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AFFINITY CHROMATOGRAPHY AND SEPARATION OF THE MOLECULAR FORMS OF MONKEY BRAIN α-L-FUCOSIDASE ON FUCOSE-LINKED SEPHAROSE

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Summary

A simple affinity system which required coupling of α-L-fucose to Sepharose 4B by epichlorohydrin treatment of Sepharose 4B in the presence of α-L-fucose under alkaline conditions has been described. A partially purified preparation of monkey brain α-L-fucosidase (α-L-fucoside fucohydrolase, EC 3.2.1.51) was resolved at pH 5.0 into two major fractions: one bound and one retarded. The enzyme bound to the affinity column and specifically eluted by 2 mM α-L-fucose at pH 5.0 appeared to be homogeneous by polyacrylamide gel electrophoresis and was constituted mainly by the tetrameric form of the enzyme. The enzyme fraction retarded by the affinity column was found to contain mainly the monomeric form of the enzyme. Additional evidence for the different molecular forms of the enzyme in the bound and retarded fractions came from pH activity profiles and heat inactivation studies. The fucose-Sepharose appeared to bind the tetrameric form of the enzyme specifically and, further, α-L-fucose helped to retain the molecular integrity of the tetrameric enzyme.

Introduction

Affinity chromatography procedures for the purification of some acid glycosides and their isoenzyme have been recently published. β-hexosaminidase, β-galactosidase, α-mannosidase and α-L-fucosidase are known to exist in forms differing in various physicochemical properties [1—3]. The enzyme, α-L-fucosidase (α-L-fucoside fucohydrolase, EC 3.2.1.51), from different sources, is known to exist in two forms on the basis of the difference in molecular weight [4—6] and up to nine forms differing in isoelectric point [7—9]. Recently, we reported that a purified preparation of α-L-fucosidase from monkey brain contains three different molecular forms: a tetramer, a dimer

and a monomer. The tetramer was observed to dissociate to the monomer via the dimer [10]. These three molecular forms also showed different pH activity profiles and thermal stability. Chester et al. [11] have also shown evidence for the existence in human liver and serum of different forms of α-L-fucosidase differing in pH optima and thermal stability. Studies on the substrate specificity and various other kinetic properties with individual forms of the purified fucosidase are not extensive and could be due in part to the non-availability of a convenient method to separate the isoenzyme in large quantities. In the present communication, we describe a convenient affinity chromatography method which purifies monkey brain α-L-fucosidase and resolves the different molecular forms reported earlier [10].

Materials and Methods

α-L-Fucose and *p*-nitrophenyl-α-L-fucoside were obtained from Sigma Chemical Co. (U.S.A.). D-Glucose, D-galactose and D-mannose were products of Pfanstiehl Laboratories, IL, (U.S.A.). Sepharose 4B and epichlorohydrin were purchased from Pharmacia Fine Chemicals, Uppsala (Sweden) and B.D.H. (U.K.), respectively. The *p*-nitrophenyl derivates of various sugars and other reagents of analytical grade were obtained from sources as mentioned earlier [10]. The brains of adult monkeys (*Macaca radiata*) were isolated under nembutal anaesthesia and stored frozen at −20°C.

Preparation of the enzyme. All the operations were performed at 0—4°C unless otherwise mentioned.

Frozen monkey brain was homogenized in 0.02 M potassium phosphate buffer (pH 7.0) in a Waring Blendor for 5 min at maximum speed. The homogenate was further processed up to the Concanavalin A-Sepharose affinity chromatography step as reported earlier [10].

Assay of enzymes. The assays of α-L-fucosidase, *N*-acetyl-β-hexosaminidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and α-mannosidase and the units of enzyme activity were as described earlier [10].

Preparation of fucose-Sepharose. To 20 ml packed, washed Sepharose 4B a solution of 500 mg α-L-fucose in 7 ml H_2O were added. To this slurry 3.0 ml of 5 M NaOH, 2.0 ml of epichlorohydrin and 40 mg of sodium borohydride were added and mixed thoroughly. The mixture was heated at 60°C for 2 h with gentle and continuous mechanical stirring. After this treatment the suspension was poured into a column and then washed, first with 80 ml water and then with excess of 1 M NaCl. The washings were collected, neutralized with HCl and used for the estimation of fucose by the cysteine-H_2SO_4 method of Dische and Schettles [12]. Epichlorohydrin treatment resulted in the coupling of about 35 μmol α-L-fucose/ml wet Sepharose 4B.

