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Review

Preparative purification of tyrosinamide N-linked oligosaccharides

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Abstract

N-linked oligosaccharides from glycoproteins can be either analyzed on a sub-nanomole scale or preparatively purified on a multi-micromole scale. Each goal necessitates a unique analytical strategy often involving oligosaccharide derivatization to enhance separation and detection. Tyrosinamide-oligosaccharides were developed to facilitate the preparative purification of N-linked oligosaccharides. These have found many uses in oligosaccharide remodeling, in the preparation of neoglycoconjugates, in developing receptor probes, and even as analytical standards in chromatography. This review discusses progress in the preparation of tyrosinamide-oligosaccharides from different glycoproteins and their utility in glycobiology research.

Contents

1. Introduction

What complicates the separation of natural mixtures of N-linked oligosaccharides is the need to resolve high molecular weight molecules that are hydrophilic, structurally similar, and that lack an intrinsic chromophore. Consequently, pre-column derivatization of oligosaccharides with a hydrophobic chromophore is the most common approach to enhance the resolution and detection of this class of biomolecules during reversed-phase (RP) HPLC [1–6].

On deciding between techniques it is impor-

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tant to determine both the scale and intended use of the resolved oligosaccharides. Many excellent analytical techniques have been developed to determine the identity and relative quantity of each N-linked oligosaccharide on one mg or less of a glycoprotein [7–9]. When applying an analytical technique, the challenge is to ascertain the relative ratio of oligosaccharides derived from a glycoprotein while avoiding manipulations that can alter the mixture. To insure accurate representation of the natural mixture, reducing-end derivatization chemistry needs to be quantitative, must produce no side products, and preferably increases the detection limit of oligosaccharides into the 10–100 pmol range [1,7]. The resolved peaks must also be collected and characterized on a sub-nmol scale since it is usually not possible to obtain enough of each oligosaccharide to carry out detailed spectroscopic analysis. Consequently, structure elucidation often employs the use of primary standard oligosaccharides which have been characterized in detail elsewhere. These are then used to assign structure to unknown peaks in the chromatogram based on their co-elution before and after enzymatic trimming. In addition to an oligosaccharide map, it may also be necessary to quantify the amount of each individual oligosaccharide and verify their recovery yield from the glycoprotein under investigation [10].

In contrast to analytical separations, a completely different strategy is required to purify multi-μmol quantities of individual N-linked oligosaccharides from gram quantities of a glycoprotein [11]. Since the N-linked oligosaccharides constitute a minor fraction of a glycoprotein (2–20 wt%), highly efficient methods are needed to remove protein which otherwise interferes with subsequent oligosaccharide derivatization and isolation procedures. Almost certainly, it is necessary to derivatize N-linked oligosaccharides prior to their separation on a preparative scale. However, the requirement for quantitative derivatization without production of side products is less stringent for preparative purifications since the aim is usually to isolate only the major oligosaccharide products from a glycoprotein. The most important criteria for a preparative purification is the simultaneous achievement of high resolution and capacity. Semi-preparative reversed-phase RP-HPLC affords sufficient capacity for isolating μmmol quantities of oligosaccharides. However, the resolution achieved is closely linked to the hydrophobicity of the aglycone which must be sufficient to contribute to retention but, at the same time, must not mask the more subtle hydrophobicity differences of structurally similar oligosaccharides.

Oligosaccharides isolated on a larger scale have many important uses. These serve as substrates for carbohydrate remodeling enzymes in order to prepare rare or novel oligosaccharide structures that are otherwise difficult to isolate from natural sources [12]. An important attribute is the reversibility of the aglycon derivatization to generate reducing-oligosaccharides if their intended use is as chromatographic standards [13,14].

However, the most compelling reason for purifying larger quantities of N-linked oligosaccharides is for their intended use in biological studies aimed at understanding the function of glycosylation on proteins. These applications may require the incorporation of a radiolabel or other probes to enhance detection of an oligosaccharide in a complex biological matrix. Probes are usually inserted at the reducing-end of an oligosaccharide in order to minimize interference with sugar residues on the outer antenna that are involved in receptor binding. Optimal designs incorporate the aglycone in a ring closed derivative which more closely resemble the natural structure linked to a protein [15]. In addition to providing sites for radiolabel incorporation, the aglycon should provide auxiliary functionality that may be coupled to without degrading the oligosaccharide [5,16].

This review describes a preparative purification scheme to isolate multi-μmol quantities of N-linked oligosaccharides following their derivatization with Boc-tyrosine. The separation of tyrosinamide-oligosaccharides from bovine fetuin [16], porcine fibrinogen [17], ovotransferrin [18], and ovalbumin [19] will be discussed along with potential biological applications for the resulting N-linked oligosaccharides.

2. Batch purification of N-linked oligosaccharides

Analytical studies often use N-glycosidase F (EC 3.2.2.18) to hydrolyze the glycosylamide linkage between asparagine and the first GlcNAc residue of an N-linked oligosaccharide [10]. Occasionally N-glycosidase F is able to completely release the N-linked oligosaccharides from a native glycoprotein, but more often, one or more of the glycosylation sites resists cleavage. This can be solved on a micro-scale using high enzyme concentrations and by denaturing the protein with detergents or mercapto ethanol [20]. The oligosaccharides can be recovered from the deglycosylated protein which is either precipitated or trapped on an ion-exchange or reversed-phase cartridge [21].

However, the expense of N-glycosidase F prevents the direct scale-up of analytical protocols to the digestion of gram quantities of glycoproteins. Fortunately, glycopeptides are much more efficiently digested by the enzyme so that large quantities of oligosaccharide can be released using micro quantities of N-glycosidase F [16]. Therefore, the first step in purifying larger quantities of N-linked oligosaccharides is to process a glycoprotein into glycopeptides (Fig. 1).

Glycoproteins such as hen ovalbumin and ovotransferrin, bovine fetuin, and porcine fibrinogen are commercially available from Sigma and represent good sources for purifying complex biantennary, triantennary, and bisecting oligosaccharides in addition to hybrid-type and certain high-mannose oligosaccharides [16–19]. Glycoproteins maybe irreversibly denatured by reduction with dithiothreitol and alkylation with iodoacetamide [16]. Reduced and alkylated (R and A) glycoproteins often precipitate during dialysis. The exception is bovine fetuin which remains soluble allowing trypsin digestion to be carried out directly. More commonly, the insolubility of R and A glycoproteins such as fibrinogen, ovotransferrin, and ovalbumin necessitates that they be re-solubilized in guanidine hydrochloride (1 to 2.4 *M*) [17–19]. Trypsin will retain its proteolytic activity even in 2.4 *M* guanidine and because of its narrow substrate specificity the resulting glycopeptides usually have a relatively long peptide sequence which is preferred for N-glycosidase F digestion.

