A PTERIDINE ADSORBENT FOR AFFINITY CHROMATOGRAPHY

R. G. H. COTTON*

Royal Children's Hospital Research Foundation, Flemington Road, Parkville, and Department of Genetics, University of Melbourne, Parkville, Victoria 3052, Australia

Received 23 May 1974

1. Introduction

Affinity chromatography [1] offers enzymologists considerable advantages over traditional methods of enzyme purification. The work described in this paper extends the range [2–7] of cofactors currently in use for affinity chromatography of enzymes with more than one substrate.

The results to be described indicate that the enzyme phenylalanine hydroxylase (EC 1.14.3.1) will bind very strongly to an adsorbent which contains the reduced pteridine cofactor, 2-amino 6, 7-dimethyl-4-hydroxy 5, 6, 7, 8-tetrahydropteridine and that it can be eluted with a strongly alkaline buffer or the cofactor itself. The use of a Sephadex G-25 column immediately below this adsorbent ensured rapid separation of the protein from the extreme pH of the elution buffer.

2. Materials and methods

2.1. *Chemicals*

CH-Sepharose 4B was purchased from Pharmacia, Uppsala (Sweden). 1-Ethyl-3 (3-dimethyl amino propyl) carbodiimide HCl (EDC) was from Calbiochem, San Diego (U.S.A.). L-[U-^{14}C] phenylalanine, 522 mCi/mmole was purchased from Amersham, England.

2.2. *Enzyme preparation*

Monkey liver extracts were prepared as described previously (except no phenylalanine or EDTA was added for the liver homogenization) and used as such or purified to the end of the ammonium sulphate step [8].

2.3. *Assays*

Enzyme and protein was assayed as previously [8] unless otherwise indicated.

2.4. *Affinity gel preparation*

The 2-amino 6, 7-dimethyl-4-hydroxy 5, 6, 7, 8-tetrahydropteridine hydrochloride (ADHT) derivative of CH-Sepharose 4B was prepared basically as recommended by the manufacturers. Precautions were taken to ensure the ADHT remained in the reduced state.

Two ml (0.5 g dry weight) of freshly washed gel (using distilled water which had been subject to nitrogen bubbling for 30 min) was added to 22 mg ADHT (a five times excess over sites available on the gel) in 2 ml of similar distilled water. Before addition the ADHT solution was gassed with N_2 for a further 30 sec. After addition the mixture was gassed for 10 min. Then 124 mg of EDC were added to 2 ml gassed water and this was added dropwise to the ADHT–gel mixture. The pH was adjusted to 5.0 with N NaOH, the solution was gassed briefly, sealed and shaken for 24 hr at room temperature.

2.5. *Affinity chromatography*

Washed gel was packed in a column (2.0 × 0.7 cm) on top of a layer of Sephadex G-25 (2.9 × 0.7 cm). The column was prepared for use by washing successively with 20 ml pH 4 buffer (0.1 M Na acetate, pH 4.0, containing 1 M NaCl, 2×10^{-3} M DTT), 20 ml

* Queen Elizabeth II Fellow.

North-Holland Publishing Company – Amsterdam

pH 9 buffer (0.1 M Tris–HCl, pH 9.0, 1 M NaCl, 2 × 10^{-3} M DTT) and 20 ml column buffer (0.05 M Tris–HCl, pH 7.6, 0.2 M NaCl, 2 × 10^{-3} M DTT). Samples of ammonium sulphate fraction (dialysed against column buffer) up to 0.7 ml (or crude homogenate up to 16 ml) were applied and washed in with the column buffer. Flow rate was 10–12 drops per min. Elution was attempted or achieved with buffers described in the text. Buffer pH was measured at room temperature. Fractions of 1.0–1.4 ml (4 min) were collected unless otherwise stated. All operations were carried out at 4°C.

2.6. *Identification of coupled ADHT*

The coupled ADHT was released from the gel by heating a weighed amount of wet gel at 100°C for 15 min in 0.1 M HCl and 2 × 10^{-3} M dithiothreitol (DTT) (pH 1.10). Standard solutions of ADHT and some unsubstituted CH-Sepharose were treated in a similar manner. Absorbance of these solutions was measured at 266 nm after dilution 1:4 in 2 × 10^{-3} M DTT (pH 1.7).

Solutions from acid-treated gels and standards were analysed after ascending chromatography on Whatman 3 mm. The chromatogram was developed by *n*-butanol–acetic acid–water (120:30:50) for $4\frac{1}{2}$ hr. The developed chromatogram was examined by UV light.

2.7. *Identification of enzyme reaction product*

Production of [^{14}C]-labelled L-tyrosine from [^{14}C]-labelled L-phenylalanine was tested as described previously [9].