Affinity chromatography of α-L-fucosidase. A portion of the enzyme eluted from concanavalin A-Sepharose was dialyzed against 300 vols. 0.02 M acetate buffer (pH 5.0) for 3 h with two changes. The precipitated protein was removed by centrifugation at 12 000 × *g* for 30 min. The supernatant (7.7 ml) containing 61 enzyme units and 2.1 mg protein, was loaded (flow rate, 10 ml/h) onto a 19 × 1.1 cm column of fucose-linked Sepharose, preequilibrated with 0.02 M acetate buffer (pH 5.0). The column was washed with 0.02 M acetate buffer

(pH 5.0) (flow rate, 20 ml/h). The column was then eluted with 2 mM α-L-fucose in 0.02 M acetate buffer (pH 5.0) (flow rate, 10 ml/h). 5-ml fractions were collected in tubes containing 0.1 ml 1 M potassium phosphate buffer (pH 7.5) and 0.2 ml 4 M NaCl. A major peak (peak I, fractions 5—8) of the enzyme activity appeared in the washing along with a minor peak at fraction 2 (Fig. 1). The fucose eluate showed another peak of activity (peak II, fractios 16—20) (Fig. 1). The enzymes from peaks I and II were pooled separately, concentrated over Aquacide II and dialyzed against 0.02 M potassium phosphate buffer (pH 7.0), 0.15 M NaCl for 3 h, with two changes. These dialyzed enzyme preparations were used for the study of various properties. The used fucose-Sepharose column was regenerated for further use by washing the column with 1 M NaCl and then reequilibrating with 0.02 M acetate buffer (pH 5.0). The regenerated column did not show any noticeable change in its binding capacity for the enzyme.

Gel filtration of the two major peaks obtained from the fucose-Sepharose column was carried out on a 42 X 1.69 cm column of Sephadex G-200 (15 ml/h) calibrated with marker proteins as reported earlier [10]. Protein was estimated according to Lowry et al. [13].

Results

The enzyme, α-L-fucosidase, from different sources has been shown to be competitively inhibited by α-L-fucose [5,14,15]. A derivative of this competitive inhibitor, fucosamine, has been used by many investigators as an affinity ligand for the purification of α-L-fucosidase [14—16]. Recently a synthetic thio-α-fucoside has also been successfully used as an affinity ligand

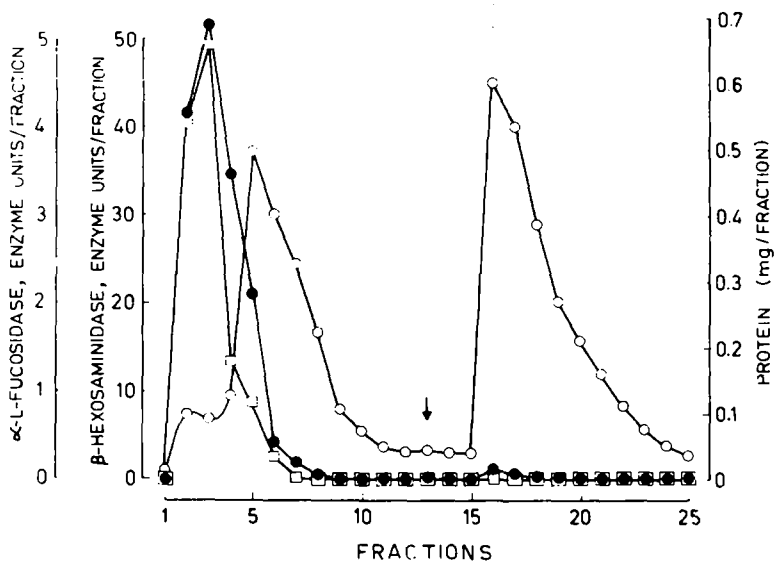

Fig. 1. Affinity chromatography of dialyzed concanavalin A-Sepharose eluate on fucose-Sepharose column. ○, α-L-fucosidase activity; □, β-hexosaminidase activity; ●, total protein. The arrow (↓) indicates the point where the elution by 2 mM α-L-fucose was started.

for the purification of rat epididymis enzyme [17]. In the present work, epichlorohydrin, a cross-linking agent for carbohydrates, was used to link fucose to Sepharose 4B. The conditions used were similar to those used by Porath et al. [18] for epichlorohydrin treatment of agarose gels, and these were claimed to cause no chemical or physical damage to the agarose matrix.

