Canine α-L-fucosidase in relation to the enzymic defect and storage products in canine fucosidosis

Christopher BARKER,* Anne DELL,† Mark ROGERS,†§ Jack A. ALHADEFF‡ and Bryan WINCHESTER*‖
*M.R.C. Human Genetic Diseases Research Group, Department of Biochemistry, King's College London, Campden Hill Road, London W8 7AH, U.K., †Department of Biochemistry, Imperial College of Science and Technology (University of London), South Kensington, London SW7 2AS, U.K., and ‡Department of Chemistry, Lehigh University, Bethlehem, PA 18015, U.S.A.

Canine liver α-L-fucosidase was purified to apparent homogeneity by affinity chromatography on agarose–ε-aminohexanoyl-fucopyranosylamine. It is composed of multiple forms of a common active subunit of 45–50 kDa, which can aggregate in different combinations to form polymers, predominantly dimers. Antiserum was raised against the purified enzyme. There is negligible residual α-L-fucosidase in the tissues of English springer spaniels with the lysosomal storage disease fucosidosis. Although no α-L-fucosidase protein was detected by Western blotting or by the purification procedure in the affected tissues, some enzymically inactive cross-reacting material was detected in both normal and affected tissues. This suggests that another protein without α-L-fucosidase activity was co-purified with the enzyme. Dog liver α-L-fucosidase was precipitated by goat anti-(human liver α-L-fucosidase) IgG, indicating homology between the enzymes in the two species. Two purified storage products isolated from the brain of a dog with fucosidosis were used as natural substrates for various preparations of canine liver α-L-fucosidase. Analysis of the digestion mixtures by t.l.c. and fast-atom-bombardment mass spectrometry suggests that canine α-L-fucosidase acts preferentially on the α-(1–3)-linked fucose at the non-reducing end and that removal of α-(1–6)-linked asparagine-linked N-acetylglucosamine is rate-limiting in the lysosomal catabolism of fucosylated N-linked glycans.

INTRODUCTION

Canine fucosidosis is a progressive neurovisceral lysosomal storage disease occurring in English springer spaniels in the United Kingdom (Littlewood et al., 1983) and Australia (Hartley et al., 1982). It is characterized biochemically by a deficiency of α-L-fucosidase and the accumulation in tissues and excretion in the urine of fucoglycoconjugates (Kelly et al., 1983; Abraham et al., 1984). The disorder is inherited in an autosomal recessive manner (Healy et al., 1984), and eradication programmes based on the detection of heterozygotes by the measurement of plasma and leucocyte α-L-fucosidase have been instituted in Australia (Healy et al., 1984) and the United Kingdom (C. Barker & B. Winchester, unpublished work). The consistent clinical and pathological features in affected animals make it an excellent model for human fucosidosis and for evaluating experimental forms of enzyme replacement therapy, such as bone-marrow transplantation (Taylor et al., 1986a,b). Although both human and canine fucosidosis result from a similar deficiency of α-L-fucosidase, there are differences in the clinical signs and cellular involvement in the two forms of the disease (Durand et al., 1982; Alroy et al., 1985; Taylor et al., 1987). In particular, the structures and tissue distribution of the storage products are different (Abraham et al., 1984; Alroy et al., 1985). We have characterized normal canine α-L-fucosidase and investigated the tissue distribution of its multiple forms to relate the enzymic defect in fucosidosis to the structures of the storage products.

MATERIALS AND METHODS

Tissues

Samples of the brain, liver kidney and spleen of a 3-year-old English springer spaniel, diagnosed biochemically and clinically as having fucosidosis, were removed at post-mortem and stored at −20 °C. The corresponding organs from an unaffected dog were also stored frozen. Homogenates (20%, w/v) were prepared in distilled water in a Potter–Elvehjem homogenizer and then centrifuged at 37000 g in an MSE high-speed centrifuge for 30 min at 4 °C. The resultant supernatant fluid was used for enzymic studies. Cultures of fibroblasts were established from explants of skin taken from the affected dog at post-portem and maintained as described previously for primary human fibroblasts (Burditt et al., 1978).

