Oligosaccharides from placenta: early diagnosis of feline mannosidosis

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High-pressure liquid chromatography analysis of oligosaccharides from placentas allowed the diagnosis of α-mannosidosis in three litters of kittens. The chromatography also afforded a detailed comparison of the oligosaccharide pattern and levels in placenta, liver, brain, urine and ocular fluid of the affected animals. In all cases, two series of compounds were observed, with one or two residues of *N*-acetylglucosamine at the reducing terminus, respectively, and between two and nine mannose residues. This pattern is unlike that of human mannosidosis, and resembles that of ruminants, except that the major oligosaccharide contains three mannose residues instead of two.

(Kitten tissue) *Oligosaccharide* *Mannosidosis* *Diagnosis* *Glycoprotein*

1. INTRODUCTION

α-Mannosidosis results from a genetic deficiency of α-D-mannosidase [1]. It causes massive accumulation in tissues and excretion in urine of oligosaccharides resulting from the incomplete catabolism of glycoprotein saccharide chains [2,3]. The disease is characterized by progressive neurological impairment, as well as visceral, ocular, and skeletal involvement [4]. Early diagnosis [5] is critical for observation of the disease over its entire course and for the initiation of attempts at therapy [6].

α-Mannosidosis occurs in humans [1,4], cattle [7], and cats [8,9], and 'induced' mannosidosis occurs in animals that ingest swainsonine, an inhibitor of α-mannosidase [10]. In a previous study of feline mannosidosis, oligosaccharides from tissues and urine of a single kitten were partially characterized and compared with those of the human and bovine diseases, but were not used for diagnosis [11].

An 11-week-old Blue Persian female kitten was diagnosed with α-mannosidosis by lectin histochemistry [12]. Assays of α-mannosidase activity in plasma and leukocytes of related cats suggested that they were carriers. These animals were rebred, and oligosaccharides extracted from the placentas, brain, liver, urine and ocular fluid of the offspring were examined by high-pressure liquid chromatography (HPLC) [10,13]. By correlation of the HPLC results with α-mannosidase levels and light microscopy, it was possible to divide the kittens into affected and normal individuals, and to distinguish presumptive heterozygotes from normal animals.

2. MATERIALS AND METHODS

Placentas were obtained at birth, from 3 litters; nos 40–42 (1st), nos 56–59 (2nd), and nos 34–38

Abbreviations: M, D-mannose; G, *N*-acetyl-D-glucosamine

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(3rd). Other tissues, and urine, were obtained at autopsy. All tissues were frozen until used.

2.1. *Extraction of oligosaccharides from tissues*

Tissues were thawed, dried, weighed and scissored, then homogenized with water (5 ml/g) at 0°C (Polytron PT10 ST, Brinkman, Westbury, NY). The homogenate was centrifuged at 49000 × *g* for 45 min at 4°C, and the supernatant lyophilized. The residue was treated with ethanol/water (1:1, 5 ml), mixed virorously, and allowed to stand for 15 min after which the liquid was transferred into clean tubes and recentrifuged. The supernatant was evaporated to dryness (N_2), and the residue redissolved in water (1 ml).

2.2. *Analysis of oligosaccharide alditols by HPLC*

Tissue extract or body fluid (1 ml) was applied to a column of Bio-Gel P-2 (1.2 × 14 cm) and eluted with water. The first fraction (6 ml) was deionized by passage through coupled columns (0.5 × 2.5 cm) of AG50W (H^+) and AG1-X8 (HCO_2^-) ion-exchange resins (Bio-Rad, Richmond, CA). The solution was evaporated (N_2) to 1 ml and oligosaccharides reduced with $NaBH_4$ (20 mg) overnight at room temperature [3]. Isocratic, normal-phase HPLC was performed on a 5 μm Amino Spherisorb column as described [10].

For gradient reversed-phase HPLC, a portion of the sample solution (50 out of 200 μl) was dried, treated with an internal standard of sucrose (25 ng) and perbenzoylated [13,14]. Processing of the sample to remove excess reagents, and HPLC on a C-8 column, were performed as in [14].