Fucose-Sepharose bound a portion of the monkey brain α-L-fucosidase which was not removed by excessive washing of the column, but could be specifically eluted by 2 mM α-L-fucose (peak II, Fig. 1). The specific activity (652 enzyme units/mg protein) of the peak II enzyme showed a 22-fold increase as compared to the value for the dialyzed concanavalin A-Sepharose eluate (29 enzyme units/mg protein). This value is close to the reported value for the homogeneous enzyme [10]. The purity of the enzyme was also confirmed by polyacrylamide gel electrophoresis at pH 7.4, which showed an pattern identical to that of the reported homogeneous enzyme [10]. It was free of the other glycosidases tested except β-hexosaminidase (which was less than 4% by activity). The fucose-Sepharose column also retarded another portion of the loaded enzyme activity, which could be removed from the column by excessive washing (peak I, Fig. 1). This fraction was distinct from the very small amount of activity that was not bound (fraction 2, Fig. 1) to the fucose-Sepharose column and was eluted within the first bed volume of the washing, accompanied by other contaminating proteins. The specific activity of the Peak I enzyme did not show any significant increase. Of the total activity recovered from the fucose-Sepharose column (70—75% of the original activity) 67% appeared in peak II, 30% in peak I and 3% in the minor peak (fraction 2) before peak I. To show that the peaks of α-L-fucosidase are distinct entities, the elution profile of another glycosidase, β-hexosaminidase is also shown in Fig. 1. A major portion of β-hexosaminidase was found in fraction 2 (ratio of β-hexosaminidase to α-L-fucosidase was 55), peak I showed much less β-hexosaminidase activity (ratio of β-hexosaminidase to α-L-fucosidase, 6) and peak II was minimally contaminated by β-hexosaminidase (less than 4% by activity).

The specificity of the affinity column and of the elution of the retained enzyme fraction (peak II) from the fucose-Sepharose was demonstrated by the following experiments. Nonspecific sugars like glucose, galactose and mannose upto a concentration of 10 mM at pH 5.0 were unable to release the bound enzyme from the column, whereas fucose (at a concentration as low as 1 mM) effectively eluted the enzyme. The fucose-Sepharose column did not show any affinity for the enzyme at pH 7.0 (a pH at which the enzyme shows markedly reduced activity). Sepharose 4B or epichlorohydrin-treated Sepharose 4B (in the absence of fucose) at pH 5.0 did not show any retention or retardation of the enzyme and the total fucosidase from the column appeared at a position corresponding to the minor peak (fraction 2).

In an attempt to characterize the molecular composition of the two major peaks of the enzyme obtained from the affinity column, they were subjected to gel filtration on Sephadex G-200 (Fig. 2). The elution pattern of the peak I enzyme indicated that over 85% of the total activity was the small molecular weight form (Fig. 2A) which was previously characterized as the monomeric form (molecular weight 75 000) of α-L-fucosidase. The peak II enzyme showed that the major portion of the activity (75% of the total) was of higher

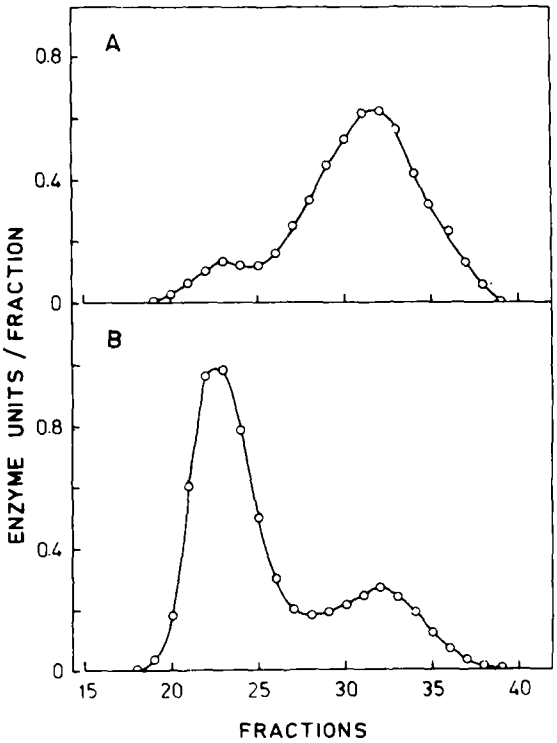

Fig. 2. Sephadex G-200 gel filtration of the two major fractions (peak I and II) obtained from the fucose-Sepharose affinity column. A, Elution profile of peak I enzyme (5 enzyme units); B, elution profile of peak II enzyme (7.2 enzyme units).