Enzyme assays

α-L-Fucosidase was assayed routinely with the fluorigenic substrate 4-methylumbelliferyl α-L-fucopyranoside (Koch–Light, Haverhill, Suffolk, U.K.) at pH 5.5 with a final substrate concentration of 0.5 mM (Abraham et al., 1984). The pH-dependence of α-L-fucosidase was measured with the use of the McIlvaine sodium phosphate/citrate buffer system over the pH range 3.7–7.0 and a Tris buffer system over the pH range 7.7–8.9 with a substrate concentration of 0.5 mM. The value of K_m for α-L-fucosidase acting on 4-methylumbelliferyl α-L-fucopyranoside was measured at pH 5.5 by the Line-

weaver–Burk (1934) graphical procedure with a 0.08–0.5 mM range of substrate concentrations. A computer program was used to obtain the line of best fit. One unit of enzyme activity is defined as the amount that transforms 1 μmol of substrate/min under the conditions specified.

Purification of canine liver α-L-fucosidase

α-L-Fucosidase was purified from normal dog liver by modification of procedures previously described for human liver α-L-fucosidase (Robinson & Thorpe, 1974; Alhadeff et al., 1975). The fraction precipitated from a 20 % (w/v) homogenate of dog liver between 30 % and 60 % saturation with $(NH_4)_2SO_4$ was applied to a column (10 cm × 1.1 cm diam.) of Sepharose–concanavalin A (Pharmacia). The material eluted with 0.5 M-methyl α-D-mannopyranoside was dialysed against 50 mM-sodium phosphate buffer, pH 6.8, and applied to a column (10 cm × 1.1 cm diam.) of agarose–ε-amino-hexanoyl-fucopyranosylamine (Miles Laboratories, Stoke Poges, Bucks., U.K.) equilibrated in the same buffer. α-L-Fucosidase was eluted with 1 % (w/v) fucose (Robinson & Thorpe, 1974).

Native gel electrophoresis in a gradient of polyacrylamide

Electrophoresis was performed under non-denaturing conditions in slab gels (80 mm × 80 mm × 3 mm) containing a gradient of polyacrylamide (4–30 %, w/v) (Pharmacia). Gels were pre-run for 15 min at 125 V in a buffer system consisting of 90 mM-Tris, 80 mM-boric acid and 2.5 mM-EDTA, pH 8.3. Samples that had been equilibrated in the same buffer were made 10 % (v/v) with respect to glycerol, and 10–40 μg of protein per lane was applied to the gel. After electrophoresis for 24 h at 150 V at 4 °C, the samples reached their pore limits. Electrophoresis was stopped and the gel was stained for α-fucosidase activity by incubating the gel overlaid with filter paper saturated with 1 mM-4-methylumbelliferyl α-L-fucopyranoside, pH 5.5, for 60 min at 37 °C. The enzyme reaction was stopped with 0.5 M-glycine/NaOH buffer, pH 10.4, and the fluorescent bands were viewed under u.v. light. Protein bands were detected by staining with 0.5 % (w/v) Coomassie Blue R-250 in methanol/acetic acid/water (55:7:43, by vol.). Standard protein molecular-mass markers were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was performed in 10 % (w/v) polyacrylamide gels (165 mm × 195 mm × 1.5 mm) with a 3 % (w/v) stacking gel according to the method of Laemmli (1970). Gels were run in an SE60 vertical slab-gel unit (Hoefer Scientific Instruments) with a discontinuous buffer system. Each suitably diluted protein sample (10–40 μg) was made 10 mM with respect to 2-mercaptoethanol, and to ensure that disulphide bonds were not re-formed the reduced polypeptides were carboxymethylated with 0.5 M-iodoacetamide (Roberts & Lord, 1981). Electrophoresis was performed at 15 mA for 2 h, then at 30 mA for 6 h. Protein bands were detected by staining with 0.2 % (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (55:7:43, by vol.) for a minimum of 2 h. The gels were destained by repeated washing with

methanol/acetic acid/water (4:1:6, by vol.). Standard protein markers were bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (35 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soya-bean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Ion-exchange chromatography on DEAE-cellulose

Ion-exchange chromatography was carried out on DEAE-cellulose (Whatman DE-52) at pH 6.5 as described previously (Abraham et al., 1984).

Protein determination

Protein was measured by the Bradford (1976) method, with bovine serum albumin as a standard.

