AFFINITY CHROMATOGRAPHY OF β-GLUCURONIDASE

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1. Introduction

For a number of years there has been interest in the role of β-glucuronidase (EC 3.2.1.31) in the mammalian system [1–4] and methods have been published describing the purification and characterization of the enzyme from a variety of sources [5–8]. There is also current interest in the role of lysosomal acid hydrolases in the destruction of host material at the invading edge of many tumours [9–11]. Our interest in the mechanism of tumour invasion and the desire to isolate homogeneous preparations of individual lysosomal enzymes from a variety of tumour tissues has led us to investigate the technique of affinity chromatography for this purpose.

This report describes the enrichment of β-glucuronidase by affinity chromatography utilising the powerful competitive inhibitor saccharo-1, 4-lactone (K_i 5.4 × 10^{-7}M) [12] attached covalently to Sepharose 4B through an α–ω diamine "extention arm" [13] the length of which has proved to be important for optimum enzyme immobilisation. A synthetic substrate, *o*-aminophenyl-β-D-glucuronide, has also been used successfully as an affinity absorbent and its usefulness is discussed.

2. Material and methods

Sepharose 4B was obtained from Pharmacia (G.B) Ltd., London. Cyanogen bromide was obtained from Eastman Kodak Co. Rochester, N.Y., USA. Saccharo-1, 4-lactone (A grade) was supplied by Calbiochem Ltd., London. Pure 3, 3′-diamino dipropylamine and *o*-aminophenyl-β-D-glucuronide were obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, U.K. Bovine liver β-glucuronidase, *p*-nitrophenyl-β-D-glucuronide and 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide were all obtained from Sigma Chemicals Ltd., London. 1, 2-diamino-ethane was obtained from B.D.H. Chemicals Ltd., Poole, England. All buffers and solutions were made up in double glass-distilled water and analytical grade reagents were used throughout.

The saccharo-1, 4-lactone affinity chromatography adsorbents were prepared, essentially, as described by Cuatrecasas [13]. A two-step reaction was performed, for both a "short arm" and a "long arm" derivative, the initial step yielding the aminoalkyl derivative of Sepharose 4B. The saccharo-1, 4-lactone was then coupled to the aminoalkyl derivative through its free carboxyl group using a water-soluble carbodiimide. The following conditions have been used a number of times (they apply to the "long arm" derivative but the "short arm" derivative was simply obtained by substituting 2 mmole/ml of 1, 2-diamino-ethane for the diamino dipropylamine) and have produced an absorbent having a high affinity and capacity for β-glucuronidase.

50 ml of well washed packed Sepharose 4B were activated using 250 mg of CNBr/ml of Sepharose. The reaction mixture was raised to and maintained at pH 11 or above with 4 M NaOH; the temperature of the reaction was kept at 20° or below by the addition of crushed ice. The activated Sepharose was washed rapidly on a Buchner funnel with about 1.5 ℓ of 0.1 M $NaHCO_3$ buffer pH 9.0 and 13.1 g of 3, 3′-diamino dipropylamine (2 mmole/ml of packed Sepharose in an equal volume of distilled water brought to pH 10.0 with 6 M HCl) was added to the activated Sepharose as soon as possible after the activation procedure. The reaction was allowed to proceed overnight at 4° after which the gel was washed with at least 3 ℓ of cold distilled water.

