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Short Communication

Purification of aldehyde oxidase from liver by affinity chromatography and FPLC*

A.J. WARNE and J.G.P. STELL†

Pharmaceutical Chemistry, School of Pharmacy, University of Bradford, Bradford BD7 1DP, UK

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Introduction

Aldehyde oxidase (EC 1.2.3.1) is a molybdoflavo enzyme which is present in the cytosol of mammalian liver. Together with the closely related xanthine oxidase, which has an overlapping substrate specificity, it has the ability to catalyse the oxidation of a wide variety of *N*-heterocycles and aldehydes [1], including a number of drugs [2]. In studies relating to drugs it is desirable to use a highly purified enzyme preparation in order to obtain unambiguous results.

Aldehyde oxidase and xanthine oxidase have many similar physical and structural features. The similarities in structure result in characteristic UV–vis absorption spectra for both the purified enzymes with maxima at 280, 335 and 450 nm. A_{280}:A_{450} absorbance ratios reported for the enzymes are 4.8 for xanthine oxidase [3] and 5.2–5.3 for aldehyde oxidase [4] thought to be 99% pure [5].

A_{280}:A_{450} values can be used as a criterion of purity for highly purified preparations of either enzyme. High values of this ratio will reflect contamination by proteins absorbing only at 280 nm.

Previous work described the development of a new combined affinity chromatography/FPLC method for the purification of rabbit liver aldehyde oxidase [6]. Benzamidine sepharose 6B was used as the affinity medium and further purification was achieved using a Mono-Q anion exchange column. This new method was advantageous with respect to speed and recovery of enzyme activity when compared with previously published methods. However, the purity of the enzyme fractions isolated by this technique was variable and was estimated to lie between 90–99% on the basis of A_{280}:A_{450} values. Electrophoretic analysis of fractions with 90% purity revealed the presence of traces of both low and high molecular weight protein impurities.

The present communication reports an improvement to the above method by the incorporation of a gel permeation chromatography step into this new method in order to remove the contaminating proteins and improve the purity of the aldehyde oxidase fractions obtained.

Experimental

Materials

The following materials were obtained from the suppliers as listed below: *p*-dimethylaminocinnamaldehyde (DMAC), benzamidine, xanthine and bis-tris propane from Sigma (Poole, Dorset, UK); benzamidine sepharose 6B, PD-10 column, C 10/10 column, Mono Q HR 5/5 column, Superose 6 HR 10/30 column and FPLC apparatus from Pharmacia (Milton Keynes, UK); Bio-Rad protein assay reagent from Bio-Rad Labs (Watford, UK).

Determination of aldehyde oxidase activity

Enzyme solution was added to DMAC

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†Author to whom correspondence should be addressed.

(25 μM) as substrate in pH 7 phosphate buffer (0.067 M) at 30°C. The decrease in absorbance at 398 nm resulting from the oxidation of substrate was monitored in a cell of 1 cm light path. A molar extinction coefficient of 30,500 was used to convert absorbance to units of enzyme activity (IU) under the above conditions [7].

Determination of activity:A_{450} values

Activity:A_{450} values for purified aldehyde oxidase were obtained by dividing the enzyme activity given by 1 ml of an enzyme fraction in the standard assay with DMAC by its absorbance at 450 nm (determined using a cell of 1 cm light path).

Determination of xanthine oxidase activity

Enzyme solution was added to xanthine (15 μM) as substrate in pH 7 phosphate buffer (0.067 M) at 30°C. The increase in absorbance due to the oxidation of the substrate to uric acid was monitored at 300 nm in a cell of 1 cm light path [8].

Protein estimation methods

The Bio-Rad dye-binding assay based on the method of Bradford [9] was used for the crude fraction and material from purification stage 1, with bovine serum albumin as standard. A spectrophotometric method using a figure of 12.4 for the $A^{1\%}$ at 280 nm for highly purified aldehyde oxidase was used for material from purification stages 2 and 3 [4].

