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PURIFICATION OF α-L-FUCOSIDASE FROM VARIOUS SOURCES BY AFFINITY CHROMATOGRAPHY

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SUMMARY

An affinity column for α-L-fucosidases was constructed by linking *p*-aminophenyl 1-thio-α-L-fucopyranoside to Sepharose 4B through linkers of succinyl 3,3'-diamino-dipropylamine. Excellent purification of α-L-fucosidase from rat epididymis, *Clostridium perfringens* and *Limulus polyphemus* (horse shoecrab) could be effected in one step with good yield. An affinity column purification step can be introduced at any point in published purification procedures. The purified enzyme is essentially free of other glycosidases and proteolytic enzymes. The column material is stable and can be reused for at least two years.

INTRODUCTION

The carbohydrate components of glycoproteins and glycolipids play an important part in the functioning of the cell and in the interaction of the cell with its environment[1]. The detailed structural and functional analyses of bound oligosaccharides have been facilitated by various glycosidases[2]. One of these, α-L-fucosidase, has been studied in mammals[3–5], abalone[6,7], *Helix pomatia*[8], Limpet[9] and in *Trichomonas foetus*[10], *Clostridium perfringens*[11], *Aspergillus niger*[12], *Rhodopseudomonas palustris*[13] and *Klebsiella aerogenes*[14]. The enzyme has been extensively purified by classical methods from rat epididymis[15], *A. niger*[12] and *C. perfringens*[11]. Generally the methods of purification are somewhat difficult and time-consuming.

Recently an α-fucosidase has been purified from human placenta[16], liver[17] and rat epididymus[18] by affinity chromatography using a column of agarose–ε-aminocaproyl fucosamine. Shah and Bahl[19] as well as Mega and Matsushima[20] have described the synthesis of a number of *p*-aminophenyl 1-thio glycosides including *p*-

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aminophenyl 1-thio-α and β-L-fucopyranosides[21]. The α form was found to compete with *p*-nitrophenyl-α-L-fucoside for the active site of clam α-L-fucosidase[21]. Although Chawla and Bahl synthesized the thio-fucoside for construction of affinity columns, they have provided no information on the exploitation of the thiofucoside derivative for enzyme purification[21]. Independently, we synthesized *p*-aminophenyl 1-thio-α-L-fucopyranoside. Despite the fact that our yield was relatively low, the properties of our products were comparable to those of Chawla and Bahl[21]. We have found that when the final product of our synthesis was linked to Sepharose 4B through succinyl 3,3'-diamino-dipropylamine, an effective, specific affinity column for α-L-fucosidase from a number of biological sources was produced.

EXPERIMENTAL

All materials were reagent grade commercial samples, unless otherwise specified. α-L(—)-fucose and the *p*-nitrophenyl glycoside, *p*-nitrophenyl α-L-fucoside, *p*-nitrophenyl α-D-galactoside, *p*-nitrophenyl α-D-mannoside, *p*-nitrophenyl N-acetyl β-D-glucosamine, and *p*-nitrophenyl α-D-fucoside used in routine assays were purchased from Sigma (St. Louis, Mo., U.S.A.). Sepharose 4B was purchased from Pharmacia (Piscataway, N.J., U.S.A.). Azocoll for proteinase assay was purchased from Calbiochem (Los Angeles, Calif., U.S.A.). 3,3'-Diamino-dipropylamine was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). Melting points were observed without corrections on compounds between glass slides with a Fisher-Johns apparatus. The rats used in these experiments were supplied by Dr. D. Kritchevsky of the Wistar Institute. A culture of *C. perfringens* was obtained from D. Aminoff and was grown according to Aminoff and Furukawa[11].

Thin-layer chromatography (TLC) was carried out using Eastman Chromatogram sheets 6060 or Baker sheet IBF (silica gel with fluorescent indicator); solvent, ethyl acetate–acetic acid–water (3:1:1) for deacetylated compounds. The spots were visualized by short wave ultraviolet light or with iodine vapor.

Synthesis of p-aminophenyl 1-thio-α-L-fucopyranoside

Of the several methods available for glycosidation[22–28], a slightly modified procedure of Levvy and McAllan[28] was used for the synthesis of the 1,2-*cis* anomer (Fig. 1) in which 1,2,3,4-tetra-O-acetyl-L-fucopyranose was condensed with *p*-nitrothiophenol in the presence of anhydrous zinc chloride (Helferich reaction). The product was purified, deacetylated and the *p*-nitro derivative was obtained; slightly yellow crystals (m.p. 218°–226° [lit.[20] 224°–226°]; $(\alpha)_D$ —343° (methanol) [lit.[20] —382°; R_F 0.85 [lit.[20] R_F 0.88]). Reduction of the deacetylated product with hydrogen gas and platinum oxide yielded a material which, upon TLC yielded one spot with an R_F of 0.65. The *p*-aminophenyl 1-thio α-fucopyranoside synthesized by Chawla and Bahl[21]

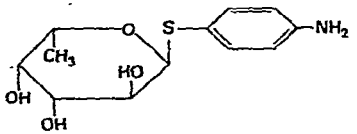

Fig. 1. Structure of *p*-aminophenyl 1-thio α-L-fucopyranoside.

had an R_F of 0.66. Since our synthetic method was unsatisfactory with regard to yield, it has not been reported in detail.

