Carbohydrate Research, 215 (1991) 211–217
Elsevier Science Publishers B.V., Amsterdam

Note

Separation of high-mannose isomers from yeast and mammalian sources using high-pH anion-exchange chromatography

R. Reid Townsend*,
Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446 (U.S.A.)

Paul H. Atkinson,
Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, NY 10461 (U.S.A.)

and Robert B. Trimble
Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201-0509 (U.S.A.)

(Received July 27th, 1990; accepted in revised form, November 20th, 1990)

The biosynthesis of oligosaccharides *N*-linked at AsnXxxThr(Ser) sequons of glycoproteins is initiated by the co-translational transfer of a $Glc_3Man_9GlcNAc_2$ from dolichyl pyrophosphate[1]. The three glucose residues are rapidly removed by two glucosidases, one $\alpha(1\rightarrow2)$- and the other $\alpha(1\rightarrow3)$-specific[1]. These first steps are identical in yeast and in man, and likely to be the same in plants[1,2]; however, the subsequent removal and addition of the peripheral mannoses are very different. The array of oligo-mannosyl structures which eventually occupy each glycosylation site are a function, in part, of the concerted action of mannosidases and mannosyl transferases on the archetypal $Man_9GlcNAc_2$ structure[3]. These enzymes often display specificity for not only the type of residue and anomeric linkage, but also for its branch location. For example, a yeast mannosidase removes a single $(1\rightarrow2)$-α-linked mannose from a specific branch of the $Man_9GlcNAc_2$ oligosaccharide[4,5]. A yeast $(1\rightarrow6)$-α-mannosyl transferase places a single mannose on one specific branch which is essential for elongation reactions[6]. Trimming by at least three mammalian mannosidases accounts for the heterogeneity of high-mannose structures at individual glycosylation sites (discussed in ref. 7). Thus, chromatographic methods which can separate not only by size and ring substitutions (linkage), but also according to branch isomerism, are required to understand the structural glycobiology of *N*-linked oligosaccharide biosynthesis.

High-pH anion-exchange chromatography (h.p.a.e.c.) has been shown to separate many oligosaccharide isomers (both neutral and charged) which differ only in a single linkage[8–14,17]). However, certain isomers were difficult to separate using

* To whom correspondence should be addressed.

0008-6215/91/$03.50

h.p.a.e.c.[12–14]. Using a more efficient stationary phase, we report the separation of high-mannose oligosaccharides, including branch isomers isolated from yeast and mammalian glycoproteins.

RESULTS AND DISCUSSION

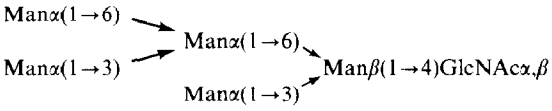
During biosynthesis of *N*-linked oligosaccharides, α-mannosidase(s) remove mannose residues from the originally transferred $Man_9GlcNAc_2$ oligosaccharide (structure **10** in Table I). In mammalian cells, any of the three terminal mannoses are removed resulting in three branch isomers[4], two of which (Man_8 isomers **8** and **9**) are shown in Table I. The third isomer is formed by removing the terminal α(1→2)-linked Man from the core α(1→3)-linked Man arm. In yeast, only one mannose is removed from the original $Man_9GlcNAc_2$ giving the mannosyl branching pattern shown in structures **7** (with an intact chitobiose core) and **8** (the endoglycosidase H product). Fig. 1, Panel A shows the h.p.a.e. separation of the mammalian $Man_8GlcNAc_2$ mixture which was released from bovine thyroglobulin with endoglycosidase H and isolated using Bio-Gel P-4 chromatography[4]. The proportional areas of the earlier to later eluting peak were 60:30. Approximately equal ratios of these two isomers and a small amount of the third possible isomer were found using ^{1}H-n.m.r. spectroscopy[4]. Panel B shows the chromatogram of compound **8** (Table I) which was isolated from *Saccharomyces* SUC2 invertase secreted by *Pichia pastoris*[15]. A 1:1 admixture of these two oligosaccharides was prepared and analyzed as shown in Panel C. It is apparent, from the increase in peak area of the earlier eluting peak, that compound **8** from both isolates co-eluted and were separated from their branch isomer **9**. These data are supported by ^{1}H-n.m.r. spectral analysis which identified structure **8** as the single component of the yeast $Man_8GlcNAc_2$ fraction (Trimble and Atkinson, unpublished data).