Preparation of a crude fraction of aldehyde oxidase

A 25% homogenate of the liver (75 g) of a New Zealand White female rabbit was prepared in 0.067 M phosphate buffer pH 7.8. The homogenate was subjected to a short heat treatment, centrifugation and ammonium sulphate precipitation of the supernatant. The precipitate was collected by centrifugation and redissolved in a minimal volume of homogenizing buffer. Enzyme activity and protein content of this fraction were determined.

To prepare this sample for affinity chromatography, aliquots were passed down a G-25M sephadex column (PD-10) to exchange into affinity chromatography buffer A.

Liquid chromatography

All liquid chromatography was carried out with the use of an FPLC apparatus with valves, mixer, columns, flow-cell and fraction collector contained in a cold cabinet maintained at 4°C.

Purification stage 1: affinity chromatography

Buffers used were as follows:

Buffer A. Glycine–NaOH (0.1125 M, pH 9) containing NaCl (0.1125 M), EDTA (0.1 mM) and cysteine (2 mM).

Buffer B. As buffer A + benzamidine (10 mM).

The crude ammonium sulphate fraction in buffer A was injected onto a C 10/10 column packed with benzamidine sepharose 6B (4.5 ml) and eluted at 1.25 ml min^{-1} as follows, with detection at 436 nm: 0–40 ml, buffer A; 40–50 ml, buffer B.

The fractions containing aldehyde oxidase activity were combined and the protein precipitated with ammonium sulphate (65%), collected by centrifugation and redissolved in 100 μl of buffer A. Assays of aldehyde oxidase activity and protein content were carried out on this sample which was then used in stage 2.

Purification stage 2: FPLC gel permeation chromatography

A 100-μl sample of the concentrated affinity purified aldehyde oxidase (from purification stage 1) was injected onto a Superose 6 HR 10/30 column and eluted with bis-tris propane–HCl buffer (50 mM, pH 7.2) containing 0.1 mM EDTA and 2 mM cysteine at 0.35 ml min^{-1} with detection at 280 nm. Fractions of 1.25 ml were collected, assayed for protein content and aldehyde oxidase activity and UV–vis absorption spectra were obtained.

The fractions containing aldehyde oxidase activity in a purified form (as indicated by A_{280}:A_{450} values) were used in purification stage 3.

Purification stage 3: FPLC anion exchange chromatography

Buffers used were as follows:

Buffer C. Bis-tris propane–HCl (20 mM, pH 6.9) containing 5% betaine and 2 mM cysteine.

Buffer D. As buffer C, pH 6.7 + 1 M NaCl.

The sample of purified aldehyde oxidase from stage 2 was injected onto a Mono Q HR 5/5

column and eluted with buffer C at 2 ml min^{-1} as follows with detection at 280 nm: 0–5 ml, buffer C; 5–30 ml, linear salt gradient 0–35% D.

Fractions of 1.25 ml were collected, assayed for protein content and aldehyde oxidase activity and UV–vis absorption spectra were obtained.

Results

Purification stage 1

The elution profile is shown in Fig. 1. The large initial response eluted between 0–30 ml contained mainly inactive material and only traces of aldehyde oxidase activity. The second, much smaller peak contained a large proportion (85%) of the applied aldehyde oxidase activity and was devoid of xanthine oxidase activity. The *p*-aminobenzamidine ligand on the benzamidine sepharose 6B is a potent inhibitor of aldehyde oxidase at pH 9 [6]. The enzyme was therefore strongly bound by this ligand when applied in buffer A, while inactive material passed through the column. Using benzamidine as a counter ligand the aldehyde oxidase could then be displaced from the matrix with a 75-fold increase in purity compared with the crude preparation applied to the column (see Table 1).

