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Review

Oligosaccharide sequencing based on exo- and endoglycosidase digestion and liquid chromatographic analysis of the products

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Abstract

Exo- and endoglycosidases are used to sequence oligosaccharides and give valuable information on the monosaccharide sequence, together with the anomericity, the stereochemistry, and in some cases, the substitution pattern of the monosaccharides. Both sequential and parallel methods of oligosaccharide sequencing are discussed.

Contents

1. Introduction

Oligosaccharides can be attached to glycoproteins in one of three different ways: (A) An *N*-glycosidic bond to the side chain N atom of asparagine, where the asparagine is part of the triplet amino acid sequence Asn–X–Ser/Thr and X is any amino acid other than proline. (B) An *O*-glycosidic linkage to the side chain O atom of serine, threonine or, in the case of collagen, to hydroxylysine and hydroxyproline. (C) Via esterification at the C terminus to the amino group of ethanolamine phosphomannoglycan which in turn is linked to phosphatidyl inositol forming a glycosylphosphatidylinositol (GPI) anchor.

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Some proteins carry just one glycan but several have more than one and the latter can include both *N*- and *O*-linked glycans with or without a GPI anchor on the same peptide chain (microheterogeneity). With very few exceptions (see e.g. [1]), the *N*-linked oligosaccharide structures are based upon a common trimannosyl chitobiose core modified by a set of glycosyltransferases and exoglycosidases in a biosynthetic pathway that is reasonably well understood [2]. Knowledge of this biosynthetic pathway is used to enable rapid sequencing of *N*-linked structures to be carried out (see section 2.6).

O-Linked oligosaccharides are much more diverse in structure, both in the saccharide–amino acid linkage and in the rest of the structure and the biosynthetic pathways involved are less clearly defined. For example, many *O*-linked structures contain a β-*N*-acetylgalactosamine–serine/threonine linkage but there are examples of structures linked to serine via xylose, fucose, glucose and *N*-acetylglucosamine (see e.g. [3–5]). This variation in monosaccharide composition and sequence makes enzymatic sequencing a more complex procedure than for the *N*-linked glycan structures.

The GPI structures have a common core structure but with considerable heterogeneity in the saccharides attached to this core. Unusual features of this core structure include a glucosamine with an unsubstituted amino group, the presence of *myo*-inositol, and an ethanolamine phosphate linking the carboxyl end of the protein chain to the 6 position of a mannose residue. Sequential enzymatic analysis has been used to elucidate the structure of a number of these GPI anchors, from mammalian and protozoal cells [6].

Enzymatic analysis should not be used in isolation for assigning a structure to a given oligosaccharide. Pure enzymes are not yet available for the hydrolysis of several monosaccharides, such as rhamnose and xylose. Additionally, the substitution profiles of many of the enzymes are ambiguous and therefore other methods, such as mass spectrometry (MS), nuclear magnetic resonance (NMR) or methylation analysis must be used in conjunction with enzymatic methods to yield the final structure [7]. The advantage of using enzymes for sequencing lies in the fact that very small quantities of the starting oligosaccharide (tens of picomoles) can be sequenced. Further, enzymatic methods may be combined with lectin probes and surface plasmon resonance techniques to give another method of sequencing glycoprotein oligosaccharides [8].

The topic of enzymatic sequencing of oligosaccharides is very large, encompassing glycoprotein carbohydrates, proteoglycans, and glycolipids from mammalian, plant, yeast and bacterial systems. For reasons of space, this review will concentrate on the enzymatic sequencing of mammalian glycoprotein oligosaccharides.

2. Enzymatic structural analysis of oligosaccharides

2.1. Exo- and endoglycosidases

Analysis of the primary structure of oligosaccharides is complicated by the number of parameters that must be determined. These include: (I) the nature, order and ring conformation of individual monosaccharides; (II) absolute stereochemistry of individual residues (D- or L-); (III) the anomericity (α- or β-linkage) of individual glycosidic bonds; (IV) substitution patterns and branch points; (V) the nature and location of chemical substituents (e.g. sulphate, phosphate, *O*-methyl) on a given monosaccharide.

