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Analysis of purified human liver α-L-fucosidase by Western-blotting with lectins and polyclonal and monoclonal antibodies

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Western-blot analysis [with lectins, polyclonal antibodies (pAbs) and four monoclonal antibodies (mAbs)] was employed to investigate the structural relationship between the separated isoforms and subunits of purified human liver α-L-fucosidase. SDS/PAGE and Western-blot analysis indicated the presence of two protein bands of 51 kDa and 56 kDa that were recognized by the pAbs. Polyacrylamide-gel isoelectric focusing (PAG-IEF) followed by blotting indicated that the pAbs and mAbs recognized at least five fucosidase isoforms (pI values 3.6–6.0). Lectin blotting indicated an enrichment of sialic acid residues in the more acidic isoforms. Western-blot analysis indicated that four mAbs recognized the 51 kDa subunit and at least two mAbs recognized the 56 kDa subunit. The subunit composition of the isoforms (separated by PAG-IEF) of human liver α-L-fucosidase was investigated by SDS/PAGE. One or two closely spaced bands were found for each isoform with a trend of increasing relative amounts of the high-molecular-mass band in the more acidic isoforms relative to the more neutral isoforms. Neuraminidase treatment of α-L-fucosidase resulted in a decrease in the amount of the high-molecular-mass subunit and an increase in the amount of the low-molecular-mass subunit, suggesting that these subunits are related at least in part by sialic acid residues. In addition, blotting with lectins indicated the presence of sialic acid residues only in the high-molecular-mass subunit. *N*-Glycanase treatment led to the disappearance of the glycosylated 56 kDa and 51 kDa protein bands and the appearance of non-glycosylated protein bands at 48 kDa and 45 kDa. The overall results indicate that (1) *N*-glycosylation contributes to, but does not account completely for, structural differences in the fucosidase subunits and (2) the more acidic isoforms of fucosidase contain enriched relative amounts of the sialylated high-molecular-mass subunit.

INTRODUCTION

α-L-fucosidase (α-L-fucoside fucohydrolase, EC 3.2.1.51) is a ubiquitous lysosomal hydrolase involved in the degradation of diverse types of fucoglycoconjugates [1]. This enzyme's importance in mammalian biochemistry is demonstrated by deficiency of its activity leading to the neurovisceral storage disorder fucosidosis in humans [2] and English springer spaniels [3].

In the present investigation, Western-blot analysis was performed on exoglycosidase- and endoglycosidase-treated human liver fucosidase, as well as on its isoforms and subunits, with lectins, polyclonal antibodies (pAbs) [4] and monoclonal antibodies (mAbs) prepared in this study against human liver α-L-fucosidase. The overall results of our study indicate that (1) variable *N*-glycosylation contributes to, but does not account completely for, structural differences between the fucosidase subunits and (2) the more acidic isoforms of fucosidase contain enriched relative amounts of the sialylated high-molecular-mass subunit.

MATERIALS AND METHODS

Purification of human liver α-L-fucosidase

α-L-Fucosidase was purified from human liver to apparent homogeneity by affinity chromatography using agarose–6-aminohexanoylfucosamine resin (Miles Scientific, Naperville, IL, U.S.A.) as described [5].

Preparation and characterization of mAbs to human liver α-L-fucosidase

mAbs were prepared by the standard hybridoma technique with the use of spleen cells of immunized rats and the Sp2/0 mouse myeloma cells for fusion according to the protocol of Fazekas de St. Groth & Scheidegger [6], slightly modified for our laboratory purposes [7]. Three rats were immunized four times at weekly intervals with 10–20 μg of purified human liver α-L-fucosidase. The rat that showed strongest immunoreactivity with α-L-fucosidase dot-blotted to nitrocellulose sheets was used as donor of spleen cells. After the fusion, antibody-secreting hybridomas were selected and cloned and four hybridomas were expanded for future work. Antibodies were collected as supernatant spent medium of cells grown *in vitro* or as the ascites fluid obtained in nude mice injected with 5×10^6 hybridoma cells. The mAbs were isotyped with the use of a Zymed MonoAb-ID EIA kit for rat monoclonals (Zymed, San Francisco, CA, U.S.A.).

