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Note

Purification of D-amino acid oxidase apoenzyme by affinity chromatography on Cibacron Blue Sepharose

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In recent years, there has been a marked increase in the use of affinity chromatography for protein purification[1]. Thus, enzymes requiring cofactors with an adenylate moiety can be purified on nucleoside or nucleotide affinity columns. It has also been shown that polyaromatic dyes can bind at the active sites of globular proteins that have a cofactor possessing a nucleotidyl residue[2]. For example, Cibacron Blue has an affinity for several dehydrogenases, kinases and phospho- or acetyltransferases[3]. Flavine adenine dinucleotide (FAD) belongs to the adenylate cofactor group and it should, therefore, be possible to purify flavoproteins by affinity chromatography on Cibacron Blue resins, especially when the cofactor is not too tightly bound to the protein. We could not find a reference to this technique in the literature. It appeared to us that such a technique would be of great value, as we needed to obtain an apoprotein in a chemically pure form in order to assay flavin analogues as electron transfer moieties.

We report here a simple and efficient method of purification of D-amino acid oxidase apoenzyme (E.C. 1.4.3.3).

EXPERIMENTAL

Blue Sepharose CL6B was obtained from Pharmacia. The disodium salt of nicotinamide adenine dinucleotide in reduced form (NADH) and the enzymes D-amino acid oxidase (15 U/mg) from hog kidney (D-AAO), catalase (65 000 U/mg) (CAT) from beef liver and lactate dehydrogenase (500 U/mg) (LDH) from rabbit muscle were obtained from Boehringer (Mannheim, F.R.G.). Flavine adenine dinucleotide was a product of Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

Apo-D-amino acid oxidase was prepared either by ammonium sulphate precipitation at acidic pH[4] or by extensive dialysis against 1 *M* potassium bromide solution[5].

The enzymatic activity was measured as follows. The concentration of pyruvic acid produced during the enzymatic reaction was continuously measured by lactate dehydrogenase activity [0.05 *M* sodium pyrophosphate buffer (pH 8.5), 0.035 *M* D-alanine, $1.8 \cdot 10^{-4}$ *M* NADH, 13 U/ml CAT, 8 U/ml LDH, D-AAO (2 μg) or D-amino acid oxidase apoenzyme (20 μg or more)] at 340 nm on a Cary 210 spectrometer.

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The chromatograms were obtained at 4°C on Blue Sepharose columns equilibrated with 0.01 *M* sodium pyrophosphate (pH 8.5) prior to the application of crude D-AAO apoenzyme. The elution was performed first with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) until a peak was eluted (void volume), then the purified D-AAO apoenzyme was eluted with 1 *M* potassium bromide in 0.01 *M* sodium pyrophosphate buffer (pH 8.5). The protein fractions were collected and concentrated by dialysis against solid polyethylene glycol (PEG 20M). The protein was then subjected to extensive dialysis against 0.05 *M* sodium pyrophosphate buffer (pH 8.5). The apoenzyme solution was stored at −20°C. The protein concentration was determined by the method of Bradford[6], using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

As we needed chemically pure D-AAO apoenzyme to assay different flavins for electron transfer experiments, we tried an affinity technique to purify the apoenzyme, which was contaminated with up to 5% of FAD. The holoenzyme was expected to elute in the void volume of the column, whereas the apoenzyme would be retained on the matrix. As Cibacron Blue is assumed to mimic an adenine moiety, Blue Sepharose appeared to be suitable for our purpose.

FAD was first dissociated from its D-AAO apoenzyme either by ammonium sulphate precipitation[4] or by extensive dialysis[5]. About 5% of the cofactor was retained on the final protein preparation. Such a biological preparation was not suitable for our electron transfer investigations and was, therefore, used as starting material for our chromatographic experiments. Chromatography on Blue Sepharose allowed its dissociation into two peaks (Fig. 1). The first peak (A), which represented about

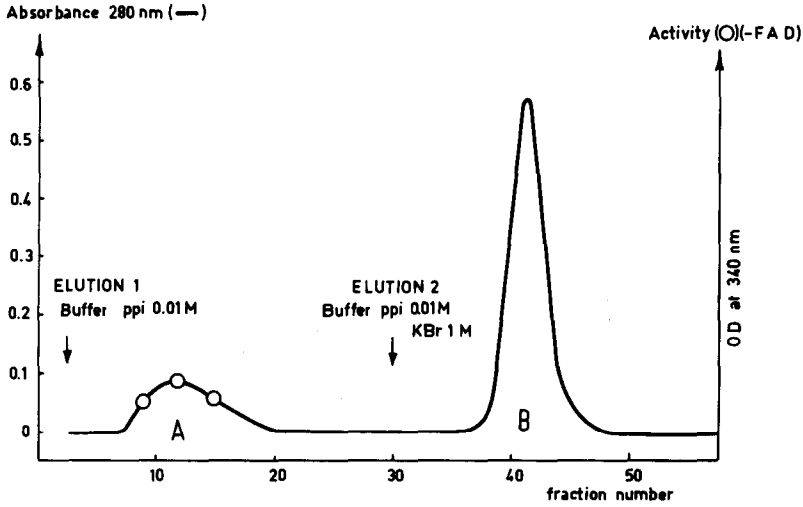

Fig. 1. Chromatography of D-amino acid oxidase holo- and apoenzymes, obtained by dialysis against 1 *M* KBr, on Blue Sepharose. The column (10 × 1 cm I.D.), equilibrated with 0.01 *M* pyrophosphate buffer (ppi) (pH 8.5), was loaded with 1 mg of proteins. The elution profile was obtained by absorbance at 280 nm. The column was eluted with 0.01 *M* sodium pyrophosphate buffer pH 8.5 (arrow 1), followed by 0.01 *M* sodium pyrophosphate buffer pH 8.5 containing 1 *M* KBr (arrow 2). Fractions of 1 ml were collected. The activity was measured (O) as described under Experimental in the absence of FAD.

