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THERMOSTABILITY OF HUMAN α-L-FUCOSIDASE

RELATIONSHIP TO FUCOSIDOSIS AND LOW-ACTIVITY SERUM α-L-FUCOSIDASE

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Summary

Thermostability studies have been performed at different preincubation temperatures (37—65°C) on human α-L-fucosidases (α-L-fucoside fucohydrolase, EC 3.2.1.51), purified serum and liver enzyme, the isoelectric forms of purified liver enzyme which were separated by preparative isoelectric focusing, crude adult and fetal liver supernatant enzyme and neuraminidase-treated enzyme. Very different thermostability curves were found for the various isoelectric forms of α-L-fucosidase. The most neutral form (I) is least thermostable and the most acidic form (VIII) most thermostable, with the intervening forms (II—VII) having intermediate thermostabilities. For the isoelectric forms of liver α-L-fucosidase there appears to be a significant trend of increasing thermostability with increasing acidity (and presumably, increasing amounts of sialic acid).

In order to determine what role, if any, sialic acid plays in determining the thermostability of α-L-fucosidase, comparative thermostability studies were performed on α-L-fucosidases from different human tissues which are reported to contain varying amounts of sialic acid. The purified sialic acid-rich serum enzyme is considerably more thermostable than the purified liver enzyme. The fetal liver enzyme (which is less acidic and may contain less sialic acid than the adult liver enzyme) is less thermostable than adult liver α-L-fucosidase. In contrast to all of the above findings which suggest that sialic acid confers thermostability to α-L-fucosidase, neuraminidase treatment of human liver α-L-fucosidase did not change its thermostability, even when considerable desialylation occurred as monitored by isoelectric focusing. The reason for these apparently

Abbreviations: Nph, *p*-nitrophenyl; 4MeUmb, 4-methylumbelliferyl.

inconsistent findings is not clear at the present time but several possible interpretations of the data are given.

Introduction

The lysosomal hydrolases are important enzymes not only because of their role in the normal catabolism of glycoproteins and glycolipids [1,2] but also because deficiency or absence of their activity leads to many human genetic diseases [1—5]. Most of the lysosomal hydrolases are glycoproteins and exist in multiple molecular forms [6—7]. Many contain the negatively charged sugar sialic acid (primarily *N*-acetylneuraminic acid) which contributes to the charge heterogeneity of these enzymes [6—8].

Human α-L-fucosidase (α-L-fucoside fucohydrolase, EC 3.2.1.51) is a sialic acid-containing lysosomal hydrolase [9—15] and deficiency or absence of its activity results in the neurovisceral storage disease fucosidosis [15]. The human enzyme consists of multiple molecular forms which appear to be related, at least in part, by sialic acid residues [9—15]. In previous studies it has been shown that the various isoelectric forms of purified liver α-L-fucosidase have different pH optimum profiles and different apparent K_m values for the *p*-nitrophenyl (Nph) substrate [9] and exhibit different gel filtration behaviors [16]. In the present investigation we have extended our previous kinetic studies of the separated isoenzymes of α-L-fucosidase to a consideration of their thermostability properties. In an attempt to define what role, if any, sialic acid plays in determining the thermostability of human α-L-fucosidase, thermostability studies were also performed on different human fucosidases containing varying amounts of sialic acid [14,15] and on neuraminidase-treated α-L-fucosidase.

Methods

General. All procedures were carried out at 0—4° C unless otherwise stated. Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as standard. Agarose-ε-aminocaproyl fucosamine was purchased from Miles-Yeda and used in the affinity chromatographic purification of α-L-fucosidase. Enzyme solutions were concentratedd by ultrafiltration using Amicon con centrators with UM-10 Diaflo membranes at 50—70 lb/inch^2 or by vacuum dialysis using a collodion bag apparatus with 25 000 molecular weight retention collodion bags (Schleicher and Schuell). α-L-Fucosidase activity was assayed under conditions where activity was linear with amount of protein and time of incubation using *p*-nitrophenyl (Nph)-α-L-fucopyranoside (Sigma) or 4-methylumbelliferyl (4 MeUmb)-α-L-fucopyranoside (Koch-Light, Ltd.) essentially as previously described [15]. A unit of α-L-fucoside activity is defined as the amount of enzyme that hydrolyses 1 nmol of substrate per min at 37° C. Human adult livers were obtained at autopsy from individuals whose livers appeared normal on gross pathological inspection and human fetal liver was obtained from aborted fetuses. Tissue was stored frozen (—20° C) until used. Liver supernatants were prepared as previously described [13]. Human plasma

