Characterization of mouse liver α -L-fucosidase

Demonstration of unusual basic isoelectric forms of the enzyme that appear to be developmentally regulated

Lisa D. LAURY-KLEINTOP,* Ivan DAMJANOV† and Jack A. ALHADEFF*‡

*Department of Chemistry, Lehigh University, Bethlehem, PA 18015, and †Department of Pathology and Laboratory Medicine, Hahnemann University School of Medicine, Philadelphia, PA 19102, U.S.A.

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Mouse tissues contain unusual basic isoelectric forms of α -L-fucosidase (with approximate isoelectric points of 8.3 and 9.0) in addition to the usual acidic and neutral forms previously described in tissues of other species. These unusual forms are very prominent in placenta and foetal tissues and comprise approx. 50–80% of total activity up to 11 days of postnatal development. By 15 days of postnatal development, the basic forms are diminished in amount and comprise not more than 25% of total activity. Neuraminidase treatment of adult mouse liver α -L-fucosidase led to significantly decreased amounts of acidic forms and increased amounts of the basic forms, suggesting that these forms are chemically related at least in part by sialic acid residues. Comparative kinetic studies on mouse liver, human liver and mouse placental α -L-fucosidases indicated that they have the same K_m (0.05–0.06 mM) for 4-methylumbelliferyl α -L-fucopyranoside but different pH optima and thermostability properties. Mouse liver α -L-fucosidase has one pH optimum (5.5) and an acidic shoulder (centred around pH 4.0) compared with two distinct optima (4.3 and 6.8) for the human liver enzyme. Mouse placental α -L-fucosidase has a pH-activity curve comparable with that of the mouse liver enzyme except that the acidic shoulder is absent. Mouse liver α -L-fucosidase is considerably more thermolabile after preincubation at 50°C than are the human liver and mouse placental enzymes, which gave similar thermodenaturation curves. Immunochemical studies indicated that mouse and human α -L-fucosidases are dissimilar antigenically but exhibit some cross-reactivity. The IgG fraction of antibody prepared in goat against human liver α -L-fucosidase was ineffective by itself in immunoprecipitating mouse liver α -L-fucosidase, but 63% and 72% of the mouse liver and placental enzymes respectively could be immunoprecipitated in the double-antibody experiments under conditions that immunoprecipitated 92% of the human liver enzyme.

The lysosomal hydrolase α -L-fucosidase (α -L-fucoside fucosylhydrolase, EC 3.2.1.51) has been studied in many mammalian tissues (reviewed in Alhadeff & O'Brien, 1977). The requirement for this enzyme in the degradation of fucose-containing glycoconjugates in humans is evidenced by the rare and fatal neurovisceral storage disease fucosidosis (Van Hoof, 1973). Natural substrates of α -L-fucosidase include both hydrophilic (e.g. oligosaccharides, glycopeptides) and hydrophobic (glycolipids) compounds with fucose in various α -glycosidic linkages to galactose, *N*-acetylgalactos-

amine and *N*-acetylglucosamine (Flowers, 1981). Although the existence of such diverse substrates suggests the presence of more than one mammalian α -L-fucosidase, only one α -L-fucosidase has been documented.

Most studies on mammalian α -L-fucosidase have demonstrated the presence of several (four to eight) electrophoretic forms of the enzyme with isoelectric points ranging from 4.5 to 7.5 (Alhadeff & O'Brien, 1977). Numerous investigators have given evidence that the isoelectric forms of α -L-fucosidase are related to one another at least in part by sialic acid residues (Turner *et al.*, 1974; Alhadeff *et al.*, 1975a,b, 1978; Thorpe & Robinson, 1975; DiMatteo *et al.*, 1976; Alhadeff & O'Brien, 1977). In the

‡ To whom correspondence and requests for reprints should be addressed.

present investigation the occurrence of unusual basic isoelectric forms of mouse α -L-fucosidase was documented. Mouse liver contains, in addition to the usual acidic and neutral forms found in tissues of most species examined to date, isoelectric forms with approximate pI values of 8.3–9.0. The presence of these unusual forms suggests that the mouse enzyme may be different from previously described mammalian α -L-fucosidases. Accordingly, mouse α -L-fucosidase was characterized kinetically, with regard to when and to what extent its unusual basic forms are expressed during development, and with regard to its antigenic relatedness to human liver α -L-fucosidase by using antibodies previously prepared in goat against purified human liver α -L-fucosidase (Andrews-Smith & Alhadeff, 1982).

