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Exoglycosidase matrix-mediated sequencing of a complex glycan pool by capillary electrophoresis

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Abstract

This paper discusses oligosaccharide sequencing by consecutive enzymatic digestion of carbohydrates using an exoglycosidase array, followed by capillary electrophoresis separation of the digests. Because of the high resolving power and good reproducibility of capillary electrophoresis, multistructure sequencing of a complex glycan pool can be performed in most instances requiring no prior isolation of the individual oligosaccharides. High sensitivity laser-induced fluorescence detection enables acquisition of complete sequence information from several picomoles of glycoproteins. Comparison of the migration times of the exoglycosidase digest fragments to the maltooligosaccharide ladder, enables calculation of migration shifts, due to cleavage based on the actual exoglycosidases used. The particular sequence of each oligosaccharide in a glycan pool can be proposed with high confidence based on the migration time shifts of the various oligosaccharide structures. However, possible combinations of various sequence fragments may have very similar charge to hydrodynamic volume ratios, resulting in electrophoretic co-migration when a mixture of different oligosaccharides is sequenced together. Then, capillary electrophoresis separations of the resulting fragments should be evaluated after each digestion step. In the instances of complex separation profiles when multiple peaks are present, the evaluation of peak shifts can get very complicated and solved only with the aid of a software program. Data about the monosaccharide composition of the glycan pool provides useful information in designing the digestion enzyme matrix. © 1997 Elsevier Science B.V.

Keywords: Oligosaccharides; Glycans

1. Introduction

The emergence of glycoproteins as pharmaceutical products increased the need for fast, efficient and reliable analytical methods for oligosaccharide sequencing [1]. Due to the augmentation in evidence that the sugar moieties of glycoproteins are important as recognition factors in receptor–ligand, receptor–cell or cell–cell interactions, in immunogenicity modulation, in the folding/unfolding process of protein molecules, and in the regulation of their bioactivity, the understanding of the role of glycoproteins in the function of normal and abnormal cells is increasingly important [2]. Slight changes in the carbohydrate structures, linkage and site occupancy can influence considerably the biological activity of glycosylated biopolymers such as glycoproteins, glycolipids, etc. The wide variety of very similar structures of the sugar moieties in glycosylated biopolymers has made it difficult in the past to obtain all the necessary information for characterization of complex carbohydrates. Analytical methods for oligosaccharide analysis include various chromatography techniques with UV, fluorescence and pulsed amperometric detection [3,4], enzyme assays [5] and nuclear magnetic resonance

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spectroscopy (NMR) [6]. Exoglycosidase digestion followed by chromatographic [7] or slab gel electrophoretic [8] separation represents the current technology for carbohydrate sequencing. Another emerging method for oligosaccharide characterization and sequencing is mass spectrometry (MS), which has already been shown as useful for molecular weight determination of individual oligosaccharide variants, and with appropriate enzymatic cleavage, it supplies detailed structure [9].

Recently, it was demonstrated that high-performance capillary electrophoresis equipped with an ultrasensitive laser-induced fluorescence (LIF) detector enables fast and high resolution separations of complex carbohydrates, allowing tentative identification of very closely related molecules [10–15]. Using this novel methodology, the extent and nature of glycosylation can easily be analyzed. Unlike other biopolymers, such as DNA and protein molecules, complex carbohydrates usually do not hold any charged groups and do not absorb UV or visible light. This makes detection of carbohydrates difficult by conventional means of UV detection in HPLC or in capillary electrophoresis (CE) [3,16]. Therefore, in most instances, pre-separation derivatization of oligosaccharides is required to provide the necessary charge for the electrophoresis based separation and UV active or fluorescent groups for efficient detection [17]. Among the various labeling agents used for sugar derivatization usually by reductive amination, 8-aminopyrene-1,3,6-trisulfonate (APTS) proved to be favorable since it requires a commonly available and stable Arion laser (488 nm) as excitation source [18].

