

Advances in the use of biotinylated diaminopyridine (BAP) as a versatile fluorescent tag for oligosaccharides

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We recently described a novel fluorescent compound, 2-amino,6-amidobiotinyl-pyridine (BAP), that allows the tagging of oligosaccharides, their fractionation by reversed-phase HPLC with picomole scale detection, and the formation of functional neoglycoprotein equivalents with (strept)avidin for the detection of receptors and the generation of monospecific antibodies (Rothenberg *et al.*, *Proc. Natl Acad. Sci. USA*, 90, 11939–11943, 1993). Here, we describe the enhancement of this approach by the following. (i) A simple one-step purification of BAP from its synthetic precursors and other reactants. (ii) Development of HPLC sizing column methods to quickly purify BAP-coupled oligosaccharides away from free BAP and other reactants. (iii) Development of anion-exchange and amine-adsorption HPLC procedures for the fractionation of BAP-oligosaccharide adducts by charge and size, respectively. (iv) Investigation of the affinity of BAP-oligosaccharides for (strept)avidin, confirming the formation of stable complexes. (v) The use of BAP for sensitive monosaccharide compositional analysis of glycoproteins. (vi) Formation of stable BAP adducts without reduction and its implications for the mechanism of adduct formation. These advances make available a multitude of techniques for the fractionation of BAP-coupled oligosaccharides based on several different physical parameters. Distinct species of BAP-coupled oligosaccharides can be isolated and subjected to detailed structural analysis. Such defined molecules form stable complexes with streptavidin that are effectively neoglycoproteins, which can be used in a variety of biological applications. Notably, all of these approaches require relatively inexpensive materials and conventional equipment available in most laboratories.

Key words: analysis/biotin/fluorescence/fractionation/neoglycoconjugates

Introduction

Oligosaccharides are involved in many diverse biological roles, such as intracellular trafficking of lysosomal enzymes (Dahms *et al.*, 1989), receptor-mediated endocytosis of asialoglycoproteins (Lodish, 1991; Lee, 1992) and cell–cell interactions mediated by the selectins (Cummings and Smith, 1992). The determination of the structure of a given oligosaccharide

and probing of its potential biological functions can be quite challenging. Frequent hurdles in structural analyses include low quantities of material, linkage and branching heterogeneity, and additional modifications (e.g. acetylation, sulphation and phosphorylation) (Baenziger and Green, 1988; Dahms *et al.*, 1989; Varki, 1993; Parekh, 1994). The purification of oligosaccharides from complex mixtures requires a variety of techniques, as well as sensitive and specific detection. Classically, the reducing end of oligosaccharides has been labelled either radioactively (Kobata, 1994) or by chromophores (Lee *et al.*, 1991; Hase, 1994) to aid in sensitive detection. The hydrophobic or anionic character of the chromophoric groups can be exploited to improve oligosaccharide fractionation by either high-performance liquid chromatography (HPLC) or electrophoresis techniques (Jackson, 1991; Lee *et al.*, 1991; Stack and Sullivan, 1992; Hase, 1994). Detection of unlabelled sugars by pulsed amperometric detection (Townsend *et al.*, 1989) is also possible, but the technique requires expensive equipment and subjects the sugars to strongly basic conditions which can cause de-*O*-acetylation and/or epimerization. Often, two or more chromatographic techniques, based on different physical properties, are required to provide resolution of complex mixtures. An example of this approach is the recently described ‘two-dimensional mapping’ of pyridylamino (PA)-coupled oligosaccharides by size and hydrophobicity (Tomiya *et al.*, 1991; Hase, 1994).

A fundamental limitation with the above techniques is that once the oligosaccharides of interest have been isolated and characterized, they are of relatively limited use for functional or biological studies. A recently described option is to convert fluorescent PA sugars to biotinylated sugars by reductive hydrogenation, followed by hydrazinolysis and subsequent rederivatization with biotin (Hase, 1992). By replacing the PA groups with biotin, (strept)avidin neoglycoproteins could be prepared for functional studies. However, this technique requires separate rederivatization and repurification of each PA-sugar of interest. Furthermore, the hydrazinolysis may cause the degradation of functionally important groups such as *O*-acetyl or sulphate esters (Patel and Parekh, 1994).

Previously (Rothenberg *et al.*, 1993), we described a novel fluorescent compound, 2-amino,6-amidobiotinyl-pyridine (BAP), that allows tagging of oligosaccharides and their fractionation by reversed-phase HPLC with picomole-level detection [see Rothenberg *et al.* (1993) for the structure of BAP and the oligosaccharide–BAP adduct]. Since biotin is already attached to the fluorophore, numerous applications could subsequently exploit the multivalent biotin–(strept)avidin interaction (Wilchek and Bayer, 1988; Shao *et al.*, 1990; Shao, 1992). The biotin moiety permits the formation of neoglycoprotein equivalents with (strept)avidin for the detection of receptors and the generation of monospecific antibodies

(Rothenberg *et al.*, 1993). However, the versatility and effectiveness of BAP as an oligosaccharide tag requires much further investigation.

Here, we present improved methods for the preparation and purification of both BAP and BAP-oligosaccharides, and techniques for fractionating BAP adducts by size and charge. A method for using BAP for accurate and sensitive monosaccharide composition analysis of glycoproteins is also presented. Further, we address the stability of BAP adducts to (strept) avidin under various conditions. Unlike other recently developed high-technology approaches, the systems described here employ conventional and relatively inexpensive materials and instrumentation.

Results and discussion

One-step purification of BAP from its synthetic precursors and other reactants

In our previous study (Rothenberg *et al.*, 1993), the reported purification method for preparing BAP was: (i) technically difficult and time consuming, involving many steps and lasting more than a week; (ii) expensive because large volumes of solvents were required for flash chromatography; and (iii) impractical for small-scale preparations of BAP. Here, we describe an improved and simplified method for purifying BAP from reactants and side products after synthesis.

BAP was synthesized using minor modifications of the previously described method (Rothenberg *et al.*, 1993). As indicated under Materials and methods, we have now found that the entire reaction mixture of substrates, side products and BAP can be directly loaded onto a 'Spice C₁₈ tube' after dilution in water, allowing a one-step purification. The only limiting factor is the capacity of the Spice tube for BAP (~50 mg) and any remaining unreacted biotin. During the water wash, a yellowish band (also fluorescent) adheres to the top of the C₁₈ tube, a blue band migrates slowly, while the strongly fluorescent excess of 2,6 diaminopyridine (DAP) is slowly eluted. As seen in lane 1 of Figure 1, *N*-hydroxy-sulphosuccinimide (NHS) and fluorescent DAP run through the C₁₈ tube during the water wash. A second wash with 10% acetonitrile is used to elute remaining traces of DAP. The pale yellow and UV-fluorescent BAP is eluted with 50% acetonitrile. This material has an identical mobility to authentic BAP, as detected by both UV and iodine visualization (see Figure 1, lane 3). Reversed-phase (RP)-HPLC and fast atom bombardment mass spectrometry (FAB-MS) confirmed the identity of the material as BAP (mol. wt 336, data not shown). No additional material was eluted with 100% acetonitrile. A blue compound that co-migrated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was eluted during a subsequent 100 ml column regeneration wash (see Figure 1, lane 5). Smaller capacity C₁₈ Spice cartridges were also effective in the purification of BAP, although BAP eluted with lower concentrations of acetonitrile. The final yield of BAP from the 50% acetonitrile eluate material was 31% (by weight, based on original biotin input). The yield increased to 38% when a 10-fold molar excess of DAP to biotin was used in the initial reaction, instead of the previously described 3-fold molar excess. The moderate yields of 31–38% may reflect the limited stability of activated EDC complexes under aqueous conditions and are currently under investigation. The purity of

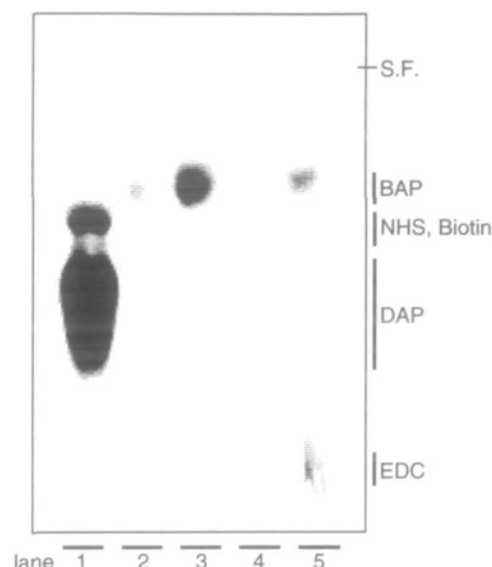


Fig. 1. HPTLC of BAP purification on a C₁₈ tube. Equal aliquots of various C₁₈ washes were spotted on a silica gel TLC plate, developed in 85% ethanol and stained with iodine vapour. Lanes 1–5 are the sequential elution of the C₁₈ tube with water, 10% acetonitrile, 50% acetonitrile, 100% acetonitrile and 2:1 chloroform/methanol in 1% glacial acetic acid, respectively. Migration positions of standards and the solvent front (S.F.) are indicated on the right. The TLC was scanned and processed using an Applescan scanner.