Preparation of antiserum and immunoprecipitation studies

Antibodies to the purified canine liver α-L-fucosidase were produced in a rabbit by intradermal injections at multiple sites. Double immunodiffusion (Ouchterlony) was carried out as described previously (Phillips et al., 1975), and active immunoprecipitin bands were detected with 4-methylumbelliferyl α-L-fucopyranoside (Robinson & Thorpe, 1974). Quantitative immunoprecipitation experiments were carried out as follows. Tissue extracts or partially purified enzyme were diluted with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4), and 100 μl samples were mixed with an equal volume of suitably diluted antiserum in 1.5 ml Eppendorf tubes. These mixtures were incubated at 37 °C for 2 h and then at 4 °C for 16 h. After this time, 100 μl of Immunoprecipitin [suspension of Staphylococcus aureus cell walls bearing Protein A (BRL) (10 %, w/v, in phosphate-buffered saline)] was added and the mixtures were incubated at 37 °C for 30 min. Immune complexes were sedimented in an MSE Microcentaur centrifuge at 12000 g for 5 min. The enzyme activity remaining in the supernatant was assayed and the volume of neat antiserum that is required to precipitate 1 unit of enzyme activity was calculated. Controls lacking antiserum were included in each experiment to determine the amount of non-specific binding to Immunoprecipitin. IgG was isolated from antiserum raised in a goat against purified normal human liver α-L-fucosidase (Andrews-Smith & Alhadeff, 1982).

Western blot

SDS/polyacrylamide-gel electrophoresis was performed as described above. The gel was washed for 10 min in 200 ml of electrotransfer buffer [containing 25 mM-Tris, 192 mM-glycine, 0.1 % (w/v) SDS and 20 % (v/v) methanol]. Electrotransfer to nitrocellulose paper (Schleicher and Schuell) was performed for 24 h at 100 mA in a Transphor apparatus (model TE-50, Hoefer Scientific Instruments) containing 5 litres of electrotransfer buffer. Excess protein-binding sites on the nitrocellulose filter were blocked by treatment with 2 % (w/v) casein in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 5.5, for 15 min. The filter was then incubated for 1 h in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 5.5 for 15 min. The filter was then incubated for 1 h in a 100-fold dilution of the first antibody [either rabbit anti-(dog liver α-fucosidase) serum or goat anti-(human α-fucosidase) IgG] in 1 % (w/v) casein in phosphate-buffered saline. After a brief washing in

distilled water the filter was then incubated for 1 h in a 1500-fold dilution of goat anti-(rabbit IgG antibody–horseradish peroxidase conjugate (Bio-Rad Laboratories) or of rabbit anti-(goat IgG) antibody–horseradish peroxidase conjugate (Sigma Chemical Co.) in 0.5% (w/v) casein in phosphate-buffered saline. The filter was then rinsed briefly with distilled water and with phosphate-buffered saline containing 0.5% (w/v) Tween 20 before being equilibrated with Tris-buffered saline (0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.5). Finally, the filter was soaked for 15 min in the colour-development solution [0.015% (v/v) H_2O_2, 0.05% (w/v) 2-chloronaphthol (Bio-Rad Laboratories) and 16% (v/v) methanol in Tris-buffered saline].

Digestion of storage products

Two fucoglycoasparagines, Fuc-GlcNAc-Asn (F.G.1) and Gal-(Fuc)Glc-NAc-Man-Man-GlcNAc-(Fuc)Glc-NAc-Asn (F.G.2), were isolated from the brain of an affected dog and characterized as described previously (Abraham et al., 1983, 1984). These storage products were incubated with various preparations of canine liver α-L-fucosidase in phosphate/citrate buffer, pH 5.6, with 0.1 unit (pure enzyme) or 0.01 unit (crude fractions) of enzymic activity per µg of fucoglycoasparagine. The digestion mixtures were analysed by t.l.c. and by fast-atom-bombardment m.s. after peracetylation and extraction of peracetylated oligosaccharides into chloroform (Abraham et al., 1984).

RESULTS

Characterization of normal canine α-L-fucosidase

α-L-Fucosidase was purified from normal canine liver by affinity chromatography (Table 1). The purified enzyme had a broad pH–activity profile for the synthetic substrate 4-methylumbelliferyl α-L-fucoside with greatest activity at pH 7 and a marked shoulder at pH 5.5. The pH–activity profile for the activity in a crude liver extract was similar. The apparent K_m value for the purified enzyme acting on 4-methylumbelliferyl α-L-fucopyranoside at pH 5.5 was 0.28 mM. The homogeneity of the preparation was analysed by electrophoresis under non-denaturing and denaturing conditions. The native enzyme gave an identical pattern of bands when stained for protein with Coomassie Blue or for activity with 4-methylumbelliferyl α-L-fucopyranoside (Fig. 1). This consisted of a predominant broad region of multiple bands with molecular masses covering the range
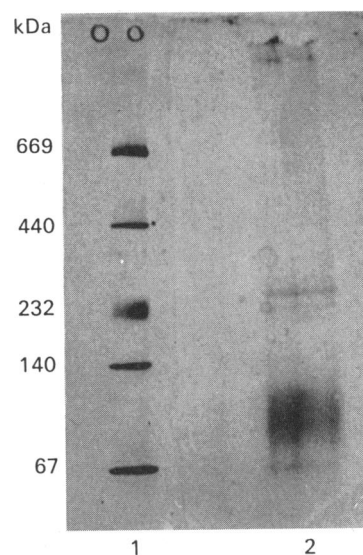