SDS/PAGE

Slab SDS/PAGE was used, according to the method of Laemmli [8], to assess purity and characterize the subunits of purified human liver α-L-fucosidase. Enzyme samples (2–10 μg) were incubated for 8 min at 100 °C in a sample buffer containing 2.0% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 62.5 mM-Tris/HCl buffer, pH 6.8, 10% (v/v) glycerol and 0.0012% Bromophenol Blue. Samples were electrophoresed on a 0.1% SDS/polyacrylamide gel (composed of a 4% stacking gel and a

Abbreviations used: SNA, *Sambucus nigra* agglutinin; GNA, *Galanthus nivalis* agglutinin; pAb, polyclonal antibody; mAb, monoclonal antibody; PAG-IEF, polyacrylamide-gel isoelectric focusing; Dig-hydrazide, digoxigenin–succinyl-6-amidohexanoic acid hydrazide.

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12% separating gel) at 100 V for 5–6 h at 4 °C in a pH 8.6 running buffer containing 25 mM-Tris, 0.2 M-glycine and 0.1% SDS. Gels were stained with 0.1% Coomassie Blue R-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) prepared in methanol/acetic acid/water (2:3:35, by vol.), and gels were destained with methanol/acetic acid/water (2:3:35, by vol.).

Polyacrylamide-gel isoelectric focusing (PAG-IEF) of α-L-fucosidase

The isoforms of purified human liver α-L-fucosidase (50 μg) were separated in 6% polyacrylamide gels containing 6% (v/v) ampholytes (Serva, pH 5–8) and 10% (w/v) sucrose in a Bio-Rad Mini-Protean II electrophoresis unit. Isoelectric focusing was performed at 150 V for 12 h at 4 °C with 0.1 M-HCl as the anode solution and 0.15 M-ethanolamine as the cathode solution. After focusing, the gels were overlaid with a strip of filter paper saturated with 1 mM-4-methylumbelliferyl α-L-fucopyranoside in 0.1 M-sodium citrate/citric acid buffer, pH 5.0, and incubated at 37 °C for 5–10 min. The individual isoforms were detected and photographed under u.v. light. The pH gradient was determined by incubating 2 mm gel segments in 0.5 ml of distilled water at 37 °C for 30 min and measuring the pH of each tube containing eluted ampholytes with a Beckman digital pH-meter.

Western-blot analysis of the subunits and isoforms of α-L-fucosidase

After SDS/PAGE or PAG-IEF, the gels were allowed to equilibrate for 30 min in 25 mM-Tris and 192 mM-glycine in 20% (v/v) methanol (SDS/PAGE) or 0.7% acetic acid (PAG-IEF). The protein was transferred to 0.2 μm-pore-size nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.), at 100 V for 1 h by using a Bio-Rad Mini-Trans-Blot electrophoresis cell according to the method described in the manual accompanying the unit. A portion of the nitrocellulose was stained for 1 min with 0.1% Amido Black 10B (Bio-Rad Laboratories) in propan-2-ol/acetic acid/water (3:1:6 by vol.) followed by destaining with propan-2-ol/acetic acid/water (3:1:6, by vol.). The remainder of the nitrocellulose was incubated in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 0.1% Tween 20 (Tris buffer) for 12 h. Before incubation with the lectin–digoxigenin conjugates, the nitrocellulose was incubated for 10 min in a 50 mM-Tris/HCl buffer, pH 7.5, without Tween 20 but containing 1 mM-$MnCl_2$, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$. The nitrocellulose was incubated with either a 10^3 or 10^4 dilution of pAb, 10^3 or 10^4 dilution of mAb 116, 10^2 dilution of mAb 100, 10^2 dilution of mAb 72, 10^2 dilution of mAb 40, in Tris buffer for 30–60 min (polyclonal, mAb 116) or 10–17 h (mAb 72, mAb 100, mAb 40), or with lectin–digoxigenin conjugates [*Galanthus nivalis* agglutinin (GNA), *Sambucus nigra* agglutinin (SNA); Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.] at a concentration of 1 μg of lectin–digoxigenin conjugate/ml of 50 mM-Tris/HCl buffer, pH 7.0, containing 0.05% NaN_3. The samples were subsequently washed three times in the Tris buffer (10 min/wash) followed by a 30–60 min incubation with a 5×10^3 dilution of rabbit anti-(goat IgG) antibody (for pAb), goat anti-(rat IgG/IgM) antibody (for mAb) or a 10^3 dilution of the Fab fragment of polyclonal sheep anti-digoxigenin (for the lectin–digoxigenin conjugates) (Boehringer Mannheim Biochemicals). All these antibodies were available coupled to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA, U.S.A., and Boehringer Mannheim Biochemicals). After three more 10 min washes in 10 mM-Tris buffer, pH 7.4, the nitrocellulose samples were placed into developing buffer (3.3 mg of Nitro Blue Tetrazolium and 1.7 mg of 5-bromo-4-chloroindol-3-yl phosphate in 10 ml of 0.1 M-Tris/HCl buffer, pH 9.5, containing 0.1 M-NaCl and 5 mM-$MgCl_2$) for 2–30 min. The colour-development reactions were stopped by immersing the nitrocellulose in water.

