AFFINITY CHROMATOGRAPHY OF HUMAN LIVER α-D-MANNOSIDASE

Donald ROBINSON, Nigel C. PHILLIPS and Bryan WINCHESTER
Department of Biochemistry, Queen Elizabeth College, London W8 7AH, UK

Received 5 March 1975

1. Introduction

At least three forms of α-D-mannosidase occur in mammalian tissues [1,2]. Two of these (forms A and B) are acid hydrolases, probably of lysosomal origin. The third (form C) has a neutral pH optimum and may be located in the soluble fraction of cell homogenates [3]. In human and bovine mannosidosis, a lysosomal storage disease in which mannose-containing oligosaccharides are excreted, [4,5] forms A and B are absent but form C is present in normal amounts [1,2]. Isolation and characterisation of α-D-mannosidase forms is therefore of considerable importance for an understanding of this genetic disorder. α-Mannosidase is also of value in the analysis of heteropolysaccharide sequences and several purification procedures have been devised to this end [6,7]. The recent successful use of a fucosylamine ligand in the affinity chromatography of human α-L-fucosidase [8] prompted this present attempt to purify α-mannosidase by an analogous procedure.

2. Experimental

2.1. *Materials*

CH Sepharose 4B and CNBr activated Sepharose 4B were purchased from Pharmacia (G.B.) Ltd., London W.5., UK, mannose was purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, UK, and 4-methylumbelliferyl-α-D-mannoside, 1-naphthyl-α-D-mannoside and the diazonium salt of 4-chloro-*o*-anisidine were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., UK.

2.2. *Preparation of affinity chromatography support*

Two forms of affinity ligand were prepared, one in which mannosylamine was directly coupled to CNBr activated Sepharose, and the other in which this functional group was separated by a hexanoyl arm from the support matrix by coupling to CH Sepharose 4B. β-1-Amino mannose was synthesised by prolonged treatment of mannose with dry ammonia in methanol as described by Isbell and Frush [9]. This was coupled in aqueous medium for 24 hr to either CNBr activated Sepharose or CH Sepharose 4B by the manufacturers recommended procedures. The activated Sepharose derivative (4 g) suspended in 30 ml of water was reacted with 5 g of mannosylamine in the presence of 0.1 M 1-ethyl(3-dimethyl-amino-propyl)carbodiimide hydrochloride. The pH was maintained between 4.5 and 6.0 for 24 hr after which the reacted support was thoroughly washed with distilled water before use. The activated support is said to have a capacity of 10–14 μ mol per ml of swollen gel (0.25 g dry wt). A large excess of mannosylamine was used and no attempts were made to determine the degree of substitution which is presumed to be virtually complete.

2.3. *Enzyme preparation*

A 50% w/v homogenate of post-mortem human liver in 50 mM sodium phosphate buffer pH 6.0 was centrifuged at 18 000 *g* for 15 min in an MSE 18 refrigerated centrifuge. The supernatant (preparation 1) contained α-D-mannosidases A, B, and C. This supernatant was fractionated by ammonium sulphate precipitation, the fraction obtained between 40 and 55% saturation containing 90–95% of the original acid α-D-mannosidases A and B and none of the neutral

 North-Holland Publishing Company – Amsterdam

activity. The precipitate was dissolved in the above phosphate buffer and dialysed against a large volume of the same buffer for 24 hr. The final solution (preparation II) contained 30 mg protein/ml, and 100 ml of this extract was obtained from 250 g of liver.

2.4. *Enzyme assay*

The enzyme solution (10 μl) was added to 1 ml of 1 mM 4-methylumbelliferyl-α-D-mannoside in phosphate citrate buffer pH 4.0 (200 mM disodium hydrogen phosphate adjusted to pH 4.0 with 100 mM citric acid), and incubated at 37°C for 30 min. The reaction was stopped by the addition of 1.5 ml of 0.5M glycine–NaOH buffer, pH 10.4, and the fluorescence of the liberated 4-methylumbelliferone measured. Activity was calculated as μmol 4-methylumbelliferone liberated/min under these conditions.

2.5. *Affinity chromatography*

Mannosylamine substituted Sepharose or CH Sepharose 4B was suspended in 50 mM sodium phosphate buffer pH 6.0 and packed under gravity in disposable plastic syringe barrels to give affinity columns of 5 ml total volume. The enzyme sample (50 ml of Preparation I or II) was applied and eluted in the same buffer at a flow rate of 20 ml/hr. Fractions (4 ml) were collected and assayed for α-D-mannosidase as described and for protein by absorbance at 280 nm. When this absorbance fell to near zero the eluant was changed to 300 mM mannose in the same buffer (24 ml). At this point acidic α-D-mannosidase could be successfully eluted. Finally 1.0 M NaCl in the same buffer was passed through the column until the absorbance again fell to near zero.

2.6. *Disc gel electrophoresis*

The protein patterns of starting material and material eluted from the affinity column were examined by disc gel electrophoresis using standard methods [10]. Enzyme activity was located by incubating the gel sticks for up to 24 hr in phosphate-citrate buffer, pH 4.0, containing 1 mg/ml 1-naphthyl-α-D-mannoside and 1 mg/ml diazonium salt of 4-chloro-*o*-anididine. Enzymically active bands were located by the appearance of the insoluble red complex formed between liberated 1-naphthol and the diazonium salt.