was purchased from the San Diego Blood Bank and sera was prepared from plasma as previously described [14]. The procedures which involved human tissues were approved by the Committee on Investigations/Activities Involving Human Subjects of the School of Medicine, University of California, San Diego.

Purification of α-L-fucosidase. α-L-Fucosidase was purified from 4.2 l human sera and 504 g human liver by affinity chromatography on agarose-ϵ-aminocaproyl fucosamine as previously described in detail (Refs. 14 and 13, respectively). The serum and liver α-L-fucosidases were purified in high yield to specific activities of 4417 and 18700 units/mg protein, respectively.

Preparative isoelectric focusing of α-L-fucosidase. Preparative isoelectric focusing was performed using a 110 ml column (LKB Produkter) on 1000 units of purified human liver α-L-fucosidase to separate the isoelectric forms essentially as previously described [9]. 1% ampholytes (pH 5—8) were used in a gradient of 0—67% (w/v) sucrose. The starting current was 2.5 mA at 400 V. Electrofocusing was conducted for 87 h after which fractions of approx. 0.8 ml were collected. For each fraction the pH was determined, α-L-fucosidase activity was assayed on 25 μl aliquots for 60 min and the separated forms were combined, dialyzed against 10 mM NaH_2PO_4, pH 5.5, containing 0.02% NaN_3 (w/v), and separately concentrated in collodion bags by vacuum dialysis. These separated isoenzymes, as well as purified liver and serum α-L-fucosidase, were used for the thermostability and other kinetic studies.

Neuraminidase treatment. Purified human liver α-L-fucosidase (37 units) and selected isoelectric forms (V and VIII, 4.1 and 0.4 units, respectively) were treated with either neuraminidase coupled to agarose (1 unit, type VI, *Clostridium perfringens*, Sigma Chemical Co.) or agarose alone (control) for 3 h at 23°C and 0.5 h at 37°C. The treated α-L-fucosidase samples were separated from the resin by centrifugation at $1500 \times g$ for 10 min and the resulting supernatants, as well as the resuspended resins, were assayed for α-L-fucosidase activity for 30 min at 37°C. Crude human liver supernatant α-L-fucosidase (100 units) was treated with soluble neuraminidase (10 units, 1.7 mg, type VI, *Clostridium perfringens*, Sigma Chemical Co.) for 24 h. at 37°C as previously described [18]. Thermostability studies were done on all these neuraminidase-treated α-L-fucosidases.

Kinetic studies. The kinetic studies were performed as previously described [9,19] on purified liver α-L-fucosidase and its separated isoenzymes. Apparent Michaelis constants (K_m values) for the 4MeUmb substrate were determined graphically by the Lineweaver-Burk method [20]. Incubations were carried out in duplicate for 30 min at 37°C in substrates of varying concentrations (0.03—0.50 mM) in 0.1 M citric acid-sodium citrate buffer (pH 5.0).

pH optima profiles using the 4 MeUmb substrate (1 mM) were determined with 0.1 M buffers (citric acid/sodium citrate for pH values 3.1—5.8; KH_2PO_4/Na_2HPO_4 for pH values 6.0—8.1) of varying pH values. All incubations were carried out in duplicate for 30 min at 37°C.