PAG-IEF–SDS/PAGE analysis of α-L-fucosidase isoforms

The isoforms of human liver α-L-fucosidase (14–45 μg) were separated in 5% polyacrylamide gels as described above except that the electrophoresis was performed in a Bio-Rad model 1405 electrophoresis cell at 4 W for 75 min with 1.0 M-H_3PO_4 as the anode solution and 1.0 M-NaOH as the cathode solution. The individual isoforms, detected with 4-methylumbelliferyl α-L-fucopyranoside, were cut from the gel, placed into test tubes containing 1.0 ml of 2.0% (w/v) SDS, 2.5% (v.v) 2-mercaptoethanol, 62.5 mM-Tris/HCl buffer, pH 6.8, and 0.0012% Bromophenol Blue and incubated at 100 °C for 20–30 min. The five treated gel slices were loaded into wells of the slab-gel apparatus containing a 4% stacking gel and a 12% separating gel. Coomassie Blue protein staining was used for detection of the fucosidase isoforms.

Neuraminidase treatment of α-L-fucosidase

A 50 μl portion of purified human liver α-L-fucosidase (75 μg) in 10 mM-sodium phosphate buffer, pH 5.5, containing 0.02% NaN_3 was incubated with 50 μl of neuraminidase (type X from *Clostridium perfringens*; Sigma Chemical Co.) (100 units/ml) in 0.1 M-sodium citrate/citric acid buffer, pH 5.0, for 48 h at 37 °C along with a control consisting of α-L-fucosidase in buffer without neuraminidase. The subunit composition of these samples was analysed by SDS/PAGE followed by staining with Coomassie Blue R-250.

N-Glycanase treatment of α-L-fucosidase

A 5 μl portion of α-L-fucosidase (10 μg) was denatured by incubation at 100 °C for 5 min in 0.5 M-sodium phosphate buffer, pH 8.0, containing 0.5% (w/v) SDS and 56 mM-2-mercaptoethanol. Nonidet P-40 (7.5%, v/v) and recombinant *N*-Glycanase (0.3 unit; Genzyme Corp., Framingham, MA, U.S.A.) in 80 mM-sodium phosphate buffer, pH 7.5, containing various reagents (see below) were added to the denatured fucosidase solution and Milli-Q water was added to a final volume of 25 μl. The final concentration of reagents was 0.1% SDS, 1.5% Nonidet P-40, 11 mM-2-mercaptoethanol, 10% glycerol, 30 mM-NaCl, 0.2 mM-EDTA and 12 units *N*-Glycanase/ml. The reaction mixture was incubated at 37 °C for 19, 24 or 72 h. Controls were also run that contained all constituents except *N*-Glycanase. The deglycosylation of *N*-Glycanase-treated α-L-fucosidase was evaluated by SDS/PAGE as described above, and by Western blotting with digoxigenin–succinyl-6-amidohexanoic acid hydrazide (Dig-hydrazide; Boehringer Mannheim) with the Fab fragment of polyclonal sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) for detection as described by Haselbeck & Hosel [9].

RESULTS

Human liver α-L-fucosidase was purified 7500-fold by affinity chromatography on the fucosamine resin [5] to a final specific activity of 14500 units/mg of protein in 80% yield.

Four hybridomas secreting antibodies against human α-L-fucosidase were produced (with the use of spleen cells from an immunized rat and mouse myeloma cells), selected and cloned. The mAbs produced by these hybridomas were isotyped and two clones (72 and 116) were found to produce IgG2a whereas the other two clones (40 and 100) produced IgM.