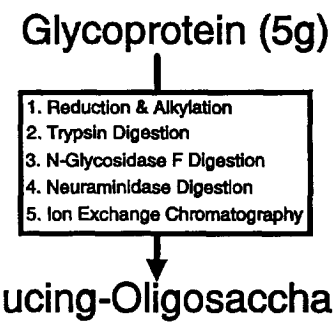

Fig. 1. Batch purification of reducing N-linked oligosaccharides. Glycoproteins (5 g) are R and A as previously described (step 1) [16]. R and A-glycoprotein is digested with trypsin (step 2) then treated with N-glycosidase F to release N-linked oligosaccharides from the tryptic glycopeptides (step 3). Neuraminidase is used to prepare asialyl-oligosaccharides (step 4) or omitted for the preparation of sialyl-oligosaccharides. Finally, ion-exchange chromatography is used to isolate reducing-oligosaccharides from the void volume of the column (step 5).

The release of N-linked oligosaccharides from glycopeptides by the action of N-glycosidase F may be monitored by high pH anion-exchange chromatography (HPAEC) [22]. The digestion is performed at a low enzyme concentration to achieve release of the oligosaccharides over a 48 h incubation [16]. Comparative analysis of the glycopeptide digest on HPAEC before and after the addition of N-glycosidase F usually identifies the presence of new oligosaccharide peaks arising from glycopeptide cleavage. An aliquot of the reaction may be digested with excess N-glycosidase F and analyzed by HPAEC to verify reaction completion. An increase in the oligosaccharide peaks on spiking with enzyme indicates that the reaction is incomplete.

Depending on the glycoprotein substrate, it may be advantageous to reduce oligosaccharide heterogeneity by removing terminal NeuAc residues. Prior knowledge of the complexity of the N-linked oligosaccharides being purified is particularly useful in making this decision. For example, bovine fetuin oligosaccharides contain numerous combinations of NeuAc linked α2–3

and α2–6 to galactose on triantennary oligosaccharides making the isolation of an appreciable quantity of any single isomer a daunting task [23]. In contrast, porcine fibrinogen possesses a simple sialylation pattern with NeuAc linked exclusively α2–6 to galactose on biantennary oligosaccharides [17].

Desialylation is achieved with neuraminidase at pH 5.0. The reaction may be monitored by HPAEC in order to observe the loss of sialyl-oligosaccharides with concomitant appearance of NeuAc. Either neuraminidase from *Clostridium perfringens* (EC 3.2.1.18) or *Arthrobacter ureafaciens* (EC 3.2.1.18) may be used to remove NeuAc residues although, for bovine fetuin oligosaccharides, these two enzymes have different selectivity which will alter the ratio of asialyl-oligosaccharides isolated from the digestion [11].

Reducing-oligosaccharides are most easily purified from the reaction mixture by ion-exchange chromatography eluted with water. A mixed bed column composed of Dowex cation (AG50WX2) and anion (AG1-X2) exchange resin separated by a glass filter is used to isolate asialyl-oligosaccharides whereas only a cation-exchange resin is used if isolating sialyl-oligosaccharides. It is important that the ion-exchange resin be prepared in the acetate form to avoid the irreversible binding of the oligosaccharide. A mixed bed ion-exchange column with dimensions of 2.5 × 40 cm has sufficient charge capacity to purify oligosaccharides from approximately 2.5 g of glycoprotein (16). The peptides are trapped on the column whereas oligosaccharides elute in the void fraction and are detected by Abs_{214nm}. The carbohydrate peak can also be detected using the phenol sulfuric acid assay [24].

The quantity of oligosaccharide recovered from each step of the batch purification is determined by quantitative glucosamine analysis [25]. The glucosamine in a weighed amount of glycoprotein is a reliable starting point for determining the purification yield. This is compared to the glucosamine in glycopeptides isolated from tryptic digestion, and with the glucosamine recovered from the reducing-oligosaccharides isolated from ion-exchange chromatography to derive yields throughout the batch purification. Some typical recoveries of N-linked oligosaccharides are presented in Table 1.

3. Formation of oligosaccharide-glycosylamines and coupling with Boc-tyrosine

Reaction of reducing-oligosaccharides with ammonium bicarbonate results in the formation of oligosaccharide-glycosylamines and glycosylamine-carbonates as first reported by Lihkoshertov et al. [26] and Kallin et al. [13] (Fig. 2).

Prior to coupling to the oligosaccharide-glycosylamine it is necessary to remove excess ammonium bicarbonate from the reaction. During this process care must be taken to minimize

Table 1
Yields[a] for reducing-N-linked oligosaccharides during batch purification

Purification step	Bovine fetuin	Porcine fibrinogen	Hen ovotransferrin	Hen ovalbumin
Glycoprotein (5 g)[b]	149	25	32	140
Tryptic glycopeptides[c]	95	23	29	98
Reducing-oligosaccharides[d]	86	21	26	85

[a] Values are μmol of oligosaccharide either four (fibrinogen) or five N-acetyl glucosamine residues per oligosaccharide.
[b] Determined from a weighed quantity of glycoprotein.
[c] Derived from tryptic glycopeptides recovered from dialysis.
[d] Isolation yield of oligosaccharides after ion exchange.

