Antibody-affinity purification of novel α -L-fucosidase from mouse liver

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Previous studies have documented the presence of a novel α -L-fucosidase in mouse liver that contains unique basic isoelectric forms and that is antigenically similar to, but not identical with, human liver α -L-fucosidase [Laury-Kleintop, Damjanov & Alhadeff (1985) *Biochem. J.* **230**, 75–82]. In the present investigation, mouse liver α -L-fucosidase was purified approx. 26 500-fold in 10% overall yield by antibody-affinity chromatography with the IgG fraction of goat anti-(human α -L-fucosidase) antibody coupled to Sepharose 4B. Native polyacrylamide-gel electrophoresis and SDS/polyacrylamide-gel electrophoresis indicated that the mouse fucosidase is highly purified if not homogeneous. Isoelectric focusing demonstrated that all enzymic forms found in crude mouse liver supernatant fluids were purified by the antibody-affinity procedure.

INTRODUCTION

α -L-Fucosidase (α -L-fucoside fucohydrolase, EC 3.2.1.51) is a lysosomal hydrolase that has been purified and characterized from many mammalian tissues (Alhadeff & O'Brien, 1977). This enzyme is essential for the degradation of fucoglyco-conjugates, and deficiency of its activity results in the rare and fatal neurovisceral storage disease fucosidosis (Van Hoof, 1973). Natural substrates of fucosidase are diverse and include hydrophilic (e.g. oligosaccharides, glycopeptides) and hydrophobic (glycolipids) compounds with fucose in various α -glycosidic linkages to galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine (Flowers, 1981). The existence of such diverse substrates suggests that more than one mammalian α -L-fucosidase exists.

Mouse liver α -L-fucosidase is different from other mammalian fucosidases studied to date because of the unique presence of basic isoelectric forms (with approximate pI values of 8.3 and 9.0) (Laury-Kleintop *et al.*, 1985a,b) in addition to the usual acidic and neutral forms previously described in tissues of other species. These unusual basic forms appear to be developmentally regulated and related to the more neutral and acidic forms by sialic acid residues (Laury-Kleintop *et al.*, 1985a,b). Immunochemical studies with antibody prepared in goat against human liver α -L-fucosidase indicated that mouse and human α -L-fucosidases were not identical antigenically although they exhibited significant cross-reactivity (Laury-Kleintop *et al.*, 1985b). This cross-reactivity has enabled the present investigation, in which mouse liver α -L-fucosidase has been purified approx. 26 500-fold in 10% overall yield by antibody-affinity chromatography with the IgG fraction of goat anti-(human α -L-fucosidase) antibody coupled to Sepharose 4B.

MATERIALS AND METHODS

General

All procedures were carried out at 0–4 °C unless otherwise stated. The method of Lowry *et al.* (1951) was used for protein determinations, with human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard. Livers were obtained from mice (Swiss Webster and BALB/c) and were stored at –20 °C until utilized.

Preparation of tissues

Tissues were homogenized 1:5 (w/v) in 10 mM- NaH_2PO_4 buffer, pH 5.5, containing 0.02% NaN_3 in a Potter–Elvehjem tissue grinder, and the homogenates were centrifuged at 27 500 *g* for 40 min. The resulting supernatant fluids were filtered through glass-wool and dialysed against 300 vol. of the 10 mM- NaH_2PO_4 buffer, pH 5.5, over a period of 5–6 days. The dialysed material was centrifuged at 27 500 *g* and the resulting supernatant fluid was filtered through glass-wool. The supernatant fluids and other enzyme preparations were assayed for enzyme activity under conditions of linearity essentially as previously described (Laury-Kleintop *et al.*, 1985b). A unit of fucosidase activity is the amount of enzyme required to hydrolyse 1 nmol of substrate/min at 37 °C.

Substrate-analogue (fucosamine) affinity chromatography

Mouse liver supernatant fluid was applied at room temperature (19–20 °C) to a 5 ml affinity column of agarose–6-aminohexanoyl-fucosamine resin (Miles Scientific, Naperville, IL, U.S.A.) (Alhadeff *et al.*, 1975) at a flow rate of approx. 15 ml/h. The eluted supernatant fluid was assayed for fucosidase activity, and the column

Abbreviations used: PBS, phosphate-buffered saline (0.15 M- NaCl /10 mM- NaH_2PO_4 , pH 5.5); PAGE, polyacrylamide-gel electrophoresis.