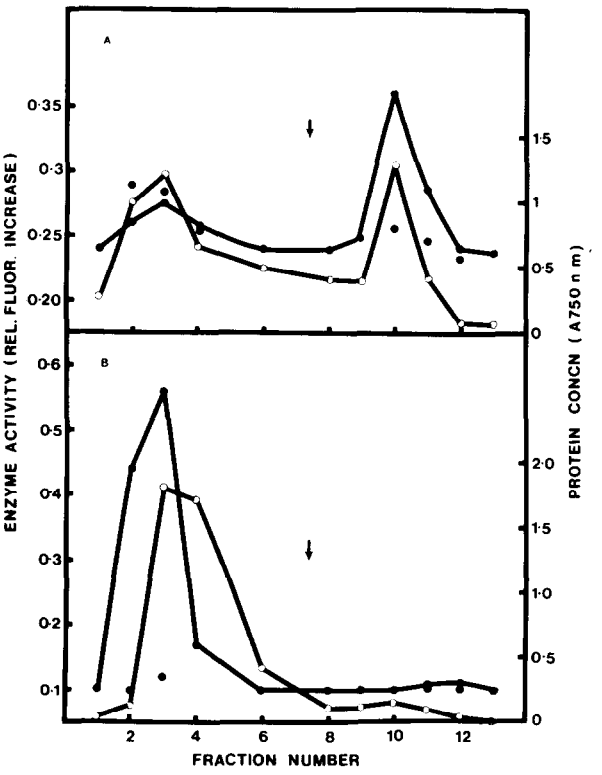

Fig. 1. Affinity chromatography of a dialysed ammonium sulphate fraction on A. ADHT-Sepharose 4B (0.6 ml sample) and B. CH-Sepharose 4B(0.4 ml sample). (•—•) Relative fluorescence of 0.3 ml of column fractions incubated in reaction mixture for 5 min (A) and 60 min (B) ; (⊙) Relative fluorescence of 0.3 ml of column fractions in reaction mixture unincubated; (○—○) Optical density of 0.3 ml of column fractions assayed by the Lowry method. The arrows indicate the point of application of the pH 11.3 buffer.

3. Results

Quantitation of the ADHT coupled in the gel revealed that it contained about 7.3 μmole/g wet gel, thus about 60% of the available sites on the gel had been derivatized.

When the acid-treated ADHT and gel solutions (75 μl) were chromatographed in *n*-butanol:acetic acid:water, a highly fluorescent spot was present in that solution derived from the substituted gel which migrated identically to that in the standard ADHT solution. This spot did not occur in the unsubstituted gel sample.

A sample, 0.6 ml of ammonium sulphate fraction dialysed into column buffer was placed on a composite column, washed with column buffer then the activity eluted with pH 11.3 buffer (0.25 M Na_2HPO_4–NaOH, pH 11.3, 2 × 10^{-3} M DTT) (fig. 1a). In two experiments recovery was about 47% and 60% with a purification of 3× in the best fraction of each. A large amount of yellow material was eluted at the void volume but the active fractions were almost colourless.

When uncoupled CH-Sepharose 4B was used instead of ADHT-Sepharose no activity or protein was retained which could be eluted with the pH 11.3 buffer (fig. 1b).

Bound enzyme could also be removed with pH 11.0 (0.25 M Na_2HPO_4–NaOH, pH 11.0, 2 × 10^{-3} M DTT)

buffer, pH 9.2 (0.25 M Tris–HCl, pH 9.2, 1 M KCl, 2×10^{-3} M DTT) buffer, or column buffer containing 2×10^{-2} M ADHT but not pH 10.0 (pH 11.0 buffer adjusted to pH 10.0 with 7.5% $NaHCO_3$) buffer or column buffer (pH 7.6) in which 1 M KCl replaced the 0.2 M NaCl.

In order to establish the identity of the activity eluted with pH 11.3 buffer the assay was carried out on the active fractions for 60 min with normal reaction mixture and with a reaction mixture from which either ADHT or phenylalanine had been omitted. No activity was seen under these conditions or when a similar fraction was boiled and assayed with a complete reaction mixture.

When crude liver homogenate was made 2×10^{-3} M with DTT and 0.2 M in NaCl and applied to the column considerable activity was removed from the extract. Activity could then be removed from the column with pH 9.2 buffer with a recovery of about 21% and the overall purification was 19-fold. Activity which was not adsorbed could be removed by another passage through a column. During these experiments it was found that recovery of enzyme was more than doubled when pH 9.2 buffer or pteridine was used instead of pH 11.3 buffer.

When ^{14}C-labelled L-phenylalanine was incubated with this purified enzyme radioactivity was found in the position of tyrosine after incubation for 120 min but not before. There was no chemical conversion in this time.

4. Discussion

An affinity adsorbent containing a reduced pteridine has been synthesized. The pteridine atom responsible for attachment to the gel is uncertain but it is likely to be via the amino group.

The adsorbent has been shown to bind phenylalanine hydroxylase. This binding is unlikely to be ionic as 0.15 M NaCl at pH 6.8 releases the enzyme from DEAE-cellulose [8]. The adsorbent thus offers a specific mode of purification for this enzyme, the optimal conditions of which are yet to be defined, but the method is much simpler than that described previously [8]. At present elution with free pteridine or pH 9.2 buffer seems most satisfactory. The loss of enzyme which occurs during the purification may well be due to a loss of the stimulating factor, Y, described previously [8].

Acknowledgements

Mr. Ian McNiece is thanked for skilled technical assistance and Dr. D. Danks and Dr. J. Connellan thanked for useful discussions. The Commonwealth Serum Laboratories (Parkville) are thanked for the monkey livers.

References

[1] Cuatrecasas, P. and Anfinsen, C. B. (1971) Methods Enzymol. 22, 345–378.
[2] Arsenis, C. and McCormick, D. B. (1966) J. Biol. Chem. 241, 330–334.
[3] Cuatrecasas, P. and Wilchek, M. (1968) Biochem. Biophys. Res. Comm. 33, 235–239.
[4] Lowe, C. R. and Dean, P. D. G. (1971). FEBS Letters 14, 313–316.
[5] Collier, R. and Kohlhaw, G. (1971). Anal. Biochem. 42, 48–53.
[6] Newbold, P. C. H. and Harding, N. G. L. (1971). Biochem. J., 124, 1–12.
[7] Allen, R. H. and Majerus, P. W. (1972). J. Biol. Chem. 247, 7695–7701.
[8] Cotton, R. G. H. (1971). Biochim. Biophys. Acta, 235, 61–72.
[9] Connellan, J. M. and Danks, D. M. (1973). Biochim. Biophys. Acta, 293, 48–55.