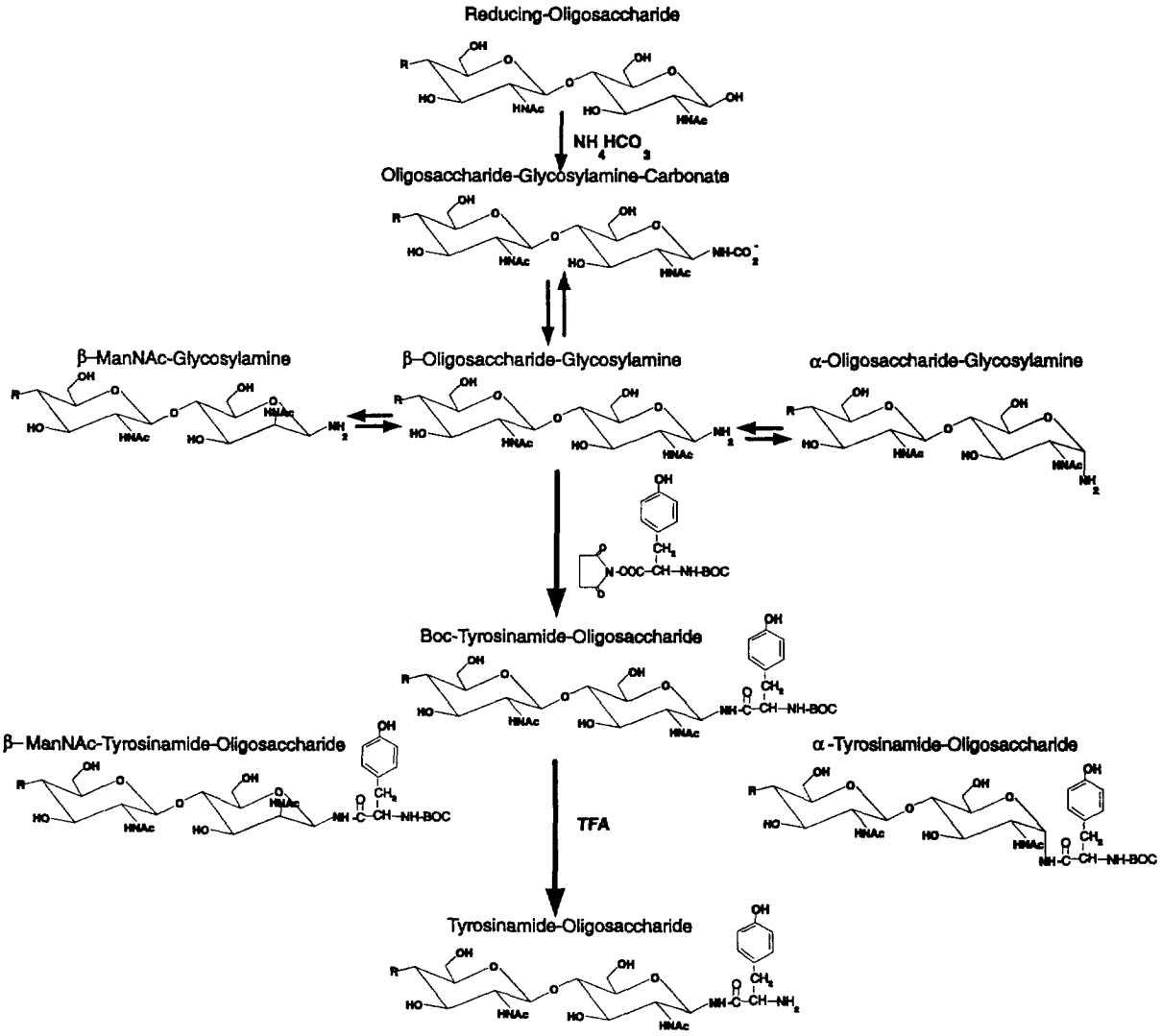

Fig. 2. Conjugation of tyrosine to reducing-oligosaccharides. Reducing-oligosaccharides react with saturated ammonium bicarbonate to form oligosaccharide-glycosylamine-carbonates and oligosaccharide-glycosylamines [16–19]. The major oligosaccharide-glycosylamine products (90%) are at an anomeric ratio (β:α) of 5:1 whereas a minor product (<5%) arises from epimerization of the C2 of the reducing-end GlcNAc resulting in the formation of β-ManNAc-glycosylamines. Boc-Tyr-NHS ester is coupled to the oligosaccharide-glycosylamines resulting in a major tyrosinamide-oligosaccharide product (80%) containing a β-linkage between GlcNAc and the tyrosine residue whereas minor quantities of a α-tyrosinamide-oligosaccharide (<3%) and ManNAc-tyrosinamide-oligosaccharides (<5%) are formed as side products [16]. Removal of the Boc group prior to RP-HPLC separation to create tyrosinamide-oligosaccharides is performed by treatment with neat TFA followed by freeze drying.

the reformation of reducing-oligosaccharide. Desalting can be accomplished by repeated freeze drying. However, this approach is unpredictable and if carried out excessively will result in the reversion of the oligosaccharide-glycosylamines to reducing-oligosaccharides. Provided the oligosaccharides are of sufficient size, a much faster and more reliable method to remove ammonium bicarbonate is gel filtration chromatography eluting with a volatile buffer having a slightly elevated pH (10 m*M* ammonium bicarbonate pH 8.0) [17–19]. The oligosaccharide-glycosylamines

should be recovered from gel filtration chromatography and freeze dried immediately to avoid reversal to reducing-oligosaccharides.

The generation of oligosaccharide-glycosylamines can also be monitored by HPAEC since this technique is very sensitive to the composition of the reducing-end [4,16]. The oligosaccharide-glycosylamines elute earlier than reducing-oligosaccharides whereas glycosylamine-carbonates carry a formal negative charge causing their later elution on HPAEC. The utility of HPAEC in monitoring glycosylamine formation is illustrated for ovotransferrin oligosaccharides in Figs. 3A and 3B.

Several N-linked oligosaccharide derivatization and separation schemes have been devised based on coupling active esters to oligosaccharide-glycosylamines to form a glycosylamide linkage [4,16,27,28]. One common approach is to generate oligosaccharide-glycosylamines as described above, which are then coupled to in a second reaction. Alternatively, some groups have found that it is equally efficient to directly couple to the glycosylamine formed by the action of N-glycosidase F [4,29].

However, beyond the method used to form an oligosaccharide-glycosylamine, the most significant difference in each approach lies in the selection of a unique aglycon. Depending on the size and reactivity of the coupling agent, varying derivatization yields and anomeric ratios of α- and β-glycosylamide products are obtained [16,28]. The chromatographic resolution and detection of the derivatized oligosaccharides is also highly dependent on the structure of the aglycone as is the utility of the isolated products [4,11,28].

Normally, an aqueous/organic solvent mixture is selected to achieve active ester coupling to the oligosaccharide-glycosylamine. During the coupling reaction, the pH must be maintained at 8.0 in order to deprotonate the amine and avoid oligosaccharide-glycosylamine reversal [27]. However, even with the addition of base it is difficult maintain the pH since hydrolysis of the coupling reagent generates a carboxyl group causing rapid acidification and glycosylamine reversal (27). To overcome this, in situ activation of the carboxyl group of a coupling agent has been used with some improvements in coupling yields [28].

An alternative solution is to react an active ester coupling reagent prepared in organic solvent directly with the dry oligosaccharide-glycosylamines. This approach has proven effective when coupling Boc-Tyr-NHS to oligosaccharide-glycosylamines in neat DMF (Fig. 2) [15,17,18]. Consequently, oligosaccharide-glycosylamine reversal is minimized by excluding water from the reaction. Also, triethylamine is added as an organic base to deprotonate the amine. The addition of base is especially important when derivatizing sialyl-oligosaccharides since these apparently form intermolecular ion pairs between the carboxyl group on NeuAc and the oligosaccharide-glycosylamine.

Initially, the oligosaccharide-glycosylamine remains insoluble but over three hours with gentle heating (50°C) it solubilizes in DMF as the reaction goes to completion. Under these conditions, Boc-Tyr-NHS not only couples to the glycosylamine but also esterifies hydroxyl groups on the oligosaccharide. During the reaction work-up with 1 *M* sodium hydroxide, the tyrosine esters are rapidly hydrolyzed whereas the desired tyrosinamide linkage is stable.