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was washed with 10 mM- NaH_2PO_4 buffer, pH 5.5, until the absorbance readings at 280 nm were not more than 0.002. The α -L-fucosidase activity was eluted from the column with 30 ml of 25 mM-fucose in 10 mM- NaH_2PO_4 buffer, pH 5.5. The fractions that contained fucosidase activity were pooled, concentrated in a collodion-bag apparatus and assayed for enzymic activity.

Binding of mouse liver α -L-fucosidase activity to the fucosamine affinity resin

Mouse liver supernatant fluids were prepared as described above by homogenizing liver in a 1:2.5 (w/v) ratio with deionized distilled water. A 500 μl portion of liver supernatant fluid was combined with 500 μl of the appropriate buffer (see below), and 50 μl of this mixture was removed for assaying. To the supernatant fluid and buffer mixture, 300 μl of agarose-6-aminohexanoyl-fucosamine suspension was added and mixed for 30 min. The resin was allowed to settle, the supernatant fluid was removed from the resin and a sample was assayed for enzymic activity. The resin was washed with the appropriate buffer, and a sample of the wash buffer was assayed for enzyme activity. The fucosidase was eluted from the resin by mixing with 500 μl of 5 mM-fucose in deionized distilled water and 500 μl of the appropriate buffer for 15 min. The fucose solution was removed from the resin, and a sample was assayed for fucosidase activity. Binding determinations were carried out with three concentrations (10, 50 and 100 mM) of four different buffers (citric acid/sodium citrate, pH 5.0, NaH_2PO_4 , pH 5.5, $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.0, and Na_2HPO_4 , pH 6.8), all containing 0.02% NaN_3 .

Preparation of goat anti-(human α -L-fucosidase) IgG-Sephacrose 4B resin (antibody-affinity resin)

The antibody-affinity resin was prepared as described in Wilchek *et al.* (1984). Goat anti-(human α -L-fucosidase) IgG (Andrews-Smith & Alhadeff, 1982) was diluted 1:30 with 0.1 M- NaHCO_3 , pH 8.0, 4.0 g of CNBr-activated Sepharose 4B (Sigma Chemical Co.) was added, and the slurry was stirred at 2–4 °C for 72 h. Then 50 ml of 1.0 M-ethanolamine, pH 8.0, was added to the slurry (in order to block any residual active groups on the Sepharose 4B), which was stirred at 2–4 °C for 24 h. The resin was then washed alternately with 0.5 M- $\text{NaCl}/0.1$ M- NaHCO_3 , pH 8.0, and 0.5 M- $\text{NaCl}/0.1$ M-sodium acetate, pH 4.0, with 10 vol. of buffer/vol. of resin, for a total of five washes in a Buchner funnel. The resin was suspended in PBS containing 0.02% NaN_3 and stored at 2–4 °C. This stored resin is stable for at least 10 months.

Stability of mouse liver α -L-fucosidase activity in possible elution buffers

Mouse liver supernatant fluids were prepared as described above by homogenizing liver 1:2.5 (w/v) with deionized distilled water. A 25 μl portion of liver supernatant fluid was added to and mixed with 25 μl of each of the following possible elution buffers and solutions: 0.30 M- NaCl with HCl, pH 2.0; 0.30 M- NaCl with NaOH, pH 11.0; 50% (v/v) ethylene glycol with NaOH, pH 11.5; 1% (w/v) Triton X-100 in 0.1 M- $\text{Na}_2\text{HPO}_4/\text{citric acid}$, pH 7.0, containing 0.02% NaN_3 (phosphate/citrate buffer); 10 M-urea in phosphate/citrate buffer; 6 M- NaSCN in phosphate/citrate buffer; 8.6 M-guanidine/HCl, pH 3.1; 8 M- MgCl_2 in phosphate/citrate buffer; 5 M-trichloroacetic acid in phosphate/

citrate buffer. After preincubation at 19–20 °C (for 5, 15, 30, 60 and 120 min), 20 μl of each solution was assayed for 20 min at 37 °C for α -L-fucosidase activity.