The principle of oligosaccharide sequencing using exoglycosidases relies upon the ability of the enzymes to remove terminal monosaccharides from the nonreducing end of oligosaccharides. Any method used to monitor this reaction must be able either to ascertain the molar ratio of the released monosaccharide(s) to the remaining glycan or to detect a change in the mass or the hydrodynamic volume (using e.g. MS or size exclusion gel permeation chromatography (SEGPC)) between the starting glycan structure and the digested glycan structure.

To achieve complete removal of the terminal monosaccharide residues(s), a relatively high concentration of enzyme is used together with an extensive incubation period. Endoglycosidases are used in a similar manner for sequencing and here reliance is placed upon the enzyme cleaving a specific internal glycon–aglycon bond(s) in the oligosaccharide under study.

The exo- and endo-glycosidases that are used in the structural analysis of oligosaccharides are very specific for the monosaccharide anomericity (α/β) of the glycosidic linkage, and the absolute stereoisomer (D/L) of the glycon. Their specificity for the ring size (pyranose/furanose), specific linkages, branch-points and the aglycon component is more variable but limited. Most of the exoglycosidases are specific for the glycon, but a few are not, notably those that remove *N*-acetylhexosamines which do not distinguish between terminal *N*-acetylglucosamine and *N*-acetylgalactosamine. The individual specificities for the better-defined exoglycosidases are summarised in Table 1. The specificities for the endoglycosidases are complex and discussions of these can be found in [9–11].

One of the problems in the sequencing of any unknown oligosaccharide using exoglycosidases is how to interpret the results of an unsuccessful enzymatic digestion. The quoted glycon specificity for any enzyme is given for particular ranges of substrate concentrations, pH, temperature, ionic strength, buffer, etc. and it cannot be assumed that the same specificity applies outside these ranges. An example of this is seen with the differential rates of hydrolysis of the different mannose $\alpha 1\rightarrow$ linkages by jack bean α-mannosidase. Under certain reaction conditions, all linkages would be hydrolysed, but under slightly different conditions, some linkages would remain intact. It is generally true that, although exoglycosidases have been used extensively against a diverse range of oligosaccharides, few in-depth kinetic studies have been performed, and K_m and V_{max} data using different oligosaccharides is virtually non-existent. The small amounts of oligosaccharide that are now being sequenced means that some of the reactions, being pseudo-first order, will not go to completion and therefore extra peaks will appear on the separation chromatogram. Should such a situation occur, one way to proceed would be to increase the amount of enzyme and to incubate the mixture for a second time in an attempt to drive the reaction to completion. If this fails, then consideration must be given to other forms of analysis of the oligosaccharide.

The converse of the above problem may also occur where an enzyme may over-digest a given oligosaccharide. An example of this is seen with β-*N*-acetylhexosaminidase from *Streptococcus pneumoniae* [12] which, at low concentrations (<10 mU/ml), can distinguish between GlcNAcβ1–2Man, GlcNAcβ1–4Man and GlcNAcβ1–6Man linkages. The enzyme is therefore of considerable value in the linkage analysis of tri- and tetra-antennary oligosaccharides. However, although the enzyme rapidly hydrolyses the GlcNAcβ1–2Man linkage at this concentration, slow hydrolysis of the other two types of linkage also occurs. Extra peaks may therefore again appear on the separation chromatogram.

The use of enzymes that are contaminated may give rise to extra peaks in the chromatogram and therefore it is important to use enzyme preparations that have been assessed for purity under a range of reaction conditions against as wide a panel of standard oligosaccharides as is practical. The purity of enzymes from various sources cannot always be guaranteed; for example, preparations of *Charonia lampas* α-L-fucosidase are sometimes contaminated with various other exoglycosidases, thus seriously impairing its use as an analytical reagent.

