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The Purification of the Three Trimannosyl-*N*-acetylchitobiose Pentasaccharides from the Urine of Swainsonine-intoxicated Sheep

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A simple method for the preparative resolution of three $Man_3GlcNAc_2$ isomers called Ia, Ib and II has been designed. It consists mainly of the use of concanavalin A-Sepharose which allowed the total purification of $Man_3GlcNAc_2$-Ia, and then of anion-exchange resin in borate buffer-gradient to separate the Ib and II isomers. The purity of each oligosaccharide was checked by two HPLC methods. The use of these oligosaccharides for different analytical and biosynthetic purposes is discussed, and the unexpected resistance of one of the $Man_3GlcNAc_2$ alditols to the action of endo-β-*N*-acetylglucosaminidase H is noted.

Swainsonine-intoxicated sheep [1] excrete three major pentasaccharides having the composition $Man_3GlcNAc_2$, which have been named $Man_3GlcNAc_2$-Ia, $Man_3GlcNAc_2$-Ib, and $Man_3GlcNAc_2$-II (Fig. 1) according to the elution pattern in high pressure liquid chromatography. $Man_3GlcNAc_2$-Ia is derived mainly from "complex" glycoprotein chains and is a major component of urine only at the beginning of swainsonine toxicosis. The major trimannosyl compounds in fully-established toxicosis are $Man_3GlcNAc_2$-Ib and $Man_3GlcNAc_2$-II, both derived by the action of residual α-mannosidase activity on $Man_5GlcNAc_2$ [2].

We were interested in the purification of the isomers of $Man_3GlcNAc_2$: (a) to obtain pure samples as reference compounds in the study of mannosidosis oligosaccharide excretion in different animal species; (b) to have well-defined substrates for the control of the endo β-*N*-acetylglucosaminidase activities D and H; (c) to have pure starting materials for the chemical synthesis of intermediates like $Man_3GlcNAc_2$-PP-Dolichol required for studies of glycoprotein biosynthesis [3]; (d) to provide pure, defined natural substrates for the study of lysosomal α-mannosidase in humans, cats, and ruminants, and (e) to provide samples of oligosaccharides for the development of analytical procedures,

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Formula	Structure
$Man_3(GlcNAc)_2$-Ia	Manα6 | Manβ4GlcNAcβ4GlcNAc | Manα3
$Man_3(GlcNAc)_2$-Ib	Manα3Manα6Manβ4GlcNAcβ4GlcNAc
$Man_3(GlcNAc)_2$-II	Manα6Manα6Manβ4GlcNAcβ4GlcNAc

Figure 1. Structure of three pentasaccharides from swainsonine-intoxicated sheep.

especially NMR and mass-spectrometry. Thus, we used a separation on concanavalin A-Sepharose, which has been shown to be convenient for the separation of various oligosaccharides derived from *N*-glycoproteins [4], and a separation by anion-exchange chromatography in borate buffer. We report herein the preparative purification in milligram amounts, before and after reduction, of the three $Man_3GlcNAc_2$ isomers, found in swainsonine intoxicated sheep.

Materials and Methods

Materials

Concanavalin-A-Sepharose was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, USA) and was used after prior washing with cold "Con A buffer" (10 mM each of $MnCl_2$, $MgCl_2$, and $CaCl_2$).

Bio-Gel P-2 was obtained from Bio-Rad Laboratories (Richmond, CA, USA) and was suspended in distilled water. Bio-Gel P-4 (400) was used in 0.1 M pyridinium acetate (pH 5). Anion-exchange chromatography was performed with AG 1-X2 resin (Bio-Rad) in boric acid/pyridine buffer. The column (10 × 0.8 cm) was eluted by a linear gradient from 0.1 M boric acid/0.15 M pyridine to 0.3 M boric acid/0.45 M pyridine, 40 ml of each, and from 0.3 M boric acid/0.46 M pyridine to 0.5 M boric acid/0.75 M pyridine, 50 ml of each for unreduced and reduced oligosaccharides, respectively. Fractions (1 ml) were collected and assayed by the anthrone reagent on 100 μl aliquots. The pooled fractions were concentrated and then washed with methanol.