Tyrosinamide-oligosaccharides are recovered free from excess reagents using a gel filtration column during which reducing-oligosaccharides and tyrosinamide-oligosaccharides co-eluted ahead of excess Boc-Tyr. The derivatization yield may be estimated by comparing the ratio of the tyrosinamide-oligosaccharide peak relative to the reducing-oligosaccharide on HPAEC (Fig. 3C) Alternatively, a quantitative assessment of reaction yield is measured from the absorbance of the tyrosine group which has a known molar absorptivity ($\epsilon = 1330\ M^{-1}\,cm^{-1}$). Both measurements compare favorable to arrive at a derivatization yield which is typically 80%.

4. Separation of tyrosinamide-oligosaccharides on RP-HPLC

Tyrosinamide-oligosaccharides are resolved on either an analytical or semi-preparative (5 μm particle) reversed-phase C8 or C18 HPLC col-

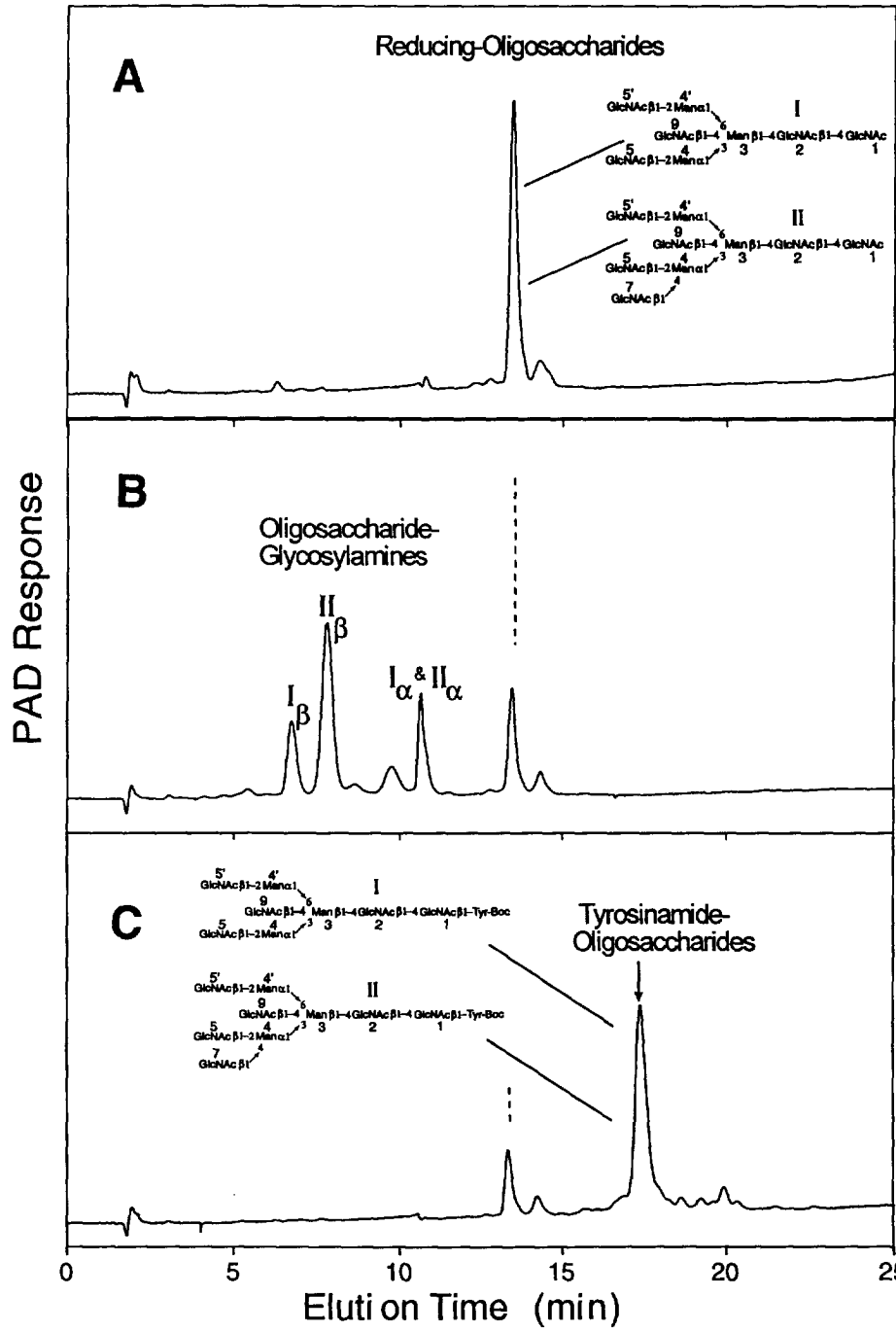

Fig. 3. HPAEC of ovotransferrin oligosaccharides. Reducing-oligosaccharides (1 nmol) from ovotransferrin were chromatographed on PA-1 column (1 ml/min, 100 m*M* sodium hydroxide with sodium acetate 0–250 m*M* in 30 min) detecting the eluting peaks electrochemically using a pulsed amperometric detector (PAD) [18]. Panel A illustrates a single major peak representing two unresolved bisecting oligosaccharides (30% I and 70% II). Panel B shows the same oligosaccharide mixture after reaction with ammonium bicarbonate and desalting. Peaks I and II were assigned as either the α or β glycosylamine [18] and approximately 15% of the oligosaccharide remained unreacted. Following derivatization with Boc-Tyr-NHS ester HPAEC revealed the presence of tyrosinamide-oligosaccharides and unreacted reducing-oligosaccharides (Panel C). The ratio of products in the chromatogram was used to estimate the coupling yield of 80%.

umn. Selection between phases is primarily dictated by the hydrophobicity of the oligosaccharides. The more hydrophobic Boc protected oligosaccharides resolve better on a C8 column whereas the hydrophilic Boc deprotected oligosaccharides are better suited for separations on a C18 column. In either case, small particle packings are essential to achieve sufficient resolution of complex mixtures of oligosaccharide. One drawback to the purification of oligosaccharides on silica based reversed-phase columns is the low recovery (50%) resulting from interactions of the hydroxyl groups on oligosaccharides with the silica column support. Polymeric RP HPLC columns provide improved recoveries (>90%) but lack sufficient resolution and are thereby reserved for less demanding purifications.

Separation of tyrosinamide-oligosaccharides on RP HPLC is performed with a mobile phase composed of either acetic acid (0.1 v/v%) or TFA (0.1 v/v%) and acetonitrile as the organic solvent. Asialyl Boc-tyrosinamide-oligosaccharides are neutrally charged and, as a result, their separations are insensitive to the solvent pH. Also, the use TFA in the mobile phase to resolve sialyl-oligosaccharides is not recommended due the reported susceptibility of this linkage to hydrolysis during chromatography [30].