Purification stage 2

The elution profile is shown in Fig. 2. The major peak eluted contained aldehyde oxidase activity with some contaminants being at least partially separated. The shaded area of the peak (2.5 ml) was then used in stage 3. Although a considerable increase in purity was achieved by the single affinity chromatography step, the preparation still contained small amounts of contaminating proteins and traces of the benzamidine counter ligand. High and low molecular weight contaminants were separated by the gel permeation step. Aldehyde oxidase fractions with high A_{280}:A_{450}

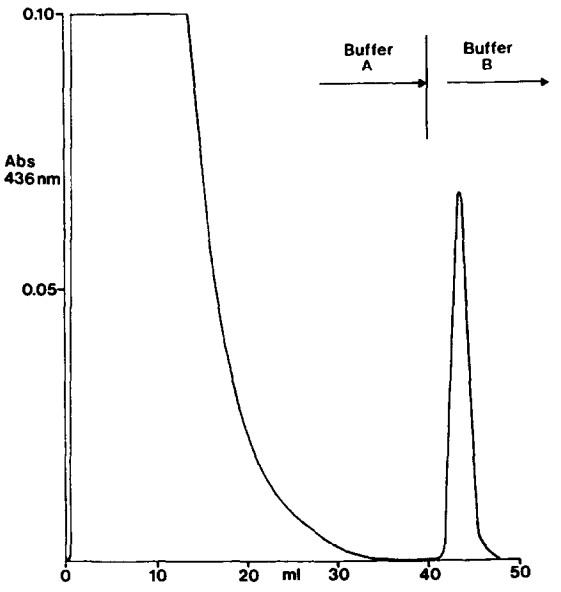

Figure 1
Purification stage 1, affinity chromatography on benzamidine sepharose 6B.

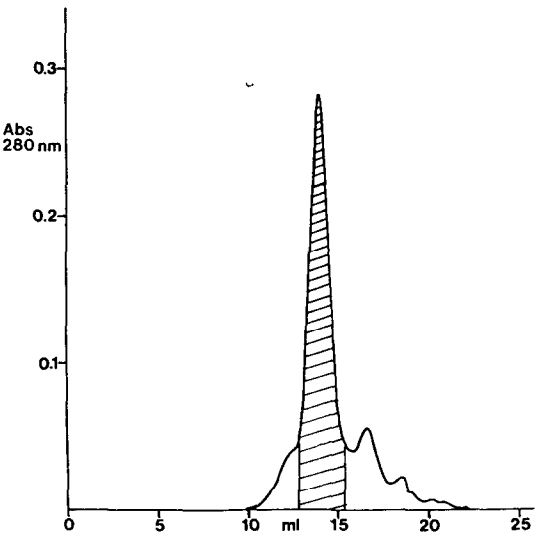

Figure 2
Purification stage 2, FPLC gel permeation chromatography.

Table 1
Purification of aldehyde oxidase from rabbit liver

	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	A_{280}:A_{450}	Yield (%)*	Purification factor
Crude fraction	116	4.65	0.04	N/A	100	1
Stage 1	1.32	3.96	3.0	N/A	85	75
Stage 2	0.785	3.32	4.2	6.05	71	105
Stage 3	0.635	3.11	4.9	4.87	66	122

*Excluding losses accounted for in sample application and in assays of enzyme activity.

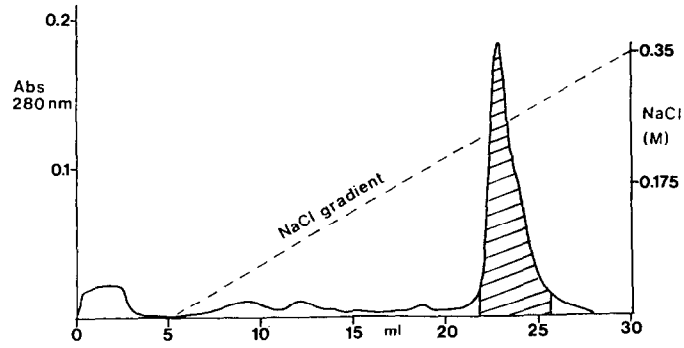

Figure 3
Purification stage 3, FPLC anion exchange chromatography.

values were discarded, the fraction retained for use in stage 3 had an A_{280}:A_{450} value of 6.05, and an activity:A_{450} value of 20.6.