BAP was slightly improved from 95% to >99% (as judged by RP-HPLC fluorescence) by a second passage over a C₁₈ tube (data not shown). The purity of the final preparation is comparable to that obtained by the previously described method (Rothenberg *et al.*, 1993).

The new method is: (i) quick, the purification takes ~1 day; (ii) inexpensive, only minimal amounts of organics and C₁₈ tubes or cartridges are required; (iii) adaptable, it can be scaled up or down as desired. Thus, reasonable quantities of BAP can now be quickly prepared at low cost.

General properties of BAP

The biotin moiety causes BAP to be slightly hydrophobic. However, the solubility of BAP in water at room temperature is ~1 mg/ml (or 3000 nmol BAP/ml). This can be increased (>10-fold) by the presence of very small quantities of organic modifiers [(e.g. <1% dimethyl sulphoxide (DMSO)]. Moreover, BAP-oligosaccharide adducts contain hydrophilic sugars which should also increase solubility. In practice, organic modifiers (e.g. acetonitrile) facilitate the fractionation of BAP adducts on 'toyopearl' (Tosohass) or silica-based supports. However, we have noticed some smearing on Sepharose and Sephadex resins due to non-specific interactions with BAP. As previously reported, the optimum pH for sensitive detection is <5 (Rothenberg *et al.*, 1993). The adducts show good stability at room temperature (1 day), and for over a year at –20°C (data not shown). Finally, it should be noted, that as with other previously described oligosaccharide-tagging methods involving reductive amination, the coupling reactions are not stoichiometric, and depend to some degree on the nature of the oligosaccharide being coupled. Using BAP, we have typically noted coupling efficiencies ranging from 40 to 90%, with the larger, more charged chains tending to be less efficient.

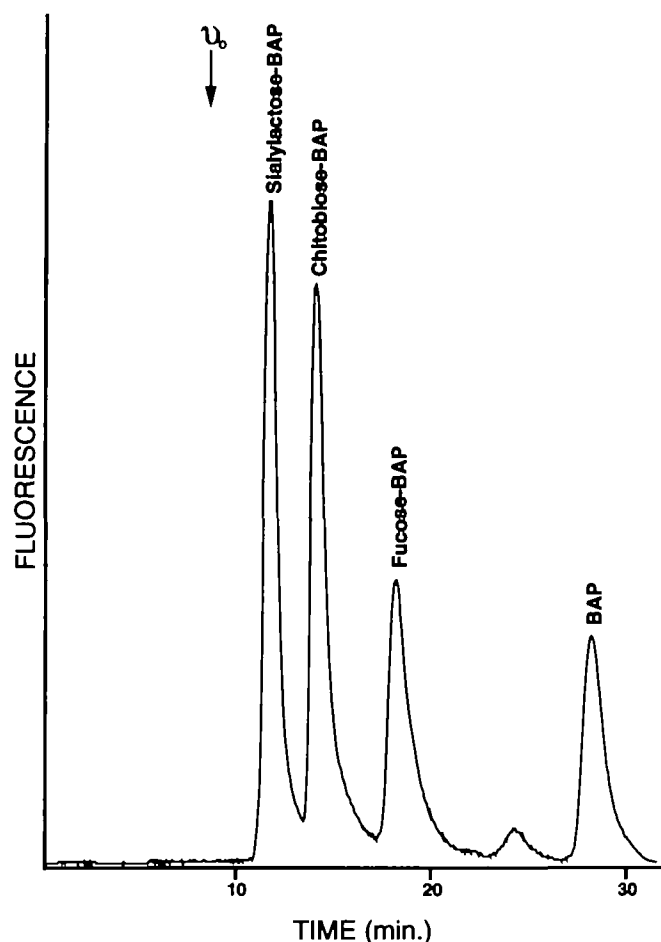


Fig. 2. Removal of BAP and fractionation of small BAP adducts by size-exclusion chromatography (SEC-HPLC). A mixture of sialylactose-BAP, chitobiose-BAP, fucose-BAP and BAP was injected onto a TSK-G3000PWxl HPLC column operating isocratically and the fluorescence was monitored. The void volume (where large BAP adducts elute) is marked by v_0 .

Development of HPLC sizing column methods to fractionate distinct species of BAP-coupled oligosaccharides away from free BAP, and from one another

Historically, after oligosaccharide derivatization, excess coupling reagents and contaminants have been removed by gel filtration, evaporation or extraction with benzene (Hase *et al.*, 1984; Iwase *et al.*, 1990; Kuraya and Hase, 1992; Linhardt, 1994). Evaporation techniques are usually only partially effective in removing excess reagents, and must be followed by gel-filtration chromatography. Slow column flow rates can necessitate lengthy run times, sometimes exceeding 24 h (Rothenberg *et al.*, 1993; Hase, 1994). To avoid these time-consuming or chemically toxic processes, an HPLC fractionation of BAP-oligosaccharides away from free BAP was performed by size exclusion on a TSK-G3000PWxl column.

As shown in Figure 2, this system gave an excellent separation of oligosaccharide adducts (mono-, di- and tri-saccharides), based on size, with high reproducibility. Larger BAP-oligosaccharides eluted close together near the void volume (data not shown). Since all coupled sugars are well separated from free BAP, the column is routinely used preparatively to remove excess BAP and other reagents from derivatized samples. The short run time, coupled with the

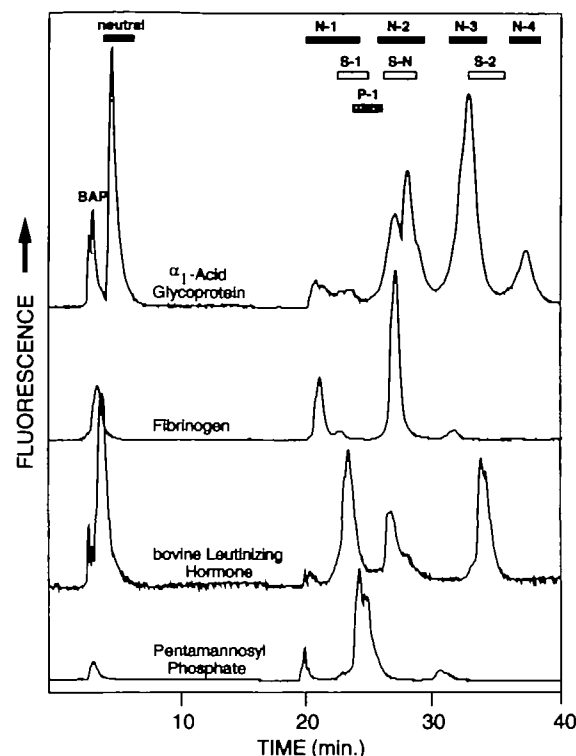


Fig. 3. Anion-exchange HPLC of BAP-oligosaccharides on a TSK-DEAE-2SW column. N-Linked oligosaccharides were released from the indicated glycoproteins with PNGaseF, coupled to BAP, and purified on a TSK-G3000PWxl column as in Figure 2. An aliquot of the v_0 eluting material was then analysed on a TSK-DEAE-2SW column eluted with an acetate gradient. The elution positions of BAP-oligosaccharides containing zero (neutral), one (N-1), two (N-2), three (N-3) and four (N-4) sialic acid residues are indicated with a solid box. BAP-oligosaccharides containing one (S-1) or two (S-2) sulphate residues, or one sialic acid and one sulphate (S-N), are indicated with an open box. BAP-oligosaccharides bearing one phosphate (P-1) are indicated with a dashed box.

ability to monitor real-time fluorescence, makes this procedure advantageous over classical gel filtration. This system also provides greater sensitivity (low picomole range), avoiding 'blind pooling' of fractions. Additionally, the high resolution of this column permits fractionation and structural analysis of small BAP-oligosaccharides. Higher molecular weight range columns made of similar support material (G4000 and G5000) were less efficient in fractionation of BAP adducts, but sufficient for removal of excess BAP. Since the columns are relatively expensive, the occasional user may still prefer to use our previously described technique of conventional gel filtration (Rothenberg *et al.*, 1993) to remove excess BAP.