An example of the semi-preparative separation of asialyl-oligosaccharides (2 μmol) from bovine fetuin is shown in Fig. 4 [16]. The elution order is dictated by hydrophobicity such that a triantennary oligosaccharide containing eleven sugars elutes 8 min earlier than biantennary with

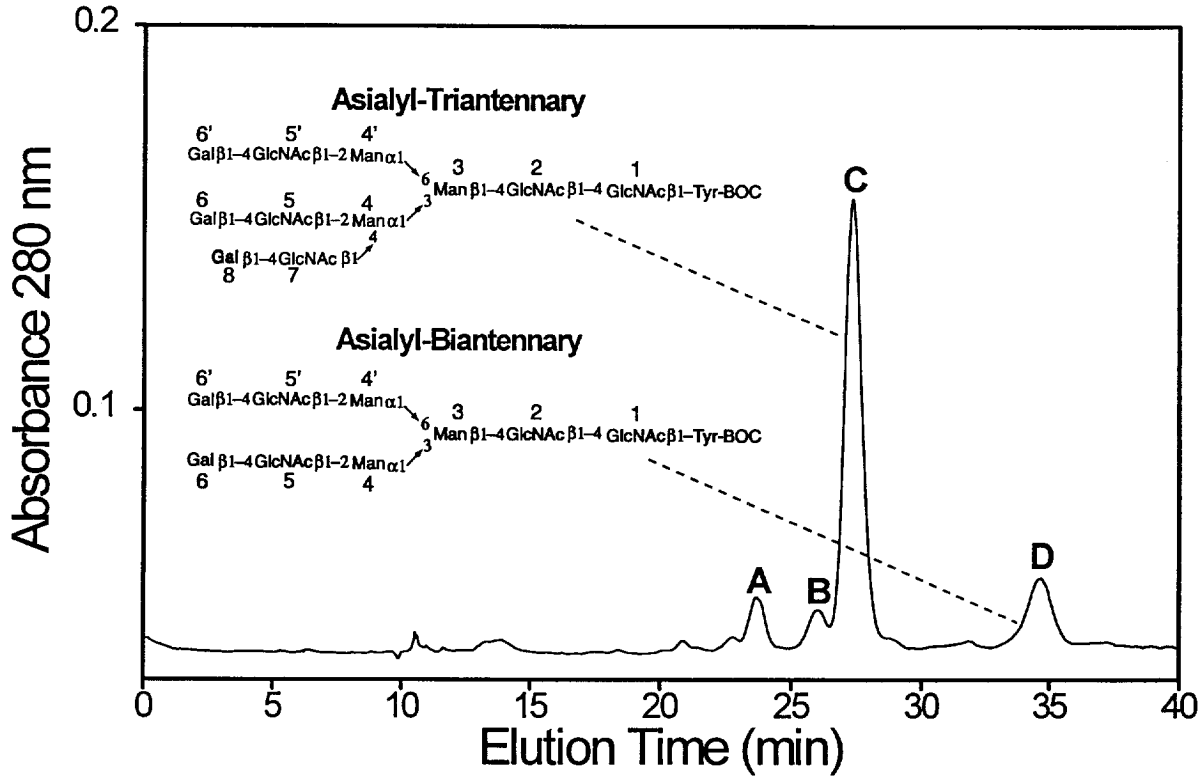

Fig. 4. Separation of bovine fetuin tyrosinamide-oligosaccharides. The chromatogram illustrates the semi-preparative RP-HPLC separation of bovine fetuin asialyl tyrosinamide-oligosaccharides (2 μmol) on a C8 column (5 μm, 2 × 25 cm, 50°C) eluted isocratically at 10 ml/min with 0.1% acetic acid 8% acetonitrile while detecting Abs_{280nm}. Asialyl-triantennary (peak C) and asialyl-biantennary (peak D) were the major products purified from bovine fetuin whereas peak A represents the ManNAc isomer of asialyl-triantennary [16]. Peak B was found to be a mixture of the α-tyrosinamide-triantennary and a β-tyrosinamide-triantennary possessing a β1–3 linkage between residues 8 and 7.

only nine sugar residues. In addition, even more subtle linkage differences (ie. β1–4 to β1–3) are resolved on two otherwise identical triantennary oligosaccharides.

Sialylated tyrosinamide-oligosaccharides also resolved on RP HPLC and their separation is strongly influenced by the number of charged NeuAc residues. When eluting with 0.1% acetic acid and acetonitrile the more highly charged disialyl-oligosaccharides elute earlier than monosialyl or asialyl-oligosaccharides (Fig. 5). In contrast to the separation of asialyl tyrosinamide-oligosaccharides, the pH does influence the separation of sialyl-oligosaccharides and consequently, increasing the acetic acid concentration slightly (0.3%) improves the column capacity (up to 2 μmols) when isolating sialyl-oligosaccharides from semi-preparative RP HPLC. Purified sialyl- or asialyl-oligosaccharides are concentrated by freeze drying or rotary evaporation of the solvent. If TFA is added to the mobile phase, freeze drying is preferred over rotary evaporation.

The chromatographic resolution of complicated mixtures of Boc-tyrosinamide-oligosaccharides is not always guaranteed. In these circumstances, the selectivity and resolution of the separation can be dramatically altered and improved by removing the Boc group in order to expose a primary amine [18,19]. Since Boc deprotection requires acidic conditions, care must be taken to avoid hydrolysis of the glycosidic linkages of the oligosaccharide. Deprotection is achieved by reacting the dry Boc-tyrosinamide-oligosaccharide with neat TFA at room temperature for 10 min. The TFA is then removed by freeze drying which also removes the tert-.butanol formed from the Boc group.

Boc deprotected oligosaccharides are resolved on RP HPLC eluted with either 0.1% acetic acid