TABLE I

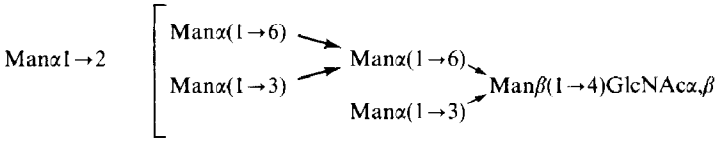
Structures of high-mannose oligosaccharides

Compound	*Structure*
1	Manα(1→6)↘ Manβ(1→4)GlcNAcβ(1→4)GlcNAcα,β Manα(1→3)↗
2	Manα(1→6)↘ Manβ(1→4)GlcNAcβ(1→4)GlcNAcα,β Manα(1→2)Manα(1→2)Manα(1→3)↗
3	Manα(1→6)↘ Manα(1→6)↘ Manβ(1→4)GlcNAcβ(1→4)GlcNAcα,β Manα(1→3)↗ Manα(1→3)↗

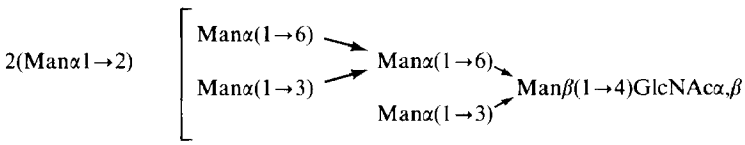
4



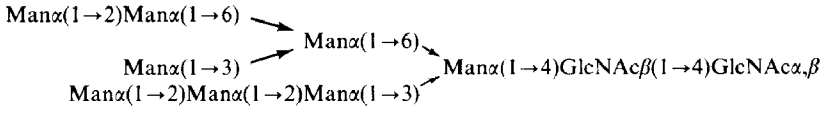
5



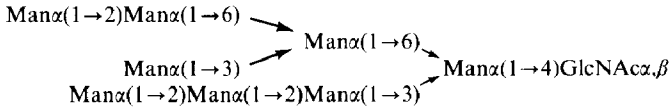
6



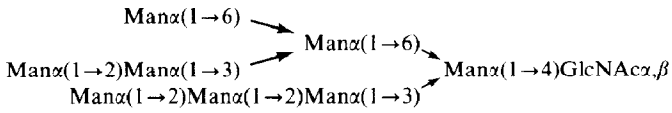
7



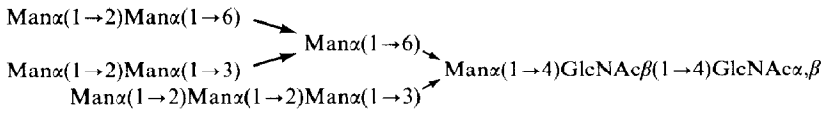
8



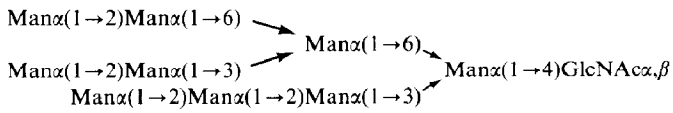
9



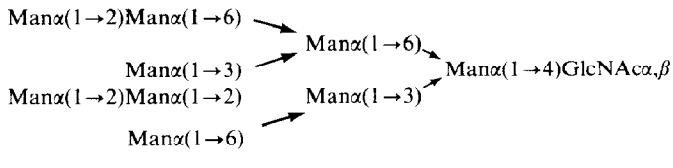
10



11



12



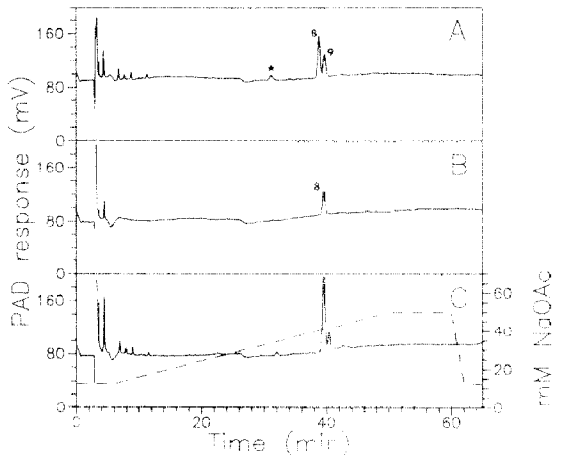


Fig. 1. H.p.a.e.c. with pulsed amperometric detection (p.a.d.) of $Man_8GlcNAc_1$ branch isomers. $Man_8GlcNAc_1$ (~ 1 nmol), which was isolated from bovine thyroglobulin as described in ref. 4, was injected into the described chromatograph in 50 μL of water (Panel A). Panel B shows the chromatography of $Man_8GlcNAc_1$ isolated from *Saccharomyces cerevisiae* invertase expressed in *Pichia pastoris* yeast cells. The $Man_8GlcNAc_1$ oligosaccharides were mixed in approximately equal proportions and analyzed as shown in Panel C. The gradient profile shown in Panel C is described in detail in the Experimental section. Detection was by pulsed amperometry, 300 nA full scale. The numbers above each peak correspond to those in Table I.

High-mannose $Man_8GlcNAc_1$ branch isomers have been separated using h.p.a.e.c. using a CarboPac PA-1 column (Dionex Corporation, Sunnyvale, CA)[14]; however, other high-mannose oligosaccharides, in which branch isomers (Man_6 and Man_8) may occur, eluted as single peaks[14]. Consistent with these findings[14], we were also unable to resolve the Man_9GlcN_1 branch isomers, with the above column using different elution conditions (Townsend and Hardy, unpublished results).