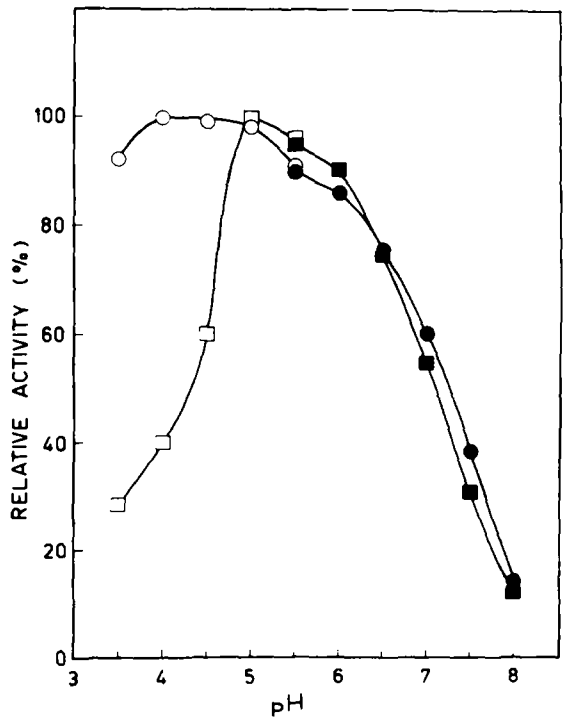

Fig. 3. pH vs. activity profiles of peak I and peak II enzymes from the fucose-Sepharose column. The enzyme (0.05 units) was assayed according to the standard assay conditions at different pH. Peak I enzyme: □, acetate buffer; ■, phosphate buffer. Peak II enzyme: ○, acetate buffer; ●, phosphate buffer.

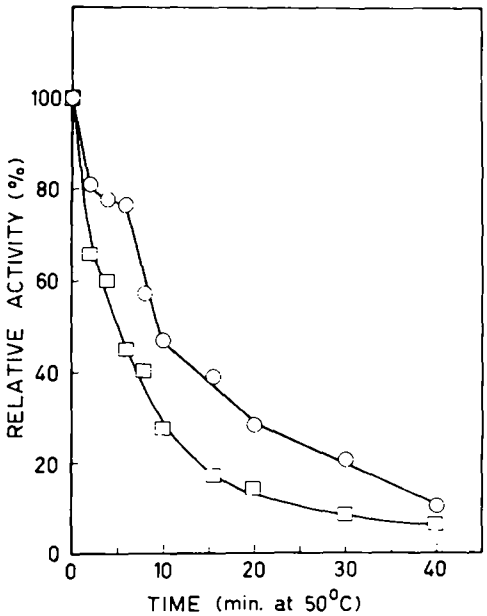

Fig. 4. Heat inactivation profiles of peak I and peak II enzymes. □, peak I enzyme (0.05 units, 0.41 mg protein/ml); ○, peak II enzyme (0.05 units, 3.92 μg protein/ml) were heated at 50°C for periods as indicated, chilled at 0°C and assayed for residual activity according to the standard assay method.

molecular weight (Fig. 2B), corresponding to the tetrameric form of the enzyme of molecular weight 285 000; the rest of the activity corresponded to the monomeric form. Additional support of the characterization of the two peaks of activity came from pH activity and thermal inactivation studies. The pH activity profile of the peak II enzyme showed a high activity in the pH range of 3.5 to 5.0 with an optimum of pH 4.0 characteristic of the tetrameric form of the enzyme (Fig. 3). The pH activity profile of peak I enzyme had a sharp optimum at pH 5.0 with a considerably lower activity in the more acidic region, which indicated the monomeric nature of the enzyme (Fig. 3). The thermal inactivation pattern of peak I enzyme showed a sharp and continuous decrease in activity (Fig. 4) which resembled the monomeric form of the enzyme. The heat inactivation profile of peak II enzyme showed (Fig. 4) an initial sharp drop, then a slower rate of inactivation followed by a triphasic inactivation pattern characteristic of a mixture of monomeric, dimeric and tetrameric forms of the enzyme.