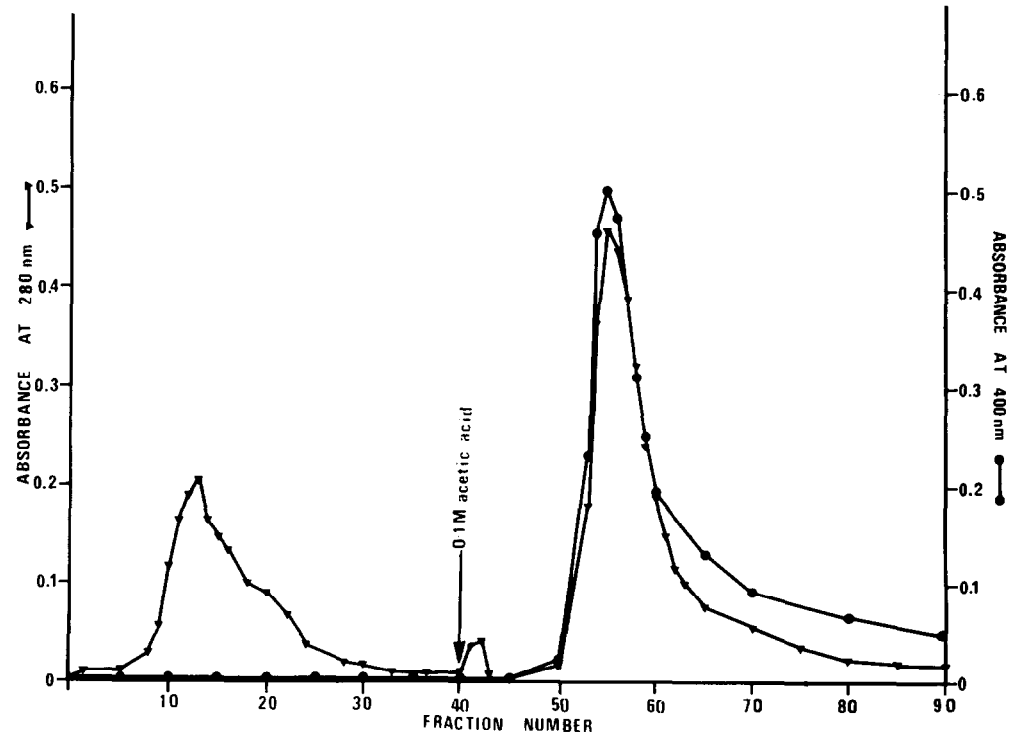

Fig. 1. Affinity chromatography of 50 mg bovine β-glucuronidase on a "long arm" saccharo-1, 4-lactone Sepharose column (2 × 15 cm). The column was equilibrated with 2 mM Tris/5 mM NaCl buffer pH 7.3 and the bovine β-glucuronidase was applied in 5 ml of the same buffer. Forty samples were collected and the pH 7.3 buffer was replaced with 0.1 M acetic acid. The volume of each fraction was 5 ml and the flow rate was kept constant at 120 ml/hr.

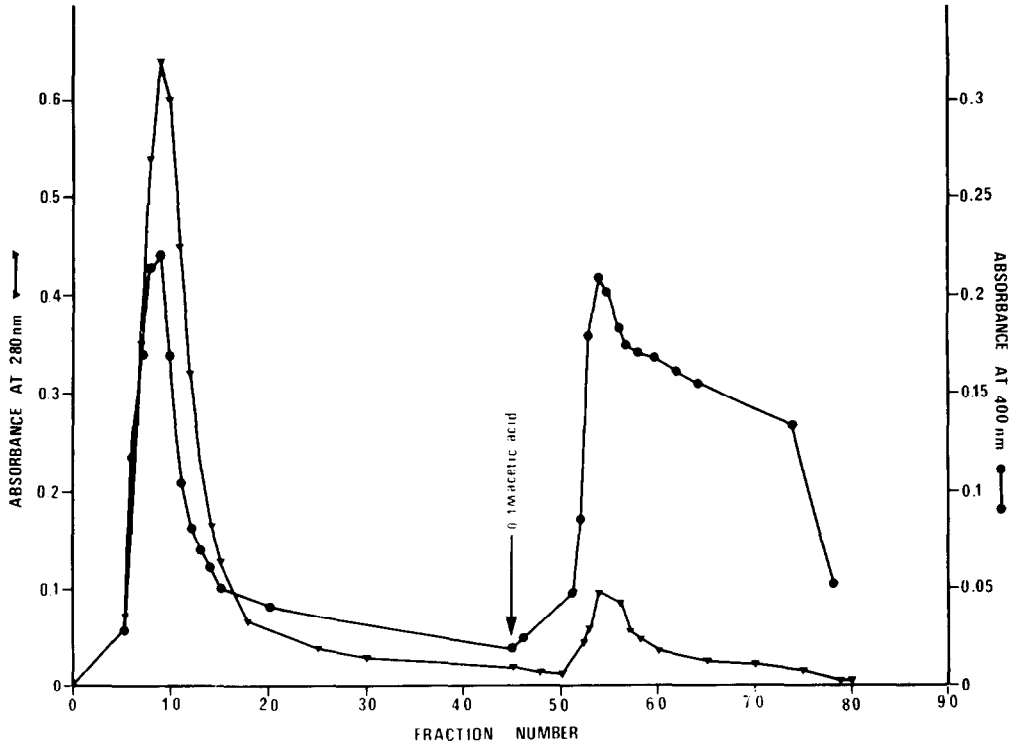

Fig. 2. Affinity chromatography of 50 mg bovine β-glucuronidase on a "short arm" saccharo-1, 4-lactone Sepharose column (2 × 15 cm). Forty five 5 ml fractions of Tris/NaCl eluate were collected in this case but all other conditions were as in fig. 1.