2.2. Labelling of the oligosaccharide pool

Prior to undertaking enzymatic analysis, labelling of the oligosaccharide pool must be performed to allow detection of the remaining glycan structure(s) after enzyme digestion. This is usually achieved by labelling the reducing terminus with either tritium (traditionally) [13] or a fluorescent label, which in practice, gives a greater improvement in sensitivity over radiolabelling. Several fluorescent labels that are

Table 1
Exo- and endoglycosidases commonly used for oligosaccharide structural analysis

Enzyme	EC number	Source	Specificity[e]	Ref.
α-D-Sialidase[a]	EC 3.2.1.18	*Arthrobacter ureafaciens*	NeuAc/NeuGcα2→6>3,8	[29]
		Clostridium perfringens	NeuAc/NeuGcα2→3,6,8	[61]
		Vibrio cholerae	NeuAc/NeuGcα2→3,6,8	[61]
		Newcastle disease virus (Hitchner B1 strain)	NeuAc/NeuGcα2→3,8	[62]
		Salmonella typhimurium	NeuAc/NeuGcα2→3>6≫8,9	[63]
		Macrobdella leech	NeuAcα2→3	[35]
β-D-Galactosidase	EC 3.2.1.23	Bovine testes	Galβ1→3>4>6	[64]
		Escherichia Coli	Galβ1→4Glc	[65]
		Jack bean (*Canavalia ensiformis*)	Galβ1→6>4≫3	[66]
		Streptococcus pneumoniae	Galβ1→4	[67]
α-D-Galactosidase	EC 3.2.1.22	Green coffee bean	Galα→3,4,6	[68,69]
β-N-Acetyl-D-hexosaminidase	EC 3.2.1.30	Jack bean (*Canavalia ensiformis*)	Glc(Gal)NAcβ1→2,3,4,6	[70]
		Streptococcus pneumoniae[b]	Glc(Gal)NAcβ1→2,3	[12]
α-N-Acetyl-D-galactosamine	EC 3.2.1.49	Chicken liver[c]	GalNAcα1→	[71]
		Porcine liver		
α-D-Mannosidase	EC 3.2.1.24	Jack bean (*Canavalia ensiformis*)	Manα1→2,3,6	[72]
		Aspergillus saitoi	Manα1→2	[73,74]
β-D-Mannosidase[d]	EC 3.2.1.25	*Helix pomatia*	Manβ1→4	[75]
		Achatina fulica	Manβ1→4	[76]
α-L-Fucosidase	EC 3.2.1.51	*Charonia lampas*	Fucα1→2,3,4,6	[77]
		Bovine epididymis	Fucα1→6>2,3,4	
		Almond emulsin II	Fucα1→2	[78]
		Almond emulsin III	Fucα1→3,4	[79]
β-D-Xylosidase		*Charonia lampas*	Xylβ1→2	[80]
Endoglycosidase H	EC 3.2.1.96	*Streptomyces plicatus/griseus*	See Ref. [10]	[81]
Endoglycosidase F1		*Flavobacterium meningosepticum*	See Ref. [82]	[9,83,84]
Endoglycosidase F2		*Flavobacterium meningosepticum*	See Ref. [82]	As above
Endoglycosidase F3		*Flavobacterium meningosepticum*	See Ref. [82]	As above

(Continued on next page)

Table 1 (*continued*)

Enzyme	EC Number	Source	Specificity[e]	Ref.
Endo-β-D-galactosidase	EC 3.2.1.103	*Escherichia freundii*	See Ref. [10]	[85,86]
		Bacteroides fragilis	See Ref. [87]	[88]
Endoglycosidase D		*Streptococcus pneumoniae*	See Ref. [10]	[89]
Endo-α-D-sialidase		KIF phage in *Escherichia coli*	See Ref. [10]	[90]
Endo-α-N-acetylgalactosaminidase	EC 3.2.1.97	*Streptococcus pneumoniae*	See Ref. [91]	[91,92]