In yeast, an α(1→6)-linked Man is added to the α(1→3)-linked Man of **8** to form **12**, a new Man_9 structure[16], whereas in mammalian systems, the originally transferred $Man_9GlcNAc_2$ structure **10** is trimmed to form smaller high-mannose oligosaccharides and precursors for hybrid and complex structure biosynthesis[1]. These two Man_9's (**11** and **12**) are different in two respects: (*i*) the branch location of a terminal mannose and (*ii*) its linkage (α1→2 *vs.* α1→6). Fig. 2 shows the h.p.a.e. chromatogram of these two Man_9 structures. The $Man_8GlcNAc_1$ from bovine thyroglobulin eluted ~ 4 min earlier than the yeast $Man_9GlcNAc_1$. The earlier elution time of the mammalian $Man_8GlcNAc_1$ is consistent with the potential for compound **12** to form an additional charged center at a 2-OH position. The later elution of a homologous series of higher d.p. yeast mannose oligosaccharides with one additional 2-OH group was recently reported[17]. However, a number of exceptions to correlating the propensity for oxyanion formation (extrapolated from monosaccharide studies[18]) and the substitution pattern in oligosaccharides have been reported[11,13,14].

Endoglycosidases have become essential reagents for the analysis of *N*-linked glycoprotein glycans (for review, see ref. 19). Two classes of enzymes have been described: (*i*) those that cleave the chitobiose core and (*ii*) those that release the intact

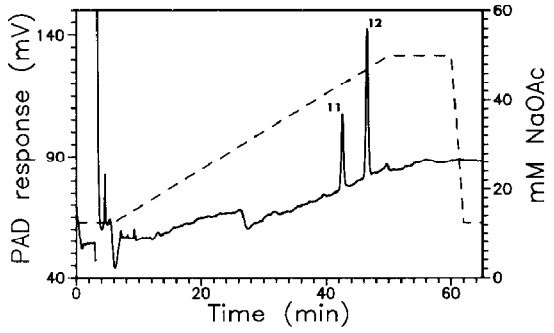


Fig. 2. H.p.a.e.c. with p.a.d. of $Man_9GlcNAc_1$ isomers from yeast and bovine sources. An admixture of $Man_9GlcNAc_1$ oligosaccharides from *Saccharomyces cervisiae* invertase, which were expressed in *Pichia pastoris* yeast cells, and $Man_9GlcNAc_1$ from bovine thyroglobulin (~300 pmol each) were mixed and injected into the chromatograph in 50 μL of water. Detection was by pulsed amperometry as described in the Experimental section at 300 nA full scale. The acetate gradient is indicated by the dashed line. The structures of the numbered peaks are given in Table I.