The washed gel, resuspended in about 200 ml of distilled water containing 2.1 g (10 mmoles) of saccharo-1, 4-lactone, was brought to pH 4.8 with 0.1 M NaOH and the coupling reaction was initiated by slowly adding 1.2 g (10 mmoles) of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide HCl in 8 ml of water. The mixture was left overnight at room temp., the adsorbent washed free of unbound saccharo-1, 4-lactone with large quantities of distilled water and finally suspended in 2 mM Tris/5 mM NaCl pH 7.3.

β-glucuronidase assays were usually performed on 0.1 ml samples of enzyme but occasionally 0.5 ml samples were assayed. In all cases the volume was made up to 1 ml with 0.2 M acetate buffer pH 5.0 containing 0.2 M NaCl. Each tube was incubated at 37° for 2 hr with 0.5 ml of *p*-nitrophenyl-β-D-glucuronide (2×10^{-4}M) in 0.2 M acetate buffer pH 5.0. The reaction was stopped with 0.5 ml of 2 M glycine buffer pH 10.4 containing 0.2 M NaCl and tubes were read at 400 nm.

3. Results and discussion

The existence of both a synthetic substrate (*o*-aminophenyl-β-D-glucuronide) and a powerful competitive inhibitor (saccharo-1, 4-lactone) both of which are capable of being substituted onto Sepharose 4B has made possible the simultaneous evaluation of both ligands for use in the purification of β-glucuronidases from a variety of sources.

Fig. 1 shows an elution profile using saccharo-1, 4-lactone attached to Sepharose 4B through a diamino dipropylamine arm. The protein peak emerging in the Tris/NaCl eluate has been consistently free of β-glucuronidase activity. Switching from Tris/NaCl buffer to 0.1 M acetic acid produces a large, single protein peak. The enzyme activity profile corresponds exactly with this protein peak. This column has been loaded with at least 10 separate 50 mg amounts of bovine β-glucuronidase and a number of times with tumour and normal tissue extracts from a variety of sources. In no case has β-glucuronidase been detected in the Tris/NaCl buffer washings but only in the 0.1 M acid eluate.

We have also investigated the substrate *o*-aminophenyl-β-D-glucuronide coupled directly onto Sepharose 4B through the amino group. A similar profile to that shown in fig. 1 was produced with 50 mg of bovine β-glucuronidase loaded and eluted in the same way. The column was reusable nine times with the application of a variety of tissue extracts but was marked by a failure to retain a further batch of enzyme under the previously favourable conditions. This was thought to be caused by exhaustion of the covalently attached substrate and although the column had behaved well the saccharo-1, 4-lactone derivative has the advantage of possessing the same strong binding characteristics and a much longer life.

It has been demonstrated [14] that successful purification of many proteins by affinity chromatography depends on placing the binding ligand at a suitable distance from the supporting gel to minimise steric interferences, particularly with protein–ligand complexes of weak affinity. Saccharo-1, 4-lactone has a strong affinity for β-glucuronidase (K_i 5.4×10^{-7}M) and yet it is necessary to place the inhibitor at the end of a diamine arm. This is approx. 12 Å for 3-3′-diamino dipropylamine.

Fig. 2 shows the elution profile of bovine β-glucuronidase on a "short arm" (1, 2-diamino-ethane) saccharo-1, 4-lactone column. Enzyme activity has always appeared in the Tris/NaCl buffer washings corresponding with the protein peak. Continuous batch-wise application of enzyme to the column caused a progressive decrease in the amount of enzyme attaching to the absorbent. The degree of substitution of the gel by the two derivatives, as determined by the 2, 4, 6-trinitrobenzene-sulphonate colour test [13] was similar, indicating that the difference in affinity is not simply due to a difference in the amount of saccharo-1, 4-lactone attached.

It is conceivable that there is stronger binding between enzyme and inhibitor in close proximity to the gel matrix which is irreversible under the conditions used for eluting the enzyme. This would account for the rapid decrease in affinity of the adsorbent for further batches of enzyme.

This aspect, the effect of using other diamine side arms on the amount of enzyme bound and the degree of enzyme purification obtained are being investigated and will be reported in a further publication.

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