B39

16 Simultaneous Fluorescent Labelling and Biotinylation of Oligosaccharides: A Versatile Approach to the Analysis of Oligosaccharide Structure and Function

Derek K. Toomre and Ajit Varki

16.1 Introduction

Oligosaccharides participate in a variety of important biological functions, including interactions with specific receptors (1–4). A prerequisite to the discovery and exploration of such interactions is the comprehensive structural analysis of homogeneous oligosaccharide species. The purification of oligosaccharides from complex mixtures requires diverse techniques, as well as sensitive and specific detection. One way to achieve sensitive detection is to attach a chromophore to the reducing sugar by reductive amination (5, 6) (see Chapters 7, 11, 15, 18 in this volume for other examples of such techniques). Fluorescent chromophores (in contrast to light-absorbing chromophores) can often permit detection in the low picomole range for high-pressure liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE) techniques, and in the femtomole range in capillary electrophoresis. Furthermore, hydrophobic or anionic properties of the chromophoric groups can be exploited to improve oligosaccharide fractionation by either electrophoresis or HPLC (5–8). Often several multidimensional chromatographic techniques, based on different physical properties, are required to adequately resolve complex mixtures. Several examples have been described of "two-dimensional mapping" with fluorescent pyridylamino (PA)-coupled oligosaccharides (5, 9). More recently, 2-aminobenzamide has been employed as an alternate fluorescent tag which gives both nonselective and highly efficient coupling to oligosaccharides (10).

A shortcoming of most structural analysis techniques is that once the oligosaccharides of interest have been isolated and characterized, they are of relatively limited use for functional or biological studies. An alternative is to label glycopeptides with biotin, allowing the formation of stable multivalent complexes with avidin or streptavidin (10, 11). Although pyridylamino sugars can be *replaced* with biotinyl groups by a series of chemical treatments (12), this technique requires separate re-derivatization and repurification of each PA sugar of interest.

Recently, we described the preparation and applications of a versatile fluorescent label, biotinylated diaminopyridine (BAP) (13, 14). It allows oligosaccharides to be tagged on their reducing terminus (Fig. 16.1) and subsequently fractionationed by several orthogonal HPLC techniques with picomole-level detection (13).

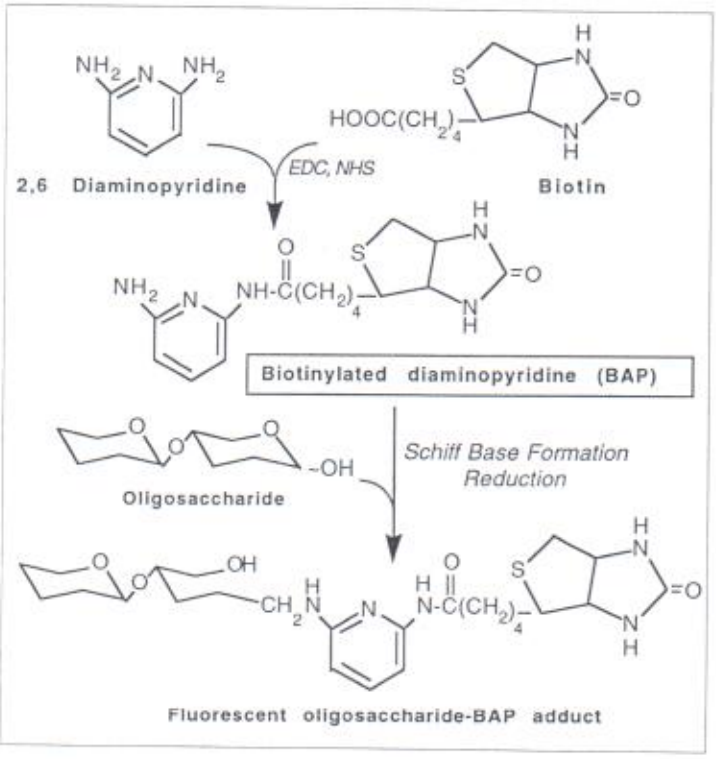


Figure 16.1 Schematic of BAP synthesis and coupling to oligosaccharides by reductive amination