HPLC was performed with a chromatograph Model 5020 (Varian Associates, Palo Alto, CA, USA), equipped with a UV detector Model ERC 7210 (Erma Optical Company, Japan). Two types of column were used: (a) Regis 5 μm Hi-chrom reversible Amino-Spherisorb column, analytical, eluted with acetonitrile/15 mM KH_2PO_4, 7/3 by vol, at a 2 ml/min flow rate; the oligosaccharide elution was monitored at 195 nm; (b) A Rainin Microsorb

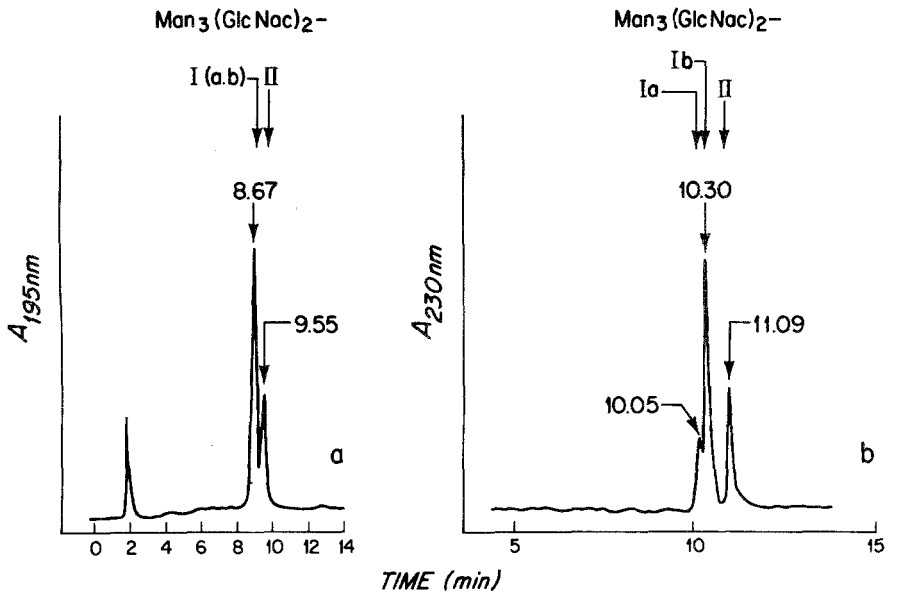

Figure 2. High pressure liquid chromatography analysis of the $Man_3GlcNAc_2$ isomers from swainsonine-intoxicated sheep urine. a: Amino-Spherisorb column. b: C-8 reversed phase column.

Short-One, 3 μm C-8 reversed phase column eluted with a gradient of acetonitrile/water (80-100% over 20 min) at a 1 ml/min flow rate. The elution was monitored at 230 nm. In that case, oligosaccharide derivatization by perbenzoylation was performed as previously described [5]. When necessary the oligosaccharides were reduced by sodium borohydride [6].

Results

The starting material was a crude oligosaccharide fraction from swainsonine-intoxicated sheep urine, isolated by preparative layer-chromatography after charcoal adsorption, P-6 chromatography, and deionization [7]. It contained mainly the $Man_3GlcNAc_2$ isomers, but was contaminated by small proportions of $Man_2GlcNAc_2$, $Man_3GlcNAc$, $Man_4GlcNAc$, and $Man_4GlcNAc_2$. The material (5.2 mg) reduced or unreduced, was applied to a column of Bio-Gel P-4 (400; 190 × 1 cm) and 0.5 ml fractions were collected. The material eluted from tubes 194 to 199 was pooled and was shown by HPLC analysis to contain only the $Man_3GlcNAc_2$ isomers (Fig. 2).