SDS/PAGE followed by transfer to nitrocellulose and detection of protein with Amido Black indicated that two human

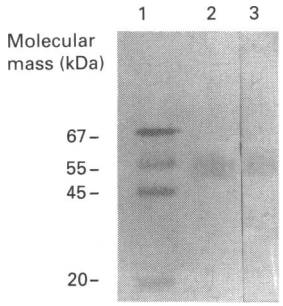

Fig. 1. Western-blot analysis of purified human liver α-L-fucosidase

See the Materials and methods section for details. Lane 1, standard protein molecular-mass markers: 2 μg of BSA (67 kDa), 4 μg of γ-globulin (55 kDa and 20 kDa) and 2 μg of ovalbumin (45 kDa) stained with 0.1 % (w/v) Amido Black; lane 2, 2 μg of human α-L-fucosidase stained with 0.1 % (w/v) Amido Black; lane 3, 0.5 μg of human α-L-fucosidase detected with a 10^3 dilution of pAb.

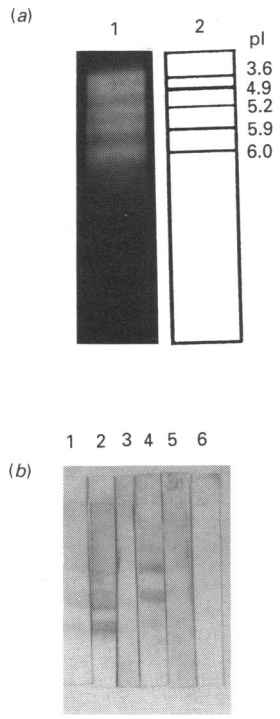

Fig. 2. (*a*) Slab PAG-IEF and (*b*) Western-blot analysis of human liver α-L-fucosidase

See the Materials and methods section for details. (*a*) Lane 1, 4-methylumbelliferyl α-L-fucopyranoside stain of human α-L-fucosidase isoforms; lane 2, interpretation of lane 1 with corresponding pI values. (*b*) Western-blot analysis of PAG-IEF of human liver α-L-fucosidase. Lane 1, pAb (10^4 dilution); lane 2, mAb 116 (10^4 dilution); lane 3, mAb 100 (5×10^2 dilution); lane 4, mAb 72 (5×10^2 dilution); lane 5, mAb 40 (5×10^2 dilution); lane 6, secondary antibody [goat anti-(rat IgG/IgM) antibody] (5×10^3) dilution (negative control).

fucosidase bands at 51 kDa and 56 kDa are present (Fig. 1, lane 2), although these bands are not as well resolved after electrotransfer as they are after just SDS/PAGE (e.g. see Fig. 6, lane 1). The pAb appears to recognize both protein bands (Fig. 1, lane 3).

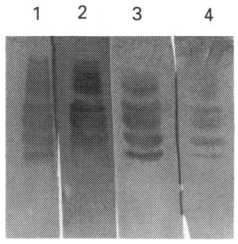

Fig. 3. Western-blot analysis of slab PAG-IEF$^+$ of human liver α-L-fucosidase

See the Materials and methods section for details. Lane 1, 14 μg of α-L-fucosidase detected with a 10^3 dilution of pAb; lane 2, 28 μg of α-L-fucosidase detected with digoxigenin-conjugated SNA; lane 3, 28 μg of α-L-fucosidase detected with digoxigenin-conjugated GNA; lane 4, 28 μg of α-L-fucosidase stained with 0.1 % (w/v) Amido Black.

The isoelectric forms of purified human liver α-L-fucosidase were separated by PAG-IEF (Fig. 2). Staining the gels with the fluorescent 4-methylumbelliferyl α-L-fucopyranoside substrate indicated that α-L-fucosidase contained at least five major isoforms with pI values between 3.6 and 6.0 (Fig. 2*a*, lanes 1 and 2). Transfer of the individual human isoforms on to nitrocellulose followed by incubation of the nitrocellulose with the pAb and four mAbs indicated that all the isoforms were recognized by pAb (Fig. 2*b*, lane 1), by mAbs 116 and 72 (Fig. 2*b*, lanes 2 and 4 respectively) and, to a much lesser extent, by mAbs 100 and 40 (Fig. 2*b*, lanes 3 and 5 respectively). Lane 6 in Fig. 2(*b*) is a negative control treated only with secondary antibody. These results are consistent with the results of dot immunoblotting experiments in which human fucosidase was recognized by pAb and mAbs (results not shown).