[a] The specificities of sialidases are complex, and are influenced both by the glycosidic linkage and substitutions to the sialic acid; see Ref. [33] for a full discussion.
[b] The specificity of the enzyme is critically dependent on the aglycon; see Ref. [12] for a full discussion.
[c] The activity of this enzyme against substrates containing non-reducing terminal GlcNAcα1→ has not been extensively investigated.
[d] The activity of the enzymes against substrates containing non-reducing terminal Manβ1→2,3,6 has not been extensively investigated.
[e] NeuNAc = N-acetyl-D-neuraminic acid; NeuNGc = N-glycolyl-D-neuraminic acid; Gal = D-galactose; GlcNAc = N-acetyl-D-glucosamine; GalNAc = N-acetyl-D-galactosamine; Man = D-mannose; Fuc = L-fucose; Xyl = D-xylose.

compatible with subsequent enzymatic digestion have been used, including 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) [14–16] or 2-aminopyridine (2AP) [17,18]. Recently, 2-aminobenzamide (2AB) has been used as the fluorescent label, and this has been shown to give non-selective, high efficiency labelling of the oligosaccharide pool and to be compatible with separation of the oligosaccharides using size-exclusion gel permeation chromatography [19]. Similarly, 2-aminoanthranilic acid (2AA) can be used to label the glycans and has been shown to have many of the advantage of the 2AB label. Separation of 2AA labelled glycans can be carried out using capillary zone electrophoresis (CZE) but now the glycans cannot be separated using SEGPC as the 2AA label is negatively charged.

2.3. Separation of charged glycan structures

Separation of the charged from the uncharged glycans in the oligosaccharide pool may be carried out by a variety of methods, such as high-performance anion-exchange chromatography (HPAEC) (see [20] and references therein) or paper electrophoresis [21]. Oligosaccharides carrying from zero to four anionic moieties in the form of sialic acid, sulphate or phosphate may be separated on the basis of charge using HPAEC separation on a weak anionic exchange (WAX) column in phosphate or acetate buffer [22,23]. The linkage of the sialic acid (α2→3 or α2→6 for example) does not significantly affect the separation under these conditions. Further separation may be achieved on the basis of size if desired by running the column in an ion-suppression mode using, for example, acetic acid and triethylamine in the mobile phase. Additional purification of each fraction may be desirable to obtain a homogeneous preparation of each oligosaccharide and this may be achieved by the use of high-pressure liquid chromatography (HPLC) and a porous graphite carbon (PGC) column [24–27].

Examples of the separation of charged and non-charged oligosaccharides using a WAX column and a PGC column are given in Figs. 1 and 2.

2.4. Enzymatic analysis of charged glycan structures

Determination of the nature of the charged glycans involves initial incubation with a general

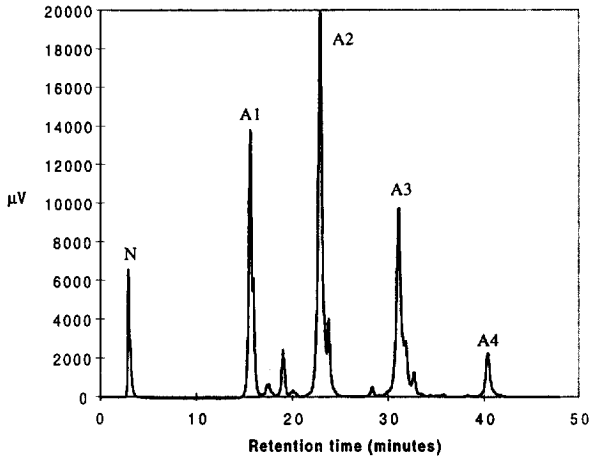

Fig. 1. Charge profile analysis on a weak anionic exchange (WAX) column (GlycoSep C, Oxford GlycoSystems, Abingdon, UK) of the 2-aminobenzamide labelled glycans released from bovine serum fetuin using hydrazinolysis. Peak N: neutral glycans; peaks A*n*: glycans carrying *n* negative charges.

sialidase, such as that from *Clostridium perfringens* [28] or *Arthrobacter ureafaciens* [29]. The latter enzyme has recently been shown [30,31] to be active despite the presence of sulphate on the glycan. Currently, sialidases are unable to hydrolyse 4-*O*-substituted sialic acids while 7-, 8- and 9-*O*-substituted sialic acids are only hydrolysed at between 20 and 50% of the rate of the unsubstituted sialic acid [32,33]. In practice, this does not prove to be a problem as most of the procedures for the isolation and purification of sialylated oligosaccharides will remove sialic acid substituents at some point [33,34].