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 as the standard. Absorbance was read on a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer. Tissues were obtained from Swiss-Webster mice and from autopsied humans (whose livers appeared normal upon gross pathological examination). The foetal mouse tissues were derived from timed pregnancies, counting the day of copulation as day zero. All tissues were stored at –20°C until utilized. All procedures involving human tissues were approved by an institutional Committee on Investigations/Activities Involving Human Subjects.

Tissues (0.5–1.0g) were homogenized 1:5 (w/v) with 10mM-sodium citrate buffer, pH 5.0, containing 0.02% NaN₃ in a Potter-Elvehjem tissue grinder, and the homogenates were centrifuged at 40000g for 40min. The resulting pellet was resuspended with the 10mM-citrate buffer, re-centrifuged as above, and the original supernatant fluid and the washed resuspended pellet were assayed for α -L-fucosidase activity under conditions of linearity essentially as previously described (Alhadeff & O'Brien, 1977) at 37°C with 1.0mM-4-methylumbelliferyl α -L-fucopyranoside (Koch-Light Laboratories, Colnbrook, Buck., U.K.) as substrate. Fluorescence was read on a Turner model 111 fluorimeter. A unit of activity is defined as the amount of enzyme that hydrolyses 1nmol of substrate/min at 37°C. All reported α -L-fucosidase activities have been corrected by subtracting appropriate tissue and substrate blanks.

Isoelectric focusing

Isoelectric focusing was performed at 2–4°C in a 40ml column essentially as previously described

(Alhadeff *et al.*, 1975b), with 2% (v/v) ampholytes (pH range 5–8; LKB Produkter, Stockholm, Sweden) and a 0–67% (w/v) sucrose gradient. Electrofocusing was conducted at starting amperages of 1.5–2.0mA (and 600V) for 15–18h, after which 0.4ml fractions were collected. The pH value of each fraction was determined at 2–4°C with a Beckman 3500 digital pH-meter, and 50 μ l samples of each fraction were assayed for 30min at 37°C for α -L-fucosidase activity. Isoelectric focusing was performed on supernatant fluids containing 4–10 units of α -L-fucosidase activity from (1) mouse foetuses of various gestational ages (11–17days), (2) mouse placenta (17days), (3) foetal mouse liver (17days gestation), (4) mouse livers (1–15days postnatal) and (5) adult mouse liver ($\geq$ 35days postnatal). In addition, isoelectric focusing was performed on 1.0–4.0ml of mouse placental (17days) and adult mouse liver supernatant fluids (containing 16–23 units of α -L-fucosidase activity) preincubated at 37°C for 8 or 12h with 10 units of proteinase-free (<0.02% proteinase activity relative to neuraminidase activity) neuraminidase activity (3.2mg; type VI, *Clostridium perfringens*; Sigma Chemical Co., St. Louis, MO, U.S.A.) or preincubated at 37°C for 12h in citrate buffer alone (as a control). The preincubated samples were centrifuged at 12000g for 15min to remove denatured particulate matter, and the resulting supernatant fluids (which contained all the α -L-fucosidase activity) were subjected to electrofocusing. The average recovery of enzyme activity after focusing was 63%.

The relative amounts of α -L-fucosidase activity associated with the basic isoelectric forms (above an approx. pI value of 7) and the more-acidic isoelectric forms (below an approx. pI value of 7.0) were determined by cutting out the respective portions of the profiles and weighing them on a Mettler analytical balance.

Kinetic studies

Comparative kinetic studies were performed on α -L-fucosidase derived from mouse placenta (17days), adult mouse liver and adult human liver supernatant fluids. Michaelis constants (K_m values) for 4-methylumbelliferyl α -L-fucopyranoside were determined graphically by the direct-linear-plot method of Eisenthal & Cornish-Bowden (1974): 0.01 unit of α -L-fucosidase activity was incubated in duplicate with 40 μ l of 0.25M-sodium citrate buffer, pH 5.5, containing 0.02% NaN₃ and 100 μ l of 4-methylumbelliferyl α -L-fucopyranoside (in the citrate buffer) at final concentrations ranging from 0.01 mM to 0.5 mM for 10min at 37°C.