In a second experiment, the fucosidase isoforms were subjected to PAG-IEF, electrotransfer to nitrocellulose and analysis with pAbs and digoxigenin-labelled lectins with specificity for terminal mannose linked $\alpha 1 \rightarrow 2$, $\alpha 1 \rightarrow 3$ or $\alpha 1 \rightarrow 6$ to mannose (GNA) or for sialic acid linked $\alpha 2 \rightarrow 6$ to galactose (SNA). The results (Fig. 3) indicate (more clearly than in Fig. 2*b*, lane 1) that several isoforms are detectable with pAbs (lane 1) despite the small amounts of protein present (lane 4), particularly for the acidic isoforms near the top of the lane. The intermediate pI and acidic isoforms, but not the most neutral isoform(s), are detectable with SNA (lane 2). On the other hand, the intermediate and neutral isoforms, but not the most acidic isoforms, are detectable with GNA (lane 3).

Western-blot analysis of human liver α-L-fucosidase indicated that pAb (Fig. 4, lane 7) and at least two of the mAbs (72 and 116, lanes 5 and 6 respectively) recognized the 51 kDa and 56 kDa bands of the enzyme (compare with lane 2 with protein staining). mAbs 40 and 100 (lanes 3 and 4 respectively) clearly recognized the 51 kDa band but the narrower banding pattern seen with these mAbs could be due to decreased recognition or non-recognition of the 56 kDa band.

PAG-IEF was performed as described above in order to separate the isoforms of human liver α-L-fucosidase for subunit composition analysis. The individual isoforms were excised from the gels, treated with 2-mercaptoethanol and SDS at 100 °C, and subjected to SDS/PAGE. Each isoform contained a slightly different subunit composition (Fig. 5) compared with one another (lanes 3–7) and the whole enzyme (lane 2). The most neutral isoform (lane 3) contained a band at 51 kDa, the intermediate isoforms (lanes 4, 5 and 6) contained two closely spaced bands between 51 kDa and 56 kDa and the most acidic isoform (lane 7)

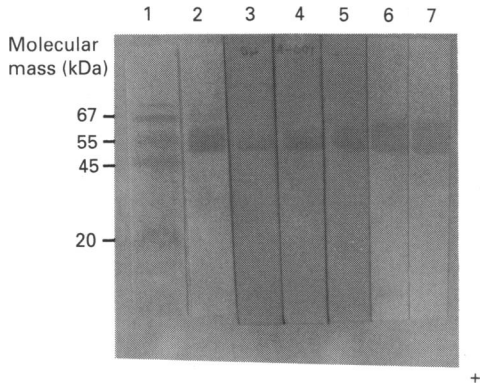

Fig. 4. Western-blot analysis of human liver α-L-fucosidase (5 μg/lane)

See the Materials and methods section for details. Lane 1, standard protein markers: 1 μg of BSA (67 kDa), 1 μg of γ-globulin (55 kDa and 20 kDa) and 1 μg of ovalbumin (45 kDa) stained with 0.1% (w/v) Amido Black; lane 2, human liver α-L-fucosidase stained with 0.1% (w/v) Amido Black; lane 3, mAb 40 (10^2 dilution); lane 4, mAb 100 (10^2 dilution); lane 5, mAb 72 (10^2 dilution); lane 6, mAb 116 (10^4 dilution); lane 7, pAb (10^4 dilution).

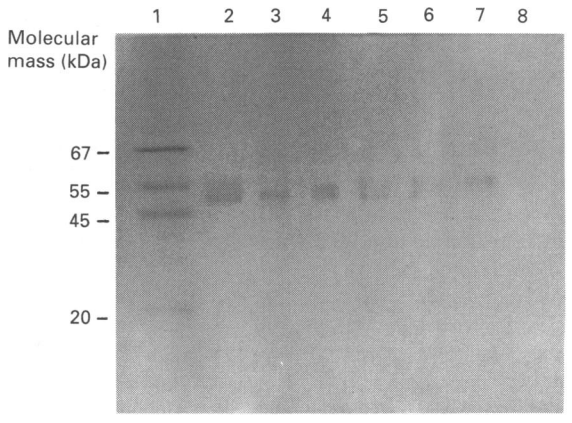

Fig. 5. SDS/PAGE of isoforms of human liver α-L-fucosidase previously separated by PAG-IEF stained with 0.1% (w/v) Coomassie Blue R-250

See the Materials and methods section for details. Lane 1, standard protein molecular-mass markers: 2 μg of BSA (67 kDa), 4 μg of γ-globulin (55 kDa and 20 kDa) and 2 μg of ovalbumin (45 kDa); lane 2, 2 μg of human liver α-L-fucosidase; lanes 3–7, human liver α-L-fucosidase isoforms with pI values 6.0, 5.9, 5.2, 4.9 and 3.6 respectively; lane 8, control gel slice.