Since biotin is already attached to the fluorophore, numerous biological applications can subsequently exploit the multivalent biotin-(strept)avidin interaction (11, 15, 16). Furthermore, high-affinity interactions between BAP oligosaccharides and (strept)avidin permit the formation of neoglycoprotein equivalents for the detection and affinity purification of receptors, and the generation of monospecific antibodies (13) (Fig. 16.2). Thus, BAP provides a comprehensive approach, uniting both structural and functional carbohydrate studies. This chapter focuses on:

(1) Synthesis and p
(2) Derivitization o
(3) Removal of exc
(4) Fractionation a
HPLC techniqu
(5) Preparation of
(6) Comparison of
Detailed guide
cans by HPLC
book. Extensiv
lated protocols

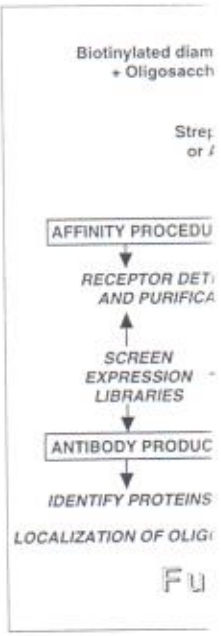


Figure 16.2 Schemat in biological applic functional studies

(1) Synthesis and purification of BAP
(2) Derivitization of oligosaccharides with BAP
(3) Removal of excess reagents after coupling
(4) Fractionation and structural analysis of BAP-oligosaccharide adducts by HPLC techniques
(5) Preparation of neoglycoproteins with strept(avidin) and applications
(6) Comparison of BAP with other currently available fluorescent labels

Detailed guides, e.g. ref. 17, are available on the analysis of derivatized glycans by HPLC in addition to procedures described in Chapters 1, 11, 15 of this book. Extensive guides on the multitude of avidin-biotin applications and related protocols are also available (16, 18).

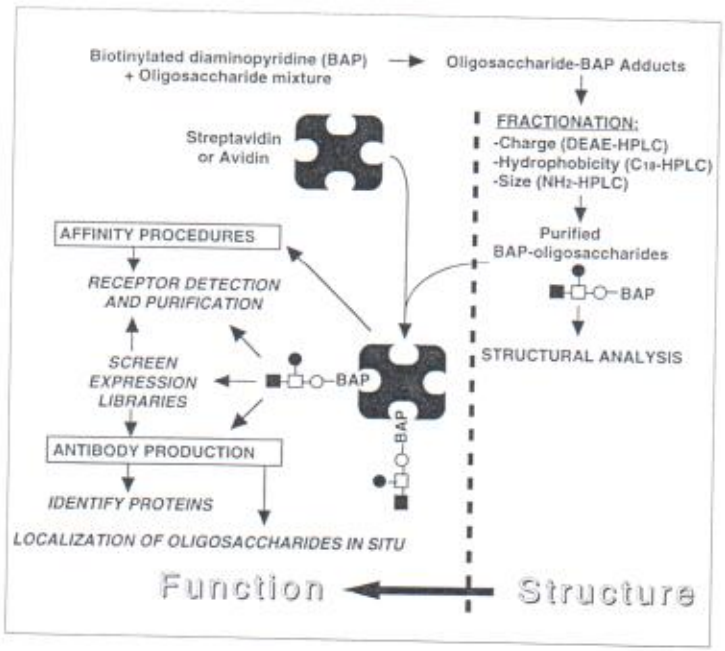


Figure 16.2 Schematic of the versatility of BAP-coupled oligosaccharides in biological applications; creating a direct bridge from structural to functional studies

16.2 Synthesis and Purification of BAP

The carboxylic acid of biotin is activated by mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/*N*-hydroxysulfosuccinimide (NHS), facilitating nucleophilic attack by excess 2,6-DAP to create BAP (Fig. 16.1). Thereafter, BAP can be purified from reactants and by -products by passage over disposable C_{18} cartridges or tubes, and monitored by thin-layer chromatography (TLC).

