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Structural Analysis of the Carbohydrate Moieties of α-L-Fucosidase from Human Liver

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Acid α-L-fucosidase (EC 3.2.1.51) was obtained from human liver and purified to homogeneity. The enzyme consists of four subunits; each of these has a molecular mass of 50 kD_a and bears one *N*-linked carbohydrate chain. The structures of these chains were studied at the glycopeptide level by methylation analysis and 500-MHz ^{1}H-NMR spectroscopy. Oligomannoside-type chains and *N*-acetyllactosamine-type chains are present in an approximate ratio of 3:1. While the oligomannoside-type chains show some heterogeneity in size ($Man_{5-8}GlcNAc_2$), the *N*-acetyllactosamine-type chains are exclusively bi-α(2-6)-sialyl, bi-antennary in their structure.

These observations on the carbohydrate moieties of α-L-fucosidase substantiate our hypothesis [Overdijk *et al.* (1986) Glycoconjugate J 3:339-50] with respect to the relationship between the oligosaccharide structure of lysosomal enzymes and their residual intracellular activity in I-cell disease. For the series of enzymes examined so far, namely, β-*N*-acetylhexosaminidase, α-L-fucosidase and β-galactosidase, the relative amount of *N*-acetyllactosamine-type carbohydrate increases, while the residual intracellular activity in I-cell disease tissue decreases in this order. The system which is responsible for preferentially retaining hydrolases with (non-phosphorylated) oligomannoside-type chains both in I-cells and in normal cells has yet to be identified.

Acid hydrolases are a special subset of glycoproteins that are unique with respect to the oligosaccharide processing they undergo in the Golgi apparatus. Newly synthesized acid hydrolases generally acquire one or more phosphorylated mannose residues as part of their oligomannoside-type carbohydrate chains, which enables them to be recognized by the Golgi-membrane-located Man-6-phosphate receptor and to be

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localized properly, within the lysosomes. An *N*-acetylglucosaminylphosphotransferase attaches GlcNAcα-1-phosphate to the 6-position of one or more mannose residues in the common Man9GlcNAc2 structure. Subsequently, any α-GlcNAc residues are removed by a phospodiesterase, resulting in terminal Man-6-phosphate groups that act as the recognition markers [1, 2].

In I-cell disease, no phosphate groups are attached to the mannose residues due to the absence of the *N*-acetylglucosaminyl-phosphotransferase. In cells of I-cell disease patients, one would thus expect the acid hydrolases not to be routed to the lysosomes, since they cannot be recognized by the Man-6-phosphate receptors. The most likely alternative pathway for such enzymes is secretion from the cell. Despite the lack of Man-6-phosphate, however, most acid hydrolases show substantial activity within the I-cells. Only a few of the lysosomal enzymes are completely absent [3].

The difference in residual intracellular I-cell activity of the different enzymes might be due to differences in their oligosaccharide chains. We speculate that an acid hydrolase such as β-galactosidase, which is completely deficient in I-cells, should have carbohydrate chains that allow, or even favour, secretion of the enzyme from the cell. Acid hydrolases such as β-*N*-acetylhexosaminidase, which exhibit a considerable residual activity, are thought to possess carbohydrate chains that may be recognized by another system than the Man-6-phosphate receptor at the (lysosomal) membrane, and thus be retained inside the cell.

The carbohydrate structures of β-galactosidase and β-*N*-acetylhexosaminidase have been shown to be considerably different from each other [4, 5]. The majority of the chains of β-galactosidase are of the *N*-acetyllactosamine-type, whereas the chains of β-*N*-acetylhexosaminidase appeared to be almost exclusively (> 90%) of the oligomannoside type.

α-L-Fucosidase is another enzyme that, similar to β-galactosidase, is virtually absent from I-cells [3, 6]. In order to substantiate the apparent correlation between the carbohydrate structures and the residual activity of acid hydrolases in I-cell disease, we have undertaken the purification of α-L-fucosidase and the structural characterization of its carbohydrate moieties. We demonstrate here that, indeed, an appreciable part (~ 25%) of the α-L-fucosidase carbohydrate side chains have *N*-acetyllactosamine-type structures.