Fig. 1. Polyacrylamide-gradient-gel electrophoresis of purified dog liver α-L-fucosidase run under non-denaturing conditions

For experimental conditions see the text. Track 1, molecular-mass marker proteins; Track 2, purified α-L-fucosidase (20 µg). The gel was stained for protein with Coomassie Blue.

80–120 kDa with minor components of molecular mass approx. 240 kDa and 65 kDa.

Electrophoresis in the presence of SDS showed that the purified enzyme contained a mixture of subunits with a molecular mass of 45–50 kDa and traces of larger components (Fig. 2). This suggests that the predominant native form of the enzyme is a dimer and that its polydispersity is due to different combinations of subunits that differ slightly in size. A similar subunit structure has been reported for human liver α-L-fucosidase (Alhadeff et al., 1975).

Further insight into the molecular structure of the enzyme was obtained by chromatographic analysis. The α-L-fucosidase in a crude extract of normal dog liver was separated into two forms by ion-exchange chromatography at pH 6.5 on DEAE-cellulose: α-L-fucosidase I (bound) and α-L-fucosidase II (unbound) by analogy with human liver α-L-fucosidase (Robinson & Thorpe, 1973). The two forms had similar kinetics and pH–acti-

Table 1. Purification of dog liver α-fucosidase

For experimental details see the text.

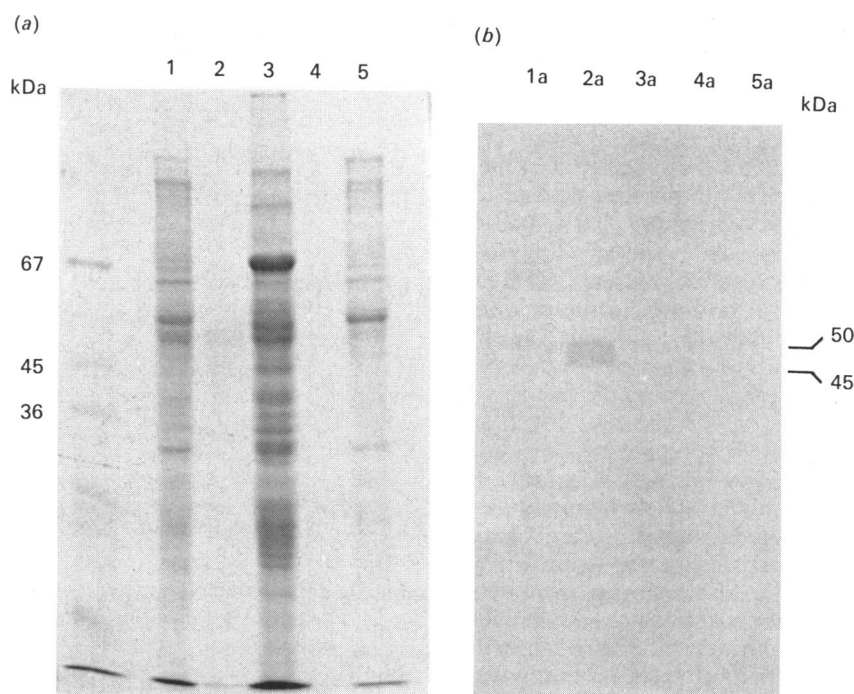|	Total protein (mg)	Total fucosidase activity (munits)	Specific activity (munits/mg)	Purification factor	Yield (%)
Original supernatant	6850	4111	0.6	–	–
(NH₄)₂SO₄ fractionation (30–60% satn.)	165	3700	22.4	37	90
Chromatography on Sepharose–concanavalin A	18	2800	155.0	258	68
Chromatography on agarose–ε-aminohexanoyl-fucosylamine	1.4	2400	1714	2857	58

Fig. 2. (a) **SDS/polyacrylamide-gel electrophoresis and** (b) **immunoblot with rabbit anti-(dog α-fucosidase) serum as first antibody**

For experimental details see the text. Tracks 1 and 1a, normal concanavalin A-purified dog liver material (50 μg); tracks 2 and 2a, normal affinity-purified dog liver α-L-fucosidase (15 μg); tracks 3 and 3a, concanavalin A-purified fucosidosis dog liver material (100 μg); tracks 4 and 4a, fucosidosis dog liver affinity column material eluted with L-fucose; tracks 5 and 5a, affinity-column-unbound fucosidosis dog liver material (50 μg).