Materials-Methods-Equipment

- ~1 dozen C_{18} "Spice" tubes (2 g of resin; Analtech, Newark, DE).
- HP-TLC silica plates (plastic-backed silica gel 60, Art 5748; Merck)
- 2,6-DAP (Aldrich; *CAUTION use gloves and avoid inhalation*)
- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride EDC (Pierce)
- *N*-hydroxysulfosuccinimide NHS (Pierce)
- d-Biotin (Sigma)
- Dimethyl sulfoxide (DMSO; Aldrich)
- Distilled water
- 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (pH 6.5)
- Other chemicals are of the highest grade commercially available
- TLC chamber
- Iodide vapor chamber
- Hand-held long-wavelength ultraviolet (UV) lamp
- UV light box (optional)
- Vacuum (house)
- Electric stirrer

yl-3-(3-dimethy-
ulfosuccinimide
reate BAP (Fig.
ducts by passage
er chromatogra-

resin; Analtech,

lica gel 60, Art

and avoid

odiimide

)

acid (MES)

commercially

IV) lamp

Protocol 2.1

The following protocol yields ~50 mg of BAP (~30% yield based on biotin conversion), which is sufficient for ~50 standard coupling reactions (1 mg each). The protocol has been successfully scaled up by a factor of 10.

1. DAP, 0.327 g (300 mM final), is dissolved in 8.5 ml of 50 mM MES (pH 6.5) and stirred in a small beaker at room temperture.
2. Biotin, 0.244 g (100 mM final), is added to a separate polypropylene tube containing 1.5 ml of DMSO and dissolved by heating in a water bath at 60oC.
3. NHS, 57 mg (50 mM final), and 296 mg of EDC (150 mM final) are added simultaneously to the DAP solution while stirring, and the warm biotin solution is immediately added dropwise.
4. The solution is covered with foil and stirred overnight at room temperature.
5. To purify BAP from substrates and by-products, 2 ml aliquots of the reaction (corresponding to ~50 mg of biotin) are dissolved into 20 ml of water, loaded onto separate C_{18} Spice tubes (prewashed with 20 ml of 50% acetonitrile, then 50 ml of water), and drawn through the tubes by vacuum as per the maufacturer's instructions.
6. The C_{18} tubes are each washed extensively with >100 ml of water. Most of the strongly fluorescent DAP is removed in the water wash. Each is then eluted with ~40 ml of 10% acetonitrile, and then with ~20 ml of 50% acetonitrile. Each wash is saved in separate vessels, and similar washes from each tube are pooled.
7. Aliquots from the washes are then analysed on silica gel TLC plates (see Fig. 16.3) developed in 85% ethanol (BAP Rf 0.75–0.80, and DAP Rf 0.50; DAP has a stronger relative fluorescence at a pH >5.0).
8. Following separation, the plates are air-dried and placed in a chamber containing trifluoroacetic acid fumes for ~3 min to acidify and aid in fluorescent visualization of BAP (usually in the 50% acetonitrile wash). After detection, the plates are stained for several hours in an iodine vapour chamber to detect nonfluorescent compounds.

9. The 50% acetonitrile pools containing BAP are concentrated by rotary evaporation, resuspended in a minimal volume of 10% acetonitrile and lyophilized (yields a fluffy yellow powder).
10. The purity of the BAP is additionally monitored by reverse-phase (RP)-HPLC as described below. Fast atom bombardment (FAB)-MS or other mass spectrometry techniques can also be used, if available (13, 14).
11. If a BAP preparation is not >95% pure by TLC, HPLC or FAB-MS, it is passed over fresh C_{18} tubes and processed as described above.
12. BAP is typically stored at -20°C in an airtight container with desiccant. It is stable for at least 3 years under these conditions.