Preparation of affinity columns

Sepharose 4B was activated with cyanogen bromide and coupled with 3,3'-diamino-dipropylamine according to the method of Cuatrecasas[29]. Succinylation of the 3,3'-diamino-dipropylamine Sepharose 4B was also done by the method of Cuatrecasas. To couple *p*-aminophenyl 1-thio α-L-fucopyranoside to the succinyl derivate, 0.068 g of the fucoside was dissolved in 1 ml of dimethylformamide and added to 10 ml of the Sepharose derivative suspended in water. The pH was adjusted to 4.7 with 0.5 g of 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide in 1 ml of water added dropwise. The pH was maintained at 4.7 for 1 h. The suspension was stirred at room temperature overnight and was then washed with 4 l of 0.1 *N* NaCl solution in a büchner funnel (coarse disc) without using suction. Columns were stored in 0.1 *N* NaCl solution containing 0.1% sodium azide at 4°.

Preparation of enzymes

Glycosidases from rat epididymis were partially purified according to Carlsen and Pierce[15] until the 35–50% ammonium sulfate precipitation step, and stored at −20° until purified further by affinity chromatography.

C. perfringens was cultured and the enzymes in the medium were prepared by the method of Aminoff and Furukawa[11]. Preliminary purification consisted of two ammonium sulfate precipitations and chromatography on a large column of Sephadex G-150[11]. The starting material was stored at −20° until used. Serum obtained from *Limulus polyphemus* was allowed to clot. Clots were removed by filtration and the serum was stored at −20°.

Columns (8.5 × 1.2 cm) were run at room temperature since the α-L-fucosidases being studied were reasonably stable at that temperature. The column was equilibrated and developed with 0.2 *M* sodium citrate buffer (pH 6.0) containing 0.02% NaN_3. The flow-rate was 25 ml/h, and 1.4-ml fractions were collected. Aliquots of 0.05 ml were routinely assayed for α-L-fucosidase, α-D-mannosidase, β-D-galactosidase and N-acetyl β-D-glucosaminidase with the appropriate *p*-nitrophenyl glycoside as substrate. Usually 5.0 ml of enzyme solution, prepared in 0.2 *M* sodium citrate (pH 6) containing approximately 12 mg of protein, were placed on the column. After washing the column with the same buffer until no enzyme activities were detectable and no protein was present as measured by light absorption at 280 nm, 10 ml of α-L-fucose solution (40 mg/ml) in the developing buffer were added to the column.

Glycosidases were assayed essentially by the method described by Bosmann[30]. A typical incubation mixture contained 0.3 ml of 0.05 *M* sodium citrate (pH 4.5), 0.3 μmoles in 0.3 ml water of *p*-nitrophenylglycoside substrate and 0.05 ml solution of enzyme. Where α-mannoside was assayed, 1.2 μmole of the *p*-nitrophenyl α-mannoside substrate was used. After 20 min of incubation at 37° the vessels were chilled in ice and 0.6 ml of 0.2 *M* potassium borate buffer (pH 9.8) was added and the light absorbance was determined at 400 nm. A unit of enzyme can cleave 1 μmole of substrate in 1 min under the conditions of the assay. Specific activities are expressed as units per mg of protein.

To assay α-L-fucosidase from *C. perfringens* which does not cleave *p*-nitrophenyl

α-L-fucoside, 0.05 ml of enzyme solution was incubated with 2 mg hog gastric mucin and 0.05 ml of 1 *M* sodium acetate buffer (pH 6) in a final volume of 0.3 ml for 25 min at 37°. Free L-fucose was assayed by an enzymatic method[31].

Purified α-L-fucosidase was electrophoresed in 5.6% polyacrylamide gels at 10° by the method of Davis[32]. The gels were run at 2.5 mA per tube (80 V) for 2 h with bromophenol blue as tracer dye. In order to locate the enzyme activity, gels were sliced into 1-mm pieces; each slice was placed in a tube containing 0.3 ml of sodium citrate buffer (pH 6) and kept overnight at 4°. Aliquots were assayed for enzyme activities as described previously. Before cutting, gels were scanned at 280 nm in a Gilford spectrophotometer.