After being dried, these oligosaccharides were dissolved in 10 ml of cold "Con A buffer" and applied to a cold concanavalin A-Sepharose column (1 ml of gel in a Pasteur pipette). The column was eluted with 5 ml more of cold "Con A buffer". This pooled fraction was called $Man_3GlcNAc_2$-Con A^-. Then the column was eluted with 5 × 1 ml of the same buffer containing 0.1 M methyl α-D-mannoside; this fraction was called $Man_3GlcNAc_2$-Con A^+. Both fractions were lyophilized and then freed of salts (and methyl α-D-mannoside)

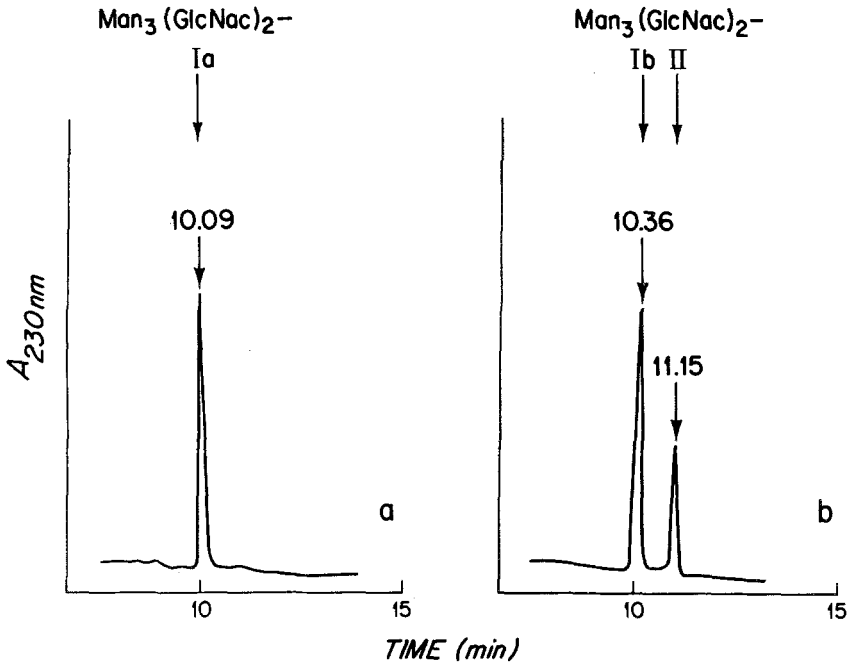

Figure 3. High pressure liquid chromatography analysis of the Con A$^+$ and Con A$^-$ fractions after concanavalin A-Sepharose affinity chromatography. The C-8 reversed phase column was used after reduction when necessary and perbenzoylation of the samples. a: Con A$^+$ fraction. b: Con A$^-$ fraction.