The pH-activity curves were determined with 0.01 unit of α -L-fucosidase activity incubated with

30 μ l of 0.75M buffer (sodium citrate buffer for pH range 2.8–6.2; sodium phosphate buffer for pH range 5.8–8.2) of different pH values and 100 μ l of 4-methylumbelliferyl α -L-fucopyranoside (1mM dissolved in distilled water) for 20min at 37°C. Reactions were performed in duplicate, and the actual pH of the tissue blanks for each pH buffer was determined with a Beckman 3500 digital pH-meter.

Thermostability curves were determined on α -L-fucosidase activity after preincubation for up to 5.5 h at 50°C. Each time point was run in duplicate, with comparable α -L-fucosidase activity (0.2 unit) and comparable amounts of protein (2 mg) in a total volume of 50 μ l. Protein concentrations were normalized with exogenous human serum albumin (fraction V; Sigma Chemical Co.) in 0.1M-sodium citrate, pH 5.0, containing 0.02% NaN_3 (citrate buffer). After preincubation 100 μ l of 4-methylumbelliferyl α -L-fucopyranoside (1mM dissolved in citrate buffer) was added to the reaction tubes, and the tubes were incubated at 37°C for 30min.

Immunochemical studies

Immunoprecipitation studies were performed on α -L-fucosidase derived from mouse placenta (17 days), adult mouse liver and adult human liver supernatant fluids by using single-antibody and double-antibody techniques. Immunoprecipitation incubation were carried out in 400 μ l microcentrifuge tubes (Cole Scientific, Calabases, CA, U.S.A.). The single-antibody experiments were performed as follows: 0.1 unit of α -L-fucosidase activity was incubated with 20 μ l of previously prepared goat anti-(human α -L-fucosidase) IgG (Ab_1) (Andrews-Smith & Alhadeff, 1982) with initial dilutions ranging from 1:10 to 1:500000. To the enzyme preparation/ Ab_1 mixture were added 60 μ l of citrate buffer containing human serum albumin (10mg/ml) and enough additional citrate buffer to yield a total volume of 200 μ l. The contents of the tubes were mixed and incubated for 16–17h at 2–4°C.

The double-antibody experiments were performed as follows: 0.1 unit of α -L-fucosidase activity was preincubated for 6–7h with 20 μ l of Ab_1 (with initial dilutions ranging from 1:10 to 1:500000), 60 μ l of citrate buffer containing human serum albumin (10mg/ml) and enough additional citrate buffer to yield a total volume of 195 μ l. After preincubation, 5 μ l of Ab_{11} [rabbit anti-(goat IgG) antibody; Research Products International Corp., Elk Grove Village, IL, U.S.A.] was added, and the tubes were mixed and incubated for 16–17h at 2–4°C. The amount of Ab_{11} used was determined from preliminary experiments optimizing the degree of immunoprecipitation (results not shown).

After the single-antibody and double-antibody incubations, the immunoprecipitation mixtures were centrifuged at 48000g for 30min. Samples (20 μ l) of the supernatant fluids were removed and assayed in duplicate for α -L-fucosidase activity.

Results

Preliminary fractionation studies on several mouse livers indicated that approx. 83% of the recovered α -L-fucosidase activity after homogenization and centrifugation was found in the supernatant fluid. All subsequent studies were carried out on this supernatant-fluid activity.

Fig. 1(a) gives the results of isoelectric focusing of α -L-fucosidase activity from adult mouse liver supernatant fluid. Approx. 78% of the total enzyme activity is found between pI values of 4 and 7 (see Table 1) and is represented by four isoelectric forms. The more-basic activity above pI 7 represents approx. 22% of the total activity, and is made up of three isoelectric forms with approximate pI values of 7.0, 8.3 and 9.0.

Fig. 1. Isoelectric-focusing profile of α -L-fucosidase from adult mouse liver supernatant fluid (a) and from 12h neuraminidase-treated adult mouse liver supernatant fluid (b)

See the Materials and methods section for details.