Antibody-affinity chromatography

A 1600 ml portion of supernatant fluid (derived from 260 g of mouse liver) was run through a 4.0 ml column of antibody-affinity resin at room temperature or 2–4 °C. [This amount of supernatant fluid was determined to be saturating by running supernatant fluid through the column until the amount of α -L-fucosidase activity eluted from the column was approximately equal to the amount of activity being applied to the column (results not shown).] The column was washed with PBS containing 0.02% NaN_3 until the absorbance readings at 280 nm were not more than 0.005. The fucosidase was eluted from the column with 50% (v/v) ethylene glycol in PBS, adjusted to pH 8.0, containing 25 mM-fucose, and 0.75 ml fractions were collected in test tubes, on ice, that already contained 0.25 ml of 0.1 M- $\text{Na}_2\text{HPO}_4/\text{citric acid}$, pH 5.5, containing 0.2% NaN_3 . The fractions with fucosidase activity were pooled and concentrated in an Amicon ultrafiltration apparatus (with a YM10 membrane) and Centricon 10 tubes (Amicon Corp., Lexington, MA, U.S.A.). Fucosidase activity and protein concentration were determined on the concentrated enzyme solution. The antibody-affinity resin was regenerated by washing successively with 50% (v/v) ethylene glycol in PBS, adjusted to pH 11.5, containing 0.02% NaN_3 , then 7 M-urea in PBS, then PBS containing NaCl at 0.25 M, and finally PBS, with 2 parts of buffer solution to 1 part of resin or until the absorbance readings at 280 nm were below 0.01. The regenerated antibody-affinity resin was found to bind the same amount of fucosidase activity as the original resin (results not shown).

Double immunodiffusion

Double-immunodiffusion experiments were carried out with 1% (w/v) agarose plates made with PBS containing 0.02% NaN_3 . The plates were spotted with 10 μl of goat anti-(human α -L-fucosidase) IgG and 20–30 μl of concentrated column eluates, placed at 2–4 °C and read after 24 h.

Polyacrylamide gels

Before SDS/PAGE and PAGE, the purified mouse liver fucosidase (0.35 ml) was dialysed against 2 litres of 10 mM- NaH_2PO_4 , pH 5.5 (containing 0.02% NaN_3 and 25 mM-fucose), in collodion bags at 0–4 °C for 3 h to remove the ethylene glycol and to concentrate it further to approx. 50 μl . Native PAGE in 5% gels was performed as reported by Davis (1964) except that a slab-gel apparatus was used. A 6–27 μg portion of α -L-fucosidase protein was run per lane on one half of the gel and 23–33 units of fucosidase activity were electrophoresed on the other half of the gel. After electrophoresis for 3–4 h at 1.8–1.5 mA per lane at 2–4 °C, the gel was sliced and one half stained for protein with 0.25% Coomassie Blue R-250 in 9.2% (v/v) acetic acid/45.5% (v/v) methanol. The other half of the gel was stained for fucosidase activity by incubating the gel overlaid with filter paper saturated with 1 mM-4-methylumbelliferyl α -L-fucopyranoside for 60 min at 37 °C.

Purity was assessed by electrophoresis in 10% polyacrylamide slab gels containing 0.1% SDS according to the method of Laemmli (1970). A 6–12 μ g portion each of bovine serum albumin (M_r 67500), ovalbumin (M_r 45500), chymotrypsinogen A (M_r 24000), cytochrome *c* (M_r 13000) (all from Schwartz/Mann, Cambridge, MA, U.S.A.) and human liver α -L-fucosidase (M_r 51000 and 56000) purified as previously described (Alhadeff *et al.*, 1975) were used as M_r standards. Before electrophoresis the protein standards and the fucosidase were treated with 1% SDS/5% 2-mercaptoethanol in 10 mM-Tris/HCl buffer, pH 8.8, at 100 °C for 10 min. Six or seven grains of sucrose and 5 μ l of 0.05% Bromophenol Blue were added after the treated samples had cooled to room temperature. Electrophoresis was conducted at 1.5–1.8 mA per lane for 3–4 h at 2–4 °C. The gels were stained with Coomassie Blue as described above for the native polyacrylamide gels. Native polyacrylamide gels and SDS/polyacrylamide gels were destained overnight in a methanol/acetic acid/water (2:3:35, by vol.) solution at 37 °C.

