AFFINITY CHROMATOGRAPHY OF HUMAN LIVER α-L-FUCOSIDASE

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1. Introduction

Purification of human α-L-fucosidase is essential for the further study of the genetic disorder of fucosidosis in which this enzyme is defective [1], and for any proposed attempts at enzyme replacement in these cases. The enzyme is also in demand from workers studying the role of fucose as an important terminal sugar in the complex carbohydrates of cell surfaces. Proteins capable of specific binding to such fucose residues have been isolated from plant materials [2] and we considered that the affinity methods used for their purification might also be applicable to the isolation of the hydrolase.

2. Experimental

2.1. *Materials*

Samples of the affinity ligand *N*(ε-amino-caproyl)-β-L-fucosylamine was kindly supplied by Miles-Yeda Ltd., Rehovoth, Israel. This ligand condensed onto Agarose is commercially available for purification of fucose-binding protein and was purchased from Miles Laboratories Ltd., Stoke Poges, Slough, England. L-Fucose was purchased (Sigma Chemical Corp., Missouri, USA), *p*-nitrophenyl α-L-fucoside was synthesised by published methods [3], and 4-methyl-umbelliferyl α-L-fucoside was purchased from Koch-Light Laboratories, Colnbrook, Bucks.

2.2. *Enzyme preparation*

A 40% w/v homogenate of post-mortem human liver in 100 mM phosphate–citric acid buffer, pH 5.5, was used for inhibition studies. The starting material for affinity chromatography was prepared as follows: A 20% w/v homogenate of human liver in 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA was centrifuged at 9000 rpm for 15 min in an M.S.E. 18 refrigerated centrifuge and the supernatant precipitated by 60% saturation with solid ammonium sulphate. This precipitate was collected by centrifugation and re-suspended in about one-tenth the original volume of 50 mM phosphate buffer, pH 6.8, and de-salted by passing through a column of Sephadex G-25 equilibrated with the same buffer. A final volume of about 100 ml of crude enzyme preparation was obtained from an initial 500 ml of liver homogenate with no appreciable loss of total activity.

2.3. *Enzyme assay*

The enzyme solution (100 μl) was added to 50 μl of 10 mM *p*-nitrophenyl-α-L-fucoside in 100 mM disodium hydrogen phosphate adjusted to pH 5.5 with 100 mM citric acid and incubated for 30 min. at 37°C. The reaction was stopped by addition of 3 ml of 0.2 M glycine–Na_2CO_3 buffer, pH 10.7, and the liberated *p*-nitrophenol measured spectrophotometrically by its absorbance at 400 nm. The results were compared with blank assays in which buffer had been substituted for the enzyme solution or substrate solution and the activity calculated as μmoles *p*-nitrophenol liberated/min under these conditions. For inhibition studies at sub-optimal substrate concentrations 20 μl of 40% homogenate of human liver prepared as above were incubated with 40 μl of the above phosphate–citrate buffer, pH 5.5, containing the required concentration of substrate and 40 μl of the inhibitor solution in the same buffer. Incubations of 1 hr at 37°C were followed by assay of liberated *p*-nitrophenol as before.

2.4. *Disc gel electrophoresis*

The protein patterns of starting material and most highly purified samples of α-L-fucosidase were examined by disc gel electrophoresis using standard methods and stained for protein with Coomassie Blue [4]. Enzyme activity was located by incubating the gels for 15 min at 37°C in 1 mM 4-methylumbelliferyl-α-L-fucoside in 100 mM phosphate–citrate buffer, pH 5.0, and viewing under UV light for the fluorescence of liberated aglycone.

2.5. *Affinity chromatography*

A 5 ml column of Agarose-ε-amino caproyl fucosamine suspended in 50 mM sodium phosphate buffer, pH 6.8, was packed under gravity in a disposal syringe barrel and washed with several bed volumes of the same buffer. The enzyme sample (10 ml of above preparation) in this buffer was applied and elution continued at a flow rate of 16 ml/hr. After discarding the void volume (1.5 ml) fractions (4 ml) were collected and monitored for α-L-fucosidase as described above and for protein by the absorbance at 280 nm. When this absorbance fell to a steady near-zero value the eluant was changed to 1% L-fucose in the same buffer (12 ml) and this was followed by 100 mM NaCl in the same buffer until no further protein could be detected in the eluate. The column was finally purged with 2 M NaCl in buffer (20 ml) before re-equilibrating in the original buffer (10 ml) ready for re-use. There appeared to be no appreciable loss in the binding capacity of the column for α-L-fucosidase after several cycles of this procedure. Because of the inhibitory effect of fucose in the enzyme assay, the column samples were dialysed for 24 hr against 6 changes of the phosphate buffer (pH 6.8) before assay. No significant change in volume was noted, and enzyme recovery was unaffected by this process.