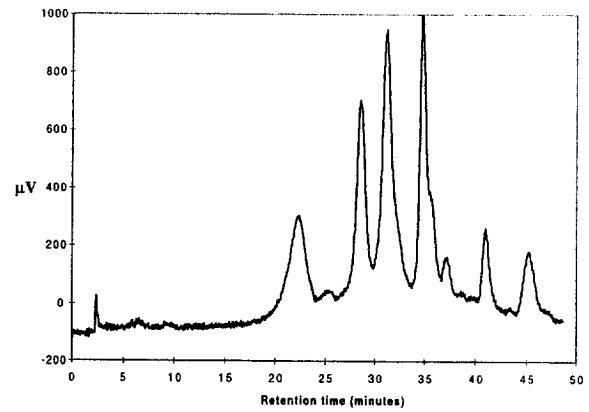

Fig. 2. Separation of the 2-aminobenzamide labelled glycans from peak A3 in Fig. 1 carried out on a porous graphite carbon (PGC) column (GlycoSep H, Oxford GlycoSystems). It serves to show the large numbers of oligosaccharide structures that can be present in one peak of the chromatogram arising from a charge profile analysis (see Fig. 1).

Desialylation is then followed by applying the glycan to the WAX column and detecting the integer decrease (if any) in the charge on the glycan. Residual charge would imply resistance to sialidase, thus indicating the presence of sulphation or phosphorylation on the glycan. Sialic acid linkage information can be determined by using linkage specific sialidases; a recent example of a sialidase that is specific for the $\alpha 2 \rightarrow 3$ linkage is given by [35]. The sialidases that are useful for sequencing purposes are given in Table 1.

Both substitution and linkage determination of phosphate groups on oligosaccharides using enzymatic analysis can be problematical. Phosphomonoesters can be hydrolysed using various alkaline phosphatases (e.g. from *Escherichia coli* [36]) but these are rather non-specific for the linkage involved. It is, however, possible to take the monophosphoglycan and sequence it until a block to further enzymatic digestion is encountered. Phosphatase treatment will then allow further enzymatic sequencing through the block, thus giving the phosphate-substituted monosaccharide unit. Complications may arise in the case of a glycan substituted with more than one phosphate group and clearly other sequencing techniques, such as MS, are required then to analyse the glycan further.

The situation with regard to sulphated glycan structures is slightly different to that for phosphorylated glycans. As yet, there is no broad specificity sulphatase available that can be used to identify the presence of sulphate groups. Baenziger et al. [37] have used what appears to be a reasonably non-specific arylsulphatase B prepared from human liver for the desulphation of the *N*-linked oligosaccharides from the glycohormones lutropin and chorionic gonadotropin but no details of its preparation or detailed specificity are available as yet. Chemical

methods for non-specific desulphation of glycans have been developed (see e.g. [38–42]) but these may be misleading because of accompanying glycosidic cleavage [43]. Currently, the most reliable method for identification of glycan sulphation is to use metabolic ^{35}S labelling. Specific sulphatases are, however, available for certain monosaccharides (see e.g. [37,44–50]). These sulphatases have not, as yet, found general usage in the sequencing of sulphated structures. Thus, Mawhinney et al. [51,52] in their analysis of sulphated oligosaccharides from tracheobronchial mucus used a combination of sequential exoglycosidase degradations, endoglycosidase digestion, permethylation analyses and specific lectin affinities to determine the carbohydrate structures. A similar approach has been taken more recently by Hemmerich et al. [30,31] in their sequencing of the carbohydrate structures on the L-selectin binding ligand GlyCAM-1. Undoubtedly recognition of the role that sulphated oligosaccharide structures play in the field of cell–cell recognition is growing, particularly in the central nervous system, and therefore it will become more important to find both specific and general sulphatases to facilitate sequencing.