Thermostability studies were performed on purified liver and serum α-L-fucosidase after preincubation at various temperatures (37, 45, 55, 60, 65°C) for up to 2.0 h. Thermostability studies were also performed after preincubation at various temperatures (45, 55, 60, 65°C) for up to 3 h on: (1) the separated isoenzymes of purified liver α-L-fucosidase; (2) crude human adult and

fetal (gestational age: 109 days) liver supernatant α-L-fucosidase; (3) agarose-neuraminidase-treated α-L-fucosidase; (4) agarose-treated α-L-fucosidase (control) and (5) soluble neuraminidase-treated crude liver supernatant α-L-fucosidase. For the thermostability studies on the separated isolelectric forms, human serum albumin (10 mg/ml) was added to give a constant, final protein concentration of 1.3 mg/ml. After thermal preincubation, the Nph or 4MeUmb substrate was added and the samples were assayed in duplicate for 4—30 min (for purified enzyme) and for 30 min (for supernatant enzyme) at 37°C.

Results

Affinity chromatography on agarose-ε-aminocaproyl-fucosamine was employed as previously described in detail [13,14] to purify the human serum and liver α-L-fucosidases. These purified enzymes were used for the comparative thermostability studies. Purified liver α-L-fucosidase was also used as the enzyme source for separating the isoenzymes of α-L-fucosidase preparative isoelectric focusing [9].

The isoelectric profile of purified human liver α-L-fucosidase is depicted in Fig. 1. The usual seven forms, with a small amount of the most neutral form, I, were found between isoelectric points (p*I* values) of 5.2 and 6.7 [9,15,18]. An additional acidic form (VIII) centered around a p*I* of 5.0 was also resolved. The fractions representing the individual isoelectric forms were pooled, dialyzed, concentrated and kinetically characterized. Each peak has previously been shown (by starch gel electrophoresis at pH 5.9) to consist primarily of one enzymatically active form with the expected electrophoretic migration [9].

Thermostability studies were performed on purified human liver α-L-fucosidase and its separated isoenzymes. After preincubation at 45°C for up to 60 min, very different thermostability curves were found for the various forms and the whole enzyme. Fig. 2 depicts selected thermostability curves and gives the

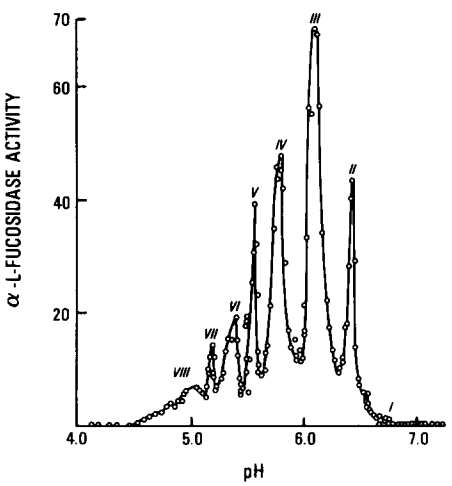

Fig. 1. Isoelectric focusing profile (pH 5—8) of purified human liver α-L-fucosidase. See Methods section for details.

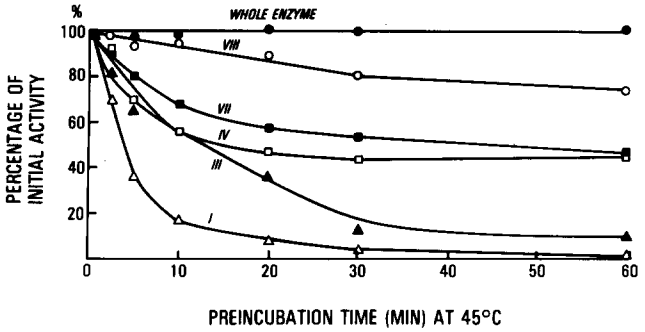

Fig. 2. Thermostability curves of purified human liver α-L-fucosidase and its separated isoelectric forms after preincubation at 45°C. See Methods section for details.

percentage of initial activity after various preincubation times for several isoelectric forms and the whole enzyme. Form I is most thermolabile and loses 100% of its activity after 60 min preincubation. Forms II and III are also quite thermolabile, losing approx. 90% of their activity after 60 min preincubation. Forms IV—VII exhibits similar thermostability curves, losing 50—60% of their activity after 60 min preincubation. Form VIII and the whole enzyme lose only 26 and 0%, respectively, of their enzymatic activity after 1 h preincubation. Table I summarizes the p*I* values and thermostability behavior for the eight isoenzymes of liver α-L-fucosidase after preincubation at 45°C. For these isoenzymes there appears to be a significant trend of increasing thermostability with increasing acidity (decreasing p*I*). Similar results were found after preincubation at 55°C for up to 60 min except that greater losses of activity were found for a given preincubation time. At this higher temperature, form I lost all of its activity after 5 min preincubation. All of the thermostability studies were done using small amounts of enzymatic activity (approx. 0.06 unit) and in the presence of a constant amount of human serum albumin to control the total protein concentration.