Development of anion-exchange HPLC procedures for the fractionation of BAP-oligosaccharide adducts

Anion-exchange HPLC on a TSK-DEAE-2SW column (Tosohass) was found to efficiently fractionate negatively charged BAP-oligosaccharides (see Figure 3). In order to avoid desalting the sample after fractionation, we chose not to use the well-established ion-exchange chromatography protocols using phosphate gradients on amino columns (Green and Baenziger, 1986). Furthermore, the DEAE columns provide greater stability than amino columns during repeated use. Mono-Q anion-exchange columns (Pharmacia) were tested, but non-specific

interactions caused excessive band broadening. Owing to the hydrophobic nature of BAP, the DEAE system was operated in the presence of 25% acetonitrile. Under the stated conditions, oligosaccharides containing all commonly found anionic charges (sialic acid, sulphate, phosphate or uronic acid) bound to the column, and were eluted by an increasing gradient of acetate. Separation of different oligosaccharides into the indicated classes was based primarily on the net negative charge. As seen in Figure 3, N-linked oligosaccharides from α_1 -acid glycoprotein, where the negative charge is due exclusively to sialic acid, were separated into five discrete regions containing molecules with 0–4 sialic acids. Moreover, structural heterogeneity within a given charge class can be seen, and most likely represents a partial separation of oligosaccharides that differ in molecular size, as has been reported previously for α_1 -acid glycoprotein (Ohta *et al.*, 1990). For unknown samples, this additional level of fractionation could potentially be utilized in structural analyses.

Since the column is run at pH ~ 4, the pK_a s of different anionic groups influence the net negative charge and hence the binding to the column. A single negative charge from sulphate shows increased retention times relative to sialic acid, although variations in sugar linkage positions can also effect elution (Green and Baenziger, 1986; Ohta *et al.*, 1990). Increased retention of P-Man₅-BAP relative to sialic acid (Figure 3) most likely results from a stronger net negative charge, due to partial deprotonation of phosphate hydroxyl groups (pK_a = 2.1 and 5.5) at pH 4.

Sulphated oligosaccharides from bovine luteinizing hormone (bLH) fall into separate, although partially overlapping, regions with sialylated oligosaccharides. Green and Baenziger (1986) previously reported separation of sialylated and sulphated oligosaccharides on an amine column at pH 1.7, but not at pH 4. The partial resolution of these oligosaccharides even at pH 4 may reflect the difference in column stationary phase or the presence of organic modifiers. By further lowering the pH, the separation of sialic acid and sulphated regions could possibly be further enhanced.

This system provides efficient and reliable separation of BAP-oligosaccharide adducts by negative charge. Each run can be completed in an hour and no column regeneration is required. Treatment of the BAP-oligosaccharides either chemically or enzymatically provides a simple means of confirming the nature of the negatively charged moiety. Unlike most systems which typically use sodium or phosphate salts, this method uses a completely volatile buffer system, avoids desalting and facilitates subsequent chromatographic or enzymatic steps.

After anion-exchange HPLC, the pooled fractions may be further purified or structurally characterized by other HPLC techniques. As a model study, structural sequencing of previously well-known biantennary BAP-oligosaccharides from fibrinogen was carried out. Analysis of these oligosaccharides isolated from the N-2 region of the anion-exchange column (Figure 3) was performed by sequential exoglycosidase digestions and RP-HPLC analysis (Figure 4). Treatment with glycosidases caused the product to become more hydrophobic and elute with increased acetonitrile concentrations, resulting in distinct shifts after each digestion.

Although very useful for such analysis, we have found that RP-HPLC has the following general limitations: (i) failure to resolve oligosaccharide species of similar relative

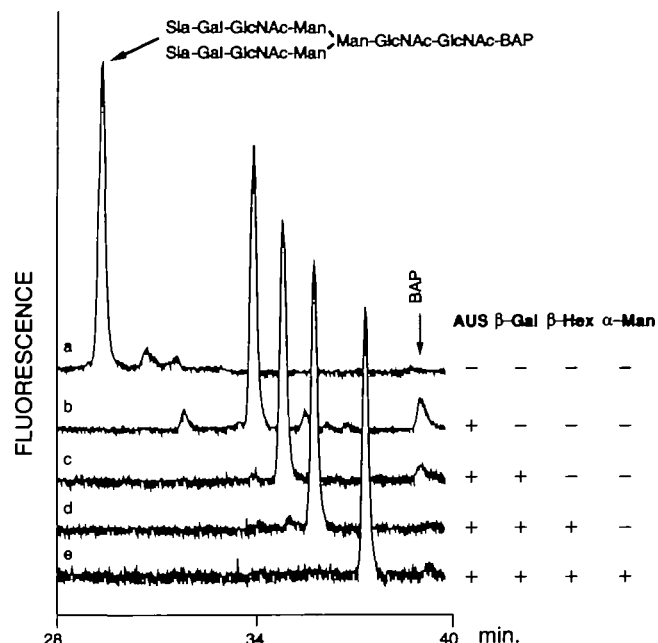


Fig. 4. Analysis of BAP adducts on RP-HPLC by sequential exoglycosidase digestions. BAP adducts of fibrinogen oligosaccharides were collected from the N-2 region on a TSK-DEAE-2SW column (see Figure 3) and treated with *A. ureafaciens* sialidase (AUS), jack bean β -galactosidase (β -Gal), *S. pneumoniae* β -N-acetylhexosaminidase (β -Hex) and α -mannosidase (α -Man) as indicated, and a fraction was analysed on a Microsorb C₁₈ column. Owing to differences in the pH optima of the enzymes the major peak, after treatment with AUS, was collected and sequentially treated with the remaining enzymes. Sham treatments without enzyme showed no effects.

hydrophobicity; (ii) difficulty in estimating the number of sugar residues of an unknown sample based solely on hydrophobicity, since factors such as branching can play important effects (Oku *et al.*, 1990); (iii) poor resolution of oligosaccharides with >12 monosaccharide units. Therefore, separation according to another physical parameter, such as size fractionation by amine-adsorption chromatography, was explored.

Development of an amine-adsorption HPLC system for size fractionation of neutral BAP-oligosaccharides

An amine-adsorption HPLC system provided separation of neutral BAP adducts based on the number of hydroxyl groups. Using an inexpensive NH₂ column (Microsorb, Rainin) and an increasing water gradient, excellent fractionation of a BAP-oligosaccharide ladder from partially hydrolysed dextran was obtained (see Figure 5a). Baseline separation of (Glc)_n-BAP adducts containing 1–35 glucose residues was achieved in <50 min. The minor peaks in between the major ones are probably due to isomers. Although the upper limits of this system were not explored, these results compare very favourably with amine-adsorption methods of size fractionation for non-derivatized oligosaccharides (Mellis and Baenziger, 1981). In the present system, BAP-oligosaccharides from RNase B yielded a pattern very similar to published reports (Rudd *et al.*, 1992). The elution position of the first major peak, corresponding to Man₅-GlcNAc₂-BAP, near the Glc₈ standard may result from increased retention of the mannose oligosaccharide containing a 1,6-linkage (Blanken *et al.*, 1985). Oligosaccharide adducts from ovalbumin contained a cluster of