2.3. *Other methods*

Hexose was determined by the phenol-sulfuric acid procedure [15]. Carbohydrate composition was determined by methanolysis followed by GLC determination of per(trimethylsilylated) methyl glycosides [16]. Permethylation of M_2G_2 (~100 μg) and GLC determination of partially methylated alditol acetates were performed as in [17,18].

Synthetic M_3G_2 was provided by Professor Hans Paulsen, University of Hamburg. M_3G was prepared by the action of *endo-β-N*-acetylglucosaminidase D on M_3G_2 as described for M_5G_2 [19]. Mannosidosis oligosaccharides (human [14], bovine [20], swainsonine-induced ovine [19,20]) were prepared as described previously. Assay of tissue α-mannosidase levels was performed with 4-methylumbelliferyl α-D-mannoside [5]. Tissue blocks of placenta (5 mm^3) were fixed in 2% Trumps solution, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, dehydrated through graded ethanol solutions, and embedded in Epon 812. Sections (1 μm thick) of Epon-embedded placentas were stained with toluidine blue for light microscopy.

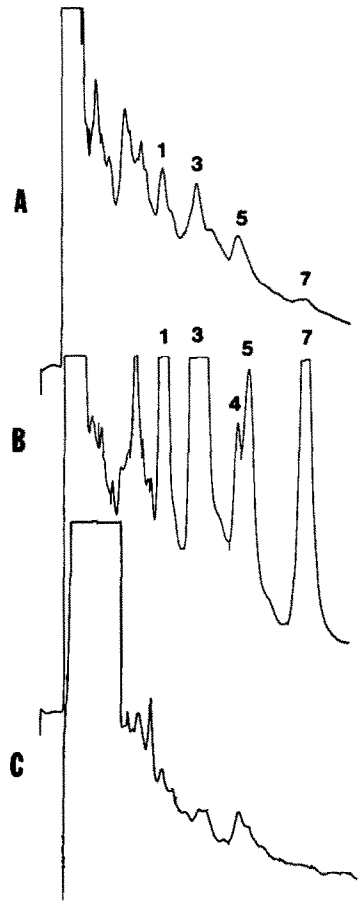

Fig.1. HPLC of heterozygote (A) (no. 40); affected (B) (no. 57), and normal (C) (no. 41) cat placenta oligosaccharides. Isocratic, normal-phase chromatography was performed as described in section 2, total elution time 25 min. For key to numbers above peaks, see legend to fig.3. For the 3 panels, the amount of extract injected was equivalent to the same weight of tissue.

3. RESULTS

Oligosaccharides from 12 placentas were analyzed by isocratic HPLC. Placentas 37, 56, 57 and 58 showed high levels of oligosaccharides with the type of pattern typical of mannosidosis, having a series of peaks with retention times showing a logarithmic relationship (fig.1B). Placentas 34, 35, 36, 38, 40, 42 and 59 showed a less well-defined pattern, with peaks having similar retention times, but at much lower levels (fig.1A). Placenta 41 showed no discernible pattern of HPLC peaks (fig.1C). It was concluded that the placentas of the first group were from affected animals, those from the second were heterozygotes, and the other placenta (41) was from a normal kitten. This conclusion was in full agreement with the results of light microscopy, which showed hypertrophy and vacuolation of fetal endothelial and mesenchymal cells in placentas 37, 56, 57 and 58 (fig.2), and α-mannosidase assay in placenta homogenates (table 1). The activity in placentas 37, 56, 57 and 58 was only 12–25% of that in 41, while in placentas 34, 35, 36, 38, 40 en 42 it averaged 60%.

α-Mannosidase activity in the brain and liver of clinically affected animals 57 and 58 was severely deficient, comprising less than 5% of the activity in a normal animal (table 2). When examined for oligosaccharides, these tissues showed a similar HPLC profile to the placentas of the affected group. It was unlike that of human mannosidosis [14], but bore some similarity to that of bovine [20], or swainsonine-induced ovine mannosidosis [10,13]. However, although the major peaks had similar retention times, the distribution was dif-