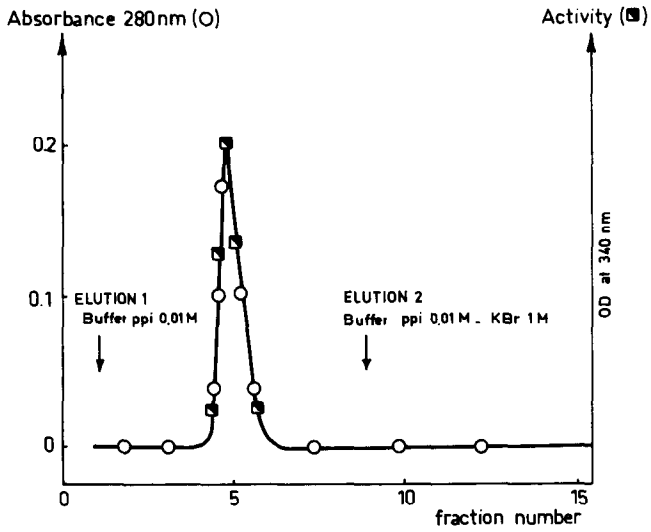

Fig. 2. Chromatography of D-amino acid oxidase holoenzyme on Blue Sepharose. The column (5 × 0.8 cm I.D.), equilibrated with 0.01 *M* sodium pyrophosphate buffer (pH 8.5), was loaded with 0.5 mg of holoenzyme. Arrow 1 indicates the beginning of elution with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) followed, at arrow 2, by the same buffer containing 1 *M* KBr.

5% of the total material loaded on the column, was eluted in the void column of the column. The second peak (B), representing 95% of the material, was eluted with 1 *M* potassium bromide in 0.01 *M* sodium pyrophosphate buffer.

Peak A had the physico-chemical properties and enzymatic activities of the D-AAO holoenzyme, and presented the same elution profile (Fig. 2) obtained when

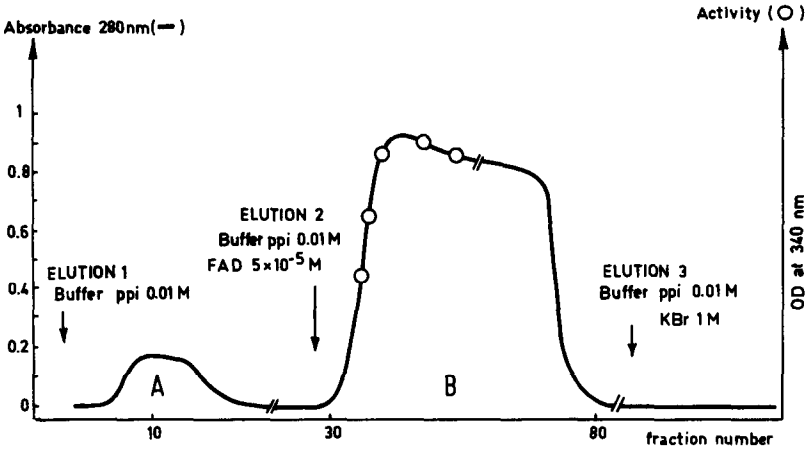

Fig. 3. Displacement of D-amino acid oxidase apoenzyme from Blue Sepharose with FAD. The column (10 × 1 cm I.D.), equilibrated with 0.01 *M* pyrophosphate buffer (pH 8.5), was loaded with a mixture D-AAO holo- and apoenzymes (2 mg). The elution profile was obtained from the absorbance at 280 nm. The column was eluted first with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) (arrow 1), followed by 0.01 *M* sodium pyrophosphate buffer containing $5 \cdot 10^{-5}$ *M* FAD (arrow 2) and by 0.01 *M* sodium pyrophosphate buffer containing 1 *M* KBr (arrow 3). Fractions of 1 ml were collected.

D-AAO holoenzyme was submitted to the same type of chromatography under the same experimental conditions.

Peak B represented the apoenzyme, as demonstrated by the experiments reported below. The material of peak B was a protein but had no absorption band in the visible spectrum, demonstrating the absence of FAD in the structure. It had no D-AAO activity. The enzymatic activity could be completely restored, however, by incubation with FAD. The specificity of the binding to the Blue Sepharose column was demonstrated as follows. The crude apoenzyme preparation was absorbed on a column equilibrated with 0.01 *M* sodium pyrophosphate buffer (pH 8.5) and the elution (Fig. 3) was carried out first with the same buffer. A first peak (A) was immediately eluted. When the UV absorbance at 280 nm reached zero, the elution was developed with the same buffer containing $5 \cdot 10^{-5}$ *M* FAD, and a second peak (B) was eluted. The same buffer, this time containing 1 *M* potassium bromide, was then applied. No additional material was desorbed from the column. The analysis of the fractions showed that peak A had D-AAO activity. Its specific activity was lower than expected, probably owing to some denaturation of the protein. Peak B had, as expected, a fully restored enzymatic activity.

This work demonstrates that the use of Cibacron Blue Sepharose for chromatography readily provides a flavoapoenzyme in a chemically pure form. The binding between Cibacron Blue Sepharose and D-AAO apoenzyme may also be an excellent tool for studying cofactor binding. Other experiments are under way to explore the potential of such resins.

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