- Step (4): Insoluble material may be observed early , but becomes soluble as the reaction progresses. Next morning, the solution appears clear forest green. The mixture can be stored at 4°C (up to 1 week).
- Step (5): Using a vacuum manifold apparatus, many tubes can be simultaneously processed. Other kinds of disposable C18 silica columns/cartridges can be used, but capacity and elution conditions may vary and require adjustments (monitored by TLC).
- Step (6): A hand-held long-wavelength UV lamp can be used to monitor the elution of fluorescent DAP (water wash) and BAP (mainly in the 50% acetonitrile wash).
- Step (9): Typically, most of the BAP is in the 50% acetonitrile pools, and other fractions can be discarded.
- Step (11): Alternatively, BAP can be recrystallized by removing acetonitrile and most of the water by rotary evaporation until it is barely soluble, cooled at 4°C overnight, and the solids collected by filtration.

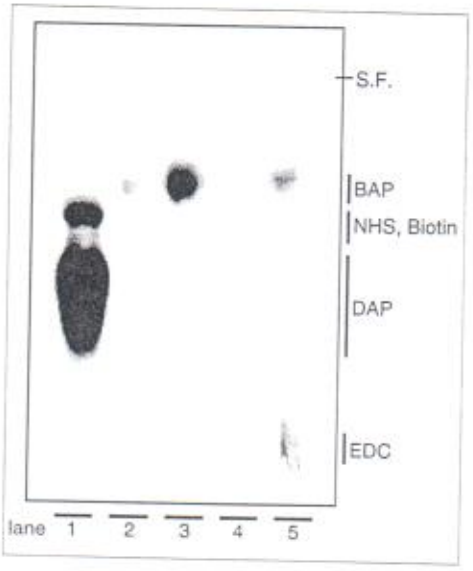


Figure 16.3 HP-TLC of BAP purification on a C_{18} tube

Equal aliquots of various C_{18} washes were spotted on a silica gel TLC plate, developed in 85% ethanol and stained with iodine vapour. Lanes 1 through 5 are the sequential elution of the C_{18} tube with water, 10% acetonitrile, 50% acetonitrile, 100% acetonitrile and 2 : 1 chloroform/methanol in 1% glacial acetic acid, respectively. Migration positions of standards (DAP, - 2,6-diaminopyridine) and the solvent front (S.F.) are indicated on the right. The TLC was scanned and processed using an applescan scanner. (From ref. 14, used with permission)

Troubleshooting guide

Removal of impurities after synthesis of BAP

- Second passage over C_{18} Spice cartridge
- Use DAP of higher purity or recrystallize
- Adjust the concentration of acetonitrile washes as necessary so that BAP is well fractionated from contaminants (monitored by TLC).

Low sensitivity when monitoring BAP synthesis by TLC

- It may be necessary to concentrate BAP prior to application onto TLC plates.
- After development, the TLC plate must be acidified to enhance fluoresence.

Low Yield of BAP

- Contamination with primary amines or carboxylic acids
- Avoid conjugation in buffers with Tris, glycine, EDTA or phosphate: use MES buffer.

EDC or NHS is inactive (obtain fresh reagents)

- EDC rapidly degrades in H_2O under acidic conditions

16.3 Coupling of Oligosaccharides to BAP

Materials-Methods-Equipment

- Clean (see below) reactivials (100 µl; Pierce, Rockford, IL) with caps and Teflon discs (Tuf-Bond; Pierce). Reactivials are rinsed clean in water with sonication if necessary, carefully heated at 60°C for at least 4 h in 50% v/v nitric acid, then rinsed extensively with pure water. After each rinse, water is completely aspirated; this is repeated three times, and vials are dried in an oven.
- Dry glacial acetic acid. Glacial acetic acid (Fisher) is carefully placed in a large test-tube on ice (or very briefly on dry ice) until most of the acid is frozen. Liquid is decanted to remove traces of water as previously described (13). The sample is thawed, and this process is repeated twice. The remaining acid is stored in Teflon-capped vials in a desiccator.
- Phosphorous pentoxide
- Pyridine (99.9% pure; Aldrich)
- Borane dimethylamine complex (Aldrich)
- Coupling reagent (prepared freshly before use): 2 parts (200µl) of dry pyridine are mixed with 1 part (100 µl) of dry glacial acetic acid in an Eppendorf tube.
- BDA reducing reagent (prepared freshly before use): 12.5 mg of borane dimethylamine (BDA) complex is dissolved in 100 µl of coupling reagent (2.1 M final).
- Lyophilizer
- Heating block
- Desiccator
- Glass or polypropylene containers (*No polystyrene; BAP is absorbed*)