Table 1. *Relative distribution of mouse α -L-fucosidase activity in the isoelectric-focusing profiles*

Sample	Relative distribution (% of recovered activity)	
	Below pI 7	Above pI 7
Adult mouse liver		
Without neuraminidase treatment	78	22
After 8 h incubation with neuraminidase	26	74
After 12 h incubation with neuraminidase	25	75
After 12 h incubation with citrate buffer	76	24
Postnatal mouse liver		
15-day	76	24
11-day	54	46
7-day	46	54
1-day	49	51
Foetal mouse tissue		
17-day foetal liver	52	48
15-day foetus	34	66
13-day foetus	32	68
11-day foetus	27	73
Mouse placenta (17-day)		
Without neuraminidase treatment	23	77
After 12 h incubation with neuraminidase	14	86

Fig. 1(b) depicts the results of isoelectric focusing of α -L-fucosidase activity from a portion of the same mouse liver as in Fig. 1(a) but after treatment with neuraminidase for 12 h at 37°C. The neuraminidase treatment produced a greatly altered α -L-fucosidase profile with decreased amounts of enzymic activity at acidic pI values and increased amounts above a pI value of 7 (approx. 75% of the total activity compared with 22% for the untreated liver preparation). The neuraminidase-treated α -L-fucosidase has three resolved isoelectric forms with approximate pI values of 6.4, 7.5, and 8.7. Neuraminidase treatment for 8 h at 37°C gave comparable results (see Table 1). In addition, a control experiment indicated that the isoelectric-focusing profile of α -L-fucosidase activity preincubated for 12 h at 37°C with citrate buffer alone was similar to that in Fig. 1(a) (with approx. 76% of the activity below a pI value of 7).

Isoelectric focusing was performed on supernatant-fluid α -L-fucosidase from mouse placenta, mouse foetuses of various early gestations and mouse liver at various developmental time points (late prenatal and postnatal) to assess whether the enzyme's isoelectric profile changes during development. The relative distribution of α -L-fucosidase activity above and below pI 7.0 determined from the profiles is summarized in Table 1, and representative profiles are given in Fig. 2 for 17-day mouse placenta (Fig. 2a), 17-day foetal mouse liver (Fig. 2b) and 11-day postnatal mouse liver (Fig. 2c). Each of these profiles appears to contain eight or nine isoelectric forms with pI values

ranging from about 4 to 9. Inspection of Fig. 2 and the data in Table 1 indicates that there is a trend of decreasing amounts of the basic forms with pI values above 7 (and a concomitant increase in amounts of acidic forms) as mouse development proceeds. Mouse placenta has 77% of its α -L-fucosidase activity with pI values above 7 (Fig. 2a), a finding very similar to that for the distribution of activity for the neuraminidase-treated mouse liver α -L-fucosidase. After neuraminidase treatment at 37°C for 12 h, mouse placenta supernatant fluid has almost all (86%) of its α -L-fucosidase activity with pI values above 7, associated with one major form at a pI value of 8.0. Up to 11 days postnatal development, approx. 50% of mouse liver α -L-fucosidase activity is associated with forms having pI values above 7. By 15 days postnatal development, the distribution of mouse liver α -L-fucosidase activity has become very similar to that of the adult liver, with approx. 25% of its activity associated with forms with pI values above 7. Mouse foetuses were used for the early prenatal time points (11–15 days; see Table 1) since liver could not be dissected from these small foetuses in large enough quantities and of assured purity.

K_m determinations performed with 4-methylumbelliferyl α -L-fucopyranoside as substrate resulted in comparable values for supernatant-fluid α -L-fucosidase derived from adult human liver (0.05 ± 0.01 mM, mean $\pm$ S.D.), adult mouse liver (0.06 ± 0.01 mM) and 17-day mouse placenta (0.06 ± 0.01 mM).

The pH-activity curves for human liver, mouse

Fig. 2. Isoelectric-focusing profile of supernatant-fluid α -L-fucosidase from 17-day mouse placenta (a), 17-day foetal mouse liver (b) and 11-day postnatal mouse liver (c). See the Materials and methods section for details.

liver and mouse placental α -L-fucosidases are shown in Figs. 3(a), 3(b) and 3(c) respectively. There were no noticeable pH buffer effects (between the citrate and phosphate buffers) for the mouse and human enzymes. The human enzyme has a broad pH-optimum range with $\geq 50\%$ maximal activity between pH 3.4 and 7.3. The pH-optimum range is not as broad for mouse fucosidase, with $\geq 50\%$ maximal activity between pH 4.6 and 7.0. The human enzyme has two distinct pH optima, with the first at approx. pH 4.3 and the second at approx. pH 6.8. The mouse liver enzyme has only one major optimum, centred around pH 5.5, and an acidic shoulder, centred around

Fig. 3. pH-activity curves of supernatant-fluid α -L-fucosidase from adult human liver (a), adult mouse liver (b) and 17-day mouse placenta (c). See the Materials and methods section for details.