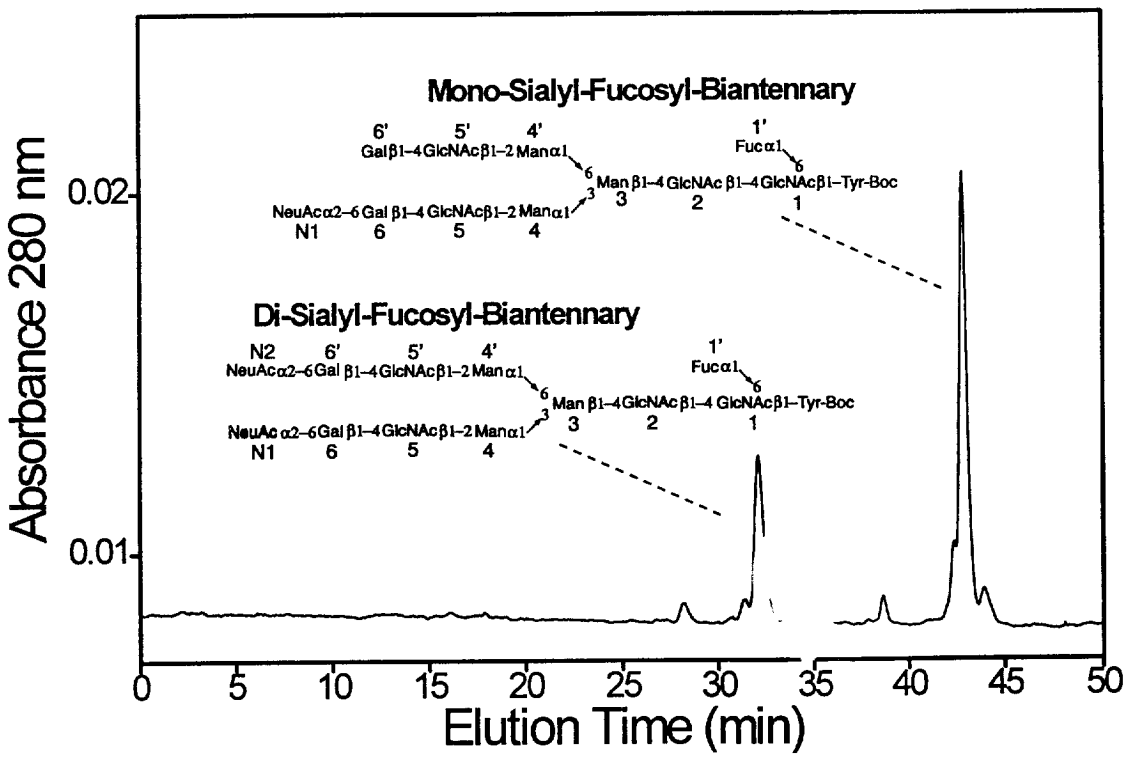

Fig. 5. Separation of sialyl-tyrosinamide-oligosaccharides from porcine fibrinogen. The analytical RP-HPLC separation of mono- and disialyl-fucosyl-biantennary was performed on a analytical C8 column (50°C) eluted isocratically at 1 ml/min with 0.1% acetic acid and 13% acetonitrile while detecting by Abs_{280nm} [17].

or 0.1% TFA. Normally, the Boc group has a significant contribution to the overall hydrophobicity of Boc-tyrosinamide-oligosaccharides. In contrast, Boc deprotected tyrosinamide-oligosaccharides require only shallow acetonitrile gradients (1–5%) to achieve resolution. The reduced hydrophobicity of the tyrosinamide moiety results in separations that are primarily influenced by the more subtle hydrophobicities of the carbohydrate portion of the molecule. The ability to alter hydrophobicity without removing the chromophore is a unique aspect of tyrosinamide derivatives. As an example of this effect, the separations of Boc deprotected tyrosinamide-oligosaccharides from ovalbumin is shown in Fig. 6A [19].

Separation conditions must be re-optimized when scaling to semi-preparative RP HPLC

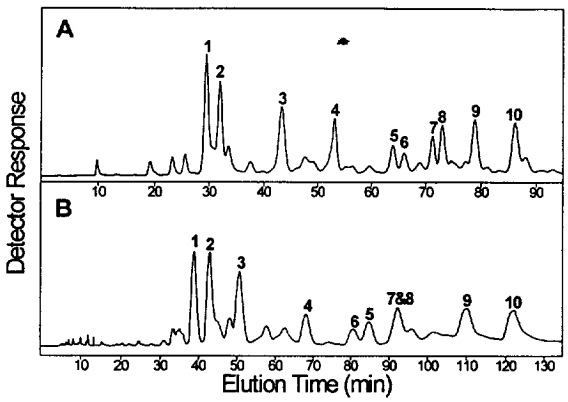

Fig. 6. Separation of ovalbumin tyrosinamide-oligosaccharides. Panel A shows the resolution of 10 major tyrosinamide-oligosaccharides from ovalbumin by injecting 3 nmol of the mixture onto an analytical C18 column eluted at 1 ml/min with 0.1% TFA and a step gradient of solvent B [10% B in 30 min, 20% B in 20 min, 35% B in 20 min, and 45% B in 20 min, where B is 2.5% (v/v) acetonitrile in 0.1% TFA] while detecting by fluorescence. Panel B shows the comparative semi-preparative separation of 3 μmol applied to a semi-preparative (2 × 25 cm) C18 RP-HPLC column eluted at 10 ml/min with 0.1% TFA and linear gradient of solvent B [20% to 50% B in 120 min, where B is 5% (v/v) acetonitrile in 0.1% TFA] while detecting by Abs_{280nm}. The semi-preparative column provided nearly equivalent resolution as the analytical column with the exception of peaks 7 and 8 which were unresolved. Tyrosinamide-oligosaccharide (1–10) were purified and characterized by proton NMR and FAB-MS. Their structures ranged from high-mannose oligosaccharides to pentaantennary complex oligosaccharides [19].

because the flow rate cannot be scaled linearly from analytical conditions while keeping the pressure within acceptable limits. Nevertheless, the resolution of 3 μmol quantities tyrosinamide-oligosaccharide on semi-preparative RP HPLC is comparable to that achieved analytically even for complicated mixtures of ovalbumin tyrosinamide-oligosaccharides (Fig. 6B).

The separation and characterization of over ten oligosaccharides from ovalbumin highlights the utility of the method for resolving a broad range of structures and also provides further insight into the selectivity of the separation [19]. High-mannose oligosaccharides elute earliest (peaks 1 and 2) in the chromatogram followed by hybrid (peaks 3–7) and complex type structures (peaks 8–10) [9]. In general, structures with increasing numbers of GlcNAc residues are retained longer presumably due to direct interaction of the acetyl group with the reversed-phase support.

Tyrosine's absorbance provides a convenient measurement of the molar quantity (Abs_{280nm}, $\epsilon = 1330\ M^{-1}$) of purified tyrosinamide-oligosaccharide and allows routine UV monitoring of the elution of tyrosinamide-oligosaccharides on gel filtration chromatography and RP HPLC. In addition, tyrosine is a fluorescent amino acid with an excitation maximum at 275 nm and emission at 305 nm. Fluorescence monitoring increases the limit of detection to 10–100 pmol on RP HPLC [14]. Tyrosine's fluorescence intensity is altered by the presence of a Boc protecting group and also by high concentrations of organic solvent such as acetonitrile [14]. However, elution of oligosaccharide mixtures from RP HPLC with shallow gradients of acetonitrile (1–5%) introduces a negligible change in the fluorescence intensity across the chromatogram.

Purified tyrosinamide-oligosaccharides are characterized using a combination of fast atom bombardment mass spectrometry (FAB-MS) and proton NMR. For FAB-MS analysis, dominant molecule ions corresponding to either the M + H or M + Na are observed in the mass range of 1500–2500 m/z which provides information of the molecular composition of the oligosaccharide

under investigation (Fig. 7A). Usually, both molecular ions are observed simultaneously which correspond to within 0.1 mass units of the calculated mass of the proton and sodium adduct for the proposed oligosaccharide structure. The presence or absence of a Boc group has a minimal influence on the sensitivity of the FAB-MS analysis.