2.5. Sequential enzymatic analysis

Analysis of an oligosaccharide structure may be performed by sequentially exposing the labelled oligosaccharide in solution to a series of highly purified exoglycosidases. For experimental details, the reader is referred to several papers in which enzymatic sequencing has been carried out [53–57]. After each incubation the oligosaccharide product is identified and the loss (if any) of monosaccharide by enzyme digestion is determined and measured. A typical set of digestions is shown in Fig. 3. Identification of the oligosaccharide product may be carried out in several ways, but use is commonly made of SEGPC which gives a reproducible measure of the hydrodynamic volume of the oligosaccharide. A decision is made as to which enzyme to use next depending on whether or not the previous enzyme digest has resulted in a decrease in hydrodynamic volume. The decision process is

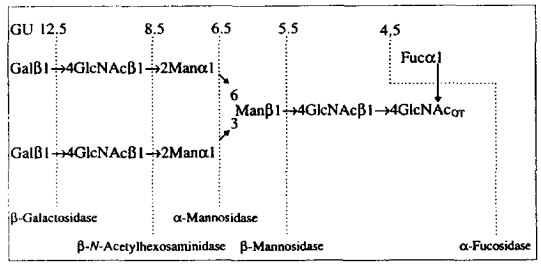

Fig. 3. Idealised sequential exoglycosidase analysis of a tritium-labelled complex biantennary carbohydrate with core fucose. The exoglycosidases used for the cleavage of each glycan linkage are indicated. The hydrodynamic volume in glucose units (GU) of the radiolabelled glycan fragment after each digestion is indicated at the top of the diagram.

speeded up if other information, such as monosaccharide content from permethylation analysis and gas chromatography–mass spectrometry (GC–MS), is available. The iterative process is continued until no further digestions can be made or the reducing terminal monosaccharide is reached. Use of techniques such as matrix-assisted laser desorption mass spectrometry (MALD-MS) or HPAEC with pulsed amperometric detection (PAD) will also allow the reaction mixture to be sampled and loss of monosaccharides to be detected without chromatographically recovering the oligosaccharide. The next exoglycosidase may then be added directly to the mixture (provided that the presence of the previous exoglycosidase does not interfere with the action of the succeeding one), but it is more usual to separate and purify the oligosaccharide chromatographically before addition of the enzyme.

2.6. Parallel enzymatic analysis

A faster method of sequencing oligosaccharides has recently been described [58] which uses enzyme digests analysed by SEGPC (the reagent array analysis method, RAAM). The technique differs from that described above in that only one chromatographic analysis is required and hence the sequencing process is speeded up considerably. Briefly, the oligosaccharide sample is subdivided into a number of different aliquots

that are incubated with precisely defined mixtures of glycosidases. After the digestions are completed, the individual reaction mixtures are pooled and the products analysed using SEGPC. Each peak on the chromatogram represents the product that is the result of digestion by one of the mixtures of enzymes. The complex profile is computer-converted into a histogram. Using a library of enzyme rules, together with a knowledge of the *N*-linked glycan biosynthetic pathways, software has been written to enable the glycan structure or structures that, when digested with the given enzyme array, most closely match the experimental histogram, to be deduced. The