Fig. 4. Thermostability curves of supernatant-fluid α -L-fucosidase from adult human liver (●), adult mouse liver (○) and 17-day mouse placenta (▲)

See the Materials and methods section for details.

pH 4.0. Mouse placental α -L-fucosidase has a pH-activity curve comparable with that of the mouse liver enzyme except that the acidic shoulder centred around pH 4.0 is absent (Fig. 3c).

Thermostability curves for human and mouse liver and mouse placental α -L-fucosidases are depicted in Fig. 4. These curves were generated after preincubation at 50°C, with comparable amounts of enzymic activity normalized to constant protein concentration by the addition of exogenous human serum albumin. The curves for human liver and mouse placental α -L-fucosidases are biphasic, with initial rapid activity losses of 35% and 50% respectively in the first 10 min followed by little loss (5–10%) in the next 320 min. The curve for the mouse liver enzyme appears to be triphasic, with an initial activity loss of 40% in the first 10 min, an additional 30% in the next 50 min and an additional 10–15% in the next 270 min. Semi-logarithmic plots of the data (not shown) confirmed the biphasic and triphasic nature of the curves.

Single-antibody and double-antibody immunoprecipitation studies were employed to assess the antigenic relatedness of human liver, mouse liver and mouse placental α -L-fucosidases. In the single-antibody experiments, which employed the IgG fraction of antibody (Ab_1) previously prepared against the human liver enzyme (Andrews-Smith & Alhadeff, 1982), little (not more than 8%) of the mouse liver α -L-fucosidase activity could be immunoprecipitated under conditions that immunoprecipitated approx. 80% of human liver α -L-fucosidase (results not shown). In the double-antibody experiments, which employed Ab_1 and a commercial antibody (Ab_{11}) prepared against goat IgG, approx. 63% and 72% of mouse liver and placental α -L-fucosidases respectively could be

Fig. 5. Double-antibody immunoprecipitation curves of supernatant-fluid α -L-fucosidase from adult human liver (●), adult mouse liver (○) and 17-day mouse placenta (▲)

See the Materials and methods section for details.

immunoprecipitated under conditions that immunoprecipitated approx. 92% of human liver α -L-fucosidase (Fig. 5).

Discussion

Numerous investigations have found only one mammalian α -L-fucosidase, which exists in multiple electrophoretic forms with pI values ranging from 4.5 to 7.5 (reviewed in Alhadeff & O'Brien, 1977). Rat liver contains about five or six forms with approximate pI values of 4.2 to 6.4 (Opheim & Touster, 1977), and human liver contains seven or eight forms with pI values of 5.0 to 6.7 (Alhadeff & Andrews-Smith, 1980). A previous study has given genetic evidence that a variant (Afuc-2) of somatic mouse tissue α -fucosidase (Afuc-1) exists in mouse spermatozoa, but this potentially different fucosidase was not characterized electrophoretically (Self *et al.*, 1978). In the present study, basic isoelectric forms of α -L-fucosidase (with pI values of 8.3 and 9.0) have been found in mouse foetuses, mouse placenta and mouse liver, suggesting the occurrence of a second mammalian α -L-fucosidase. To our knowledge, the camel is the only other organism that has been shown to contain such basic forms of α -L-fucosidase (Watkins & Alhadeff, 1981).