Materials and Methods

Isolation and Purification of α-L-Fucosidase

α-L-Fucosidase was isolated from normal human autopsy liver, stored at -40°C. The tissue (3200 g) was homogenized in water with a Polytron homogenizer. The homogenate (14.4 l) was centrifuged at 100 000 × *g* for 1 h. To the supernatant a concentrated buffer solution was added of such a composition that the final concentration equalled that of the starting buffer of the subsequent Con A-Sepharose purification step.

Con A-Sepharose chromatography was done as described earlier [7]. The column material was equilibrated in 50 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl and 20 mM of $MnCl_2$, $CaCl_2$ and $MgCl_2$. The starting buffer was the same except for the absence of $MnCl_2$, $MgCl_2$ and $CaCl_2$. The α-L-fucosidase-active fractions obtained were dialyzed against the starting buffer of the subsequent affinity chromatography step. Affinity chromatography, using agarose-ε-aminocaproyl-fucosamine as affinity matrix, was performed as described by Alhadeff *et al.* [8], using a 10 mM sodium phosphate buffer, pH 5.5 as starting buffer and a 100 mM α-L-fucose solution in this buffer as eluant.

α-L-Fucosidase activity was determined with 4-methylumbelliferyl-α-L-fucoside as substrate [9]. Sodium dodecylsulphate-polyacrylamide gel electrophoresis was done as described [7].

Structural Analysis of the Carbohydrate Chains

The carbohydrate composition of α-L-fucosidase was determined by GLC after methanolysis, trimethylsilylation and re-*N*-acetylation as described earlier [7]. Pronase digestion followed by methylation analysis and 500-MHz ^{1}H-NMR spectroscopy of the intact glycopeptides obtained, were performed as described previously [5].

Materials

The following materials were supplied by the manufacturers indicated. Con A-Sepharose (Pharmacia, Uppsala, Sweden); agarose-ε-aminocaproyl-fucosamine and α-L-fucose (Sigma, St Louis, MO, USA); Pronase (Calbiochem, San Diego, CA, USA); 4-methylumbelliferyl α-L-fucoside (Koch-Light Ltd., Poole, UK). All other chemicals were of the best quality available.

Results

α-L-Fucosidase was isolated from human liver and purified to homogeneity, as demonstrated by sodium dodecylsulphate-polyacrylamide gel electrophoresis. The preparation showed a single band, namely at $M_r \sim 50\,000$. The purification factors of the various enzyme preparations obtained for structural study of the carbohydrate chains varied between 4 300- and 6 600-fold, with recoveries of activity varying from 34-39%. Finally, in a typical experiment, 23.6 mg of purified enzyme were obtained from 3 200 g of wet tissue.

Preliminary studies had already indicated that the enzyme contained 3.7% (w/w) of sugar and that, in addition to oligomannoside-type chains, considerable quantities of *N*-acetyllactosamine-type, *N*-glycosidic oligosaccharides were present, based on the amounts of galactose and *N*-acetylneuraminic acid found [10]. Fucose was found in very low amounts (< 1% of the total amount of sugar).

Methylation analysis applied to the pronase digest of α-L-fucosidase followed by GC-MS of the resulting partially methylated methyl glycosides showed (see Table 1) that the substitution patterns of the galactose, *N*-acetylglucosamine and *N*-acetylneuraminic

Table 1. Relative amounts of partially methylated, partially acetylated glycosides obtained from human liver α-L-fucosidase by methylation analysis.

Glycoside	Relative amount[a]
2,3,4,6-Me-Man	23.8
3,4,6-Me-Man	11.2
2,4,6,-Me-Man	4.8
2,4-Me-Man	19.4
2,3,4-Me-Gal	7.5
3,6-Me-GlcNAc(Me)	21.6
4,7,8,9-Me-NeuAc(Me)	8.0

[a] Other derivatives detectable in trace amounts, together accounting for the remaining 3.7% of the total amount were: 4,6-Me-Man; 2,3,4-Me-Man; 2,3,6-Me-Man and 4-Me-GlcNAc(Me).