3. Results

Both α-L-fucose and the *N*(ε-aminocaproyl)-β-L-fucosylamine behaved as competitive inhibitors for human liver α-L-fucosidase, having K_i values of 0.8×10^{-3} M and 2.6×10^{-3} M respectively calculated from Lineweaver–Burk reciprocal plots. The apparent K_M for *p*-nitrophenyl-α-L-fucoside was 1.1×10^{-3} M determined by the same method.

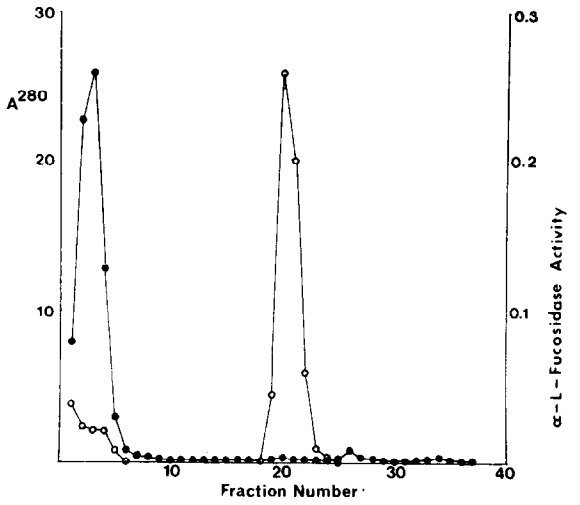

Fig. 1. Affinity chromatography of human liver α-L-fucosidase on Agarose-ε-aminocaproyl-fucosamine. For details see text. Open circles, α-L-fucosidase activity expressed as μmoles *p*-nitrophenol split/min/fraction. Closed circles absorbance at 280 nM. The 1% L-fucose solution was applied at fraction number 18 and 100 mM NaCl at fraction number 24.

There is thus a similar affinity for the proposed elutant and the ligand, despite the fact that the latter

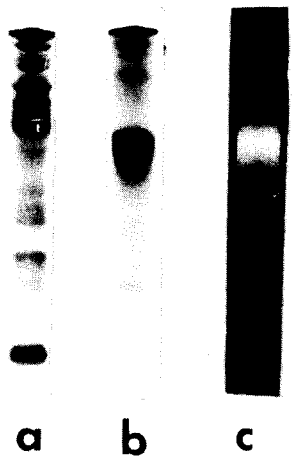

Fig. 2. Disc polyacrylamide electrophoresis of human liver α-L-fucosidase preparation in discontinuous Tris (pH 9.0)–Tris–glycine (pH 8.5) buffer using 8% gels: a) ammonium sulphate fraction of human liver; b) 1% fucose eluate from column; c) Enzyme activity located by 4-methylumbelliferyl α-L-fucoside.

has opposite anomeric configuration to the preferred substrate. The results of affinity chromatography are shown in fig. 1. In a typical experiment 90% of the α-L-fucosidase applied to the column was eluted by 1% fucose in buffer with an increase in specific activity of 250-fold. When 90 ml of the same enzyme preparation was applied in an attempt to saturate the column enzymic activity appeared in the main protein peak suggesting that saturation had taken place. After washing to a low protein level in the eluate and then applying the 1% fucose solution a total of 1.6 e.u. of activity (2.3 mg protein) were specifically eluted with a purification of 300-fold over the original preparation. Disc electrophoresis of the fucose-eluted product showed that it contained one major protein component, coinciding with the location of enzymic activity and traces of several minor constituents that were non-enzymic (fig. 2).

4. Discussion

A substantial degree of purification is obtained in one step on the affinity support described. In view of its original use for fucose-finding proteins it is not surprising that more than one protein species is eluted and these may include denatured α-fucosidase as well as other fucose-specific proteins with no hydrolytic activity. The question of anomeric specificity deserves mention. The ligand is stated to have β-configuration and it is generally thought that glycosidases are absolutely specific for the stereochemistry of the glycosidic bond. Either this is not so in this case or the ligand contains a small proportion of the α-anomer. Blumberg [2] suggested a substitution of 2 μmoles of covalently bound fucosylamine/ml resin was obtainable by the method used for preparation of the affinity support. If the lowest molecular weight form [5] of α-L-fucosidase (60 000) is assumed to have a single fucose-binding site the theoretical capacity of the column used should be approx. 600 mg protein. However, in practice many of the fucose substitutions may be inaccessible to the proteins and the working capacity of our column material was stated by the manufacturers to be 2.3 mg/ml for fucose-binding lectins with equivalent weight of 30 000. On this basis we might expect about 0.07 μmole of monovalent enzyme bound/ml of resin or a total capacity of 23 mg. The observed capacity is only 10% of this and could be explained by the presence of some α-anomer in the original ligand. This problem may be resolved if the column saturated with α-L-fucosidase retains its normal capacity for fucose-binding lectins and vice versa.

Acknowledgements

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References

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