Comparative thermostability studies were performed on purified human serum and liver α-L-fucosidase at various preincubation temperatures (37, 45,

TABLE I

THERMOSTABILITY OF ISOENZYMES OF PURIFIED HUMAN LIVER α-L-FUCOSIDASE

α-L-Fucosidase isoenzyme	Isoelectric point (p*I*)	Percentage of initial activity after preincubation at 45°C for			
		2.5 min	5.0 min	30.0 min	60.0 min
I	6.7	77	36	3	0
II	6.4	71	64	18	9
III	6.1	82	65	11	9
IV	5.8	92	69	43	45
V	5.6	79	78	41	41
VI	5.4	91	80	42	38
VII	5.2	90	80	54	46
VIII	5.0	98	94	80	74
Whole enzyme	—	98	97	100	101

55, 60, 65°C). No thermostability differences were seen after preincubation at the lower temperatures (37, 45°C) with both enzymes retaining 100% of their activity after preincubation for up to 120 min. However, with increasing preincubation temperatures (55, 60, 65°C) increasingly significant differences were found, with the serum α-L-fucosidase exhibiting increased thermostability when compared to the liver enzyme (Fig. 3). At the highest preincubation temperature of 65°C, liver α-L-fucosidase loses approx. 80% of its initial activity after 60 min preincubation compared to about only 50% for the serum enzyme.

Fig. 4 depicts thermostability curves after preincubation at 60°C for crude supernatant α-L-fucosidase from two adult and one fetal (109 days gestation) human liver. For each of the preincubation time points, fetal liver α-L-fucosidase is less thermostable than the adult liver enzyme. After 30 min thermal preincubation (and for every time point thereafter), adult α-L-fucosidase retains 15—25% more of its initial activity than the fetal α-L-fucosidase.

Thermostability studies were performed on neuraminidase-treated and buffer-treated (control) samples of purified human liver α-L-fucosidase, selected isoelectric forms (V, VIII) and crude human liver supernatant α-L-fucosidase. Recovery of α-L-fucosidase activity was comparable for neuraminidase-treated samples and their corresponding controls and ranged from 66 to 100%. As in previous studies [18,19], isoelectric focusing was employed to monitor the effectiveness of neuraminidase treatment. Neuraminidase treatment resulted in significantly decreased amounts of the more negative isoelectric forms (p*I* values 5.0—6.0), presumably by removal of sialic acid residues, and increased amounts of the more neutral forms (p*I* values 6.8—7.0) for only the crude supernatant α-L-fucosidase. These isoelectric profiles before and after neuraminidase treatment are very similar to those reported previously [18] and are not given here. It is not known why neuraminidase treatment did not affect the isoelectric profiles of the purified α-L-fucosidase and the two relatively acidic isoelectric forms (V, VIII). For each neuraminidase-treated sample of α-L-fucosidase, the thermostability curves obtained were essentially identical to the corresponding ones depicted in Figs. 2 and 3 for untreated α-L-fucosidase. Despite many repreated attempts, neuraminidase treatment did not appear to change the thermostability behavior of α-L-fucosidase.