Prior to high field proton NMR analysis, tyrosinamide-oligosaccharides are D_2O exchanged and buffered with 10 μl of 10 m*M* sodium phosphate pH 7.0. The buffer is especially important for sialyl-oligosaccharides and Boc deprotected tyrosinamide-oligosaccharides which carry a formal charge. N-linked oligosaccharides provide characteristic chemical shifts and coupling constant for the downfield (5.5–4.4 ppm) anomeric proton of Gal, Man, Fuc, and GlcNAc and the upfield N-acetyl protons of GlcNAc (2.1–1.9 ppm) (Fig. 7B) [31]. In addition to signals associated with the oligosaccharide, the tyrosinamide group is observed by the presence of phenol ring protons (7.2–6.8 ppm) and by a signal for the Boc group (1.346 ppm). The Boc protecting group also causes broadening of the N-acetyl signals of GlcNAc 1. The attachment of tyrosine to the oligosaccharide through a β-glycosylamide linkage is evident from the coupling constant (9.5 Hz) of the anomeric proton for GlcNAc 1 (5.014–5.050 ppm).

Although the β-tyrosinamide-oligosaccharide is the major product, two different reaction side products have also been isolated and characterized [16]. One of these contains tyrosine linked to the oligosaccharide in an α-glycosylamide linkage whereas the second possesses ManAc replacing GlcNAc as the first sugar residue (Fig. 2) [16]. Each of these arise from the derivatization chemistry but represent minor components (<5%) which are chromatographically resolved from the major β-glycosylamide products.

5. Applications for tyrosinamide-oligosaccharides

An important attribute of tyrosinamide-oligosaccharides is their reversal to form reducing-oligosaccharides via Edman degradation [14]. Reaction of the amine terminus with phenylisothiocyanate (PITC) results in a PTC-oligosaccharide which is rapidly cyclized to AZT-tyrosine and the reducing-oligosaccharide when treated with TFA (Fig 8A) [32]. This results in the initial formation of an oligosaccharide-glycosylamine which can either be coupled to directly or decomposed into a reducing-oligosaccharide by lowering the pH. The TFA hydrolysis steps are even mild enough to reverse tyrosinamide-oligosaccharides that possess acid label fucose or NeuAc residues. This provides a facile route to convert tyrosinamide-oligosaccharides into reducing-oligosaccharides which may then be used to prepare a variety of analytical standards for chromatography [1–9].

In another application, oligosaccharide remodeling has been performed to prepare μmol quantities of rare tyrosinamide-oligosaccharides (Fig. 8B) [12]. Glycosidases such as neuraminidase, β-galactosidase, β-N-acetylglucosaminadase, and α-mannosidase can be used partially or completely to remove the terminal residues on complex oligosaccharides. Removal of sugar residues increases the overall hydrophobicity of a tyrosinamide-oligosaccharide resulting in their longer retention on RP HPLC. Likewise, glycosyltransferases such as galactosyltransferase, fucosyltransferase, and sialyltransferase have been used to attach sugars to biantennary and triantennary oligosaccharides resulting in novel glycoconjugates [11]. The transfer of sugar residues causes a shift to earlier elution times on RP HPLC. The optimization of enzyme reaction conditions and the purification of the transformed product is simplified due to the presence of the tyrosinamide group which is detected by fluorescence and facilitates RP HPLC purifications.

One of the main attributes of tyrosinamide-oligosaccharides is their ability to be radioiodinated (Fig. 8C). This is performed using the chloramine-T reaction followed by purification on gel filtration chromatography [33]. The purity of the radioiodinated oligosaccharides can be established using TLC and quantitative autoradiography [11]. Radioiodinated tyrosin-