In this paper, an oligosaccharide sequencing strategy is presented for instances when more than one oligosaccharide structure is present in the glycan pool. Our goal was to avoid isolation of the individual structures prior to sequencing, thus introducing multistructure oligosaccharide sequencing. The reductive end of all the carbohydrates in the pool was labelled by APTS and subject to enzymatic digestion with specially designed exoglycosidase mixtures. The resulting fragments were separated after each individual digestion step by high-performance capillary electrophoresis, using LIF detection. The sequences of the individual oligosaccharides were computed by the assistance of sophisticated software, based on the electrophoretic mobility shifts resulting from digestion by the different exoglycosidase mixtures.

2. Materials and methods

2.1. Chemicals

The standard maltooligosaccharide ladder M040 (Grain Proc. Muscatine, IA, USA) labelled by 8-aminopyrene-1,3,6-trisulfonate (APTS) (Beckman Instruments, Fullerton, CA, USA) was used as a reference standard in all the separations. Reagents and recombinant exoglycosidases of neuraminidase (NANase), β-galactosidase (GALase), β-N-hexosaminidase (HEXase), α-mannosidase (MANase) and α-fucosidase (FUCase) for sequencing were obtained from Glyko (Novato, CA, USA). The test compounds of core-fucosilated-bisialo-biantennary, high mannose and asialo-tetraantennary structures were from Oxford Glycosystems (Abingdon, UK). The labelled and digested oligosaccharide samples were used directly after derivatization or stored at −20°C. All buffer solutions were filtered through a 0.45-μm pore size filter and carefully vacuum degassed at 100 mbar.

2.2. Apparatus

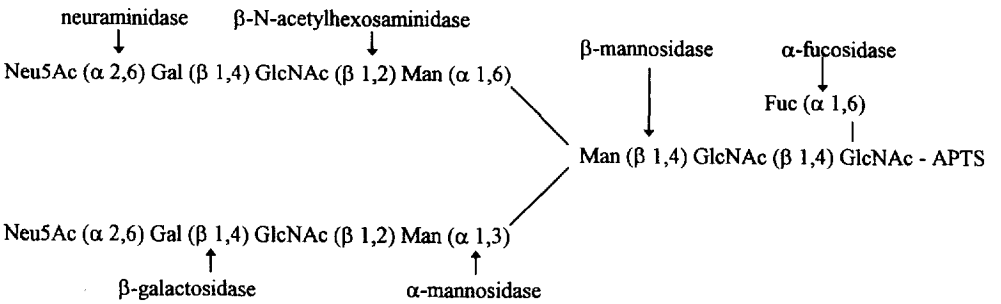
Capillary electrophoresis separations were achieved by using a P/ACE 5500 capillary electrophoresis system (Beckman Instruments), with the cathode on the injection side and the anode on the detection side (i.e., reversed polarity). A laser-induced fluorescence (LIF) detection system was used to monitor the separations employing a 4-mW argon-ion laser with 488-nm excitation wavelength and a 520-nm emission band-pass (10 nm) filter. The temperature of the cooling liquid of the capillary cartridge was controlled at 20°C±0.1°C.

2.3. Procedures

The labeling reaction of the maltooligosaccharide ladder and the test carbohydrate structures were incubated at 55°C for 90 min and diluted with deionized water ten-fold to stop the reaction, as

Table 1
Carbohydrate sequencing exoglycosidase enzyme matrix. Lower panel depicts the cleavage spots of the individual enzymes in the matrix