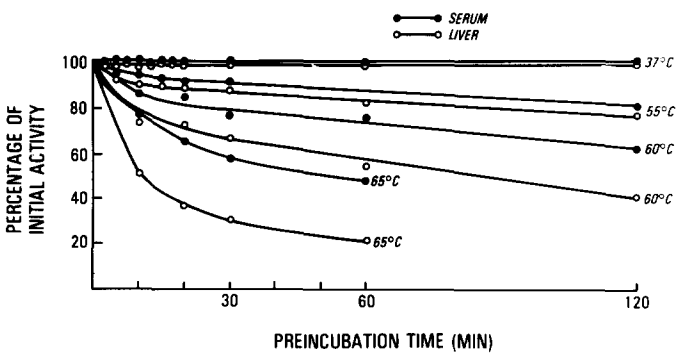

Fig. 3. Thermostability curves of purified liver and serum α-L-fucosidase after preincubation at various temperatures (37, 55, 60, 65°C). See Methods section for details.

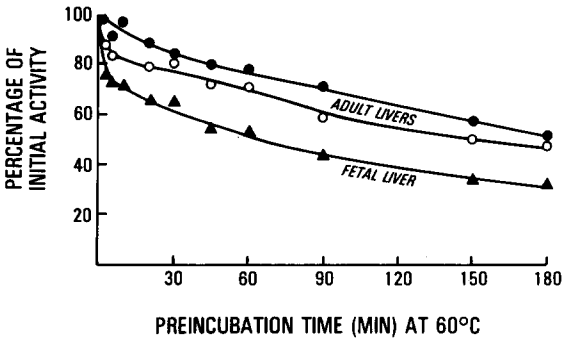

Fig. 4. Thermostability curves of crude supernatant α-L-fucosidase from adult and fetal human livers after preincubation at 60°C. See Methods section for details.

Discussion

Recently, several investigators have shown that the presence of sialic acid may serve functional roles on glycoproteins [21]. Among these roles is the protection against proteolytic attack [22], the regulation of the half-life of molecules and cells in the plasma [1,23] and the regulation of cell-surface related phenomena such as the masking of cell-surface antigens, cell-cell recognition, contact inhibition, cellular migration and malignant transformation [1,2,21,24]. The presence of sialic acid on some enzymes (e.g. alkaline phosphatase, α-galactosidase, arylsulfatase, α-L-fucosidase) has also been reported to affect such kinetic behavior as pH optimum curves, apparent Michaelis constants (K_m), maximal velocity (V) and thermostability [9,25—29]. In a previous study we have shown that the isoenzymes of purified liver α-L-fucosidase have different pH optimum profiles and different apparent K_m values for the *p*-nitrophenyl substrate [9]. In the present investigation we have extended our previous kinetic studies on the separated isoenzymes of purified human liver α-L-fucosidase. The kinetic differences previously reported using the *p*-nitrophenyl substrate [9] were also found in the present study for the 4-methylumbelliferyl substrate (data not shown): the most neutral form (I) has a higher apparent K_m and decreased activity at acidic pH values (3.0—5.0) when compared to the other isoelectric forms. In addition, significant thermostability differences were found for the isoenzymes of α-L-fucosidase. In analyzing the data (Fig. 2 and Table I), a trend of increasing thermostability with increasing acidity is apparent. The most neutral isoelectric form is least thermostable and the most acidic isoelectric form is most thermostable, with the intervening forms having intermediate thermostabilities. Since more acidic isoelectric forms of human liver α-L-fucosidase have been shown to be related to the more neutral forms, at least in part by sialic acid residues [9—15], it is possible that the increased thermostability of the more acidic isoelectric forms of α-L-fucosidase is due to the presence of sialic acid residues.

In an attempt to further define what role, if any, sialic acid has on the thermostability of α-L-fucosidase, thermostability studies were performed on fucosidases from different human tissues which are reported to contain varying amounts of sialic acid [13—15]. Although purified liver and serum α-L-fucosi-

dase have similar apparent thermostabilities at lower preincubation temperatures (37—55°C), the serum enzyme is considerably more thermostable than the liver enzyme at higher preincubation temperatures (60, 65°C). This increased thermostability of the serum enzyme may be partially due to the fact that it contains more sialic acid (1.7 μg/100 μg enzyme protein) [14] than the liver enzyme (0.96 μg/100 μg enzyme protein) [13].