Table 2. ^{1}H-Chemical shifts of pertinent structural-reporter groups of the monosaccharides present in the glycopeptides from human liver α-fucosidase (α-Fuc).

Reporter group	Residue	$Man_5GlcNAc_2$		$Man_{6-9}GlcNAc_2$		$NeuAc_2Gal_2GlcNAc_4Man_3$	
		α-Fuc	ref.[a]	α-Fuc	ref.[a]	α-Fuc	ref.[a]
H1	GlcNAc-**1**	5.08	5.07	5.08	5.07	5.08	5.088
	GlcNAc-**2**	4.62	4.61	4.62	4.61	4.61	4.616
	Man-**4**	5.095	5.099	5.341	5.342	5.136	5.133
	Man-**4'**	4.869	4.872	4.869	4.871	4.942	4.949
	GlcNAc-**5/5'**	—	—	—	—	4.604	4.603
	Gal-**6/6'**	—	—	—	—	4.446	4.445
	Man-**A**	5.095	5.093	5.398	5.401	—	—
	Man-**B**	4.909	4.908	5.136	5.141	—	—
	Man-**C**[b]	[5.055	5.052]	5.302	5.304	—	—
	Man-$\mathbf{D_1/D_2}$	—	—	5.055	5.059	—	—
	Man-$\mathbf{D_3}$	—	—	5.042	5.042	—	—
H2	Man-**3**	4.254	4.251	4.254		4.254	4.254
	Man-**4**	N.D.[c]	4.077	N.D.[c]		4.194	4.195
	Man-**4'**	N.D.[c]	4.144	N.D.[c]		4.115	4.116
H3ax	NeuAc	—		—		1.712[d]	1.716[d]
H2eq	NeuAc	—		—		2.672[d]	2.670[d]
NAc	GlcNAc-**1**	2.010	2.012	2.010	2.011	2.010	2.002
	GlcNAc-**2**	2.064	2.060	2.064	2.061	2.078	2.081
	GlcNAc-**5**	—		—		2.071	2.067
	GlcNAc-**5'**	—		—		2.065	2.063
	NeuAc	—		—		2.032[e]	2.030[e]

[a] Reference compounds: 62, 70 and 33 respectively from [11].
[b] Terminal Man-**C** does not occur in $Man_5GlcNAc_2$ but in a particular isomer of $Man_6GlcNAc_2$.
[c] N.D. = not determined.
[d] Signal from two protons.
[e] Signal from two methyl groups.

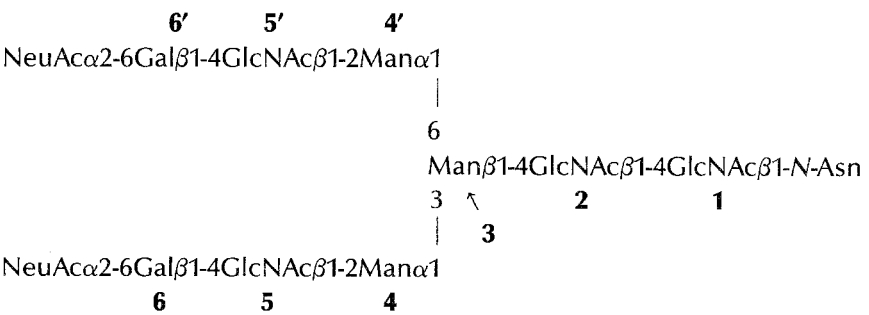
acid derivatives, along with the presence of 2-mono- and 3,6-di-substituted mannose, fitted a typical bi-antennary *N*-acetyllactosamine-type structure, in which *N*-acetylneuraminic acid was suggested to be linked to the 6-position of the galactose residues. No terminal galactose residues were found to occur, since no 2,3,4,6-Me-Gal derivative was detected, nor was there any indication of terminal *N*-acetylglucosamine or 3,4,6-trisubstituted mannose residues. The relative excess of both 2-mono- and 3,6-di-substituted mannose residues, compared to 6-substituted galactose and terminal *N*-acetylneuraminic acid (see Table 1), in conjunction with the presence of terminal mannose residues in large amounts, pointed to the additional presence of oligomannoside-type chains in the glycopeptide mixture.