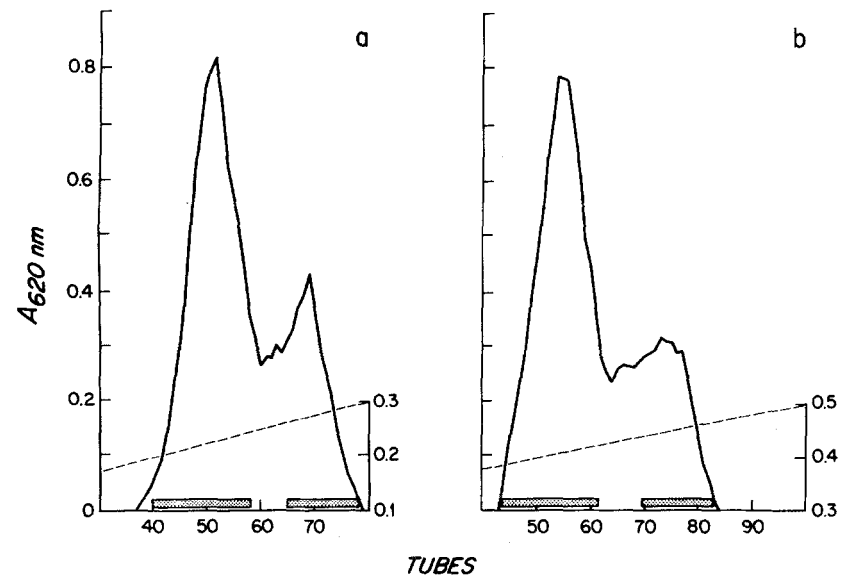

Figure 4. Preparative anion-exchange chromatography in borate buffers of the Con A$^-$ fractions. The molarity of the boric acid gradients is indicated on the right of each panel. The oligosaccharide elution was monitored at 620 nm after anthrone assay. a: Unreduced Con A$^-$ fraction. b: Reduced Con A$^-$ fraction. The tubes were pooled as indicated by the solid bars.

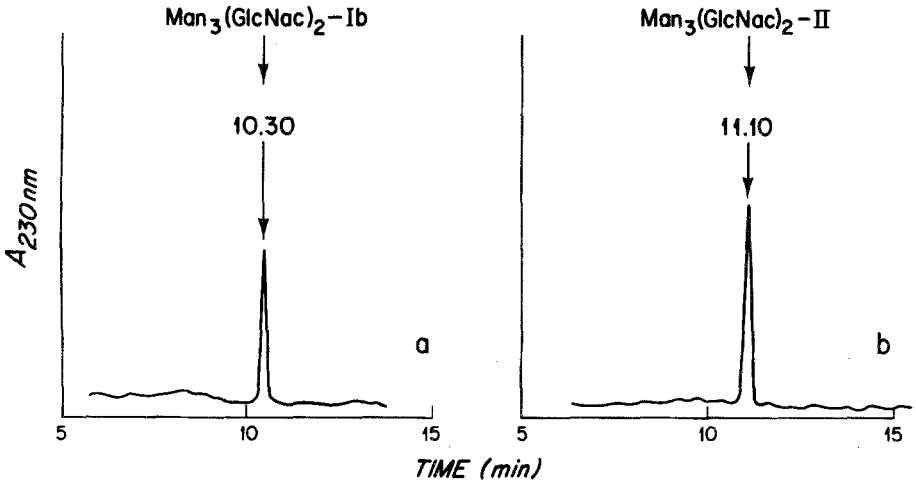

Figure 5. High pressure liquid chromatography analysis of the two peaks from the preparative anion-exchange chromatography. The C-8 reversed phase column was used after reduction and perbenzoylation of the samples. a: 1st peak. b: 2nd peak.

by filtration on a Bio-Gel P-2 column (30 × 1 cm) in water. Fractions (1.2 ml) were collected and monitored for oligosaccharide content by spotting on a silica gel plate and spraying with the anisaldehyde reagent [8]. The contents of the tubes reacting positively were pooled and evaporated.

The contents of the Con A^- and Con A^+ fractions were analyzed by HPLC on the C-8 column after perbenzoylation. Fig. 3 shows the total purification of $Man_3GlcNAc_2$-Ia contained exclusively in the Con A^- fraction. The final separation of $Man_3GlcNAc_2$-Ib and -II, contained in the Con A^- fraction, was obtained by anion-exchange chromatography in borate buffers. The sample (3.4 mg) was dissolved in 0.5 ml of the initial buffer and applied to the AG 1-X2 column which was eluted by the linear gradients desribed above (Fig. 4). The quality of the separation was monitored by analytical HPLC after reduction when necessary, and perbenzoylation of the samples (Fig. 5). It is noticeable that the reduced oligosaccharides were eluted later than the unreduced.

The overall yield of the entire process was 57.5%: $Man_3GlcNAc_2$-Ia, 140 μg; -Ib 1 400 μg and -II 560 μg from 3 600 μg, (after the Bio-Gel P-4 column) as measured by the anthrone assay.