Isoelectric focusing

Isoelectric focusing was performed as described previously (Laury-Kleintop *et al.*, 1985a,b) with approx. 10 units of fucosidase activity, a 0–67% (w/v) sucrose gradient and 2% (v/v) pH 5–8 ampholytes (LKB Produkter, Stockholm, Sweden) at 600 V (1.5–0.5 mA) for 17 h. The relative amounts of α -L-fucosidase activity associated with the basic and more-acidic isoelectric forms (above and below an approximate pI value of 7 respectively) were determined by cutting out the respective portions of the profiles and weighing them on a Mettler analytical balance.

RESULTS

Attempts to purify mouse liver fucosidase by affinity chromatography on the fucosamine resin and the conditions used for purifying human fucosidase (Alhadeff *et al.*, 1975) were unsuccessful owing to insufficient binding of mouse enzyme to the resin. Varying the buffers, their pH and molarity (see the Materials and methods section) did not significantly enhance binding of enzyme to the fucosamine resin.

Mouse liver supernatant fluid was applied to columns

(0.5 ml) of antibody-affinity resin at 21 °C until saturated with fucosidase activity. The columns were washed with PBS containing 0.2% NaN_3 , and each column was eluted with one of the solutions or buffers (see the Materials and methods section) in which fucosidase activity was stable (retaining at least 70% of initial activity) for at least 2 h. After concentration of the fractions that contained fucosidase activity, the concentrates were run on double-immunodiffusion plates to determine if any antigenic material was eluted from the columns. Precipitin lines were observed for the concentrates eluted with 0.15 M-NaCl/NaOH, pH 11.0, and 50% (v/v) ethylene glycol adjusted to pH 11.5 with NaOH. Since the concentrate containing ethylene glycol gave a more-dense precipitin line, small columns (0.25 ml) of antibody resin saturated with mouse fucosidase activity at 2–4 °C were eluted with ethylene glycol and fucose in PBS at lower pH values to optimize recovery of fucosidase activity. The results of these preliminary experiments indicated that the greatest amount of fucosidase activity was eluted at pH 8.0 (results not shown).

After a potential purification scheme was worked out for the mouse enzyme, approx. 4800 units of α -L-fucosidase were applied to a 4 ml antibody-affinity column, and approx. 91% of the activity bound to the resin. The column was washed with PBS containing 0.02% NaN_3 until no detectable protein was coming off the column. The enzyme was eluted with 50% ethylene glycol in PBS, adjusted to pH 8.0, containing 25 mM-fucose to stabilize the fucosidase activity. α -L-Fucosidase activity was found in fractions 5–40 (36 ml), which were combined, concentrated and assayed for fucosidase activity and protein content. The procedure resulted in a 26500-fold purification with a 10% overall yield of fucosidase of high specific activity (5840 units/mg of protein) (Table 1).

Purity of the mouse liver α -L-fucosidase was assessed by SDS/PAGE and native PAGE. When 30 μ g of mouse fucosidase protein was run on SDS/PAGE, two major protein bands (lane 1) (with approximate M_r values 57000 and 63000) and four very faint bands (with approximate M_r values 12000, 25000, 71000 and 76000) were observed (Fig. 1). Although it is difficult to see resolution of the two major protein bands after being

Table 1. Purification of mouse liver α -L-fucosidase by antibody-affinity chromatography

For experimental details see the Materials and methods section.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor	Overall yield of activity (%)
Mouse liver supernatant fluid	6600	30000	0.22	—	—
Dialysed and centrifuged mouse liver supernatant fluid	4800	14000	0.34	1.6	73
Pooled and concentrated fractions 5–40 from antibody affinity column	666	0.114	5840	276500	10 (15*)

* Yield relative to amount of fucosidase activity that bound to antibody-affinity column.