oligosaccharide chain. Often these two activities are found in a single source and must be separated and their substrate specificity determined. Thus, chromatography which cleanly separates oligosaccharides which differ only in the presence of one *vs.* two core GlcNAc's would be useful. We analyzed three such structural pairs, **3** and **4**, **7** and **8**, and **10** and **11**. Interestingly, the oligosaccharides with the intact chitobiose core eluted earlier than those with a remaining GlcNAc. This trend has also been found for lactosamine-type oligosaccharides[13,14]. The difference in elution times for all three pairs were found to be 3.6–3.8 min. Fig. 3 shows the chromatogram of high-mannose oligosaccharides with one (Panel A) and two (Panel B) GlcNAc's in the core. In addition to the above discussed separations, branch and linkage isomers of $Man_5GlcNAc_2$ (**2** and **3**) and isomeric forms of $Man_6GlcNAc_1$ (**5**) and $Man_7GlcNAc_1$ (**6**) were resolved.

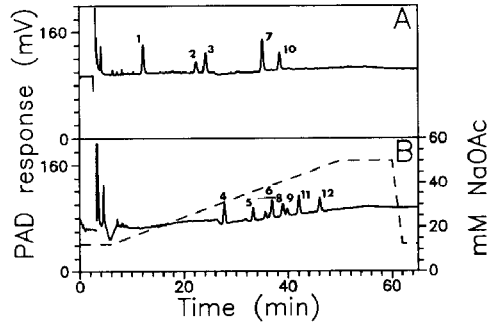


Fig. 3. H.p.a.e.c. with p.a.d. of $Man_9GlcNAc_1$ and $Man_9GlcNAc_2$ high-mannose oligosaccharides. An admixture of $Man_{3\text{-}9}GlcNAc_1$ oligosaccharides (Panel A) and $Man_{5\text{-}9}GlcNAc_2$ (Panel B) oligosaccharides were prepared. Structures corresponding to the numbered peaks are given in Table I. Each peak represents approximately 300 pmol of each individual oligosaccharide. The total volume injected for each mixture was 150 μL. Detection was by pulsed amperometry as described in the Experimental section at 300 nA full scale. The acetate gradient is indicated by the dashed line. The structures of the numbered peaks are given in Table I.

Chromatography of an admixture of the total $GlcNAc_1$ and $GlcNAc_2$ high-mannose oligosaccharides shown in Table I revealed that only two oligosaccharides in the series (**7** and an isomer of **6**) co-eluted (data not shown).

In summary, h.p.a.e.c. with a more efficient pellicular quaternary ammonium resin and increased column length (two serial columns) enabled the separation of branched isomeric forms of high-mannose oligosaccharides that are key biosynthetic intermediates. The two different core products of endoglycosidases, which are used to study glycoprotein glycans, were separated to the same extent, independent of chain length (d.p. 6–11). H.p.a.e.c. with p.a.d. should be a useful adjunct for defining the biosynthetic steps of *N*-linked oligosaccharides from various species as well as for profiling and purifying the ensemble of high-mannose oligosaccharides from glycoproteins.

EXPERIMENTAL

Materials. — Compounds **1**, **3**, and **10** were acquired from Dionex Corporation (Sunnyvale, CA). Compounds **2** and **7** were isolated from a mutant (alg3, sec18) of *Saccharomyces cerevisiae*[20]. Compounds **4**, **5**, **6**, **8**, **9**, and **11** were isolated from bovine thyroglobulin[4]. Compound **8** and **12** were also isolated from *Saccharomyces* SUC2 invertase expressed in *Pichia pastoris* yeast cells. Fifty per cent (w/w) NaOH solution was purchased from Fisher Scientific Co. (Rockville, MD). Sodium acetate was from J. T. Baker (Philipsburg, NJ). Nylon membranes were from Schleicher and Schuell (Keene, NH).