Table 2. Percentage bound (form I) and unbound (form II) α-fucosidase activity in normal dog tissues after ion-exchange chromatography

For experimental details see the text.

	Percentage bound (form I)	Percentage unbound (form II)
Liver (purified enzyme)	68.2	31.8
Liver (crude extract)	70	30
Spleen (crude extract)	69	31
Kidney (crude extract)	26.5	73.5
Brain (crude extract)	51.4	48.6
α-L-Fucosidase I	100	0
α-L-Fucosidase II	46	54

vity profiles, although the ratio of activity at pH 5.5 to that at pH 7 was slightly greater for α-L-fucosidase I. When α-L-fucosidase I was rechromatographed on DEAE-cellulose, all the activity was recovered in the bound fraction, i.e. as α-L-fucosidase I. However, when α-L-fucosidase II was rechromatographed a mixture of α-L-fucosidases I and II was consistently recovered (Table 2). The proportions of α-L-fucosidases I and II varied from tissue to tissue (Table 2).

Two peaks of activity were also separated when a crude extract of dog liver was analysed by gel filtration on Sephacryl S-200. They had molecular masses of

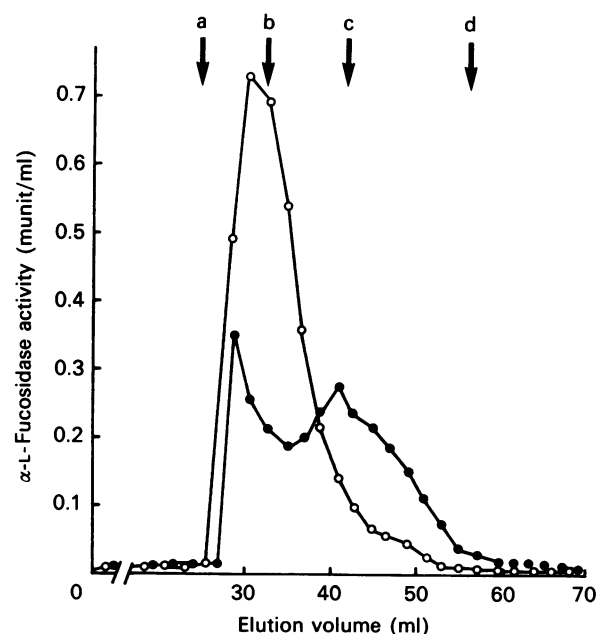**Fig. 3. Gel filtration on Sephacryl S-200 of dog liver α-L-fucosidase forms separated previously by ion-exchange chromatography on DEAE-cellulose**

For experimental details see the text. ●, DEAE-cellulose-unbound material; ○, DEAE-cellulose-bound material. Key to marker positions: a, void volume; b, lactate dehydrogenase (140 kDa); c, horseradish peroxidase (49 kDa); d, cytochrome c (12 kDa).

90–150 kDa and 50 kDa, again suggesting that the native enzyme existed in active monomeric and polymeric, largely dimeric, forms. Gel filtration of α-L-fucosidases I and II that had been separated on DEAE-cellulose showed that α-L-fucosidase I consisted exclusively of the polymeric form of the enzyme whereas α-L-fucosidase II contained a mixture of the monomeric and aggregated forms (Fig. 3). This observation suggests that the active monomeric form (α-L-fucosidase II) can readily aggregate to the polymeric form.

The enzymic defect in canine fucosidosis

The α-L-fucosidase activity in different tissues of a normal and an affected dog is given in Table 3. There is a severe deficiency of α-L-fucosidase in all the affected tissues, less than 2% residual activity being detected. Ion-exchange chromatography of extracts of all the affected tissues showed that both α-L-fucosidases I and II were absent, suggesting a close structural and genetical relationship between the forms. To test whether any α-L-fucosidase protein was present in canine fucosidosis, an attempt was made to purify the residual activity from the liver of the affected dog by exactly the same procedure as used for the normal enzyme. This purification was carried out 'blindly', as no activity was detected in the fractions obtained in the chromatographic steps. No α-L-fucosidase activity was specifically eluted with fucose and no α-L-fucosidase was detected in the unadsorbed fraction or washings. All the protein applied to the agarose–ε-aminohexanoyl-fucopyranosylamine affinity column was either unadsorbed or eluted with the

Table 3. α-L-Fucosidase activities in normal and affected dog tissues

For experimental details see the text.