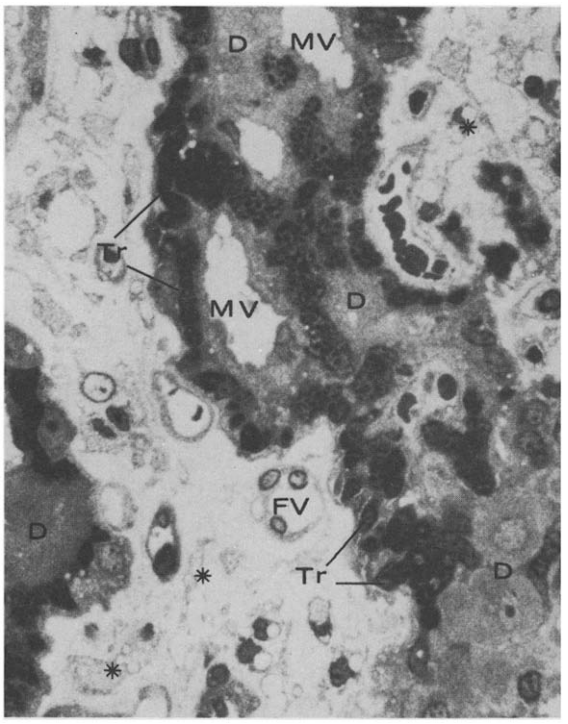

Fig.2. Light micrograph of Epon-embedded placenta obtained from kitten 57, illustrating maternal cells including 'decidual' (D) cells and blood vessels (BV) and fetal cells including trophoblasts (Tr), blood vessel (FB) and mesenchymal cells (asterisks). The blood vessel endothelium and mesenchymal cells are vacuolated. × 550.

Table 1

Feline placenta α-mannosidase[a] and oligosaccharide[b] levels

Component	Normal	Heterozygote[c]	37	56	57	58
α-Mannosidase	3487	2084 ± 385	417	616	442	882
M_2G_2			19	59	60	92
M_3G			5	19	30	40
M_3G_2		0.2 ± 0.14[d]	37	238	315	412
M_4G_2 + M_4G			10	42	38	60
M_5G			11	21	21	47
M_5G_2				41	55	47
M_6G_2			2	15	17	13
M_7G_2			1	6	7	2
Total[e]			85	441	543	713
M_3G_2 (%)			44	54	58	58
Hexose[f]	255	281 ± 117	1440	1120	1400	2300

[a] nmol substrate cleaved/mg protein per h, using 4-methylumbelliferyl α-D-mannoside as substrate [5]

[b] Levels (nmol/g tissue) based on integrated area of major HPLC peaks after perbenzoylation and calculated by reference to an internal standard of sucrose, assuming absorbance is directly proportional to the number of benzoyl groups [14]. For explanation of the formulas, see scheme 1

[c] Mean of 6 values ± SD

[d] Measured by reference to an external standard of sucrose

[e] Derived by addition of the calculated figures, does not include minor peaks

[f] μg/g tissue, measured by the phenol-sulfuric acid procedure [15]

Table 2

α-Mannosidase and oligosaccharide levels[a] in feline liver, brain, urine[b] and ocular fluid[c]

Component	Liver 57	Brain 57	Liver 58	Brain 58	Urine 58	Ocular fluid 58
α-Mannosidase	2.1	2.2	0.4	4.2		
M_2G_2	126	23	188		20	
M_3G	117	39	103	9	26	5
M_3G_2	2880	216	653	45	279	44
M_4G_2 + M_4G	484	69	144	13	31	16
M_5G	956	59	98	22	19	5
M_5G_2		43	36		26	9
M_6G_2	128	15	21	4	8	4
M_7G_2	77	7	7	2	6	2
Total[d]	4768	473	1250	95	415	85
M_3G_2 (%)	60	46	52	47	67	52
Hexose[d]	13 600	600	10 000	1400	1300	200

[a] For methods of measurement, see table 1, footnotes a and b; α-mannosidase activities are expressed as percentages of values in tissues of a control animal; extracts of liver and brain from this control showed no peaks with retention times corresponding to any of the oligosaccharides

[b] nmol/ml, measured by reference to an external standard of raffinose

[c] nmol/ml

[d] Derived as stated for table 1

ferent, the chromatograms for the cat tissues showing a very high peak corresponding to M_3G_2. This assignment has been confirmed by the chromatographic isolation of a pure sample from liver and methylation analysis [17,18], which indicated the branched structure shown in scheme 1. Compositional analysis of the total extract from salivary gland of 58 showed the presence of mannose, *N*-acetylglucosamine, and *N*-acetylglucosaminitol, in the ratio 3.6:0.6:1.0.