Purification stage 3

The elution profile is shown in Fig. 3. The major peak which was eluted contained the aldehyde oxidase activity (shaded area). This final purification step successfully removed further contaminants. In particular, contaminants with isoelectric points greater than pH 7 were not retained and were eluted during the sample application. A reduction in A_{280}:A_{450} to 4.87 accompanied by an increase in specific activity of the aldehyde oxidase fraction was observed (see Table 1). The UV–vis absorption spectrum of the material from this final purification stage is shown in Fig. 4. The activity:A_{450} value of this preparation was 19.3, indicating slight deactivation of the enzyme from stage 2.

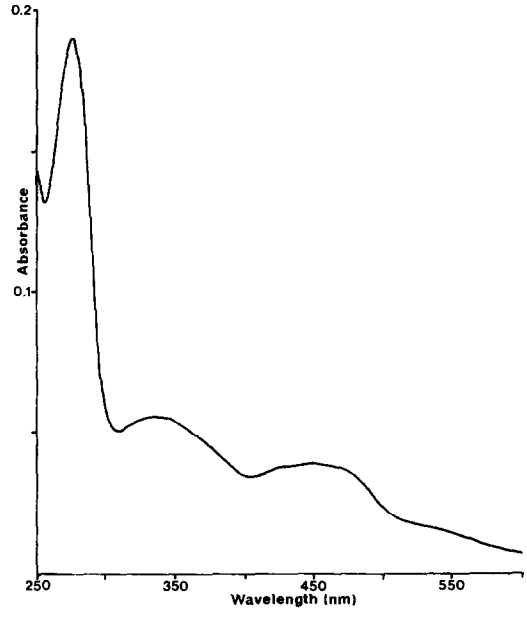

Figure 4
UV–vis absorption spectrum of aldehyde oxidase from purification stage 3.

Conclusions

Incorporation of a gel permeation step into the previously described affinity chromatography/FPLC anion exchange method for the purification of aldehyde oxidase [6] successfully removed traces of contaminating protein.

The final product had an A_{280}:A_{450} value of 4.87 which is one of the lowest so far reported, indicating that a high degree of purity had been achieved. The figure of 4.87 for this absorbance ratio was in good agreement with values reported for highly purified xanthine oxidase [3] and further illustrated the close similarity of these two related enzymes. The speed of the process and the yield were not significantly compromised by the addition of the gel permeation step. A comparison of the activity: A_{450} value of the enzyme from stage 3 with previously obtained values indicated that 57% of the enzyme was in a catalytically active form, a result comparable to the best reported to date [10].

References

[1] T.A. Krenitsky, *Biochem. Pharmacol.* **27**, 2763–2764 (1978).
[2] C. Beedham, *Drug Metab. Rev.* **16**, 119–156 (1987).
[3] C.H. Sullivan, I.H. Mather, D.E. Greenwalt and P.J. Madara, *Mol. Cell. Biochem.* **44**, 13–22 (1982).

[4] R.L. Felsted, A.E.-Y. Chu and S. Chaykin, *J. Biol. Chem.* **248**, 2580–2587 (1973).
[5] R.C. Bray, in *The Enzymes* (3rd edn) Vol. 12B, pp. 299–419. Academic Press, New York (1975).
[6] J.G.P. Stell, A.J. Warne and C. Lee-Woolley, *J. Chromatogr.* **475**, 363–372 (1989).
[7] J. Kurth and A. Kubiciel, *Biomed. Biochem. Acta* **43**, 1223–1226 (1984).
[8] T.A. Krenitsky, T. Spector and W.W. Hall, *Arch. Biochem. Biophys.* **247**, 108–119 (1986).
[9] M. Bradford, *Anal. Biochem.* **72**, 248–254 (1976).
[10] U. Branzoli and V. Massey, *J. Biol. Chem.* **249**, 4346–4349 (1974).

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