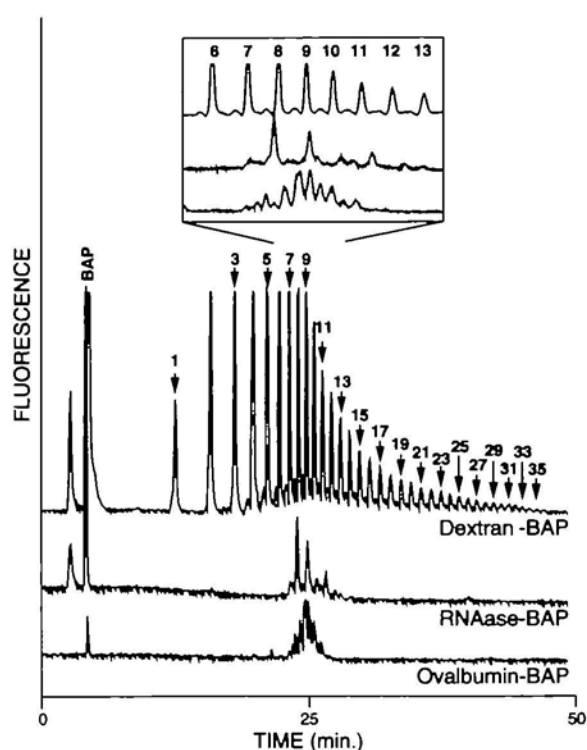


Fig. 5. Amine-adsorption HPLC fractionation of neutral BAP-oligosaccharides. Aliquots of various neutral BAP-oligosaccharides were analysed on a Microsorb NH_2 column. A dextran-BAP ladder was prepared by partial acid hydrolysis and coupling to BAP. Coupled sugars were purified on a C_{18} cartridge. The number of glucose residues is indicated above the HPLC peaks. RNase B and ovalbumin N-linked oligosaccharides were released by PNGase F, coupled to BAP and initially purified on a TSK-G3000PWxl column. The inset shows an expanded view of the region including the ovalbumin and RNase B adducts.

over a dozen different neutral species which were partially resolved consistent with known structural heterogeneity. Since oligosaccharides show a relatively predictable behaviour on amine columns, it is possible to determine the relative oligosaccharide size by comparison with glucose standards. Based on these findings, it is likely that an amine-adsorption/ion-suppression system (Green and Baenziger, 1986) could also be developed as an alternative method for the fractionation of negatively charged BAP-oligosaccharides. Together, the combination of amine-adsorption HPLC with anion-exchange HPLC and RP-HPLC represent a powerful approach for the fractionation and analysis of heterogeneous oligosaccharide mixtures.

Determination of the stability of BAP-oligosaccharide adducts attached to avidin and streptavidin

The original purpose of including biotin in the fluorophore was to have an oligosaccharide tag that permitted the formation of neoglycoproteins for functional studies. In order for BAP compounds to be practical in such functional studies, it is important that the affinity for (strept)avidin be high, as seen with unmodified biotin ($K_d = 10^{-12}$ – 10^{-15}) (Livnah *et al.*, 1993). Prior literature has shown that, in some cases, the derivitization of biotin without an additional spacer arm may sterically interfere with binding to (strept)avidin (Green, 1990). Also, the (strept)avidin-biotin complex could possibly be destabilized by the proximity of the positively charged pyridinium ring.

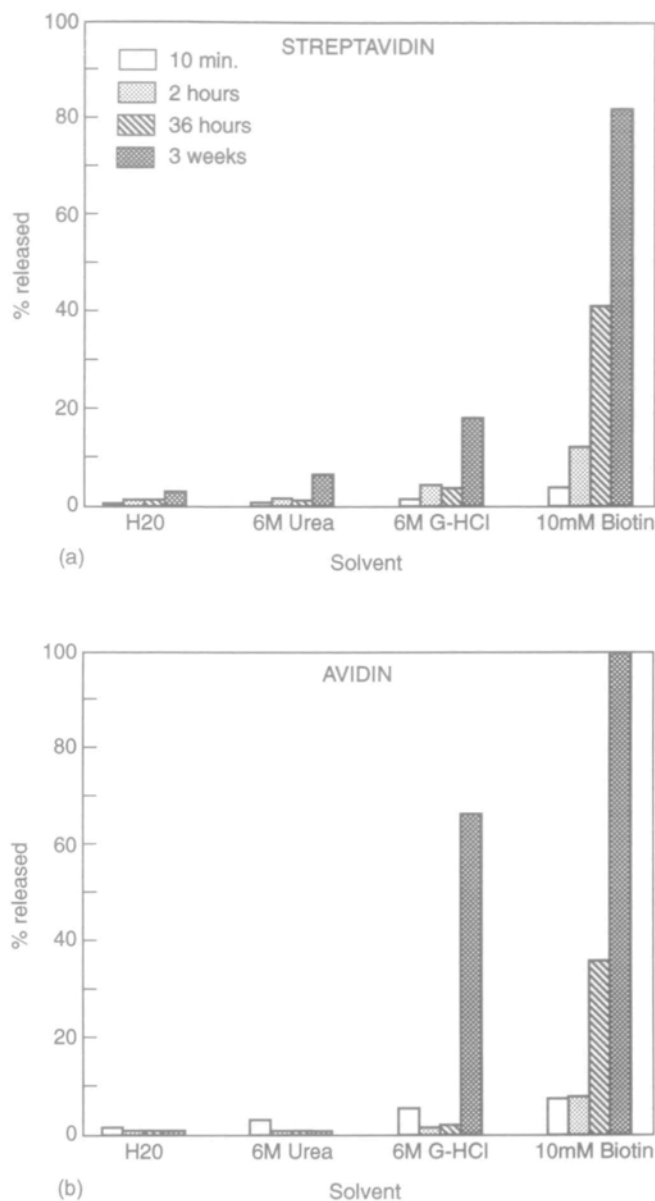


Fig. 6. Stability of BAP adduct attachment to streptavidin and avidin. [^3H]Gal-chitobiose-BAP was bound to either streptavidin (a) or avidin (b). Centricon-10 ultrafiltration tubes were used to remove unbound adducts. The release of oligosaccharide adducts was monitored after incubation with water, 6 M urea, 6 M guanidine-HCl (G-HCl) or 10 mM biotin for various times, and was expressed as a percentage of released radioactive material from remaining bound material at each step.

[^3H]Galactose-chitobiose-BAP was chosen as a model compound to investigate the stability of BAP adducts to streptavidin and avidin (see Figure 6). The stability of binding to either denaturation or competition with cold biotin was examined. After incubation in water for 3 weeks at room temperature, <3% of the BAP adduct was released in each case. Even in the presence of 6 M urea, relatively little material was released. However, 6 M guanidine-HCl caused release of 18 and 67% of BAP adduct from streptavidin and avidin after 3 weeks, respectively. The differences between the two proteins may reflect the fact that in 6 M guanidine-HCl streptavidin is tetrameric, while avidin is known to dissociate into a lower-affinity monomeric form (Green, 1990). For avidin, the binding

of BAP adducts versus [^3H]biotin was directly examined by competition with 10 mM biotin, corresponding to a 50 000 M excess of unlabelled biotin. After 36 h, only 3% of the free [^3H] biotin was displaced (data not shown), whereas 37% of the BAP adduct was displaced. Although BAP adducts show a slightly reduced affinity, by a factor of ~ 10 , these values indicate very stable complexes of BAP adducts with streptavidin or avidin. Thus, the pyridinium ring does not markedly reduce the affinity of BAP adducts for (strept)avidin and the interaction can be considered practically covalent, even in rather severe conditions. To release bound BAP adducts, it is necessary to either denature the protein into the monomeric form or use a large excess of free biotin at room temperature for long durations of time. The realistic implication is that the binding of BAP adducts to streptavidin or avidin is practically non-reversible in all but the harshest conditions, thus forming stable neoglycoproteins for functional and biological studies. Note, however, that binding of BAP adducts to columns of monomeric avidin (Pierce) is reversible with mild acid (Kohanski and Lane, 1990), allowing the use of such columns to retrieve BAP-oligosaccharide adducts from reaction mixtures if necessary.

Use of BAP for sensitive monosaccharide compositional analysis of glycoproteins

The procedures described below provide a convenient and sensitive means of analysing both neutral and amino monosaccharides from glycoproteins by RP-HPLC. Although there are many methods for monosaccharide analysis by pre-column derivatization, several require additional chromatographic steps or specialized equipment to remove excess reagents (Suzuki, 1991; Spiro and Spiro, 1992; Hase, 1994). Furthermore, the majority require specialized columns as well as heating or cooling jackets (Muramoto *et al.*, 1987; Suzuki, 1991; Spiro and Spiro, 1992; Kwon and Kim, 1993).