Slab gels were also run in the presence of 0.1% sodium dodecyl sulfate (SDS) using the discontinuous system of Laemmli[33]. The separating gel was 10% acrylamide and the stacking gel 5%. Electrophoresis was conducted at a current of 20 mA for 4 h. Pyronine Y was used as a marker. Gels with and without SDS were stained according to Fairbanks *et al.*[34].

RESULTS

α-Fucosidase from rat epididymis

As seen from Fig. 2, α-L-fucosidase of rat epididymis is weakly but competitively inhibited by *p*-aminophenyl 1-thio α-L-fucoside (K_I 12.5 × 10^{-4} *M*); L-fucose itself is more inhibitory (K_I = 2.85 × 10^{-4} *M*). The K_m of *p*-nitrophenyl α-L-fucoside is 2.77 × 10^{-4} *M*[37]. Chawla and Bahl[21] found a K_I of 7.1 × 10^{-4} *M* for *p*-aminophenyl 1-thio α-L-fucoside and a K_m of 0.85 × 10^{-4} *M* for *p*-nitrophenyl α-L-fucoside for the α-L-fucosidase of clam under somewhat different assay conditions. D-fucose, D-galactose, L-arabinose and D-arabinose at 1-m*M* concentration did not inhibit α-L-fucosidase.

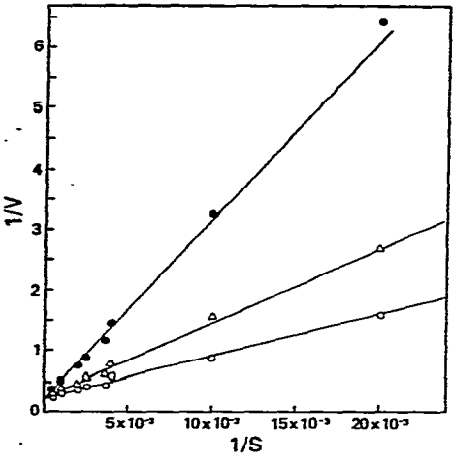

Fig. 2. Lineweaver and Burk[37] plots of the reaction catalyzed by α-fucosidase of rat epididymis with and without inhibitors. Various amounts of substrate (0.05 × 10^{-3} *M* to 0.5 × 10^{-3} *M*) were incubated with the enzyme purified on the affinity column under conditions explained in Experimental. ●, α-Fucosidase; △, α-fucosidase + 1 m*M* *p*-aminophenyl 1-thio α-L-fucopyranoside; ○, α-fucosidase + 1 m*M* α-L(−)-fucopyranose.

In Fig. 3 is seen the results of a typical run of the affinity column using enzyme from rat epididymis. Virtually all of the α-L-fucosidase is retained and essentially all of the activity is recovered (Table I) with a 59-fold purification in the one step and a final purification of over 400-fold. The specific activity is 15.6 which compared favorably with the value of 12.8 obtained by Carlsen and Pierce[15] after chromatography on columns of CM-cellulose and DEAE-cellulose and entailing considerable loss of activity.

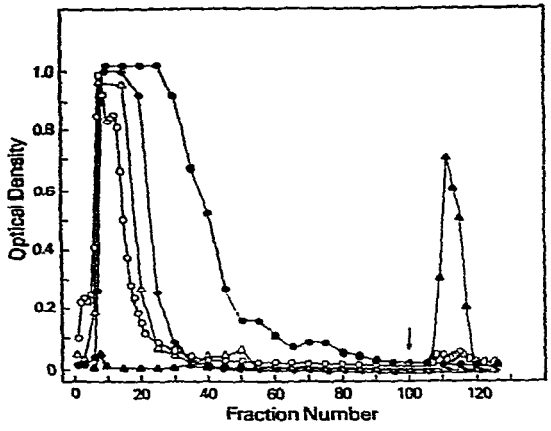

Fig. 3. Affinity chromatography of the 35–50% ammonium sulfate fraction of rat epididymis extract. The column (1.2 × 7.5 cm) was equilibrated and developed with 0.2 *M* sodium citrate buffer (pH 6) containing 0.02% sodium azide, and run at room temperature at a flow-rate of 33 ml/h. Samples 105–120 were dialyzed to remove the sugar before being assayed for enzyme activities. At fraction 101 (arrow), 10 ml of 40 mg/ml L-fucose were added to the column. Fractions (2 ml) were collected and aliquots were assayed for absorbance at 280 nm (●) and for α-L-fucosidase (▲), α-D-mannosidase (△), β-D-galactosidase (○), and β-N-acetyl-D-glucosamine (◆).