Fig. 1. SDS/PAGE of purified mouse liver and human liver α -L-fucosidases and protein standards

Lane 1, mouse liver α -L-fucosidase; lane 2, human liver α -L-fucosidase. Proteins were stained with Coomassie Blue. The positions of the fucosidase protein bands and the M_r standards are indicated by arrows. See the Materials and methods section for details.

photographed, two closely spaced but distinct bands (designated by arrows) were observed on the gel (Fig. 1). SDS/PAGE of 12 μ g of mouse liver fucosidase revealed the presence of only the two major protein bands (results not shown). Native PAGE indicated that mouse liver fucosidase migrates as a broad band of protein-positive material that corresponds to the region of enzymic activity. Isoelectric focusing demonstrated that all isoelectric forms of mouse fucosidase were purified by the antibody-affinity procedure. Approx. 23% of the recovered activity was associated with forms with pI values above 7, very similar to results for crude adult mouse liver fucosidase (Laury-Kleintop *et al.*, 1985a,b).

DISCUSSION

A number of α -L-fucosidases have been purified and characterized from various mammalian tissues, including human liver (Robinson & Thorpe, 1974; Alhadeff *et al.*, 1975; Kress *et al.*, 1980), placenta (Alhadeff *et al.*, 1974; DiMatteo *et al.*, 1976; Turner, 1979), spleen (Chien & Dawson, 1980), brain (Alhadeff & Janowsky, 1977) and serum (Alhadeff & Janowsky, 1978; DiCioccio *et al.*,

1982), rat liver (Opheim & Touster, 1977), epididymis (Wright *et al.*, 1976; Jain *et al.*, 1977) and cerebral cortex (Bosmann & Hemsworth, 1971), monkey brain (Alam & Balasubramanian, 1979) and pig thyroid (Grove & Serif, 1981). With the exception of the early study on rat cerebral cortex, all of the above purifications utilized affinity chromatography with substrate analogues of fucose (primarily fucosamine) for the ligand. Attempts to purify mouse α -L-fucosidase with the fucosamine resin were unsuccessful even after varying the buffer type, pH and ionic strength. These results give further evidence that mouse α -L-fucosidase is different from other mammalian fucosidases.

Since mouse liver α -L-fucosidase is cross-reactive with the antibody prepared against human liver fucosidase (Laury-Kleintop *et al.*, 1985b), an antibody-affinity resin was prepared that successfully bound mouse fucosidase from crude supernatant fluids. After extensive washing, the bound fucosidase was eluted from the column by using ethylene glycol in PBS, adjusted to pH 8.0, containing fucose. This procedure resulted in a 26500-fold purification with a 10% yield. The fold-purification results of the antibody-affinity resin are comparable with or better than those of the fucosamine resin in purifying several mammalian α -L-fucosidases. The purification yield was, in general, not as good as for the fucosamine resin, but this is probably due to the small amount of fucosidase purified, whose activity is unstable at low protein concentrations. It is hoped that scaling up the procedure will lead to higher final fucosidase protein concentrations, greater stability of enzymic activity and, consequently, to significantly improved yields. Isoelectric focusing demonstrated that the antibody-affinity procedure purifies all fucosidase forms found in crude liver supernatant fluid.

The purity of the mouse fucosidase was assessed by SDS/PAGE and native PAGE. SDS/PAGE revealed the presence of two major, closely spaced, protein bands, which appear to be the subunits of mouse liver α -L-fucosidase. Other very faint protein bands were observed, which could represent very minor amounts of contaminating proteins and/or fucosidase at various stages of post-translational processing or degradation. The SDS/PAGE results indicate that mouse liver fucosidase is highly purified, if not homogeneous. Native PAGE of mouse α -L-fucosidase also revealed a highly purified enzyme in which the protein-staining and activity-staining material corresponded. The broadness of the fucosidase bands after PAGE is due to the mouse enzyme being comprised of seven or eight differentially migrating isoelectric forms (Laury-Kleintop *et al.*, 1985b).

A new antibody-affinity procedure for purifying mouse α -L-fucosidase has been described in this study that is based on antigenicity rather than catalytic-activity properties. This procedure should be useful for purifying abnormal fucosidases (e.g. derived from tissues of patients with fucosidosis), which might not bind to the fucosamine resin as a consequence of altered active sites.

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