In our studies, inexpensive Microsorb-MV C_{18} columns provided good peak resolution of different BAP-monosaccharides at ambient temperatures under isocratic conditions. The monosaccharides frequently found in glycoproteins are well resolved: Gal, Man, Xyl, Fuc, GlcNAc and GalNAc (see Figure 7). Glucose, a common environmental contaminant, eluted as a shoulder behind galactose. Other C_{18} columns as well as C_4 columns (authors' unpublished observations) also work well, although optimal acetonitrile concentrations vary between columns. This simple method requires no additional chromatography or workup of BAP adducts prior to HPLC analysis, and is sensitive from the low picomole range (~ 20 pmol) up to 10 nmol of reducing sugar.

To minimize sample loss and maintain good reproducibility, it is preferable not to remove excess BAP before HPLC analysis. Thus, to avoid column overload, only 100 nmol of BAP were used in each reaction. This corresponds to a ≥ 5 -fold molar excess of BAP over the total monosaccharides analysed. When 25% of such a sample was injected, there was no peak overlap between BAP-monosaccharides and free BAP. The standard curves for different monosaccharides show a linear response from <100 pmol to ~ 3 nmol of monosaccharide mixtures (see Figure 8). If greater quantities of sugars are present, they will be underrepresented. However, this can easily be avoided by serial dilutions prior to derivatization. The variations in peak area for different monosaccharides, particularly the lower reactivity of the 2-amino-acetyl sugars,

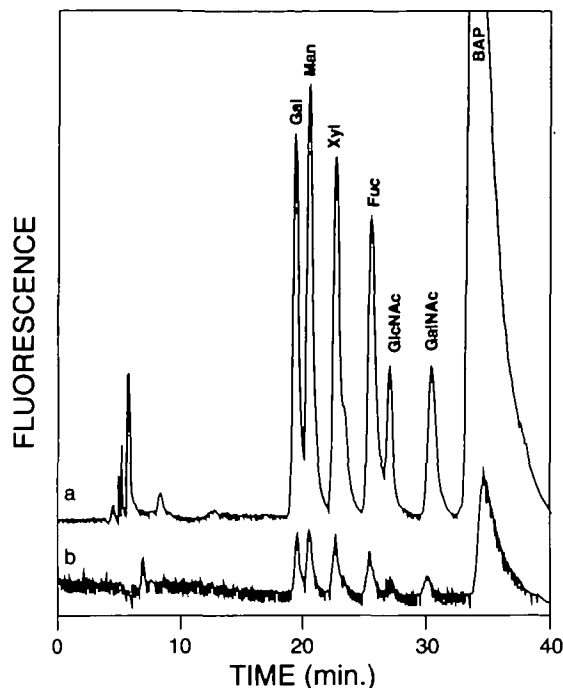


Fig. 7. RP-HPLC analysis of reduced BAP-monosaccharide adducts. A monosaccharide mixture containing equal molar amounts of galactose (Gal), mannose (Man), xylose (Xyl), fucose (Fuc), *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) was coupled to BAP and subsequently reduced. Samples corresponding to 1 nmol (a) or 20 pmol (b) were injected onto a Microsorb C_{18} HPLC under isocratic conditions of 74% Buffer A and 26% Buffer B (resulting in a final acetonitrile concentration of 13%) at a flow rate of 0.5 ml/min, and the fluorescence was monitored. The BAP-monosaccharide peaks are marked, while unmarked peaks are byproducts of the coupling reaction. The sensitivity of the range was increased for trace (b).

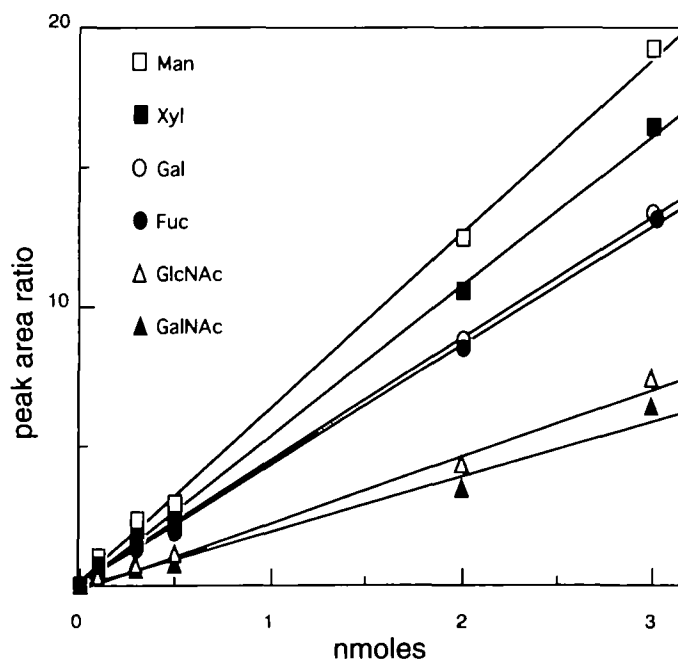


Fig. 8. Standard curve of coupling monosaccharides to BAP. A mixture of monosaccharides (100 pmol to 3 nmol) was coupled to BAP and reduced; 10% of each aliquot was analysed by RP-HPLC, as described in Figure 7.

Table I. Monosaccharide compositional analysis of glycoproteins

Glycoprotein*	Monosaccharide ($\mu\text{g}/\text{mg}$)					
	Gal	Man	Xyl	Fuc	GlcNAc	GalNAc
Ovalbumin	6.1 (0–15)	24 (20–28)	0 (0)	0 (0)	22.9 (12–27)	0 (0)
Fetuin	28.4 (35–46)	26.6 (23–30)	0 (0)	0 (0)	30.8 (26–56)	10.2 (5.4–7)
Mucin (bovine submaxillary)	9.6 (10–36)	5 (0–5.4)	0.7 (0–1.1)	4.2 (3–18)	11.2 (13–69)	73.6 (30–168)

*1 mg each of ovalbumin, fetuin and bovine submaxillary mucin were acid hydrolysed, the monosaccharides re-*N*-acetylated (Kwon and Kim, 1993), and a fraction (1%) was coupled to BAP and subsequently reduced. Aliquots (10%) of each were analysed by RP-HPLC as described under Materials and methods. The results (average of two experiments) are expressed as $\mu\text{g}/\text{mg}$ relative to known monosaccharide standards. The values in parentheses correspond to the range of published values from prior literature [summarized in Kwon and Kim (1993)].

reflects variable coupling efficiency under these conditions of limited BAP excess. This is not due to a decreased detector response, since 25 pmol of purified Xyl and GlcNAc adducts (measured by biotin content) had similar RP-HPLC fluorescent peak areas (data not shown). Thus, under these conditions of low BAP concentrations there is a linear but incomplete coupling to some monosaccharides. It should be noted that under conditions where higher concentrations of BAP are used (i.e. in coupling to oligosaccharides), the efficiency of coupling of GlcNAc as a reducing terminal monosaccharide can be as high as 90%.

To test the applicability of this system to monosaccharide analysis of intact glycoproteins, we acid hydrolysed several previously well-characterized glycoproteins, re-*N*-acetylated and BAP-coupled the monosaccharides. The values of monosaccharide composition obtained (by comparison to monosaccharide standards) from the various glycoproteins correlated well with published values (Table I). The amount of material injected per HPLC run typically represented $\sim 1 \mu\text{g}$ of glycoprotein. Thus, the method is useful for monosaccharide analysis of glycoproteins.

Monosaccharide analysis of samples containing glucose

In the samples studied above, glucose contamination was not a problem. Since most glycoproteins do not contain much glucose (Kornfeld and Kornfeld, 1985; Kwon and Kim, 1993), analysis of such molecules can be accomplished in a single run. However, in some cases the fact that the glucose adduct partially overlaps in elution with the galactose adducts could pose a potential problem. Likewise, glucose can arise from hydrolysis of contaminating polysaccharides from the environment. In order to separate galactose and glucose derivatives (a problem frequently encountered in RP-HPLC monosaccharide analysis) (Eggert and Jones, 1985; Muramoto *et al.*, 1987; Kwon and Kim, 1993), monosaccharides were coupled to BAP and the subsequent reduction step was omitted. Under these conditions, the elution order and resolution of the monosaccharide adducts was altered such that galactose and glucose adducts are readily resolved (see Figure 9). Although non-reductive coupling standard curves of the linear range are still needed, samples with glucose contamination could presumably be analysed in two runs, with and without adduct reduction.