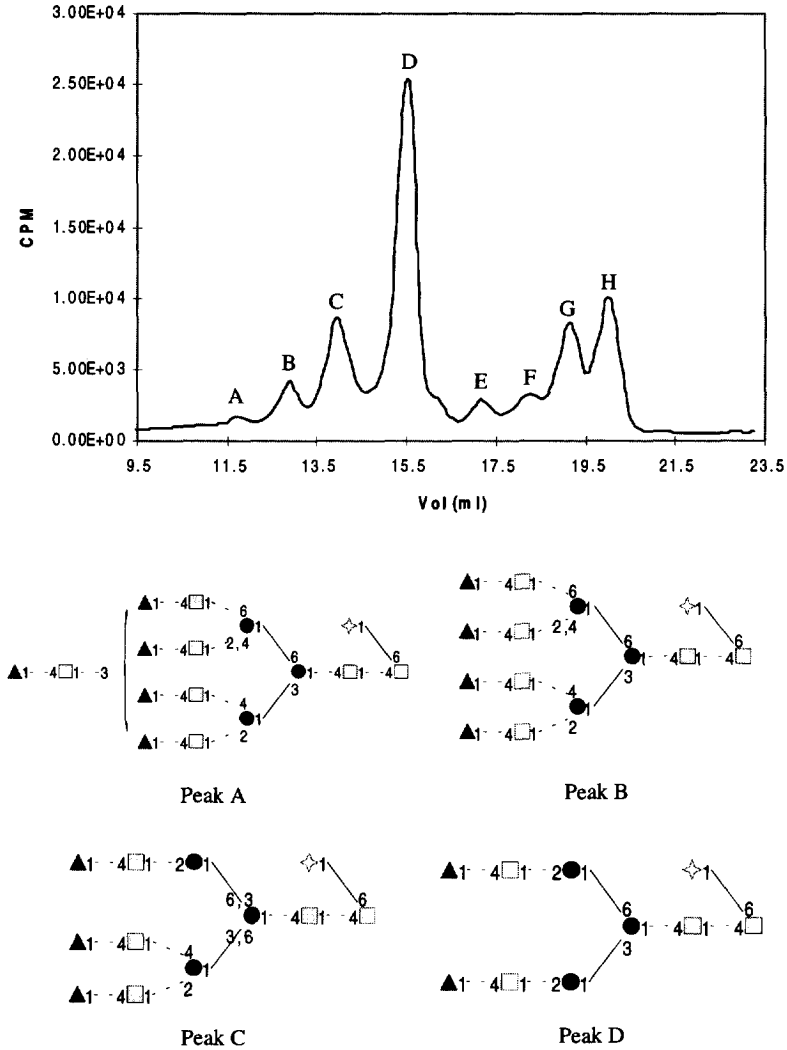

Fig. 4. Example of the use of the reagent array analysis method (RAAM) for the analysis of the *N*-linked carbohydrate structures on recombinant tissue plasminogen activator (rtPA). The glycans were released from the glycoprotein using hydrazinolysis, labelled at the reducing terminus with tritium and separated using size exclusion gel permeation chromatography. Individual peaks were then isolated and sequenced using an automated RAAM sequencer (RAAM 1000, Oxford GlycoSystems). Four of the structures are shown, corresponding to the structures in peaks A, B, C and D. Peaks E, F, G and H contain, respectively, a hybrid structure, Man-7 structures, Man-6 structures and Man-5 structures. The time for removal, isolation and sequencing of all eight peaks was approximately ten days.

advantages of the method are that it is very fast, reproducible and there are internal checks in the mixtures of enzymes to ensure that the oligosaccharide is sequenced completely. The method is also somewhat tolerant of impurities (≤15%) within the sample. The amount of oligosaccharide that can be analysed may be as little as a few picomoles, depending upon the labelling and detection method used. An example of the use of RAAM for the analysis of the oligosaccharides from recombinant tissue plasminogen activator (rtPA) is shown in Fig. 4.