In order to obtain independent evidence for these findings, the glycopeptide preparation derived from α-L-fucosidase was subjected to high-resolution ^{1}H-NMR spectroscopy. The 500-MHz ^{1}H-NMR spectrum was obtained for a 0.15 μM ^2H_2O-solution at p^{2}H 6 and 27°C. As anticipated from the results of sugar and methylation analysis, the spectrum revealed that the preparation indeed contained a mixture of *N*-acetyllactosamine-type and oligomannoside-type glycopeptides, in the approximate molar ratio of 1:3.

The chemical shifts of pertinent structural-reporter groups have been listed in Table 2. The ^{1}H-NMR spectrum revealed first of all that the carbohydrates are *N*-glycosidically linked to asparagine forming part of a short peptide chain. This was inferred from the occurrence of the H-1 and the *N*-acetyl resonances of GlcNAc-**1** at δ 5.08 and 2.010 ppm, respectively [11].

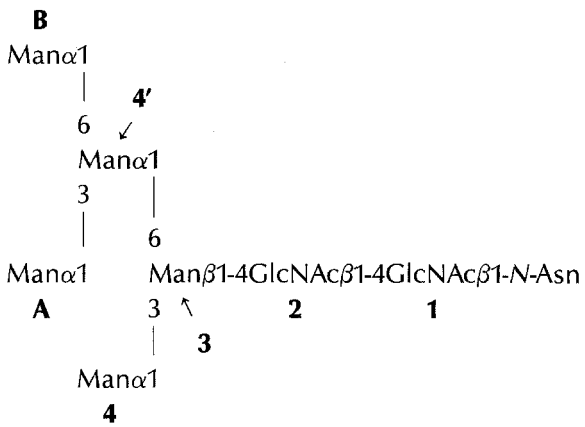
The structures of the oligomannoside- and *N*-acetyllactosamine-type components were determined by comparison of the chemical shifts of the structural-reporter groups with those of previously characterized, pure compounds [11]. This approach has been successfully applied before, *inter alia* to the structural elucidation of the glycopeptides obtained from β-*N*-acetylhexosaminidase [5] and from β-galactosidase [4].

The *N*-acetyllactosamine-type glycopeptides in the mixture appeared to be homogeneous in their carbohydrate portion. Their structure could be disclosed because those signals in the ^{1}H-NMR spectrum attributed to the *N*-acetyllactosamine-type chains matched perfectly the spectrum of the bi-sialyl, bi-antennary glycopeptide (see Table 2)



Thus, the signals attributable to the *N*-acetyllactosamine-type compounds indicate that there is virtually no heterogeneity in this type of chain, neither in degree of branching nor in the presence and the type of linkage of the sialic acid residues. Both branches of the bi-antennary compounds are exclusively α(2-6)-sialylated. The additional presence of monosialyl analogues of this structure in the mixture was definitely ruled out because of the absence of a signal attributable to H-1 of a terminal β-galactosyl residue (to be expected at δ 4.47 ppm) [11]. Also, no terminal *N*-acetylglucosaminyl residues were found to occur (H-1 signal to be expected at δ 4.55, but no doublet observed at that position).