3. Results

Negligible binding of α-D-mannosidase from either preparation I or II was observed with the mannosylamine directly bound to Sepharose. Similarly the corresponding CH Sepharose 4B derivative in which fucosylamine replaced mannosylamine [8] was unable to retard α-mannosidase. We may therefore conclude that non-specific hydrophobic binding to the hexanoyl arm is not taking place. In contrast the activity of both preparations I and II was bound to the mannosylamine column prepared with CH Sepharose 4B. This column was re-cycled several times without loss of efficiency or enzyme binding capacity and appears to be stable under the conditions of use.

When preparation I was used the A and B forms of the enzyme were unretarded but α-D-mannosidase C was strongly bound and could only be eluted with the salt solution. This preferential binding of form C appears to be a competitive phenomenon since both forms A and B were successfully bound on the column from preparation II in which form C is absent. The results of such an affinity purification are shown in fig.1. Typically 0.21 e.u. of mixed α-D-mannosidases

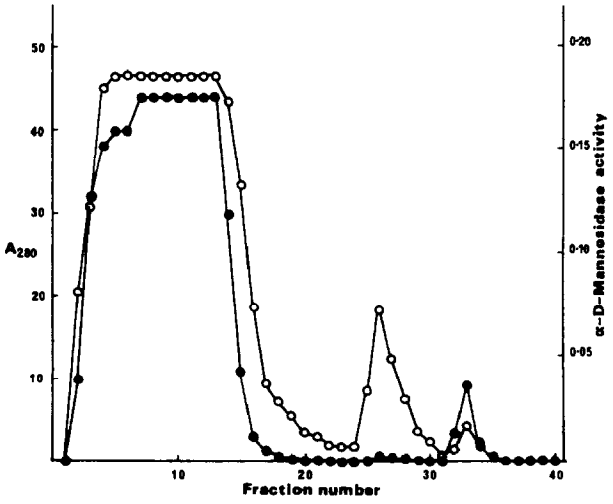

Fig.1. Affinity chromatography of human liver α-D-mannosidase preparation II on CH-Sepharose-mannosylamine. For details see text (o–o–o) Acidic α-D-mannosidase activity expressed as μmol 4-methylumbelliferone liberated/min/fraction. (•–•–•) Absorbance at 280 nm. The 300 mM mannose was applied at fraction number 24 and 1.0 M NaCl at fraction number 30.

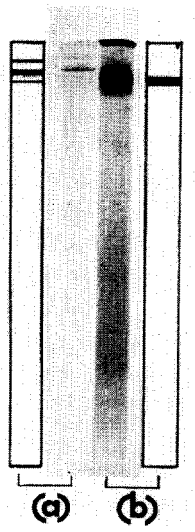

Fig.2. Disc polyacrylamide electrophoresis of human α-D-mannosidase preparation in discontinuous Tris (pH 9.0)–Tris-glycine (pH 8.5) buffer using 7% gels. a) 300 mM mannose eluate from column; b) Enzyme activity located by 1-naphthyl-α-D-mannopyranoside.

A and B were specifically eluted from the 5 ml column by 300 mM mannose, giving a purification of 360-fold over the original homogenate. The sample contained 1.36 mg protein which was shown by disc electrophoresis to be heterogeneous. The enzymic activity was not associated with the major protein band but with one of the two minor components (fig.2).
A large number of protein species was eluted with the final NaCl solution, several of them having weak α-D-mannosidase activity.

4. Discussion

The use of glucosylamines as affinity ligands has been extensively used by Sharon and co-workers for the isolation of sugar specific lectins [11]. A fucosylamine derivative has previously been used for isolation of α-L-fucosidase [8] and the general method has been shown here to be effective for α-D-mannosidase. The neutral form of the enzyme (C) appears to bind more strongly and to exclude the acidic forms, A and B, under these conditions. However, when form C is eliminated by a prior $(NH_4)_2SO_4$ fractionation a purification of the A and B forms is possible and they can be eluted in good yield by 300 mM mannose. Removal of the C form from the affinity support is more difficult and requires much higher mannose concentrations or 1 M NaCl. Although a considerable one step purification is effected by the method the eluted protein is not homogeneous as was noted in earlier papers using similar ligands [8,11]. In particular a major protein without α-D-mannosidase activity is present. Several explanations may be put forward for these contaminants. The system would be expected to separate any protein species with mannose-binding capacity and these may include mannosyl transferases, denatured mannosidases, and binding subunits of oligomeric enzymes. The presence of lectin-like proteins specific for mannose in mammalian cells is a possibility remaining to be investigated.

References

[1] Carroll, M., Dance, N., Masson, P. K. Robinson, D. and Winchester, B. G. (1972) Biochem. Biophys. Res. Commun. 49, 579–583.
[2] Phillips, N. C., Robinson, D., Winchester, B. G. and Jolly, R. D. (1974) Biochem. J. 137, 363–371.
[3] Marsh, C. A. and Gourlay, G. C. (1971) Biochim. Biophys. Acta 235, 142–148.
[4] Nordén, N. E., Lundblad, A., Öckerman, P. A. and Jolly, R. D. (1973) FEBS Lett. 35, 209–212.
[5] Nordén, N. E., Öckerman, P. A. and Szabo, L. (1973) J. Pediat. 82, 686–688.
[6] Snaith, S. M. and Levvy, G. A. (1969) Biochem. J. 114, 25–33.
[7] Chu, C. Y. T. and Turner, M. D. (1974) Biochem. J. 139, 469–477.
[8] Robinson, D. and Thorpe, R. (1974) FEBS Lett. 45, 191–194.
[9] Isbell, H. S. and Frush, H. L. (1958) J. Org.Chem. 23, 1309–1319.
[10] Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
[11] Lotan, R., Gussin, A. E. S., Lis, H. and Sharon, N. (1973) Biochem. Biophys. Res. Commun. 52, 656–662.