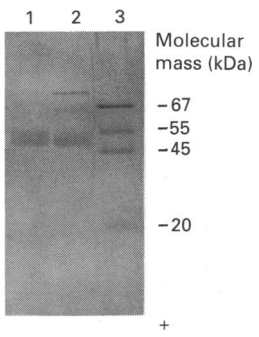

Fig. 6. SDS/PAGE of human liver α-L-fucosidase and neuraminidase-treated human liver α-L-fucosidase stained with 0.1% (w/v) Coomassie Blue R-250

See the Materials and methods section for details. Lane 1, 2 μg of α-L-fucosidase; lane 2, 2 μg of α-L-fucosidase treated for 48 h at 37 °C with neuraminidase; lane 3, standard protein molecular-mass markers: 2 μg of BSA (67 kDa), 4 μg of γ-globulin (55 kDa and 20 kDa) and 2 μg of ovalbumin (45 kDa).

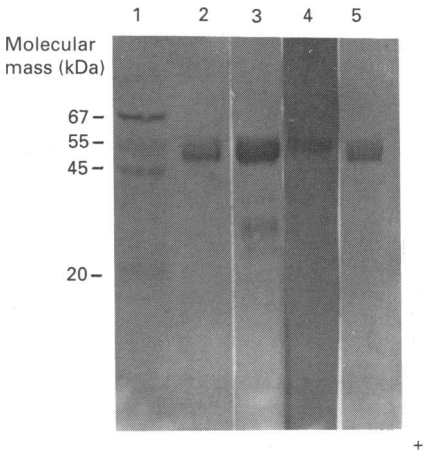

Fig. 7. Western-blot analysis of human liver α-L-fucosidase with digoxigenin-labelled lectins

See the Materials and methods section for details. Lane 1, standard protein molecular-mass markers: 5 μg of BSA (67 kDa), 10 μg of γ-globulin (55 kDa and 20 kDa) and 5 μg of ovalbumin (45 kDa) stained with 0.1% (w/v) Amido Black; lanes 2 and 5, 5 μg of α-L-fucosidase stained with 0.1% (w/v) Amido Black; lane 3, 10 μg of α-L-fucosidase detected with digoxigenin-conjugated GNA; lane 4, 10 μg of α-L-fucosidase detected with digoxigenin-conjugated SNA.

contained a band at 56 kDa. A trend is present in going from the most neutral to the most acidic isoform with increasing amounts of the 56 kDa, and decreasing amounts of the 51 kDa, bands being seen. No protein bands are present in the control (lane 8), which contained a gel slice from PAG-IEF without any α-L-fucosidase.

The subunit composition of neuraminidase-treated human liver α-L-fucosidase was determined by SDS/PAGE (Fig. 6). Staining with Coomassie Blue R-250 indicated that neuraminidase-treated α-L-fucosidase (lane 2) contained less of the 56 kDa band and more of the 51 kDa band when compared with untreated fucosidase (lane 1) and a control in which fucosidase was treated in buffer at 37 °C for 48 h without neuraminidase (results not shown). The high-molecular-mass bands seen in lane 2 are presumably from the neuraminidase preparation.

A Western-blot analysis of the subunits of human liver α-L-fucosidase with digoxigenin-labelled lectins is shown in Fig. 7. The results indicate that both fucosidase subunits, as indicated by protein staining in lanes 2 and 5, are recognized by GNA (lane 3) whereas only the 56 kDa band is recognized by SNA (lane 4). Minor amounts of GNA-positive low-molecular-mass bands that are not detectable by protein staining (lanes 2 and 5) appear to be present in lane 3. These GNA-positive bands are probably due to minute amounts of carbohydrate-rich protein contaminants.