Protocol 16.2

1. Oligosaccharide samples (< 50 nmol) containing a free reducing terminus and a sample blank (important negative control) are placed in 100 µl tapered glass reactivials and

Troubles

Poor co

- Impur cans: salts
- Sampl dards.

lyophilized overnight. (2) BAP (0.5–1 mg, >50-fold molar excess) is added to the vials and further dried for several hours in a P_2O_5 desiccator, or until ready for use.

2. Ten microlitres of fresh coupling reagent is added to the vials, which are capped, vortexed and heated for 1 h at 80°C in a heating block. They are additionally vortexed after the first 5 min. (4) An equal volume (10 ml) of fresh BDA reducing reagent is added to the samples, which are vortexed and reduced for 1 h at 80°C.

- Step (1): When larger amounts of glycans are used, the sample is divided into several vials.
- Step (3): Some large neutral or highly acidic oligosaccharides may require the addition of minimal amounts of DMSO or water to solubilize.
- Step (4): The samples can then be immediately purified, as described in the next section, or stored for up to several days at 4°C.
- The coupling efficiency of standards can be monitored as a positive control, as previously described (14). Basically, nonreduced radiolabelled oligosaccharides are coupled, passed over a C_{18} cartridge in water, eluted with acetonitrile and monitored for radioactivity. The free glycans are run through the water wash, while bound BAP adducts are eluted with acetonitrile. Nonradiolabelled samples can be similiarly treated and monitored by colorimetric assays to detect saccharides. 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%; the larger, more charged chains tend to be less efficient.

Troubleshooting guide

Poor conjugation of oligosaccharides to BAP

- Impurities (especially amines and aldehydes) may compete with BAP or the glycans: Samples should be gel-filtered or dialysed to assure that they are free of salts or contaminants.
- Samples or solvents not dry: Excess water decreases coupling efficiency in standards. Thus, samples must be adequately dried and only high-grade reagents

used. For very acidic/hydrophilic samples, small amounts of DMSO or water sufficient to dissolve the samples may improve coupling.

16.4 Purification of BAP Oligosaccharides

After coupling, excess reagents (BAP; by-products; uncoupled sugars, *optional*) must be removed either by (i) HPLC size-exclusion chromatography or (ii) gel filtration. HPLC provides higher resolution, short run times and the ability to monitor real-time fluorescence. However, the gel filtration method is inexpensive and can handle larger samples. Both UV absorption and fluorescence can be monitored, but fluorescence offers a hundredfold or greater improvement in sensitivity (low picomole range).

Materials-Methods-Equipment

- 1 M stock of NH_4COOH (pH 3.7): 2 M formic acid is brought up to the correct pH with aqueous ammonia, then diluted to 1 M with water.
- Acetonitrile, HPLC grade
- Toyopearl HW40S resin (TosoHass, Montgomeryville, PA)
- TSK-G3000PWx1 column (*optional*; 7.8 x 300 mm; TosoHass)
- C_{18} "Spice" cartridge (Analtech; Newark, DE)
- HABA (4'-hydroxyazobenzene-2-carboxylic acid) dye-displacement method for detection of biotin (19) (ImmunoPure HABA kit from Pierce).
- Detection, Quantitation of BAP adducts
  - HPLC: The area of BAP-oligosaccharide peak of interest is compared to that of BAP-labelled standard of known concentration (determined by the HABA assay for biotin). The standard should either be run internally or in the preceding run under identical conditions, since lamp intensity can vary over time. The limit of sensitivity is in the low picomole range

using a fluorescent detector and ~100 pmol using UV absorbance.