When the dialyzed, concentrated eluate containing α-L-fucosidase was electrophoresed on polyacrylamide gel[32] only one band could be detected by absorption at 280 nm and by staining with Coomassie blue. This band contained the α-L-fucosidase activity. Upon electrophoresis in an SDS gel system[33] two bands were seen which when compared to standards had molecular weights of 50,000 and 57,000. Carlsen and Pierce[15] have reported the presence of two subunits of molecular weights 47,000 and 60,000 for α-L-fucosidase of rat epididymis.

The effectiveness of the column depends on the presence of the *p*-amino 1-thio-α-L-fucopyranoside residues. The enzyme is not retained on columns of Sepharose 4B to which are attached succinyl 3,3'-diamino-dipropylamine groups alone nor is it taken up by the column if *p*-aminophenyl 1-thio α-L-fucopyranoside residues are substituted for by the anomeric β compound. Details of the synthesis of *p*-aminophenyl 1-thio β-L-fucopyranoside and the construction of affinity columns with it are described elsewhere[35].

Once the enzyme is retained by the column it cannot be removed by shifting the pH values between 6 and 4, nor by an NaCl gradient up to 2 *M* nor by passing a solution of D-fucose or D-galactose (40 mg/ml) through the column.

While the original eluate contained more than 90% of the α-mannosidase,

TABLE I

PURIFICATION OF α-L-FUCOSIDASE FROM RAT EPIDIDYMIS

Fraction	*Volume (ml)*	*Concentration (mg/ml)*	*Total (mg)*	*Sp. activity μmole/min per mg protein)*	*Total activity*	*Purification of step*	*Total purification*	*Yield (%)*
Homogenate after heating at 37°	545	3.86	2104.0	0.037	77.9	1		100
Homogenate after heating at 60°	540	3.16	1708.0	0.046	78.6	1.2	1.2	100
$(NH_4)_2SO_4$ ppt. (35–50%)	7	20.3	142.0	0.264	37.5	5.7	6.8	48.1
After elution with L-fucose and dialysis	172	0.014	2.4	15.57	37.4	59.0	420.8	48.0

β-galactosidase and N-acetyl β-D-glucosaminidase activities applied, these could not be detected in the pooled fractions eluted by L-fucose. Further, this fraction contained no detectable protease (azocoll assay), β-L-fucosidase or α-D-fucosidase activities. The recovery of protein as assayed by the method of Lowry *et al.*[36] was quantitative (102%).

As much as 1.03 units of α-fucosidase of rat epididymus in 2.11 mg of protein in 2.5 ml of buffer of the 35–50% ammonium sulfate fraction[15] could be applied to 1 ml of column material without overloading it. This is about 2.5 times more than was usually used in routine runs. The purified material off the column retained full activity for 2 months when stored at 4° while material frozen at −20° retained only half of its activity after 6 months. The enzyme could be concentrated without appreciable loss of activity after 6 months. The enzyme was concentrated by dialyzing against a solution of 20% polyethylene glycol in 0.2 *M* sodium citrate solution (pH 6).

Excellent purification of α-L-fucosidase from *C. perfringens* and *Limulus polyphemus* were effected on the same affinity column by the same procedure used for the enzyme from rat epididymis (Table II). For unknown reasons the yield of enzyme of *Limulus* was variable although purification was always good. The enzyme from *C. perfringens* clearly differs from the others in that it does not cleave *p*-nitrophenyl α-L-fucoside and yet behaves similarly on the affinity column. Assay of this enzyme was accomplished by measuring the L-fucose released from hog gastric mucin[31].

TABLE II

PURIFICATION OF α-L-FUCOSIDASE FROM VARIOUS SOURCES

	Rat epididymis	*C. perfringens*	*Limulus polyphenus**
Preparation for column	30–50% $(NH_4)_2SO_4$ fraction	Eluate from Sephadex G-50 column	Serum
Specific activity (μmole/min per mg protein)			
before column	0.26	0.014	0.001
after column	15.57	2.61	0.394
Purification	59	186	394
Yield on step (%)	100	100	24
Ref. for preliminary preparation	15	11	

* The serum was obtained at the Marine Biology Laboratory (Woods Hole, Mass., U.S.A.). Some of the work using serum of *Limulus* was carried out at the M.B.L.

DISCUSSION

It is apparent that α-L-fucosidases from a variety of sources (bacteria, invertebrate, mammal) can be specifically bound to an affinity column and extensively purified and freed of other glycosidases and proteases in one step. The column binds α-fucosidases of different specificities; that from *C. perfringens* which does not cleave *p*-nitrophenyl α-L-fucopyranoside and the enzyme from other sources which does split this substrate.

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