	α-L-Fucosidase activity (munits/mg)	
	Normal dog	Affected dog
Liver	6.86	0.01
Brain	0.53	0.01
Spleen	5.40	0.01
Kidney	3.90	0.01
Fibroblasts	1.10	0.01
Leucocytes	1.05	0.01
Plasma (munits/ml)	1.60	0.01

washings. When the fraction eluted with fucose was analysed by SDS/polyacrylamide-gel electrophoresis no protein bands were observed (Fig. 2). This observation suggests that if any α-L-fucosidase protein is present in the liver of the fucosidosis dog it is enzymically inactive and does not bind to the affinity column. Immuno-chemical techniques were used to test whether any enzymically inactive but cross-reacting material was present.

The rabbit anti-(dog liver α-L-fucosidase) serum immunoprecipitated normal dog liver α-fucosidase acti-

Fig. 4. Immunoprecipitation of canine and human liver α-L-fucosidase activity by anti-α-L-fucosidase serum

For experimental details see the text. ●, Normal dog liver α-L-fucosidase immunoprecipitated by rabbit anti-(dog α-fucosidase) serum; ▼, human liver α-L-fucosidase immunoprecipitated by rabbit anti-(dog α-fucosidase) serum; ■, pre-immune serum; ○, normal dog liver α-L-fucosidase immunoprecipitated by goat anti-(human α-L-fucosidase) IgG.

vity with an equivalence point of 0.1 ml of antiserum per unit of enzyme activity (Fig. 4). None of the following lysosomal glycosidases was immunoprecipitated by more than 3 %: α-D-mannosidase, β-D-mannosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, N-acetyl-β-D-hexosaminidase and α-glucosidase. This rabbit anti-(dog α-fucosidase) serum also immunoprecipitated normal human liver α-fucosidase activity with an equivalence point of 2 ml of antiserum per unit of enzymic activity. A sample of DEAE-cellulose-purified goat anti-(human liver α-fucosidase) IgG was also used to investigate the enzymic defect in canine fucosidosis. This IgG specifically immunoprecipitated normal human liver α-fucosidase activity with an equivalence point of less than 0.1 ml and normal dog liver α-fucosidase activity with an equivalence point of 0.2 ml. These results provide good evidence that antigenic similarity exists between dog and human α-fucosidases.

Double-immunodiffusion experiments with the rabbit anti-(dog α-fucosidase) serum showed a major precipitin line with normal dog liver crude extract. This precipitin line was enzymically active when stained with 4-methylumbelliferyl fucopyranoside. Some minor and very faint precipitin lines, which were enzymically inactive, were also detected and raise the possibility of the presence of minor contaminants in the purified fucosidase preparation. Such faint enzymically inactive precipitin lines were also observed when the anti-(dog α-fucosidase) serum was diffused against a crude extract of affected dog liver but the major precipitin line was not observed.

F.G.1: Fuc-GlcNAc-Asn

F.G.2: Gal-GlcNAc-Man-Man-GlcNAc-GlcNAc-Asn

α(1-3) α(1-6)

Fuc Fuc

Fig. 5. Structures of two fucoglycoconjugate storage products isolated from brain of a dog affected with fucosidosis (Abraham et al., 1984)

Preincubation of the rabbit anti-(dog α-fucosidase) serum with an extract of fucosidosis dog liver, before immunoprecipition of normal dog liver α-fucosidase activity, did not diminish the antibody titre. This suggests that α-L-fucosidase protein that cross-reacts with antiserum raised against the normal enzyme is essentially absent in fucosidosis. The absence of such cross-reacting material was confirmed by Western blotting. Immuno-blotting of electrophoretograms obtained in the presence of SDS of normal and affected dog liver samples, with the use of the rabbit anti-(dog α-fucosidase) serum, demonstrated specific cross-reacting components of 45–50 kDa in the purified normal dog liver α-fucosidase (Fig. 2, track 2a). Such cross-reactivity was not observed in a normal dog liver extract processed to the stage of chromatography on Sepharose–concanavalin A, presumably because the amount of α-L-fucosidase present was below the level of detection. No major cross-reacting material could be detected in the fucosidosis dog liver