Enzymes\vials	1	2	3	4	5
Neuraminidase (NANase)	x	x	x	x	x
β-Galactosidase (GALase)	-	x	x	x	x
β-N-Acetylhexosaminidase (HEXase)	–	–	x	x	x
α-Mannosidase (MANase)	–	–	–	x	x
α-Fucosidase (FUCase)	–	–	–	–	x



described elsewhere [19]. The sequencing enzyme digestion was completed by the application of several exoglycosidase enzyme mixtures (Table 1) to five equal-volume aliquots of the labelled test compounds. The digestion tubes contained the following mixtures of exoglycosidases in 0.1 *M* phosphate buffer (pH 5.5): (1) 3.4 mU NANase, (2) 3.4 mU NANase and 3 mU GALase; (3) 3.4 mU NANase, 3.0 mU GALase and 140 mU HEXase; (4) 3.4 mU NANase, 3.0 mU GALase, 140 mU HEXase and 20 mU MANase; (5) 3.4 mU NANase, 3.0 mU GALase, 140 mU HEXase, 20 mU MANase and 10 mU FUCase. After overnight incubation at 37°C, the reactions were stopped by diluting the individual digests with 10 volumes of ice-cold CE-grade water in an ice bath. All digests were immediately subject to capillary electrophoresis analysis using a neutrally coated capillary column of 50 μm I.D. with 40-cm effective length (47 cm total length, eCAP N-CHO Coated Capillary) with the eCAP Carbohydrate Profiling Kit separation buffer (Beckman Instruments). The samples were injected by the pressure injection mode of the automated capillary electrophoresis system, typically for 5–10 s at 3.45 kPa.

Sequence information was computed based on the migration time shifts using the Cseq carbohydrate sequencing software package (ComGenex, Budapest, Hungary).

3. Results and discussion

Sequencing of a mixture of glycans was preceded by monosaccharide composition analysis [20], to provide adequate information about the enzymes necessary in the digestion matrix. The individual sugars found in the analyte glycan pool were: sialic acid, galactose, N-acetylglucosamine, mannose and fucose (data not shown) suggesting the use of the following enzymes for digestion: neuraminidase (NANase), β-galactosidase (GALase), β-N-hexosaminidase (HEXase), α-mannosidase (MANase) and α-fucosidase (FUCase) (Table 1). Before the application of the array of enzymes, the various oligosaccharides in the pool were derivatized via reductive amination using the fluorescent label 8-aminopyrene-1,3,6-trisulfonate (APTS). APTS has proven high derivatization yield (>95%) with unbiased labeling of a large variety of complex carbohydrate structures [21]. After completion of derivatization, the reaction mixture was aliquoted into five equal portions and specially designed mixtures of exoglycosidases were applied to each of the aliquots (Table 1, upper panel). The first reaction vial contained NANase which cleaves all α 2–3, 6 and 8 linked sialic acids, as it is shown in the lower panel of Table 1. The second vial contained the mixture of NANase and GALase, cleaving all α 2–3,

6, 8 linked sialic acids and β 1–4 linked galactoses (Table 1, lower panel). Reaction vial 3 contained the mixture of NANase, GALase and HEXase, cutting all α 2–3, 6, 8 linked sialic acids, β 1–4 linked galactoses and β 1–2, 3, 4, 6 linked N-acetylglucosamines (Table 1, lower panel). Vial 4 contained a mixture of NANase, GALase, HEXase and MANase, cutting all α 2–3,6,8 linked sialic acids, β 1–4 linked galactoses and β 1–2, 3, 4, 6 linked N-acetylglucosamines and α 1–2,3,6 linked mannoses (Table 1, lower panel). Finally, vial 5 had all the enzymes necessary to cleave all sugars from the N-linked trimannosil core structure including NANase, GALase, HEXase, MANase and FUCase, cutting all α 2–3, 6, 8 linked sialic acids, β 1–4 linked galactoses and β 1–2, 3, 4, 6 linked N-acetylglucosamines, α 1–2, 3, 6 linked mannoses and α 1–6 linked fucose (Table 1, lower panel).