Isoelectric focusing has been employed to determine the relative abundance of the basic forms of α -L-fucosidase during development of the mouse. These basic forms constitute approx. 70–80% of the total enzymic activity in mouse placenta and mouse foetuses (11–15 days) and approx. 50% of the total activity in mouse livers from older foetuses and newborn pups up to approx. 11 days

postnatally. Mouse livers sampled on day 15 *post partum* show the same distribution of activity as the adult mouse liver, with the basic forms comprising about 25% of the total activity. It thus appears that the relative amount of the acidic forms of α -L-fucosidase increases during prenatal and postnatal development. This finding is similar to previous findings in human liver in which the neutral forms of α -L-fucosidase were converted into more acidic forms as development proceeded (Alhadeff *et al.*, 1975b). It is likely that the developmental change in mouse liver α -L-fucosidase is due at least in part to the addition of sialic acid residues, as has been demonstrated for the human liver enzyme (Alhadeff *et al.*, 1978), since treatment of adult mouse α -L-fucosidase with proteinase-free neuraminidase converted two-thirds of the acidic activity into basic forms with pI values greater than 7. However, it should be emphasized that none of the human tissues previously investigated (including placenta, foetal livers of various gestational ages and 12 h postnatal liver) contained forms of α -L-fucosidase with pI values above 7 (Alhadeff *et al.*, 1975b; Alhadeff & O'Brien, 1977).

Comparative kinetic and immunochemical studies were carried out on mouse and human liver α -L-fucosidases as well as on mouse placental α -L-fucosidase (which contains a preponderance of basic isoelectric forms) to determine whether any differences between the human and mouse enzymes are due to the presence of the basic forms that are developmentally expressed. Although the K_m values for the 4-methylumbelliferyl substrate were comparable (0.05–0.06 mM) for the fucosidases, significant differences were found for their pH-activity curves. The mouse liver enzyme had considerably less activity than the human liver enzyme at acid pH values (3.0–4.5) and one major optimum (pH 5.5) and a minor acidic shoulder (pH 4.0) compared with two distinct optima for human liver α -L-fucosidase (pH 4.3 and 6.8). The fact that mouse placental α -L-fucosidase had a pH-activity curve comparable with that of the mouse liver enzyme but missing the acidic shoulder (centred around pH 4.0) suggests that this shoulder is due to the more-acidic isoelectric forms found in abundance in adult mouse liver α -L-fucosidase but deficient in the placental enzyme. The pH-activity curve and pH optimum of mouse liver α -L-fucosidase are quite similar to the pH optima of mouse spermatozoal α -L-fucosidase (major and minor peaks at pH 5.4 and 3.5 respectively) (Self *et al.*, 1978) and to the pH-activity curve and optimum (5.5–5.8) previously found for rat liver α -L-fucosidase (Opheim & Touster, 1977), even though the rat enzyme did not contain any basic isoelectric forms.

Significant differences were also found between human liver and mouse liver α -L-fucosidase with regard to their thermostability curves. The multiphasic nature of these curves suggests that both human and mouse α -L-fucosidases consist of more than one molecular species with differing thermostability properties. It is clear that mouse liver α -L-fucosidase is considerably more thermolabile than the human enzyme. The thermostability differences between mouse and human liver α -L-fucosidases do not appear to be due to the presence of the basic forms in the mouse, since the thermostability curve of the mouse placental enzyme was quite similar to that of the human liver enzyme.

The most compelling evidence that mouse α -L-fucosidase may be different from human and other mammalian α -L-fucosidases came from the immunochemical studies. Despite the fact that α -L-fucosidases have been shown to be antigenically similar across species lines (Alam & Balasubramanian, 1978; Watkins & Alhadeff, 1981), the antibody previously prepared against human liver α -L-fucosidase was ineffective by itself in immunoprecipitating mouse liver α -L-fucosidase. This suggested that these two enzymes are dissimilar antigenically. However, partial antigenic cross-reactivity was demonstrated in the more rigorous double-antibody studies, in which 63% and 72% of mouse liver and placental α -L-fucosidases respectively could be immunoprecipitated under conditions that immunoprecipitated 92% of the human liver enzyme. The unexpected finding that more of the mouse placental α -L-fucosidase could be immunoprecipitated than of the mouse liver enzyme suggests that the presence of the basic isoelectric forms in the mouse is not responsible for the antigenic differences between human and mouse liver α -L-fucosidases. It is possible that the partial cross-reactivity is due to the fact that mouse and human α -L-fucosidases share a common polypeptide chain in addition to each enzyme possessing a unique polypeptide chain. Further studies on the polypeptide composition, subunit composition and substrate specificity of purified mouse liver α -L-fucosidase are required before any definite conclusions can be made about its exact structural and functional relationship to other mammalian α -L-fucosidases.

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