– Fluorometer/spectrophotometer: Quantitation is by fluorescence spectrophotometry (15 pmol is detectable in a cuvette holding 1 ml) or by UV absorbance spectrophotometry ($\varepsilon = 7000\ M^{-1}\ cm^{-1}$ in water; practical limit ~10 nmol in a 1-ml cuvette).

Biotinylated standards can be calibrated using the Pierce HABA assay (19).

- HPLC apparatus with Rheodyne injector and ability to perform gradient elutions
- In-line fluorescence dual monochromater spectrophotometer (Spectrovison: ex = 345, em = 400) or UV absorbance spectrophotometer ($Od_{max} = 342$). Values given are for Galβ1-4-GlcNAcβ1-4-GlcNAc-BAP in 100 mM NH_4COOH (pH 3.5) containing 25% acetonitrile. The wavelength shifts up as the pH is decreased (D. K Toomre and A. Varki, unpublished observations).
- Integration software (e.g. Dynamax)
- Fluorometer or UV-spectorophotometer (*optional*)
- Glass or polypropylene containers (*No polystyrene; BAP is absorbed*)

Protocol 16.3

Purification of BAP oligosaccharides on HPLC-sizing column

Column: TSK-G3000PWxl column (TosoHass; 7.8 x 300 mm) with PWxl guard column (TosoHass; 6 x 40 mm) and pre-guard column replaceable filter (A-101X; Upchurch Scientific)
Solvent A: 10 mM ammonium formate, pH 4.0
Solvent B: 10 mM ammonium formate, pH 4.0, in 50% acetonitrile
Elution: Isocratic, ratio of A : B of 50 : 50 (v/v%) at a constant flow rate of 0.8 ml/min

1. 180 µl of water is added to BAP coupled samples (in 20 µl reaction buffers), vortexed, spun in an Eppendorf (10,000 rpm for 3 min) to remove any precipitate.
2. All or part of the sample is injected, the fluorescence is monitored, and peaks of interest are pooled (Fig. 16.4), collected, and lyophilized in polyproplyene Eppendorfs other tubes.

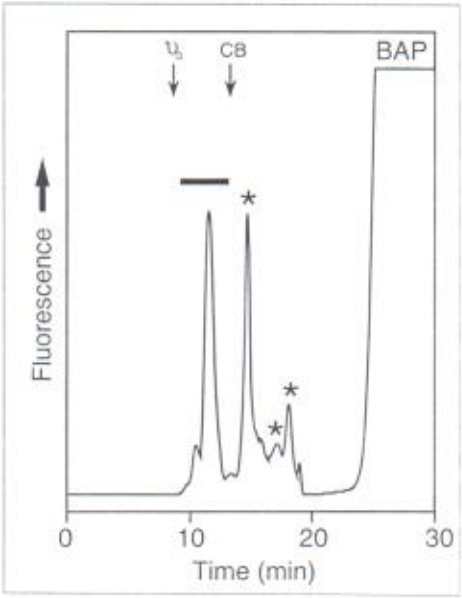


Figure 16.4 Purification of BAP oligosaccharides from BAP and by-products by size exclusion HPLC

After coupling ovalbumin oligosaccharides to BAP, the reaction mixture was injected onto a TSK-G3000PWxl column while monitoring the fluorescence, and the area indicated by a solid bar, corresponding to BAP oligosaccharides, was collected. The void volume is marked by υ_0, an N,N'-diacetyl-chitobiose-BAP standard is marked by "CB", and the asterisks indicate small reaction by-products.

Protocol 16.4

Alternate purification of BAP oligosaccharides by gel filtration

Column:	**Toyopearl HW40S (e.g. 1.5 cm x 48 cm)**
Buffer:	**10 mM ammonium formate, pH 4.0 in 50% acetonitrile**
Flow rate:	**1 ml/min with a peristaltic pump**

1. Samples in 0.5 ml of 50% acetonitrile are loaded and run on a pre-equilibrated column.
2. Fractions (1 ml) are collected and analysed in a fluorometer or spectophotometer.
3. Fractions eluting ahead of unreacted BAP and by-products are pooled and lyophilized.