M —
F —
S —

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 6. T.l.c. of oligosaccharide digestion products

For experimental details see the text. Tracks 1 and 15, dextran hydrolysate; track 2, F.G.1; track 3, F.G.1 incubated with normal dog liver crude extract; track 4, F.G.1 incubated with concanavalin A-purified normal dog liver extract; track 5, F.G.1 incubated with normal dog liver lysosomal preparation from subcellular fractionation; track 6, F.G.2; track 7, F.G.2 incubated with normal dog liver crude extract; track 8, F.G.2 incubated with concanavalin A-purified normal dog liver extract; track 9, F.G.2 incubated with normal dog liver lysosomes; track 10, normal dog liver crude extract; track 11, concanavalin A-purified, normal dog liver extract; track 12, normal dog liver lysosomes; track 13, L-fucose; track 14, F.G.2 incubated with affinity-purified normal dog liver α-L-fucosidase. The solvent system was propan-1-ol/acetic acid/water (3:3:2, by vol.), and the t.l.c. plate was stained with orcinol. Key to markers: M, methyl α-D-mannopyranoside from affinity chromatography on Sepharose–concanavalin A; F, fucose; S, sucrose in lysosomal fraction.

samples (Fig. 2, tracks 3a, 4a and 5a). However, minor bands of cross-reacting material could be seen faintly in normal dog enzyme tracks. These may be due to the presence of the enzymically inactive contaminants in the antigen preparation, previously observed by immuno-diffusion. Immunoblotting of a duplicate SDS/poly-acrylamide-gel electrophoretogram with anti-(human α-fucosidase) IgG gave an identical result except that the minor cross-reacting bands were not detected. Thus the immunochemical data indicate that there is no detectable cross-reacting material corresponding to the polypeptides of the normal enzyme present in fucosidosis dog liver.

Digestion of storage products by dog liver α-fucosidase

Two purified fucoglycoconjugates F.G.1 and F.G.2 (Fig. 5), which had been isolated from an affected dog brain, were incubated with various preparations of liver α-L-fucosidase. This resulted in their partial digestion, albeit slowly (Fig. 6). The greatest breakdown of both substrates occurred with the crude tissue extract, but the presence of all the lysosomal glycosidases in such reactions precluded analysis of the steps in the catabolic pathway. A discrete intermediate, not F.G.1, was observed in all the digestions of F.G.2. Its mobility suggested that more than one residue had been removed from the substrate. F.G.1 and F.G.2 were also incubated with the purified liver α-L-fucosidase. No hydrolysis of F.G.1 was detected and only approx. 5–10 % of F.G.2 was degraded after incubation at 37 °C for 65 h. The digestion mixture of F.G.2, which contained the same intermediate produced by the crude enzyme, was analysed by fast-atom-bombardment m.s. after peracetylation. The molecular ion and fragment ions observed for the undigested substrate were all detected. In addition, a signal at m/z 2058, corresponding to the fully per-acetylated pseudomolecular ion $(M+H^+)$ with the com-position FucHex$_3$(HexNAc)$_3$-O-acetate, was prominent. This indicates the loss of asparagine from the reducing end and one fucose residue during the digestion. The detection of an A-type fragment ion, m/z 618, corre-sponding of HexHexNAc shows that it is the peripheral (1–3)-linked fucose at the non-reducing end that has been removed. The loss of fucose and asparagine is consistent with the mobility of the intermediate observed by t.l.c.

To test whether the specificity of the enzyme was related to its state of aggregation, α-L-fucosidases I and II separated on DEAE-cellulose were incubated sepa-rately with F.G.2. Although comparable activity towards the synthetic substrate was used in both digestions, hydrolysis was only observed with α-L-fucosidase I. A time course over 6 days showed the progressive accumulation of the same intermediate produced in digestion mixtures with the purified or crude enzyme.

DISCUSSION

Canine liver α-L-fucosidase was purified to apparent homogeneity by affinity chromatography on agarose-ε-aminohexanoyl-fucopyranosylamine. The purified enzyme exists in multiple active forms, which can be separated by ion-exchange chromatography, gel filtration and electrophoresis. The basis of this heterogeneity appears to be the existence of a group of active subunits of 45–50 kDa, which can aggregate in different com-binations to give polymeric forms, predominantly dimers.