The results of SDS/PAGE and Western-blot analysis of human liver α-L-fucosidase before and after *N*-Glycanase treatment are depicted in Fig. 8. After *N*-Glycanase treatment for 24 h, two protein-staining bands are seen (lane 3) at 48 kDa and 45 kDa rather than the 56 kDa and 51 kDa bands seen before endoglycosidase treatment (lane 2). Western-blot analysis with the

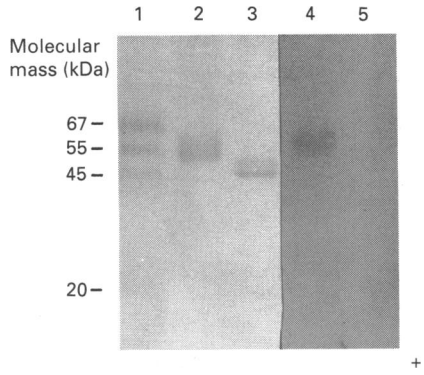

Fig. 8. SDS/PAGE and Western-blot analysis of human liver α-L-fucosidase (3 μg/lane) before and after *N*-Glycanase treatment

See the Materials and methods section for details. Lane 1, standard protein molecular-mass markers: 3 μg of BSA (67 kDa), 6 μg of γ-globulin (55 kDa and 20 kDa) and 3 μg of ovalbumin stained with 0.1% (w/v) Coomassie Blue R-250; lane 2, α-L-fucosidase stained with 0.1% (w/v) Coomassie Blue R-250; lane 3, α-L-fucosidase treated for 24 h at 37 °C with *N*-Glycanase and stained with 0.1% (w/v) Coomassie Blue R-250; lane 4, α-L-fucosidase Western-blotted and detected by the Dig-hydrazide method [9]; lane 5, α-L-fucosidase treated for 24 h at 37 °C with *N*-Glycanase, Western-blotted and detected by the Dig-hydrazide method [9].

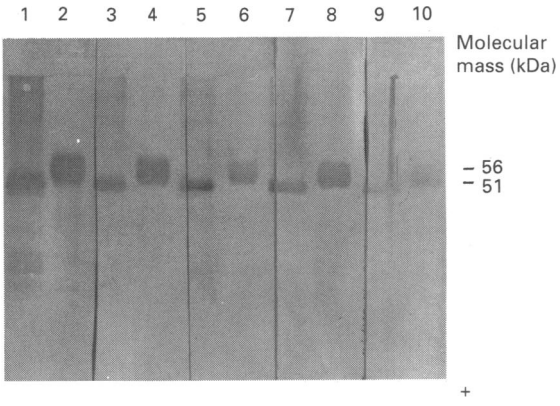

Fig. 9. Western-blot analysis of human liver α-L-fucosidase (2 μg/lane) before and after *N*-glycanase treatment for 19 h at 37 °C using mAbs and pAbs

See the Materials and methods section for details. Lane 1, α-L-fucosidase treated with *N*-Glycanase and detected with a 10^3 dilution of pAb; lane 2, α-L-fucosidase detected with a 10^3 dilution of pAb; lane 3, α-L-fucosidase treated with *N*-Glycanase and detected with a 10^3 dilution of mAb 116; lane 4, α-L-fucosidase detected with a 10^3 dilution of mAb 116; lane 5, α-L-fucosidase treated with *N*-Glycanase and detected with a 10^2 dilution of mAb 100; lane 6, α-L-fucosidase detected with a 10^2 dilution of mAb 100; lane 7, α-L-fucosidase treated with *N*-Glycanase and detected with a 10^2 dilution of mAb 72; lane 8, α-L-fucosidase detected with a 10^2 dilution of mAb 72; lane 9, α-L-fucosidase treated with *N*-Glycanase and detected with a 10^2 dilution of mAb 40; lane 10, α-L-fucosidase detected with a 10^2 dilution of mAb 40.

Dig-hydrazide technique [9] indicated the presence of carbohydrate before (lane 4), but not after (lane 5), *N*-Glycanase treatment. *N*-Glycanase treatment of fucosidase for 72 h gave the same results as the 24 h treatment (results not shown).