Additional circumstantial evidence for the role of sialic acid in conferring increased thermostability to α-L-fucosidase was provided by study of the human fetal liver enzyme. Previous studies have shown that prior to 123—124 days fetal gestation, the human liver contains smaller amounts of acidic and greater amounts of the most neutral form(s) of α-L-fucosidase. It was suggested that sialylation of the most neutral form(s) of α-L-fucosidase might account, at least in part, for the changes on the isoenzyme pattern (i.e. increased relative amounts of acidic forms) seen during development [18]. The significantly decreased thermostability of α-L-fucosidase from a fetal liver of 109 days gestation when compared to that of the adult liver enzyme (Fig. 4) supports the idea that the presence and amount of sialic acid in α-L-fucosidase may be important in determining its thermostability behavior. Although the two adult liver thermostability curves are representative of many samples studied, the above comparative results must be interpreted with caution since only fetal liver was investigated.

In contrast to all of the above studies which suggest that sialic acid confers increased thermostability to human α-L-fucosidase, neuraminidase treatment of α-L-fucosidase did not decrease its thermostability. This is true even for the crude supernatant enzyme where it appears from isoelectric focusing profiles that considerable removal of sialic acid was achieved. This finding is similar to a previous one from our laboratory in which neuraminidase treatment of human serum α-L-fucosidase did not alter its thermostability [19]. One possible interpretation of the neuraminidase studies is that only certain sialic acid residues (perhaps with specific linkages) are involved in the thermostability of α-L-fucosidase and that these residues are not adequately hydrolyzed by the bacterial neuraminidase used in our studies. Another possible interpretation is that sialic acid residues may be only one factor which indirectly affects thermostability by changing the aggregation state of α-L-fucosidase. This is a reasonable idea since we have previously shown in gel filtration experiments that the presence of sialic acid appears to affect the three-dimensional structure of α-L-fucosidase [16], possibly through a state of aggregation. The fact that the whole unfractionated purified α-L-fucosidase was entirely stable after 60 min preincubation at 45°C whereas isoenzymes II, III and IV (which form the major part of the enzyme) lost considerable activity by the same treatment can be interpreted to mean that the aggregation state of α-L-fucosidase affects its thermostablility. Other investigators have shown that the disaggregated, low-molecular weight form of α-L-fucosidase (Fuc II) exhibits significantly decreased thermostability when compared to the aggregated, high-molecular weight form (Fuc I) [30,31]. Still another possible interpretation is that sialic acid residues have little or no effect on the thermostability of α-L-fucosidase, an interpretation which does not appear consistent with the circumstantial evidence given in the present study for the involvement of the sugar in this kinetic property.

Even if sialic acid residues are not involved in the thermostability of α-L-fucosidase, the present findings on the differential thermostability of the isoelectric forms of human liver α-L-fucosidase may be relevant for better understanding the nature of the molecular defect in fucosidosis [15] and in that group of phenotypically normal individuals who have low-activity serum α-L-fucosidase [19]. In both of these heritable conditions, the residual α-L-fucosidase activity exhibits properties (decreased thermostability and decreased activity at acidic pH values) [32—35] which are similar to those of th most neutral isoelectric form (I) of α-L-fucosidase [9,32]. In addition, α-L-fucosidases from the liver [32] and sera [33,36] of fucosidosis patients have been shown to exhibit increased apparent K_m values for synthetic substrate similar to that seen for the most neutral isoelectric form of normal liver α-L-fucosidase [9]. Furthermore, the antigenicity of α-L-fucosidase from the liver of a fucosidosis patient [32] and from low-activity serum individuals [19] is similar to that of the normal liver enzyme. This similarity of properties for these α-L-fucosidases suggests that the heritable traits leading to low-activity serum α-L-fucosidase and fucosidosis may be related and may involve retention of a selective, small portion of antigenically normal α-L-fucosidase with altered kinetic properties. Since the isoenzyme pattern of α-L-fucosidase has been shown to change during fetal development, probably as a result of sialylation [18], and since the properties of the most neutral isoelectric form of α-L-fucosidase are very similar to those of α-L-fucosidase in fucosidosis and low-activity serum, it is possible that decreased sialylation of α-L-fucosidase during development (and a consequent alteration of certain kinetic properties) may be involved in both conditions.

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