Reversed-phase HPLC after perbenzoylation, and measurement of total hexose, provided a detailed comparison of the patterns and levels of oligosaccharides in the 3 groups of placentas (table 1) and other tissues and fluids (table 2). These results, together with the compositional analysis, and the structure of M_3G_2, made possible a provisional assignment of formulas and structures for the peaks, based on co-chromatography with synthetic compounds and with oligosaccharides of known structure isolated from bovine or swainsonine-induced ovine mammosidosis urines [20]. The patterns from placenta, brain, and liver (kitten 57), together with that from urine (kitten 58) are shown (fig.3), and the tentative structures corresponding to 7 of the numbered peaks are shown in scheme 1. Apart from the very high M_3G_2 peak, the profile from liver also showed higher levels of M_4G and M_5G relative to those in ruminant mannosidosis [20].

4. DISCUSSION

We have shown that HPLC analysis of oligosaccharides from placentas provides a rapid, sensitive, diagnostic procedure for animals affected with feline mannosidosis. It corroborates the results of α-mannosidase assay [5] and the histopathological findings, and will provide a non-invasive procedure to monitor the course of the disease in surviving kittens, and to monitor therapeutic trials. We have recently used a similar procedure to observe the pattern and levels of oligosaccharides in the allantoic fluid of goats with β-mannosidosis in order to develop a procedure for prenatal diagnosis applicable to human α-mannosidosis [21].

The detailed analysis of oligosaccharides makes possible a comparison of glycoprotein breakdown in humans, cats, and other species. The oligosaccharides from feline mannosidosis tissues and urine consist of 2 homologous series M_nG_2 and M_nG, as in induced and genetic α-mannosidosis of ruminants [10,20], but unlike human mannosidosis where only the M_nG series is present [2,3]. Only the first series was previously identified in cat tissues and urine [11]. Furthermore, the compounds of the M_nG series apparently have structures different from those of the M_nG_2 series, and may be derived from glycoproteins by an *endo*-β-*N*-acetylglucosaminidase having a substrate specificity different from that of human tissues [22].

A unique feature of the oligosaccharides from feline mannosidosis urine, ocular fluid, or tissues is the occurrence of very high levels of M_3G_2, suggesting that the mutant α-mannosidase of cats is impaired in its ability to hydrolyze either (1 → 3) or (1 → 6) linkages in the substrate once the pentasaccharide stage is reached. This makes it unlike either