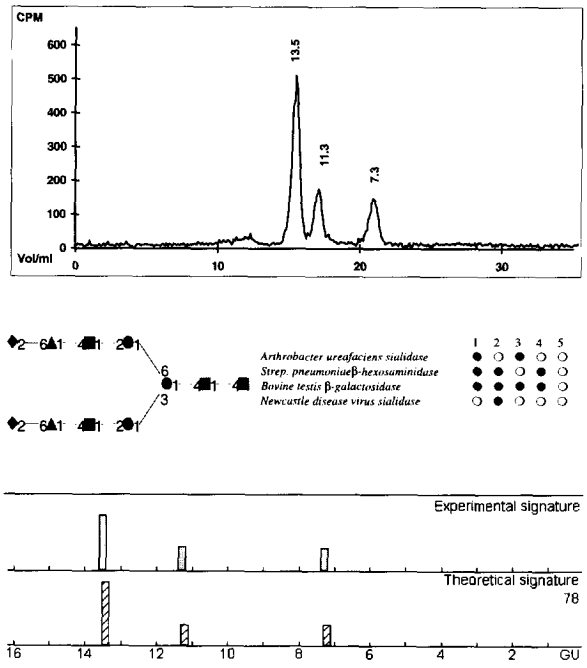

Fig. 5. RAAM digestion of a disialylated complex biantennary carbohydrate using the enzyme array shown. The figures in the chromatogram refer to the hydrodynamic volumes of the peaks in glucose units with respect to a partial hydrolysate of dextran. The experimental SEGPC data obtained from the enzyme digestions is converted into a histogram that can then be compared to a computer-generated theoretical histogram and a "match quality" obtained. The match quality is a measure of the quality of fit between experimental data and the theoretical histogram for the structure depicted. The symbols in the glycan structure are as follows: ◆ = *N*-acetyl-D-neuraminic acid; ▲ = D-galactose; ■ = *N*-acetylglucosamine; ● = D-mannose. In the enzyme array, ● indicates the presence of an enzyme, ○ indicates the absence of an enzyme.

For carbohydrates that are known to contain terminal sialic acids, an enzyme array is available that will yield details of the sialic acid linkages. Such an array is made possible by the large number of sialidases with different specificities available, as was discussed above. Results from such an array are shown in Fig. 5.

Several caveats to this method must be noted. First, the exoglycosidases used must not be contaminated, otherwise a spurious peak or peaks in the chromatographic profile will be obtained, thus leading either to a false structural assignment or, more likely, to no assignment at all. Second, more than one structure may be assigned to a given profile, thus giving rise to the concept of degeneracy. This is due to the glycosidases in the array being unable to distinguish between, for example, the oligomannose structures shown in Fig. 6. Third, the computer analysis when assigning the structure or structures, takes into account the biochemical pathways that lead to the synthesis of the oligosaccharide. In the case of the *N*-linked glycans, these pathways are well-known [2,59,60] and give rise to a particular set of structures but for *O*-linked glycans this is not true in a general sense. Enzyme arrays that can deal with certain subsets of the *O*-linked structures are being worked out but a general array for sequencing this type of glycan is probably not achievable owing to the large diversity of structures that have been found on mammalian glycoproteins.

3. Conclusions

Enzymatic analysis is a well-proven technique for the sequencing of *N*-linked and *O*-linked oligosaccharides and has also been used successfully for the sequencing of GPI anchors. It has the advantages of yielding structural information quickly and without necessarily using high-cost equipment. Future advances will see the automated analysis of products from enzyme arrays using techniques such as LD-MS, electrospray mass spectrometry, surface plasmon resonance and capillary zone electrophoresis.

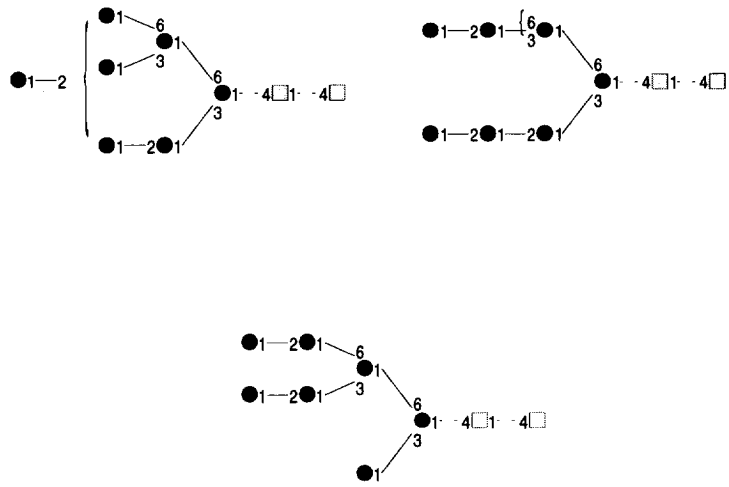

Fig. 6. Set of oligomannose oligosaccharides that cannot be distinguished using the standard array of exoglycosidases for *N*-linked glycans. The symbols in the diagram are as for Fig. 5.

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