The assembly of the remaining signals in the spectrum was strongly reminiscent of the previously described ^{1}H-NMR features of oligomannoside-type glycopeptides, like $Man_5GlcNAc_2$,



$Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ (compounds 62-72 in [11]). In Table 2, the chemical-shift data of the different oligomannoside-type components of the glycopeptide preparation from α-L-fucosidase are compared to the corresponding characteristics of the aforementioned reference glycopeptides [11]. In the carbohydrate portion of the oligomannoside-type compounds some heterogeneity is observed with respect to the presence of additional mannose residues α(1-2)-linked to Man-**4**, Man-**A** and/or Man-**B** (see Fig. 1). In addition to the signals compiled for $Man_5GlcNAc_2Asn$ in Table 2, low-intensity signals are observed at δ 5.341 ppm (related to Man-**4** substituted by Man-**C**), δ 5.398 (Man-**A** substituted by Man-$\mathbf{D}_2$) and δ 5.136 (Man-**B** substituted by Man-$\mathbf{D}_3$) [4, 11, 12]. The additional low intensity signal at δ 5.302 ppm indicates the presence in the mixture of a small amount of a structure containing Man-**C** substituted by Man-$\mathbf{D}_1$ [11, 12].

From the ratio of the intensities of the H-1 signals of Man-**4'**, at δ 4.869 ppm for the oligomannoside-type chains, and at δ 4.942 for the *N*-acetyllactosamine-type chains, it can be concluded that the glycopeptide mixture obtained from α-L-fucosidase consists of the above bi-antennary *N*-acetyllactosamine-type structure and the oligomannoside components in a ratio 1:3. The relative amount of $Man_5GlcNAc_2Asn$ compared to

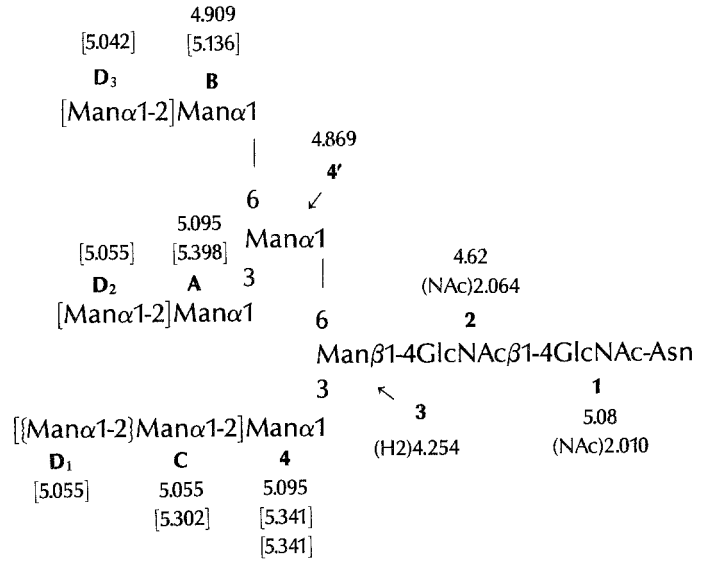


Figure 1. Comprehensive structure of the oligomannoside-type sugar chains of human liver α-L-fucosidase, along with its characteristic ^{1}H-NMR data. Chemical shift values indicated are those of the H-1 atoms (unless otherwise specified) of the residues without [and with] the pertinent substitution, respectively (compare [11]).

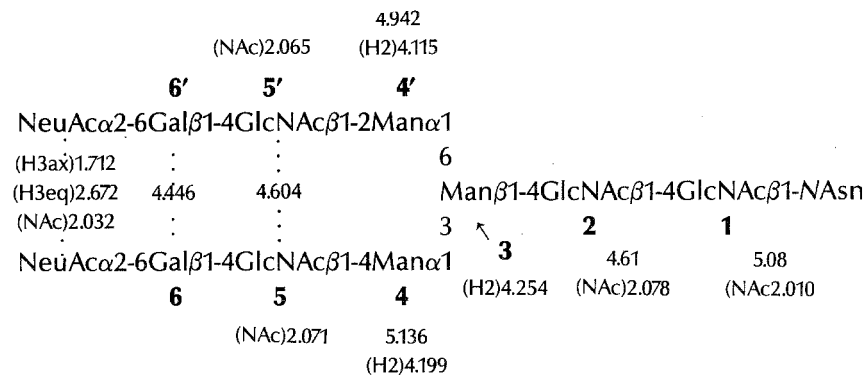


Figure 2. Structure of the *N*-acetyllactosamine-type sugar chains of human liver α-L-fucosidase, along with the characteristic ^{1}H-NMR features decisive for the structural assignment. Chemical shift values indicated are those of the H-1 atoms (unless otherwise specified) of the residue without [and with] the pertinent substitution, respectively (compare [11]).