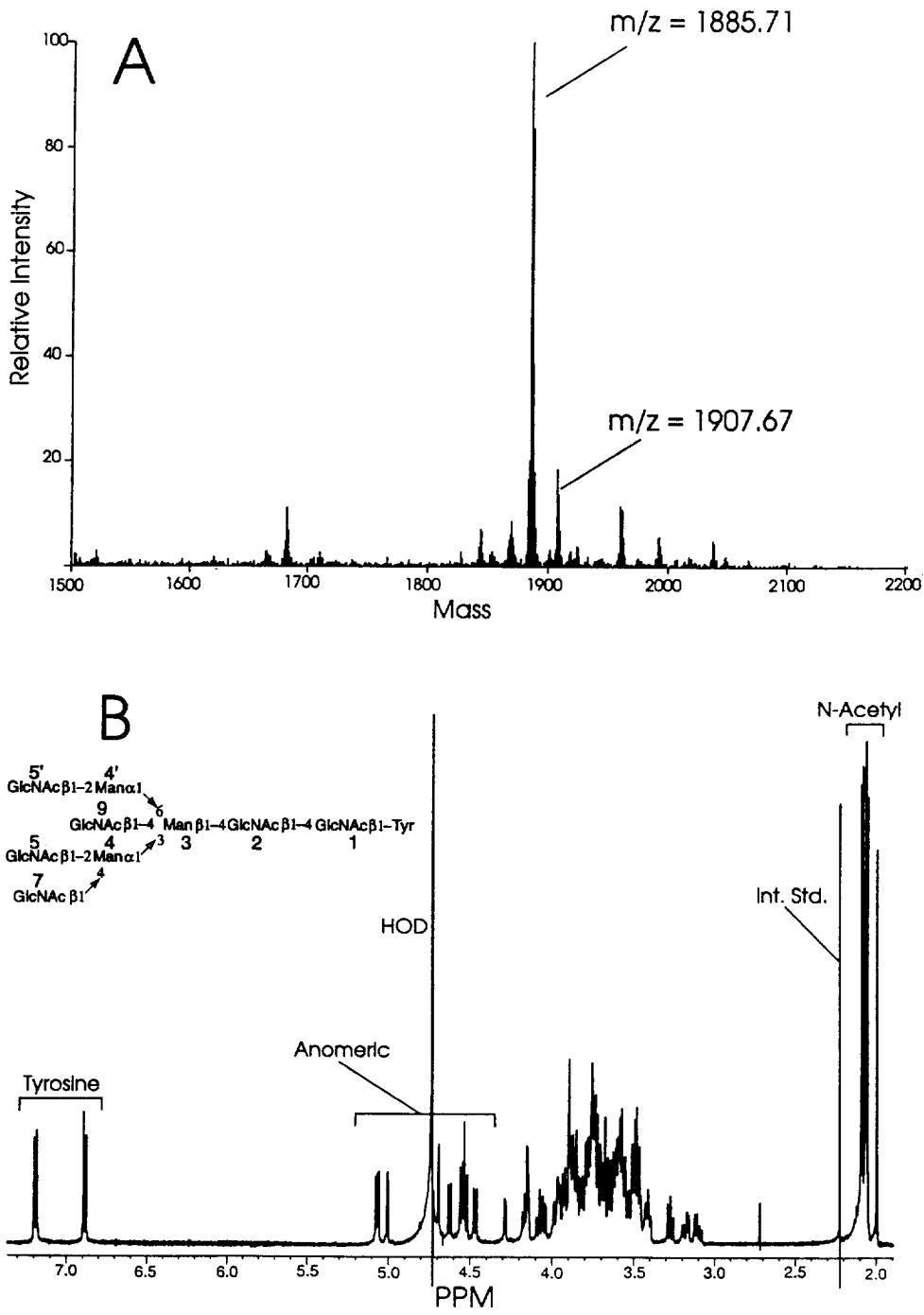

Fig. 7. Proton NMR and FAB-MS of tyrosinamide-oligosaccharides. FAB-MS analysis of the oligosaccharide provides a molecular ion corresponding to the M + 1 (m/z = 1885.71) and M + 23 (m/z = 1907.67) adducts (panel A) corresponding to a molecular composition of $GlcNAc_6Man_3Tyr$. The proton NMR spectra presented in panel B displays structural reporter group anomeric protons and N-acetyl protons consistent with the bisecting triantennary tyrosinamide-oligosaccharide shown [18].

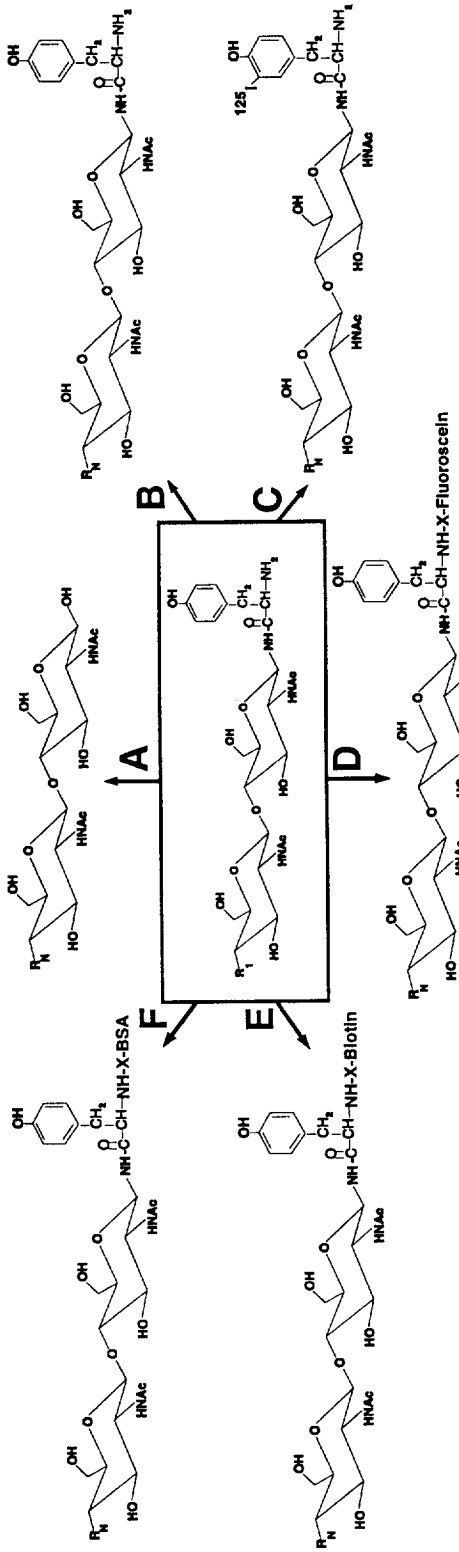

Fig. 8. Applications for tyrosinamide-oligosaccharides. After removing Boc a tyrosinamide-oligosaccharide can be reversed to a reducing-oligosaccharide using a modified Edman degradation protocol (A) [14]. Oligosaccharide remodeling enzymes can be used to alter the structure of the outer antenna of the oligosaccharide in order to convert R_1 to R_N (B) (N represents a remodeled structure) [11]. Prior to biological studies, radioiodination of tyrosine is performed with chloramine-T to generate oligosaccharides with a single ^{125}I group (C) [11]. Either fluorescein (D) or biotin (E) can be attached to tyrosine's amine terminus to create highly fluorescent or retrievable oligosaccharide probes. Oligosaccharides also may be attached to polypeptides or proteins such as BSA by conjugation of tyrosine's amine to the side chain of amino acids in the polymer (F). X represents different linker groups.

amide-oligosaccharide have a high specific activity (100 μCi/nmol) which is advantageous for receptor binding studies in vitro and for monitoring oligosaccharide biodistribution and pharmacokinetics in vivo. The tyrosinamide group provides good metabolic stability relative to natural glycopeptides [12]. Should it be necessary to incorporate a radiolabel with a longer half-life, it is also possible to remove Boc and acetylate the amine terminus with ^{3}H or ^{14}C labeled acetic anhydride.

Once the amine terminus of tyrosine is unmasked it can be used as a coupling site for attaching a variety of probes. Fluorescein can be added to the oligosaccharide by reaction with 6-(fluorescein-5-carboxyamido)hexanoic N-hydroxysuccinimide ester (Molecular Probes, Eugene, OR, USA) (Fig. 8D). In addition, tyrosinamide-oligosaccharides can be biotinylated by reaction with bioiamidocaproate N-hydroxysuccinimide ester (Fig. 8E). It is often desirable to attach oligosaccharides to macromolecules such as BSA or poly-lysine. This can be accomplished using a variety of chemical modification schemes that take advantage of the amine terminus on tyrosine [34] (Fig. 8F).

6. Conclusions

An approach to purify multi-μmol quantities of N-linked oligosaccharides from glycoproteins is described. The presence of a Boc-tyrosinamide group on each oligosaccharide product offers the opportunity to alter separation selectivity, to detect by absorbance or fluorescence, to regenerate reducing-oligosaccharides, to radioiodinate oligosaccharides, and to further derivatize the amine terminus. These attributes make tyrosinamide-oligosaccharides among the most versatile glycoconjugates developed to date. Practical applications include the generation of primary standards used to analyze oligosaccharide heterogeneity on glycoproteins and the preparation of glycoconjugate probes used to decipher the function of N-linked oligosaccharides in biological systems.

7. List of abbreviations

Boc-Tyr	*tert.*-Butoxy-carbonyl-L-tyrosine
NHS	N-Hydroxysuccinimide
PITC	Phenylisothiocyanate
ATZ	Anilinothiazolinone
PTC	Phenylthiohydantoin
DMF	Dimethyl formamide
TFA	Trifluoroacetic acid
RP-HPLC	Reversed-phase high-performance liquid chromatography
MWCO	Molecular weight cut off
HPAEC	High pH anion-exchange chromatography
PAD	Pulsed amperometric detector
NMR	Nuclear magnetic resonance
FAB-MS	Fast atom bombardment mass spectrometry
R and A	Reduced and alkylated

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