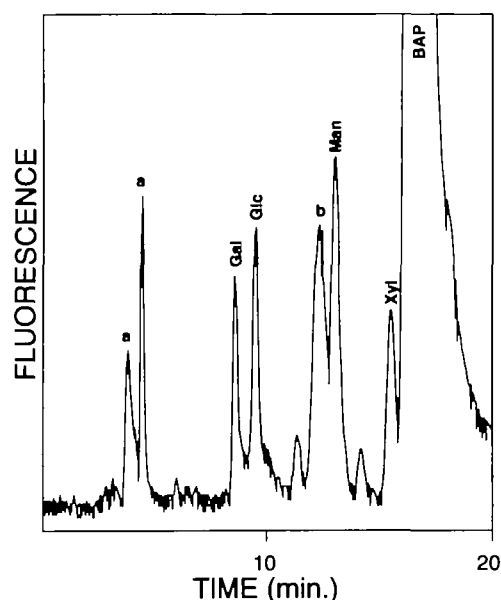


Fig. 9. RP-HPLC resolution of Glc from Gal and Man monosaccharides coupled to BAP under non-reducing conditions. A mixture containing Gal, Glc, Man, Xyl, Fuc, GlcNAc and GalNAc was coupled to BAP without subsequent reduction. An aliquot was injected onto a Microsorb C₁₈ column under isocratic conditions of 72% Buffer A and 28% Buffer B (resulting in a final acetonitrile concentration of 14%) at a flow rate of 1 ml/min. Peaks corresponding to Gal, Glc, Man and Xyl adducts are marked, while the other monosaccharides co-elute with BAP. Peaks marked a and b correspond to reaction byproducts and monosaccharide anomers, respectively.

This procedure for monosaccharide analysis has the advantage that the process can be easily performed in a single reaction vial. Furthermore, for laboratories conducting only occasional monosaccharide analysis, BAP provides an alternative route without requiring the acquisition of additional equipment, other than a fluorescence HPLC detector.

BAP-oligosaccharide adducts can be stable without subsequent reduction

Traditionally, the tagging of sugars by reductive amination is accomplished in two steps: the formation of a reversible Schiff base, and subsequent reduction to obtain an irreversible covalent adduct. In the section above, we noted the somewhat

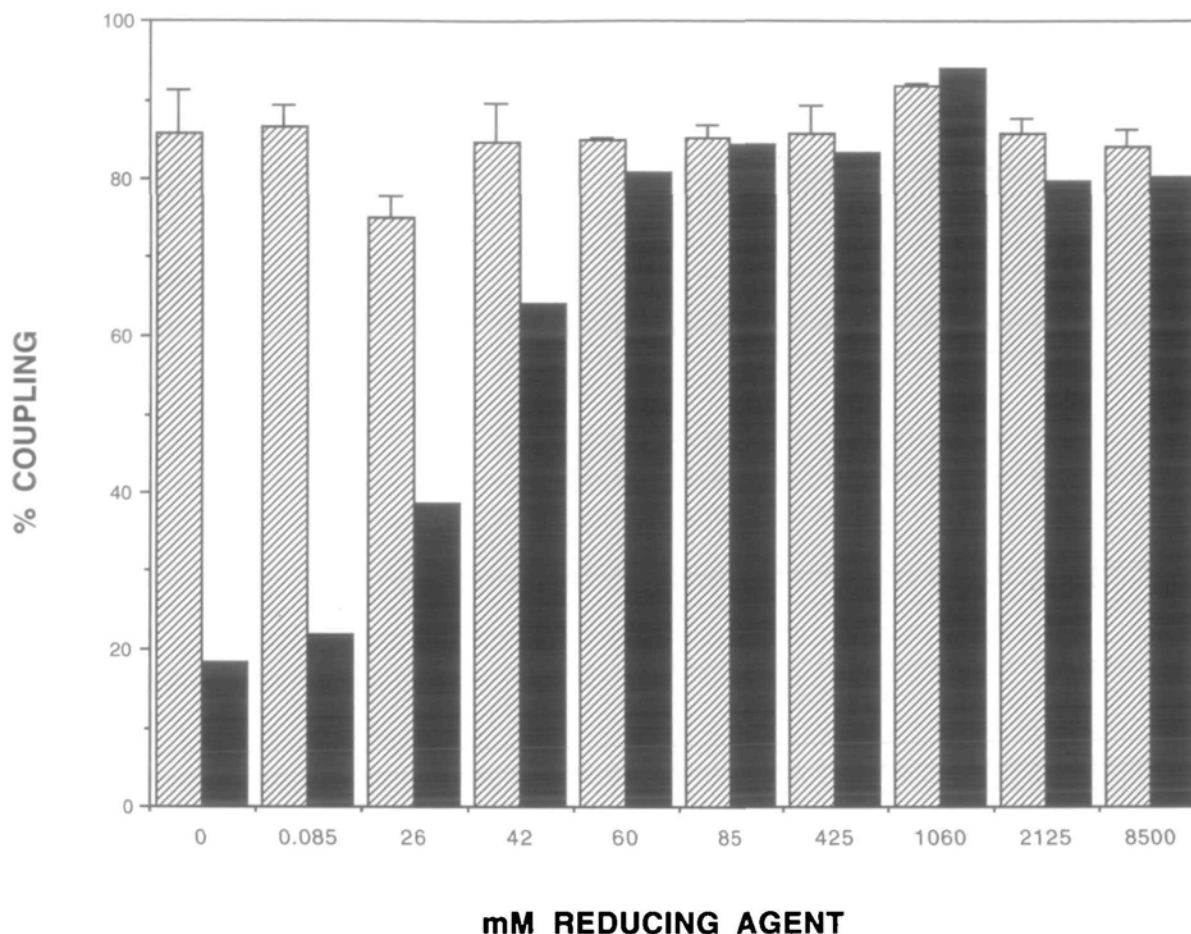


Fig. 10. BAP forms stable, but acid-labile adducts without reduction. [^3H]Gal-chitobiose was coupled to BAP and subsequently reduced with varying amounts of BDA. Half of the sample was loaded onto a C_{18} cartridge, washed with water, eluted with 50% acetonitrile and fractions were counted. The percentage of coupling is shown with dashed bars and refers to the amount of bound radioactive material relative to the total. The error bars show the SD of the mean of three experiments. The other half was dried, treated with 2 M acetic acid for 3 h at 80°C (to break glycosylamine adducts), and similarly passed over a C_{18} cartridge and counted and shown with solid bars.

surprising finding that monosaccharide-BAP adducts appeared to be relatively stable, even without reduction. [^3H]Gal β 1-4GlcNAc β 1-4GlcNAc ([^3H]Gal-chitobiose) was used as a model compound to study the effects of increasing amounts of reducing agent on coupling efficiency. As shown in Figure 10, a maximum coupling efficiency of 92% was observed with the addition of ~ 1 M borane dimethyl amine (BDA). Even in the absence of added reducing agent, most of the radioactive material remained bound to the C_{18} cartridge, a characteristic of a hydrophobic BAP-oligosaccharide complex. Furthermore, the non-reduced adduct was stable in water for >10 min at 100°C (data not shown), suggesting that it may not simply be an unreduced Schiff base. In the absence of reducing agents, the formation of stable cyclic glycosylamines complexed with 2-aminopyridine has been reported (Li and Her, 1993). To determine if the non-reduced material showed the acid lability typical of glycosylamines (Her *et al.*, 1987), half of the variously reduced samples were heated in 2 M acetic acid at 80°C for 3 h, and then analysed. As seen in Figure 10, the acid treatment greatly diminished the C_{18} -bound material in the partially reduced or non-reduced samples. Additional acid treatment of the non-reduced sample caused a further 50% decrease in binding (data not shown). The stability of the non-reduced BAP adduct in aqueous conditions, and its degradation

under acidic conditions (Amadori rearrangement products are stable to acid hydrolysis; Her *et al.*, 1987), would suggest that it is not a Schiff base nor an Amadori rearrangement product, respectively. This suggests that the adduct is most likely a glycosylamine. Further work is needed to verify this proposal.