oligomannoside-type structures larger than $Man_5GlcNAc_2Asn$ in the mixture was estimated to be 2:1, from the intensity ratio of the signals at δ 5.095 ppm on the one hand, and δ 5.341, 5.398 and 5.136 ppm on the other hand.

The finding of 2,4,6-Me-Man in the methylation analysis suggests a somewhat higher degree of heterogeneity of the oligomannoside-type chains than shown in the comprehensive structure presented in Fig. 1. However, this low amount is below the detection level of ^{1}H-NMR spectroscopy.

Discussion

The purification factor obtained for α-L-fucosidase from human liver in the present study (~ 5500 on average) is the same as that obtained by others [8, 13, 14]. The carbohydrate content of the purified α-L-fucosidase (3.7%) lies within the range given in the literature (1% [8] to 7% [13]). The reason for the differences observed in carbohydrate content is unclear. Degradation of the oligosaccharide part of the enzyme during isolation is unlikely since lysosomal α-mannosidase is not very active against oligomannoside-type glycans on glycoproteins [15]. Moreover, the intactness of the *N*-acetyllactosamine-type chains demonstrates that these chains have definitely not been degraded.

The results obtained by Alhadeff *et al.* [8] strongly suggest that α-L-fucosidase from human liver is a tetramer, with each of the subunits having a molecular weight of 50 000. Given the carbohydrate content of α-L-fucosidase to be 3.7%, each subunit is calculated to bear, on average, one glycan chain. This means that a complete tetrameric enzyme molecule contains either different subunits with respect to their carbohydrate portion, or that different pools of the enzyme exist, having either only oligomannoside-type, *N*-acetyllactosamine-type, or a mixed-type of glycosylation pattern.

A substantial part (~ 25%) of the glycan chains of α-L-fucosidase was found to be of the *N*-acetyllactosamine-type, as shown both by ^{1}H-NMR spectroscopy and by methylation analysis. The relative amount of *N*-acetyllactosamine-type chains in α-L-fucosidase is lower than in β-galactosidase [4]. This result is in line with the higher residual activity of α-L-fucosidase compared to β-galactosidase in fibroblasts from patients with I-cell disease [6].

The structures of the carbohydrate chains of lysosomal enzymes reported in the literature to date [4, 7, 16-18] raise the question why various lysosomal enzymes end up during their biosynthesis with different ratios of *N*-acetyllactosamine-type and oligomannoside-type chains. The generalization with respect to the function of these chains, that *N*-acetyllactosamine-type chains would cause/promote a glycoprotein to be excreted from the cell is at least not applicable to β-glucocerebrosidase; 80% of the carbohydrate of that enzyme consists of *N*-acetyllactosamine-type chains, while it is present in cells of normal individuals in high activity [17], as well as in fibroblasts from patients with I-cell disease [6]. It could be possible that this strongly membrane-bound enzyme is targeted *via* a Man-6-phosphate-independent mechanism, such as that proposed for the lysosomal membrane protein that was described by Krentler *et al.* [19]. Our findings on the carbohydrate structures of lysosomal enzymes in relation to I-cell disease, where the Man-6-phosphate recognition marker is not synthesized but where

the lysosomes in tissues are not (fully) depleted of most hydrolases, suggest the existence of an alternative system in the cell that is specific for non-phosphorylated oligomannoside-type chains, as has been proposed earlier [4, 20, 21]. The identification of such a system in I-cell patients and in normal individuals is a challenge for future research.

Acknowledgements

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