Discussion

Previously, the isomers of $Man_3GlcNAc_2$ have been separated by HPLC [2]. But, as shown in Fig. 2, $Man_3GlcNAc_2$-Ia and -Ib have very similar retention times and are almost indistinguishable, depending on the high pressure liquid chromatography method used. However, $Man_3GlcNAc_2$-I(a,b) could be separated from $Man_3GlcNAc_2$-II on an

"aminopropyl"-modified silica column. This result would be expected according to the recent report of Blanken *et al.* [9], because the latter isomer contains two α-(1-6)-linked mannosyl residues.

To separate $Man_3GlcNAc_2$-Ia from $Man_3GlcNAc_2$-Ib, it was necessary to perbenzoylate the sample and use reversed-phase HPLC on a C-8 column [2]; even with the best column available, the resolution of these two isomers was very difficult because they both contain an α-(1-3) and an α-(1-6) linkage [9]. Furthermore, for all separations by HPLC, it was necessary to reduce the compounds into alditols to avoid the added complexity of mixtures of anomers [10].

To achieve the separation of isomers Ia and Ib, we took advantage of the narrow specificity of concanavalin A which binds the structures R-2Manα-(R-2Manα-)X and R-2Manα2Manα-X, R being H or any other sugar residue and X a sugar residue [11]. In the present case, only $Man_3GlcNAc_2$-Ia would bind to concanavalin A. However, this isomer represented only a small part of the starting $Man_3GlcNAc_2$ mixture and the first attempt at purification was unsuccessful because of the presence of $Man_3GlcNAc_2$-Ib and -II which together represented more than 90% of the applied material. Despite the highest affinity of the branched trimannosyl core for concanavalin A [12], the α-mannosyl residues of the $Man_3GlcNAc_2$-Ib and -II isomers hindered the binding of $Man_3GlcNAc_2$-Ia to concanavalin A-Sepharose. However, dilution of the sample to a concentration of about 1 mM in terms of α-mannosyl residues allowed $Man_3GlcNAc_2$-Ia to bind totally to concanavalin A-Sepharose and to be recovered by elution with methyl α-mannoside.

The separation of $Man_3GlcNAc_2$-Ib and -II was performed by anion-exchange chromatography, taking advantage of the different negative charges gained by these two isomers upon binding to borate ions. This result emphasizes the structural difference between $Man_3GlcNAc_2$-Ib and -II, the latter exhibiting an easier accessibility and binding of the borate ions because of the conformational freedom allowed by rotation about the C5-C6 linkage between the two α-(1-6)-linked mannosyl residues, leading to a stronger negative charge. The unreduced and reduced oligosaccharides were also different, the reduced being more anionic. This difference is similar to that observed in the electrophoresis of *N*-acetylglucosamine and *N*-acetylglucosaminitol in borate buffer [13], where the latter compound is much more acidic.

These experiments also indicated that reduction to alditols was a necessary step prior to the HPLC analysis of oligosaccharides. Owing to the excellent separation also obtained on a preparative amino column in HPLC, this method could have been used for the purification of $Man_3GlcNAc_2$-Ib and -II. But the reduction of the oligosaccharides makes them unusable as starting materials for chemical syntheses and as substrates for endo-β-*N*-acetylglucosaminidases D and H. We have, indeed, found that these enzymes can hydrolyze only the unreduced forms of $Man_3GlcNAc_2$-Ia and -Ib, respectively. This result was unexpected for the action of endo-β-*N*-acetylglucosaminidase H on $Man_3GlcNAc_2$-Ib, because for larger substrates, e.g., $Man_5GlcNAc_2$, reduction did not affect the activity of the enzyme. Presumably, for small substrates, it is critical that both *N*-acetylglucosamine residues exist in the pyranose ring form because $Man_3GlcNAc_2$-Asn is a good substrate for endo-β-*N*-acetylglucosaminidase H [14].

In conclusion, we have shown that three isomers of $Man_3GlcNAc_2$ could be efficiently separated by two rapid and convenient chromatographic techniques. Because the

structures of these compounds correspond to all or part of the *N*-glycoprotein saccharide "core" and adjacent residues, the pure oligosaccharides may be used for a variety of studies of the biosynthesis and catabolism of glycoprotein saccharide chains in progress in our laboratory.

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