After overnight incubation of the reaction mixtures at 37°C, the individual reactions were subject to capillary electrophoresis analysis. The migration times of the resulting peaks were compared to the standard maltooligosaccharide ladder depicted in Fig. 1, Trace (a). The glucose unit (GU) values of the various carbohydrate structures and their fragments were calculated by:

$$GU_x = G_n + \frac{MT_x - MT_n}{MT_{n+1} - MT_n}$$

where GU_x is the glucose unit value of the peak of interest, G_n is the degree of polymerization of the maltooligosaccharide unit immediately preceding the peak of interest, MT_x is the migration time of the peak of interest, and MT_n and MT_{n+1} are the migration times of the maltooligosaccharides immediately preceding and following the peak of interest, respectively.

The capillary electrophoresis separation of the APTS-labelled intact glycan pool is depicted in Fig.

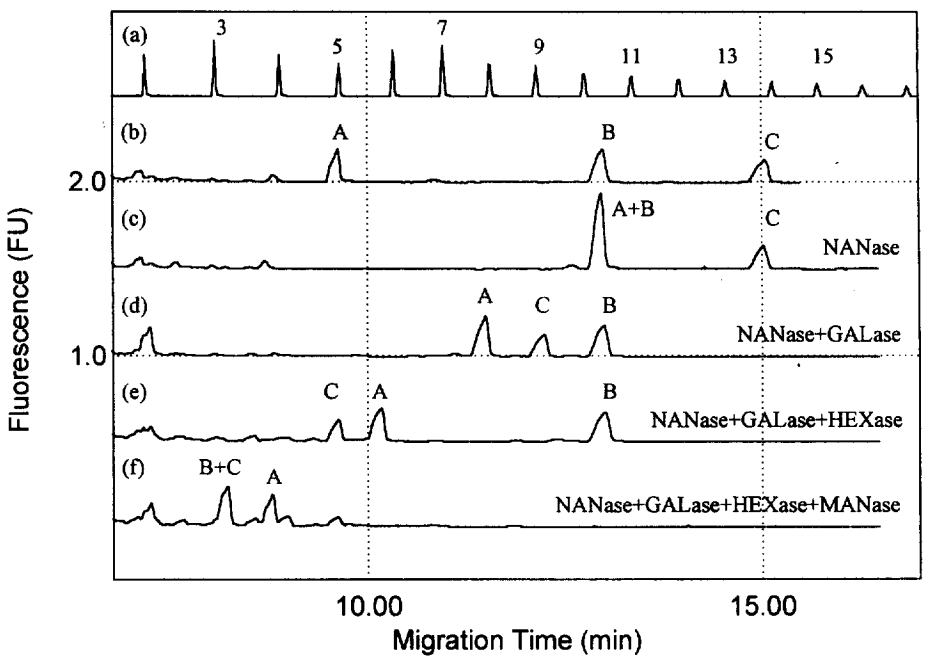


Fig. 1. Multistructure sequencing of oligosaccharides by CFE–LIF/enzyme matrix digestion assay. (a) CE–LIF separation of the APTS-labeled maltooligosaccharide ladder standard. Numbers above peaks correspond to the degree of polymerization; (b) CFE–LIF separation of the APTS-labeled intact oligosaccharide mixture; (c–f) after the application of the different enzyme mixtures of NANase (c), NANase + GALase (d), NANase + GALase + HEXase (e) and NANase + GALase + HEXase + MANase (f). Conditions: eCAP N-CHO neutrally-coated capillary (I.D.: 50 μm): 47 cm (40 cm to the detection point); applied electric field: 500 V/cm; LIF detection: excitation: 488 nm/emission: 520 nm; eCAP Carbohydrate Profiling Kit separation buffer, temperature: 20°C.

