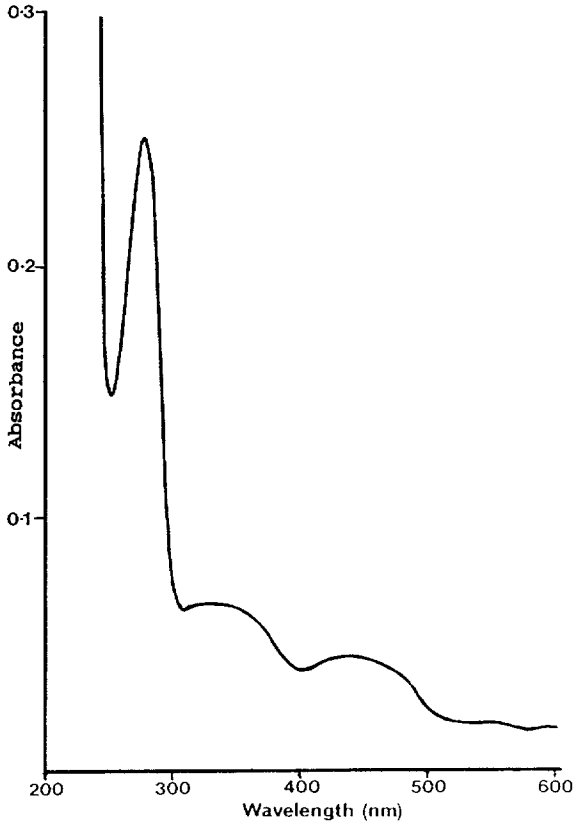

Fig. 5. Absorption spectrum of the purified aldehyde oxidase eluted from the Mono Q column.

CONCLUSION

The work presented in this paper describes a new affinity ion-exchange chromatography method for the isolation of aldehyde oxidase from rabbit liver. The method has the advantage of being considerably simpler and faster than methods hitherto employed and produces a much higher yield of the highly purified enzyme.

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