One advantage of coupling oligosaccharides to BAP under non-reducing conditions is that the closed ring of a glycosylamine can impart different physical properties on the adduct. For instance, others (Her *et al.*, 1987) reported improved HPLC resolution of PA-glycosylamines compared to open ring reduced Schiff bases. We have shown above that non-reduced BAP monosaccharides display different elution profiles compared to their reduced counterparts. This can be advantageous for separating monosaccharides containing galactose, glucose and mannose, a problem sometimes encountered in monosaccharide analysis by pre-column derivatization methods (Eggert and Jones, 1985; Kwon and Kim, 1993). For the most part, glycosylamines were purposely avoided in the past, due to the additional complexity of the anomeric linkage to the sugar (α or β); however, there are instances wherein glycosylamines can be a useful alternative for extracting structural information. Furthermore, the oligosaccharides can be released reversibly with mild acid for the recovery of free oligosaccharides that have an available reducing end. Finally,

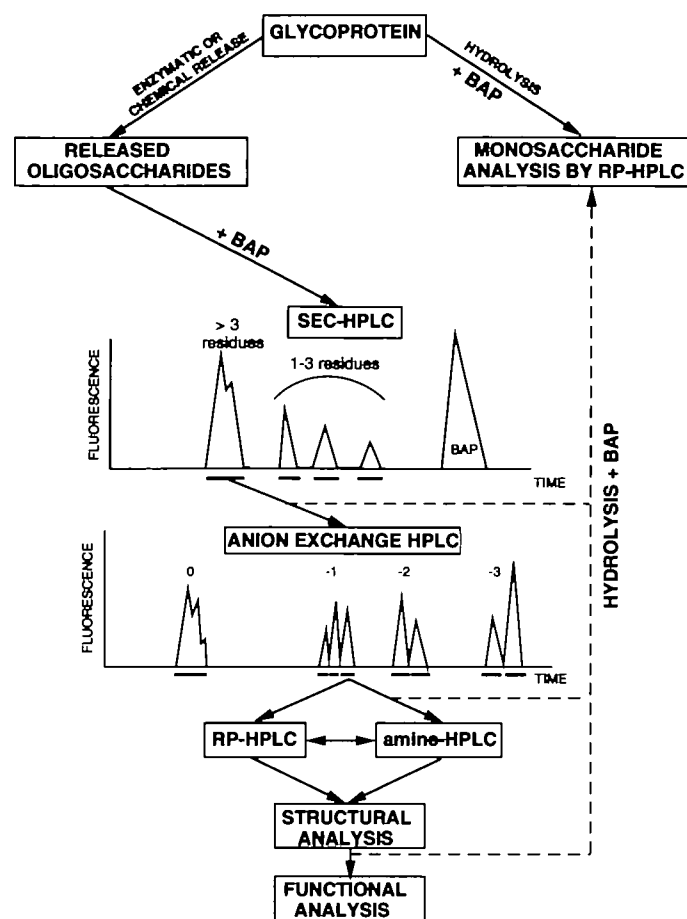


Fig. 11. Schematic of the versatility of BAP for structural analysis of oligosaccharides. Note that SEC-HPLC (TSK G3000PWxl) can alternatively be replaced by a HW40S gel filtration column to remove excess BAP.

there may be cases where it is desirable to maintain a closed ring at the 'reducing' terminus, such as when the acceptor needs to be recognized by a glycosyltransferase.

Conclusions

As outlined in Figure 11, BAP can now be used for a comprehensive approach to oligosaccharide analysis. Many discrete steps, described in this paper, can be applied in unison for studying oligosaccharide structures. Monosaccharide compositional analysis of the glycoprotein(s) of interest in the picomole range can be conducted using BAP and inexpensive HPLC columns and buffers. After releasing the oligosaccharides from the glycoprotein, and derivatization with BAP, the newly developed HPLC sizing column can quickly fractionate large BAP-oligosaccharides from smaller adducts and free BAP. Alternatively, a HW40S gel filtration column with gravity flow can be employed as previously described to isolate the adducts (Rothenberg *et al.*, 1993). An HPLC anion-exchange system can then be used to fractionate the molecules into populations of neutral or variously charged BAP-oligosaccharides. Further fractionation and structural analysis is then possible by RP-HPLC and/or amine-adsorption HPLC, while monosaccharide composition data can suggest further analyses by sequential exoglycosidase digestions. Monosaccharide analysis can also be performed on isolated, homogeneous species. The techniques described herein permit

HPLC fractionation of BAP-oligosaccharides by three distinct parameters, namely charge, hydrophobicity and size. It should now be possible to accomplish two- and three-dimensional oligosaccharide mapping similarly to that described for PA adducts. Moreover, BAP adducts possess the advantage that when purified they are already biotinylated. As expected, they possess high affinity to both streptavidin and avidin, and hence allow the formation of functional neoglycoproteins for a variety of possible functional studies [see Rothenberg *et al.* (1993) for details]. Finally, the prospect of potential BAP-glycosylamine adducts could be useful for extracting structural information, recognition by transferases and recovery of free oligosaccharides after fractionation. Taken together, these advances make BAP a versatile tool for carbohydrate studies, making it possible not only to quickly obtain pure BAP-oligosaccharides, but also providing an array of orthogonal techniques for fractionation and analysis. Finally, all of the methods described herein can be carried out with relatively inexpensive materials and equipment available in many laboratories.

Materials and methods

Materials

Biotin, monosaccharides, human fibrinogen, and ovalbumin, were obtained from Sigma. DAP and pyridine were from Aldrich. EDC and NHS were from Pierce. C₁₈ Spice cartridges and tubes were from Analtech (Newark, DE). [6-³H]Galβ1-4GlcNAc₂ ([³H]Gal-chitobiose) was prepared as described previously (Hayes *et al.*, 1993). [³H]Biotin was from New England Nuclear. α₁-Acid glycoprotein was from Calbiochem. Bovine submaxillary mucin was from US Biochemicals. bLH was from the National Pituitary Hormone Service (NIH). Avidin and streptavidin were from Scripps Laboratories. Dextran (5 mg/ml) was partially acid hydrolysed in 100 mM HCl for 1.5 h at 100°C. Peptide: N-glycosidase F (PNGaseF) was prepared as described previously (Plummer and Tarentino, 1991). All other reagents were of the highest grade commercially available.

Modified synthesis, purification and analysis of BAP

BAP was synthesized as previously described (Rothenberg *et al.*, 1993) with the following modifications: EDC and NHS were increased to 150 and 50 mM, respectively. After overnight incubation at room temperature with stirring, the reaction mixture can be stored at 4°C (up to 1 week). To purify BAP from substrates and byproducts, aliquots of the reaction (corresponding to ~50 mg biotin) are dissolved in 20 ml of water, loaded into C₁₈ Spice tubes (Analtech), and drawn through the tubes by vacuum. Using a vacuum manifold apparatus, many tubes can be simultaneously processed. A hand-held long-wavelength UV lamp is used to monitor the elution of fluorescent DAP and BAP. The C₁₈ tubes are each sequentially washed with 500 ml of water, 100 ml of 10% acetonitrile and 100 ml of 50% acetonitrile. Each wash was saved and similar washes were pooled. The C₁₈ tube can be regenerated by washing with 100 ml of 2:1 chloroform/methanol containing 1% glacial acetic acid. Aliquots from different washes were analysed by high-performance thin layer chromatography (HPTLC) analysis on plastic silica backed plates run in 85% ethanol as described previously (Rothenberg *et al.*, 1993). Following separation, the plates were placed in a chamber containing trifluoroacetic acid (TFA) fumes for 3 min to decrease the pH and aid in fluorescent visualization of BAP. After detection, the plates were stained for several hours in an iodine vapour chamber to detect non-fluorescent compounds. The 50% acetonitrile pools containing BAP were concentrated by rotary evaporation, resuspended in a minimal volume of 10% acetonitrile and lyophilized. The purity of the BAP was analysed by RP-HPLC. Additional confirmation of BAP purity was obtained by FAB-MS as previously described (Rothenberg *et al.*, 1993). If a BAP was not >95% pure by HPTLC, HPLC or FAB-MS, it was passed over a fresh C₁₈ tube and processed as described above.

Purification of N-linked oligosaccharides from glycoproteins

N-Linked oligosaccharides were released from denatured glycoproteins with PNGaseF as described previously (Hayes *et al.*, 1993). To remove proteins and detergents, the reaction mixtures were passed over C₁₈ Spice cartridges in water

and washed with an additional 10 ml of water. The pooled run-through material was concentrated by shaker evaporation, extensively dialysed (molecular weight cut-off = 500) against water and lyophilized.