1, Trace (b). Three well-separated peaks appeared, with glucose unit values of $GU_A = 4.96$, $GU_B = 10.38$ and $GU_C = 13.81$. Trace (c) of Fig. 1 shows the separation of the fragments after the first digest with NANase. Here only two peaks can be observed with very similar migration times/GU values as on Trace (b), $GU_{A-B} = 10.38$ and $GU_C = 13.81$. This lack of migration time shift for peaks B and C suggests that the corresponding structures in Fig. 1, Trace (b) probably were not subject to NANase mediated cleavage, while the structure corresponding to peak A lost its terminal sialic acids (see shift peak A). This loss apparently caused an electrophoretic mobility shift of this fragment to the exact same position as peak B, resulting in an increase in the area of peak (A+B). Based on the shift data in Table 2, the $\Delta GU = -5.42$ of peak A suggests the loss of two terminal Neu5Ac (−2.7 GU each). The lack of change in migration times of peaks B and C suggests that there were no sialic acid residues on these structures.

Reaction mixture (2) contained GALase in addition to the previously used NANase. The application of this mixture apparently cleaved off the terminal galactose residues from structures corresponding to peaks A (or B) and C. Subsequent digestions with mixtures (3) and (4) proved that in this instance the structure corresponding to peak A was cleaved by the GALase (shifted to $GU_A = 7.9$), and peak B was not affected (remained at $GU_B = 10.38$). Cutting off galactose residues caused the migration shift of peak C to $GU_C = 9.14$. Therefore, after this digestion step, three well-separated peaks resulted, as depicted in Fig. 1, Trace (d). The shift values compared to Trace (c), Fig. 1, ($\Delta GU = 2.48$ and 4.67) suggested the loss of two and four galactose units (Table 2) for peaks A and C, respectively.

The third exoglycosidase digestion matrix contained the enzyme mixture of NANase, GALase and HEXase, cleaving all terminal sialic acid, galactose and N-acetylglucosamine residues. This resulted in further shifts of peaks A and C to $GU_A = 5.78$ and $GU_C = 5.0$ ($\Delta GU_A = 2.12$ and $\Delta GU_C = 4.14$), but caused no shift of peak B ($GU_B = 10.38$). This suggests the loss of two and four N-acetylglucosamine residues from peaks A and C, respectively, but no GlcNAc of structure B (Fig. 1, Trace (c)).

Table 2
Glucose unit values (GU) of the various monosaccharide units and core structures

Core structures/monosaccharides	GU
APTS-$GlcNAc_2Man$ (trisaccharide-core)	3.21
APTS-$GlcNAc_2Man_3$ (N-linked core)	5.00
Man	0.9
GlcNAc	1.05
Gal	1.2
Fuc	0.7
Neu5Ac	−2.7

Reaction 4 included the four enzymes NANase, GALase, HEXase and MANase, cleaving all terminal sialic acid, galactose, N-acetylglucosamine and α-linked mannose residues. The application of this enzyme mixture effected all three peaks A, B and C and shifted them to $GU_A = 3.89$, $GU_{B+C} = 3.21$, as depicted in Fig. 1, Trace (f). The shift of peak B ($\Delta GU_B = 7.17$) corresponds to the loss of 8 mannoses, suggesting an original high-mannose structure. The fact, that this peak did not shift until MANase was applied, strongly supports this hypothesis. Considering the $GU_{Man} = 0.9$ from Table 2, the shifts of peaks A ($\Delta GU_A = 1.89$) and C ($\Delta GU_C = 1.79$), correspond to the loss of two mannose residues for each structure. The $\Delta GU = 0.68$ between peaks B+C and A on Trace (f), Fig. 1 suggests the presence of a core fucose unit ($GU_{Fuc} = 0.7$) on the structure corresponding to Peak A. Unfortunately, the application of reaction mixture 5 containing all the above used enzymes in addition to FUCase apparently did not effect the migration time of peak A, and, therefore did not cleave the fucose residue. The removal of this core fucose unit could only be accomplished by using the FUCase enzyme on the original unlabeled glycan pool. The reason for this unsuccessful cleavage is probably due to the large APTS label which connects to the very same GlcNAc unit as the fucose (see Fig. 2A), possibly causing steric hindrance for enzyme access to this core fucose residue.