Discussion

With the fucose-linked Sepharose affinity column, the monkey brain α-L-fucosidase could be isolated into two major fractions, a retarded peak I enzyme fraction and a peak II enzyme fraction bound by the column and specifically eluted by fucose. The present affinity system (which involved a simple method of preparation) was found to retain its full binding capacity for the enzyme and permitted elution with high yields even after multiple use, unlike the ε-amino-

caproyl fucosamine-linked Sepharose which is reported to show a low yield and reduced efficiency with repeated use [15].

The molecular nature of the retarded peak I enzyme proved to be the monomeric form as judged by gel filtration on Sephadex G-200, pH activity profile and the thermal inactivation pattern. The enzyme which was bound to the column and which could be eluted specifically by fucose (peak II) appeared to be the tetrameric fucosidase, as evidenced by its gel filtration, pH activity and thermal stability profiles. The presence of a small amount of the monomeric fucosidase in peak II enzyme as seen in the gel filtration profile could be due to the time-dependent dissociation of the tetrameric enzyme to the monomeric form as pointed out earlier [10]. This point was further substantiated by the following experimental results. The relative proportion of the tetramer and monomer was found to be in the ratio of 1 : 2 after one month of storage of the concanavalin A-Sepharose eluate at 4°C in 0.02 M phsophate buffer (pH 7.0)/0.15 M NaCl, in contrast to the initial ratio of 1 : 1. In a separate experiment it was noticed that when a mixture of tetramer and monomer (ratio 1 : 1) was stored in the presence of 2 mM α-L-fucose at pH 7.0 with 0.15 M NaCl, the tendency of the tetramer to dissociate into monomer was inhibited and the ratio of the two forms was maintained at the original value, at least upto a period of one month. Therefore it may be presumed that the tetrameric enzyme retains its molecular integrity after binding to fucose-Sepharose and that fucose elutes the enzyme in the tetrameric form. The appearance of the monomer in peak II enzyme must therefore be a post-dialysis phenomenon which removes the fucose. The percentage of contamination in peak I enzyme by the tetrameric fucosidase was relatively low. It is, however, not clear whether this minor contamination occurs because of incomplete binding of the tetrameric fucosidase to the column under the experimental conditions or due to leakage of the bound enzyme during the prolonged washing.

The appearance of a small peak (fraction 2, Fig. 1) accompanied by other proteins, consisting of 3—4% of the total fucosidase activity was repeatedly observed. The possibility of this activity being due to the overloading of the column with enzyme was ruled out by obtaining an essentially similar elution profile with four times less than the usual amount of the dialyzed concanavalin-A-Sepharose eluate applied on the same column. Due to the low activity of this minor peak, which did not show any affinity towards the fucose-Sepharose column, the data on the molecular nature of this enzyme are inconclusive.

The precise mechanism by which the fucose-Sepharose differentiates between the tetrameric and monomeric forms of the enzyme is not clearly understood. The tetrameric and monomeric species of the enzyme isolated by the affinity chromatography described when studied for their inhibition by fucose at various concentrations, showed an identical pattern of inhibition. The nature of the inhibition and the values of inhibition constant were also similar in the two cases. The differentiation between the tetrameric and the monomeric forms of the enzyme by fucose-Sepharose can be explained in two ways: (a) the differences in the three dimensional structures between the tetrameric and monomeric enzymes are responsible for a possible differential steric hindrance of the enzyme in approaching the matric-bound fucose which allows the tetrameric form to interact with the affinity ligand more freely as com-

pared to the monomer: (b) depending upon the position of the carbon atoms of fucose which are sterically available to interact with the enzyme, two active classes of the affinity ligands are generated by the present method of the preparation of fucose-Sepharose which are recognised by the tetrameric and monomeric enzymes, respectively. If the latter is true, a difference in the substrate specificities of the tetrameric and the monomeric forms of the enzyme with respect to the linkage of fucose in its natural substrates is expected. Though the substrate specificity of the multiple forms of fucosidase has not been studied extensively, certain differences observed with enzymes prepared from various sources [4,14,15,19] cannot be overlooked.

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