Western-blot analysis of α-L-fucosidase before and after *N*-Glycanase treatment for 19 h was done using the pAbs and mAbs for detection (Fig. 9). All the antibodies recognized both the glycosylated (even-numbered lanes) and deglycosylated (odd-numbered lanes) forms of α-L-fucosidase even though the banding pattern was wider for glycosylated than for deglycosylated enzyme. The banding pattern for glycosylated fucosidase with mAbs 100 and 40 (lanes 6 and 10 respectively) was narrower than when using pAbs and mAbs 116 and 72 (lanes 2, 4 and 8 respectively), consistent with the Western-blot results in Fig. 4.

DISCUSSION

α-L-Fucosidase is a lysosomal glycosidase that is present in many mammalian tissues [1]. This enzyme has been of considerable interest because of the diverse group of fucoglycoconjugate substrates [10] that it normally hydrolyses and the neurovisceral storage disease fucosidosis that results from deficiency of its enzymic activity [2,3].

In the present investigation we have employed Western-blot analysis and exoglycosidase and endoglycosidase digestions to study the structural relationship between the separated isoforms and subunits of purified human liver α-L-fucosidase. SDS/PAGE indicated the presence of two pAb-immunoreactive protein bands of 51 kDa and 56 kDa, similarly to previous results [11]. The human liver enzyme has been shown by high-speed-sedimentation-equilibrium analysis to have a molecular mass of 230 kDa [5] and is presumably tetrameric.

PAG-IEF indicated the presence of multiple fucosidase isoforms. Western-blot analysis after PAG-IEF separation of fucosidase isoforms indicated that the pAbs and at least two of the mAbs clearly recognized all the isoforms. In addition, the most acidic isoforms were recognized by SNA, a lectin with specificity for sialic acid, and not by GNA, a lectin with specificity for mannose, whereas the most neutral isoform(s) was recognized by GNA and not by SNA. These lectin blotting studies are consistent with previous studies [5,12–17] that have indicated that the fucosidase isoforms are related, at least in part, by sialic acid residues.

Western-blot analysis indicated that the pAbs and the four mAbs recognized the 51 kDa subunit of human α-L-fucosidase. In addition, the pAbs and possibly only two of the mAbs recognized the 56 kDa human fucosidase subunit. This immunochemical recognition was not eliminated by *N*-Glycanase treatment, suggesting that the pAbs and mAbs are not directed against carbohydrate epitopes. The differential recognition of the two human subunits by mAbs not directed against carbohydrate epitopes suggests that polypeptide differences exist between the subunits.

Analysis by SDS/PAGE of the subunit composition of the separated fucosidase isoforms indicated enriched relative amounts of the high-molecular-mass subunits as the isoforms became more acidic. This suggested that the high-molecular-mass subunits contain most or all of the sialic acid residues. In order to provide more evidence for this suggestion, human liver α-L-fucosidase was treated with neuraminidase and analysed by SDS/PAGE. The results indicate that neuraminidase treatment led to a decreased relative amount of the high-molecular-mass subunit and a concomitant increase in the low-molecular-mass subunit. Western-blot analysis of human liver α-L-fucosidase with lectins was consistent with the neuraminidase digestion results in that only the high-molecular-mass subunit was recognized by SNA whereas both subunits were recognized by GNA. These results suggest that the high-molecular-mass subunit contains an enrichment of all of the sialylated *N*-acetyl-lactosamine-type oligosaccharides that, along with oligomannoside-type chains, have been shown to be present in human liver α-L-fucosidase [18]. *N*-Glycanase treatment of α-L-fucosidase resulted

in the disappearance of the 56 kDa and 51 kDa carbohydrate-positive protein bands and the appearance of two carbohydrate-negative protein bands at 48 kDa and 45 kDa. This indicates that the two subunits of α-L-fucosidase are not related solely by *N*-glycosylation and that other post-translational modifications and/or polypeptide differences may exist. To date, cloning studies have provided evidence for only one polypeptide for human fucosidase [19,20] and one polypeptide for rat fucosidase [21]. These polypeptides contain 82% sequence identity and share four conserved potential *N*-glycosylation sites.

In conclusion, Western-blot analysis indicates that variable *N*-glycosylation contributes to, but does not account completely for, structural differences in the subunits of fucosidase. In addition, the more acidic isoforms of fucosidase contain enriched relative amounts of the sialylated high-molecular-mass subunit. *N*-Terminal and peptide mapping studies need to be carried out to determine whether primary amino acid sequence alterations and /or post-translational modifications other than glycosylation also contribute to the observed subunit differences of fucosidase.

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