The molecular nature of the microheterogeneity of the subunits is not known, but it is probably due to differences in glycosylation or partial proteolysis of a common polypeptide rather than multiple gene products, because all the forms are deficient in fucosidosis. The differences in the kinetics, thermal stability and isoelectric points of the multiple forms of human liver α-L-fucosidase have been attributed to the existence of at least two distinct subunits (Chester et al., 1977; Alhadeff & Andrews-Smith, 1980; Chien & Dawson, 1980).

There is negligible residual α-L-fucosidase in the tissues of a dog with fucosidosis, as is the case in human fucosidosis. Two techniques were used to investigate whether this deficiency was due to a lack of synthesis of α-L-fucosidase protein. Firstly, an extract of the liver of the affected dog was taken through the same purification procedure used to isolate the enzyme from normal liver. Secondly, antiserum was raised against purified canine liver α-L-fucosidase for immunological detection of cross-reacting material in fucosidosis tissues. Both techniques were unable to detect, in tissues from the affected dog, the polypeptides that constitute the enzyme in normal dog liver. This suggests either that α-L-fucosidase proteins are not synthesized or that an unstable mutant protein is produced that is rapidly degraded. Cross-reacting material is also not detectable in most of the cases of human fucosidosis that have been investigated in this way (Alhadeff et al., 1975; MacPhee & Logan, 1977; Robinson & Thorpe, 1978). Andrews-Smith & Alhadeff (1982) showed by a very sensitive radio-immunoassay that there was less than 1 % of the normal protein in the liver of a patient with fucosidosis. This residual enzyme has different kinetic and physio-chemical properties (Alhadeff, 1981). More recently, it has been found that the fibroblasts of some patients with no detectable α-L-fucosidase activity synthesize normal amounts of a precursor that is not processed to the mature form (Johnson & Dawson, 1985). This demon-stration of molecular heterogeneity in human fucosidosis is consistent with the previously reported clinical and biochemical variation among cases. In contrast, canine fucosidosis appears to be homogeneous, as all the known affected dogs are closely related (C. Barker & B. Winchester, unpublished work).

Digestion of the storage products F.G.1 and F.G.2 (Fig. 5) in vitro with various preparations of canine α-L-fucosidase was very slow, emphasizing the importance of the lysosomal environment for the efficient turnover of glycoproteins. The state of aggregation may also be important as the polymeric form, I, had greater activity towards the natural substrates. Similar results have been obtained for other mammalian α-L-fucosidases acting in vitro on natural substrates (Wiederschain & Rosenfeld, 1971; Dawson & Tsay, 1977; Thorpe & Oates, 1978; Chien & Dawson, 1980). Our studies using model systems to restore lysosomal enzymic activity in lysosomal storage diseases show that dispersal of storage products is much more rapid intralysosomally (Cenci di Bello et al., 1983, 1986).

The failure of the purified dog α-L-fucosidase to hydrolyse F.G.1 suggests that this molecule does not occur normally as an intermediate in the catabolic pathway, i.e. the core fucose residue is removed at an early stage. Purified human serum α-L-fucosidase also has very low activity towards this substrate (DiCioccio et al., 1982). The structure of the intermediate that

accumulates in the digestion of F.G.2 by the purified dog enzyme suggests that the peripheral (1–3)-linked fucose residue is removed preferentially and that this preparation of α-fucosidase also has aspartoamido hydrolase activity. Further, as the same compound was the only detectable intermediate in digestions of F.G.2 with crude tissue extracts, the removal of the core (1–6)-linked fucose must be the rate-limiting step in the pathway, and be a prerequisite for further breakdown at the non-reducing terminal. The resulting fucose-free oligosaccharide must be broken down rapidly by the exoglycosidases. The catabolism of fucoglycoconjugates provides further evidence for the bidirectional pathway for the catabolism of N-glycans (Abraham et al., 1983; Kuranda & Aronson, 1986; Baussant et al., 1986). The nature of the enzymic activity in pure α-L-fucosidase that hydrolyses the aspartamido linkage is puzzling. Perhaps a single activity or two physically linked activities that co-purify by affinity chromatography are responsible for hydrolysing the core fucosidic linkage and aspartamido linkage. The activity would be specific for an extended oligosaccharide–asparagine because F.G.1 is not hydrolysed. It is tempting to speculate that the protein without α-L-fucosidase activity that is co-purified with the enzyme may have a role in this step.

The great similarity between dog and human liver α-L-fucosidases (including immunological cross-reactivity) and between the enzymic defects in canine and human fucosidosis make the animal disease an excellent model for studying the human disorder and mammalian glycoprotein catabolism.

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