Coupling of oligosaccharides to BAP

Dry N-linked oligosaccharides or [³H]Gal-chitobiose were coupled for 1 h at 80°C with 1 mg of BAP in 10 µl of 2:1 pyridine/dry glacial acetic acid (traces of water were removed from the glacial acetic acid by placing on ice until frozen and pouring off any liquid; this process was repeated twice) as previously described (Rothenberg *et al.*, 1993). Oligosaccharide adducts were then reduced for 1 h at 80°C with 10 µl of 2.1 M BDA in 2:1 pyridine/dry glacial acetic acid. In some experiments, various concentrations of BDA were tried for [³H]Gal-chitobiose samples. Elimination of water in samples and other reagents was found to be important in order to obtain high coupling efficiencies. Thus, prior drying of samples was performed as indicated under monosaccharide compositional analysis. If solubility of sugars is a problem (e.g. dextran), minimal amounts of dry DMSO can be added.

Analysis of BAP coupling efficiency using [³H]Gal-chitobiose

After coupling [³H]Gal-chitobiose to BAP using various amounts of reducing agent, 50% of each sample was transferred to a tube containing 5 ml of water. The samples were loaded with a syringe onto pre-washed Spice C₁₈ cartridges (Analtech). The run-through was reloaded onto the cartridge once. The cartridge was washed three times with 5 ml of water to elute unbound material, and four times with 5 ml of 50% acetonitrile to elute BAP adducts. Aliquots of 0.5 ml were removed from the fractions, added to 4.5 ml of scintillation cocktail (Ecoscint) and radioactivity monitored. For acid treatment, the other 50% of the coupled [³H]Gal-chitobiose samples were taken to dryness, resuspended in 2 M acetic acid and heated for 3 h at 80°C. They were subsequently taken to dryness again, dissolved in 5 ml of water and analysed with C₁₈ cartridges as described above.

Size-fractionation HPLC analysis

A TSK-G3000PWxl (Tosohass; 7.8 × 300 mm) column was used isocratically with Solvent A (10 mM ammonium formate, pH 4.0) and Solvent B (10 mM ammonium formate, pH 4.0 in 50% acetonitrile) in a ratio of A:B of 50:50 (v/v%) at a flow rate of 0.8 ml/min.

Anion-exchange HPLC analysis

For fractionation by anion exchange, a TSK-DEAE-2SW (Tosohass; 4.6 × 250 mm) column was used with Solvent A (0.5 mM pyridine acetate, pH 4.0 in 25% acetonitrile) and Solvent B (2 M pyridine acetate, pH 4.0 in 25% acetonitrile). After injection, Solvent A was washed through at a flow rate of 0.8 ml/min for 10 min, after which a 30 min linear gradient up to a 50:50 ratio of A:B was applied.

Amine-adsorption HPLC

Fractionation of BAP adducts by amine adsorption was performed on a Microsorb NH₂ column (Rainin; 4.6 × 250 mm). A biphasic aqueous gradient was performed over 60 min with various ratios of solvent A (10 mM ammonium formate, pH 3.0 in 95% acetonitrile) and solvent B (10 mM ammonium formate, pH 3.0 in water) at a flow rate of 1 ml/min. After injection, the ratio of A:B was linearly changed from 95:5 (v/v%) to 60:40 over the first 20 min, and then to 40:60 over the remaining 40 min.

RP-HPLC analysis

RP-HPLC was conducted on a Microsorb-MV C₁₈ column (Rainin; 4.6 × 250 mm). Elution was performed isocratically with various ratios of Solvent A (10 mM ammonium formate, pH 4.0) and Solvent B (10 mM ammonium formate, pH 4.0 in 50% acetonitrile) at flow rates of 0.5–1 ml/min. For reduced monosaccharide adduct analysis after reduction, a ratio of A:B of 74:26 (v/v%) at a flow rate of 0.5 ml/min was typically used, while analysis of non-reduced monosaccharide adducts used a ratio of A:B of 72:28 (v/v%) at a flow rate of 1 ml/min. RP-HPLC analysis of BAP adducts of fibrinogen was performed using a 50 min linear gradient from 100% A to a ratio of A:B of 50:50 (v/v%) at a flow rate of 1 ml/min.

HPLC detection of BAP adducts

Detection of BAP adducts on all HPLC systems was performed using a Spectronics fluorescence HPLC detector with ex = 345 nm and em = 400 nm.

Monosaccharide compositional analysis

Glycoproteins were hydrolysed in 2 M TFA for 4 h and dried. Re-N-acetylation was performed by the addition of equal volumes of water, saturated sodium bicarbonate and 10% acetic acid, and incubation for 30 min at room temperature (RT) as previously described (Kwon and Kim, 1993). Aliquots of hydrolysed re-N-acetylated or pure monosaccharide standards were placed in 200 µl tapered glass reactivials (Pierce), lyophilized and dried further for several hours in a P₂O₅ desiccator. Then 10 µl of a 10 mM stock BAP solution in 2:1 pyridine/dry glacial acetic acid were added to each sample. The vials were capped with Teflon 'tuff-bond' septae (Pierce), vortexed and heated for 1 h at 80°C with an additional vortexing after the first 5 min of heating. Some samples were removed, cooled to RT, dissolved in water and analysed by RP-HPLC. Other samples (to be reduced) were cooled to RT, and 10 µl of a fresh 2.1 M BDA solution in 2:1 pyridine/dry glacial acetic acid added. The samples were capped, vortexed and heated for one additional hour at 80°C. Samples were subsequently stored at 4°C and were dissolved in 80 µl of water before HPLC analysis. Typically, 10–20% of each sample was injected onto a RP-HPLC column for analysis.

Enzymatic digestions of BAP adducts

Sequential enzymatic digestions were performed on purified bisialylated biantennary fibrinogen-BAP oligosaccharides. ~100 pmol of fibrinogen-BAP were treated overnight with 1 mU of *Arthrobacter ureafaciens* sialidase (AUN, Calbiochem) in 50 mM cacodylate buffer (pH 5.8) containing 0.1% bovine serum albumin (BSA). The sample was then boiled for 3 min, centrifuged in a microcentrifuge, and the supernatant removed for analysis. The desialylated product was isolated by RP-HPLC (see the section on RP-HPLC analysis above for details), dried and similarly treated sequentially with jack bean β-galactosidase (Oxford Glycosystems), *Streptococcus pneumoniae* β-N-acetylglucosaminidase (Oxford Glycosystems) and α-D-mannosidase (V-labs) as per the manufacturers' instructions. RP-HPLC was used to study the product after each digestion.

Determination of relative off-rates of [³H]Gal-chitobiose-BAP from avidin and streptavidin

One hundred picomoles of either [³H]Gal-chitobiose-BAP (1600 c.p.m.) or [³H]biotin (NEN; 3700 c.p.m.) were incubated for 10 min at 4°C with 1 nmol of either avidin or streptavidin in Centricon-10 ultrafiltration tubes (Amicon) in a total volume of 0.5 ml. The samples were centrifuged for 30 min at 4°C in a Sorval SS-34 rotor at 5000 g. Unbound molecules were completely removed by repeated ultrafiltration (four times) in water (residual volume of bound complexes ~50 µl). A 0.5 ml volume of solvent containing either water, 6 M urea, 6 M guanidine hydrochloride or 10 mM biotin was added, the samples incubated for 10 min at RT, centrifuged again and the filtrate monitored for radioactivity. An additional 0.5 ml of water was added to the samples, and they were centrifuged again to recover all released counts. Following the addition of 0.5 ml of the various solutions above, the remaining complexes were incubated at RT for 2 h, upon which released radioactivity was determined as described above. Two successive rounds of incubation and analysis were carried out for 36 h and 3 weeks, respectively. The percentage of released radioactive material from the remaining bound material at each step was determined.

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Abbreviations

BAP, 2-amino,6-amidobiotinyl-pyridine; BDA, borane dimethyl amine; bLH, bovine luteinizing hormone; BSA, bovine serum albumin; DAP, 2,6 diaminopyridine; DMSO, dimethyl sulphoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; FAB-MS, fast atom bombardment mass spectrometry; Gal-chitobiose, Galβ1-4GlcNAcβ1-4GlcNAc; HPLC, high performance liquid chromatography; HPTLC, high-performance thin layer chromatography; NHS, N-hydroxysulphosuccinimide; PA, pyridylamino; PNGaseF, peptide: N-glycosidase F; RP, reversed phase; RT, room temperature; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid.

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