4. Conclusions

This paper discussed a novel approach to sequence a mixture of oligosaccharides using CE–LIF and exoglycosidase matrix digestion without the need of

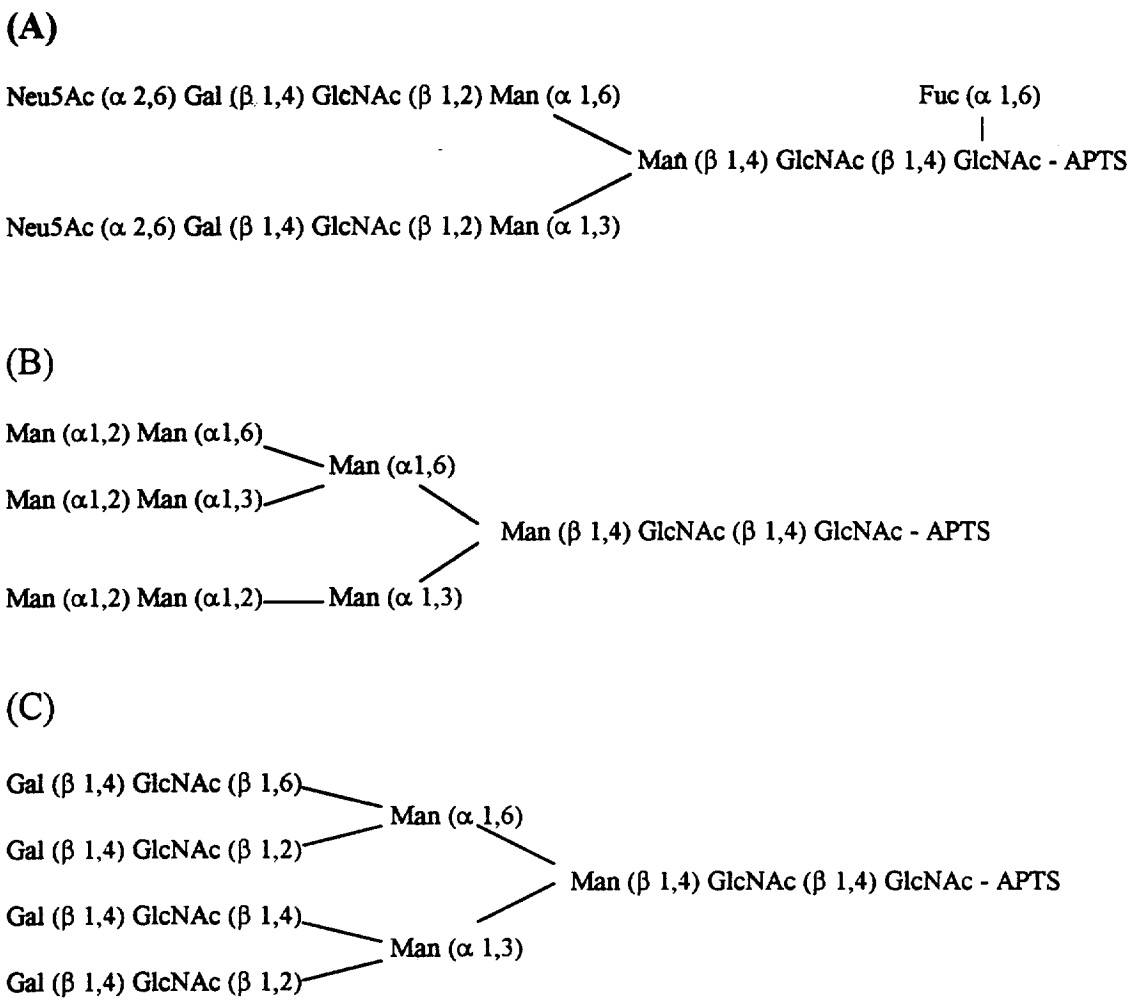


Fig. 2. Structures of the individual oligosaccharides in the mixed glycan pool: (A) core-fucosilated-bisialo-biantennary, (B) high mannose and (C) asialo-tetraantennary.