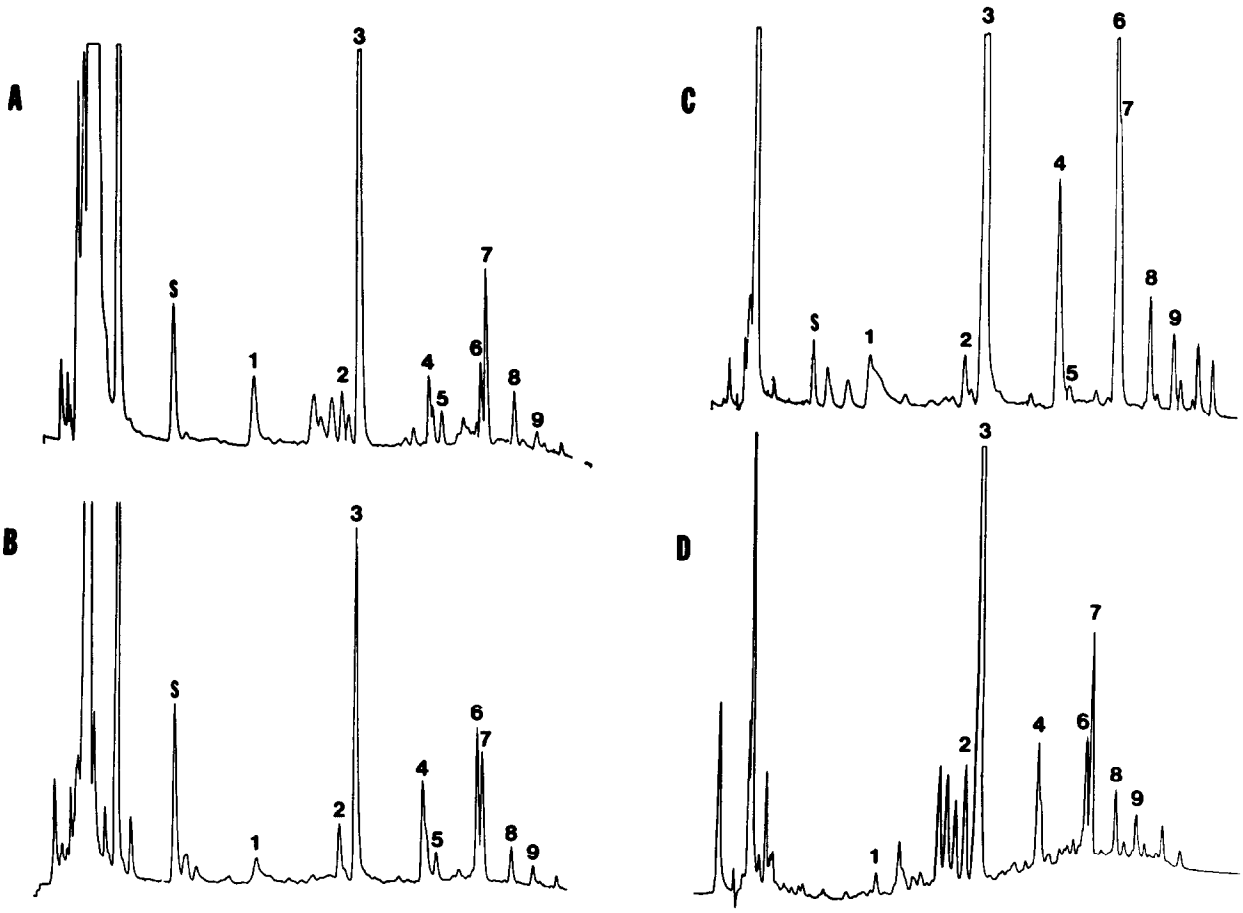

Fig.3. HPLC of oligosaccharides from placenta no. 57 (A), brain no. 57 (B), liver no. 57 (C) and urine no. 58 (D), after perbenzoylation. Chromatography was performed on a reversed-phase column as described in section 2. Key to the numbers above the peaks: S, internal standard of sucrose; 1, M_2G_2; 2, M_3G; 3, M_3G_2; 4, M_4G; 5, M_4G_2; 6, M_5G; 7, M_5,G_2; 8, M_6G_2; 9, M_7G_2. The retention time of M_3G_2 was 10.3 min, and the total elution time 20 min.

M_2G_2 Manα1 → 6Manβ1 → 4GlcNAcβ1 → 4GlcNAc

M_3G Manα1 ↘ $^{6}_{3}$Manβ1 → 4GlcNAc
Manα1 ↗

M_3G_2* Manα1 ↘ $^{6}_{3}$Manβ1 → 4GlcNAcβ1 → 4GlcNAc
Manα1 ↗

M_4G Manα1 ↘ $^{6}_{3}$Manβ1 → 4GlcNAc
Manα1 → 2Manα1 ↗

M_4G_2 Manα1 ↘ $^{6}_{3}$Manα1 ↘ $^{6}_{3}$Manβ1 → 4GlcNAcβ1 → 4GlcNAc
Manα1 ↗ Manα1 ↘ $^{6}_{3}$Manβ1 → 4GlcNAc

M_5G Manα1 → 2Manα1 → 2Manα1 ↗

M_5G_2 Manα1 ↘ $^{6}_{3}$Manα1 ↘ $^{6}_{3}$Manβ1 → 4GlcNAcβ1 → 4GlcNAc
Manα1 ↗ Manα1 ↗

Scheme 1. Proposed structures of oligosaccharides from feline mannosidosis tissues and urine based on comparison with compounds isolated from bovine mannosidosis and swainsonine-induced ovine mannosidosis [20]. * This structure has been proven by methylation analysis on a purified sample.

the human enzyme, which can hydrolyze the (1→6) linkage [11], or the bovine or ovine enzymes, which can readily hydrolyze the (1→3) linkage [20].

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