Chromatographic apparatus. — The system used for h.p.a.e. with p.a.d. consisted of a Dionex GPM pump and a model PAD II detector which was controlled using AI450 software. The Dionex Eluent Degas Module was employed to sparge and pressurize the eluents with helium. Eluent 1 was 250mM sodium acetate. Eluent 2 was water and Eluent 3 was M NaOH. Eluent 1 was filtered through 0.2 μ nylon membranes before use. The water for all eluents was glass-distilled using a Corning Mega-Pure system and collected directly into a glass container. Eluent 3 was prepared by suitable dilution of 50% NaOH solution with water. Sample injection was *via* a Spectra-Physics 8880 autosampler equipped with a 200-μL sample loop. The Rheodyne injection valve on the autosampler was fitted with a Tefzel rotor seal to withstand the alkalinity of the eluents. Oligosaccharides were separated using two serial Dionex CarboPac PA-100 pellicular anion-exchange columns (4 × 250 mm). The flow rate was 1 mL/min at ambient temperature. The sample was introduced into a system equilibrated with Eluent 1, 15%, Eluent 2, 85% and Eluent 3, 10%. This proportion was maintained for 5 min, at which time the proportion of Eluent 1 was increased linearly to 20% at 50 min. Isocratic elution was maintained for 10 min with re-equilibration to initial conditions in 2 min. The time between injections was 72 min.

Detection was by p.a.d. using a gold working electrode and triple-pulse amperometry. The pulse potentials and durations for the PAD II were $E_1 = 0.05$ V, $t_1 = 480$ ms (Range 2, position 5); $E_2 = 0.60$ V, $t_2 = 120$ ms (position 2); $E_3 = -0.60$ V, $t_3 = 60$

ms (position 1). The time constant was set to 3 s. Chromatographic data were collected and analyzed using AI450 software (Dionex Corporation).

ACKNOWLEDGMENTS

The authors thank Chris Pohl (Dionex Corporation, Sunnyvale, CA) for supplying two columns (CarboPac PA100's) and Dr. Mark Hardy (Dionex Corporation, Sunnyvale, CA) for invaluable technical assistance.

REFERENCES

1 A. J. Parodi and L. F. Leloir, *Biochem. Biophys. Acta*, 459 (1979) 1–37.
2 B. Esmon, P. C. Esmon, and R. J. Schekman, *J. Biol. Chem.*, 259 (1984) 10 322–10 327.
3 R. Kornfeld and S. Kornfeld, in W. J. Lennarz (Ed.), *The Biochemistry of Glycoproteins and Proteoglycans*, Plenum Press, New York, 1980, pp. 1–34.
4 J. C. Byrd, A. L. Tarentino, F. Maley, P. H. Atkinson, and R. B. Trimble, *J. Biol. Chem.*, 257 (1982) 14 657–14 666.
5 S. Jelinek-Kelly and A. Herscovics, *J. Biol. Chem.*, 260 (1988) 14 757–14 763.
6 P. A. Romero and A. Herscovics, *J. Biol. Chem.*, 264 (1989) 1946–1950.
7 C. Hubbard, *J. Biol. Chem.*, 263 (1988) 19 303–19 317.
8 M. R. Hardy and R. R. Townsend, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 3289–3293.
9 L.-M. Chien, M.-G. Yet, and M.-C. Shao, *FASEB J.*, 2 (1988) 2819–2824.
10 W. T. Wang and D. Zopf, *Carbohydr. Res.*, 189 (1989) 1–11.
11 M. R. Hardy and R. R. Townsend, *Carbohydr. Res.*, 188 (1989) 1–7.
12 R. R. Townsend, M. R. Hardy, D. A. Cumming, J. P. Carver, and B. Bendiak, *Anal. Biochem.*, 182 (1989) 1–8.
13 G. Pfeiffer, H. Geyer, I. Kalsner, P. Wendork, and R. Geyer, *Biomed. Chromatogr.*, 4 (1990) 193–199.
14 L. J. Basa and M. W. Spellman, *J. Chromatogr.*, 499 (1990) 205–220.
15 J. F. Tschopp, G. Sverlow, R. Kosson, W. Craig, and L. Grinna, *Biotechnology*, 5 (1987) 1305–1308.
16 L. M. Hernandez, L. Ballou, E. Alvarado, B. L. Gillece-Castro, A. L. Burlingame, and C. E. Ballou, *J. Biol. Chem.*, 264 (1989) 11 849–11 856.
17 L. M. Hernandez, L. Ballou, and C. E. Ballou, *Carbohydr. Res.*, 203 (1990) 1–11.
18 J. A. Rendlemen, *Adv. Chem. Ser.*, 117 (1971) 1–69.
19 F. Maley, R. B. Trimble, A. L. Tarentino, and T. H. Plummer, *Anal. Biochem.*, 180 (1989) 195–204.
20 M. F. Verostek, P. H. Atkinson, and R. B. Trimble, *J. Biol. Chem.*, 266 (1991) 5547–5551.