isolating the individual components for the analysis. The mixture of fluorescently-tagged oligosaccharides were readily subject to exoglycosidase digestion, except for the core-linked fucose, which was not accessible by the relevant enzyme, probably due to steric hindrance by the large tagging molecule APTS. After the application of the specifically designed enzyme matrix, the resulting fragments were separated and analyzed by CE–LIF. Capillary electrophoresis provided an excellent oligosaccharide sequencing/separation tool that is able to resolve differences in oligosaccharide size, net charge as well as variances in linkage positions [21]. The high sensitivity laser-induced fluorescent detection system allowed trace level (pmol) analysis of the APTS-labelled glycans. It is important to note that monosaccharide analysis of the glycan pool to be sequenced can provide important information about the enzymes needed in the digestion matrix. A sophisticated software program was written and used to evaluate and interpret the CE analysis results and used for constructing the suggested structures with the highest confidence level.

Sequence determination of the individual oligosaccharides in this particular mixture of glycans was accomplished by hand calculation and by a sophisti-

cated carbohydrate sequencing software. Peak A shifted −5.42 GU by the application of NANase, suggesting the loss of two terminal sialic acid residues. Further digestion with NANase+GALase resulted in the shift of 2.39 GU which corresponds to the loss of two galactose residues. Addition of further enzymes to the matrix, such as HEXase and MANase cleaved two GlcNAc and two Man residues based on the migration shifts of 2.12 and 1.88, respectively, leaving intact the characteristic N-linked trisaccharide core structure (Man-GlcNAc-GlcNAc) with 3.21 GU value. Application of the above exoglycosidase mixture with the addition of FUCase did not have any additional cleaving effect, most likely due to steric hindrance by the large APTS label on the same GlcNAc unit that holds the fucose residue. Indirect determination of this core fucosylation was accomplished by digesting the underivatized structure followed by the derivatization protocol. Based on the experimentally obtained migration shift values and Table 2, the structure of this complex carbohydrate molecule can be easily reconstructed as shown in Fig. 2A. Similar results were obtained using the Cseq software with 97+2.6% probability of this structure, based on the migration time data.

Peak B remained intact by the application of all enzymes of NANase, GALase and HEXase, until MANase was applied in the exoglycosidase mixture. The GU shift of 7.17 suggests the loss of eight mannose units connected to the trimannosyl core structure, that corresponds to a high mannose (Man-9) structure as shown in Fig. 2B. The similar conclusion of the carbohydrate sequencing software showed a precision of 99±0.15% on the structure of Peak B.

Peak C did not shift by the application of NANase, suggesting no terminal sialylation. Digestion with NANase+GALase resulted a change of 4.67 GU, corresponding to the loss of four galactose units. Application of the next enzyme mixture of NANase, GALase and HEXase shifted Peak C further to the lower migration time regime with a shift of 4.14 GU, equal to the loss of four GlcNAc units. The loss of four Gal and GlcNAc residues suggests that this structure might be tetraantennary. Application of MANase in addition to the previously used exoglycosidases yielded the common trisaccharide core structure (GU=3.21) with the loss of two mannose residues. The cutting enzymes along with the shift values predicted an asialo-tetraantennary structure, which was verified by means of the computer program with a precision of 99±1.1%.

Our data proves that multistructure sequencing of oligosaccharides is feasible from a complex glycan pool without the necessity of isolation of the individual components. The labelled oligosaccharides are subject to enzymatic digestion, and the electrophoretic migration time shift values between the undigested and the digested structures provide the means for sequence determination. If the mass to hydrodynamic volume ratios of different fragments are apparently similar, increase of the peak area can provide useful hints. We have also proved that sophisticated software can be applied to sequence determination of a complex carbohydrate mixture and, based on the migration time shifts of the individual digests, the software can precisely suggest the possible structures with the corresponding confidence levels in instances where more than one structure is suggested by the software.

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