- BAP fluorescence is pH sensitive (fluorescence decreases rapidly above pH ~4) (13) and decreases in high concentrations of acetonitrile.
- When using the HPLC-sizing column for purification of BAP oligosaccharides, it is often a good idea to initially run only a portion (e.g. 5%) of both (i) a sham coupled blank (BAP and reagents only) and (ii) the sample(s) of interest on the HPLC. Partially included, noncarbohydrate fluorescent by-product peaks are often seen (Fig. 16.4). However, the oligosaccharide adducts (trisaccharide or larger) elute near the void, away from the smaller by-products.
- To remove nonderivatized oligosaccharides (optional) from BAP oligosaccharides (purified by either method), adducts are bound in water to a Sep-Pak C_{18} cartridge, washed with 20 ml of H_2O, and then eluted with 50% acetonitrile.
- Storage: BAP oligosaccharides should be stored in polypropylene Eppendorfs other tubes at -20°C in the presence of 10–50% acetonitrile, or at 4°C for shorter times. Under these conditions, they are stable for several years.

Troubleshooting guide

Low recovery or smearing of BAP adducts

- BAP is nonspecifically absorbed to Sepharose, Sephadex and polystyrene- (e.g. Dowex) and polyacrylamide- (e.g. BioGel) based supports. However, good recovery and resolution are seen on silica-based or Toyopearl (TosoHass) supports in the presence of acetonitrile (e.g. 25%).

Degradation of BAP-conjugated oligosaccharides

- Under extended acidic conditions BAP adducts can partially break down (D. K. Toomre and A. Varki, unpublished observations). Thus, avoid acid treatment, contact with strong cation exchangers and extended rotary evaporation that may cause concentration of acids or impurities.

16.5 Fractionation and Structural Analysis of BAP Adducts

After removal of excess BAP, the BAP oligosaccharide adducts can be fractionated by combinations of anion-exchange, reverse-phase, and amino-HPLC techniques until adequate purification is achieved (see Figs. 16.5, 16.6 and 16.7 for examples). Purified adducts can be further analysed by exoglycosidase enzymatic digestions as well as classical techniques such as MS and nuclear magnetic resonance (NMR). Procedures for separating adducts by charge, hydrophobicity and size are described below. An example of the structural sequencing of a purified adduct by exoglycosidase digestion and HPLC analysis is also shown.

All three HPLC methods use volatile buffers to avoid desalting between procedures.

Materials-Methods-Equipment

- HPLC apparatus and in-line fluorescent detector are as described above.
- Individual HPLC columns are indicated below.

Protocol 16.5

The following applies for all three HPLC columns:

1. Column buffer is rinsed through the injector, and a "blank" run is conducted to insure that no fluorescent contamination is present from previous runs.
2. The BAP oligosaccharides are dissolved in 10–500 ml of column buffer, and all or part of the sample is injected, the fluorescence monitored, and the peak(s) of interest pooled and lyophilized.
3. The sample is redissolved in the appropriate column buffer, and steps 1 and 2 can be repeated on another column.

BAP oligosaccharide standards of known concentration should be run periodically to calibrate the column's performance and as a measure of the relative fluorescence.

Figure 16.5 Anion
N-linked oligosacc
F (PNGaseF), coup
of the Ω_0 eluting m
dient. The elution p
(N-3) and four (N-4)
one (S-1) or two (S-2
box. BAP oligosacc
rinogen and bovine
glycoprotein conta
permission)

? Adducts

i be fractionat-
o-HPLC tech-
nd 16.7 for ex-
: enzymatic di-
ietic resonance
ity and size are
ified adduct by

)etween proce-

iector are as de-

elow.

olumns:

and a "blank"
t contamination

l–500 ml of col-
jected, the fluo-
st pooled and

column buffer,
er column.

tion should be run
nd as a measure of

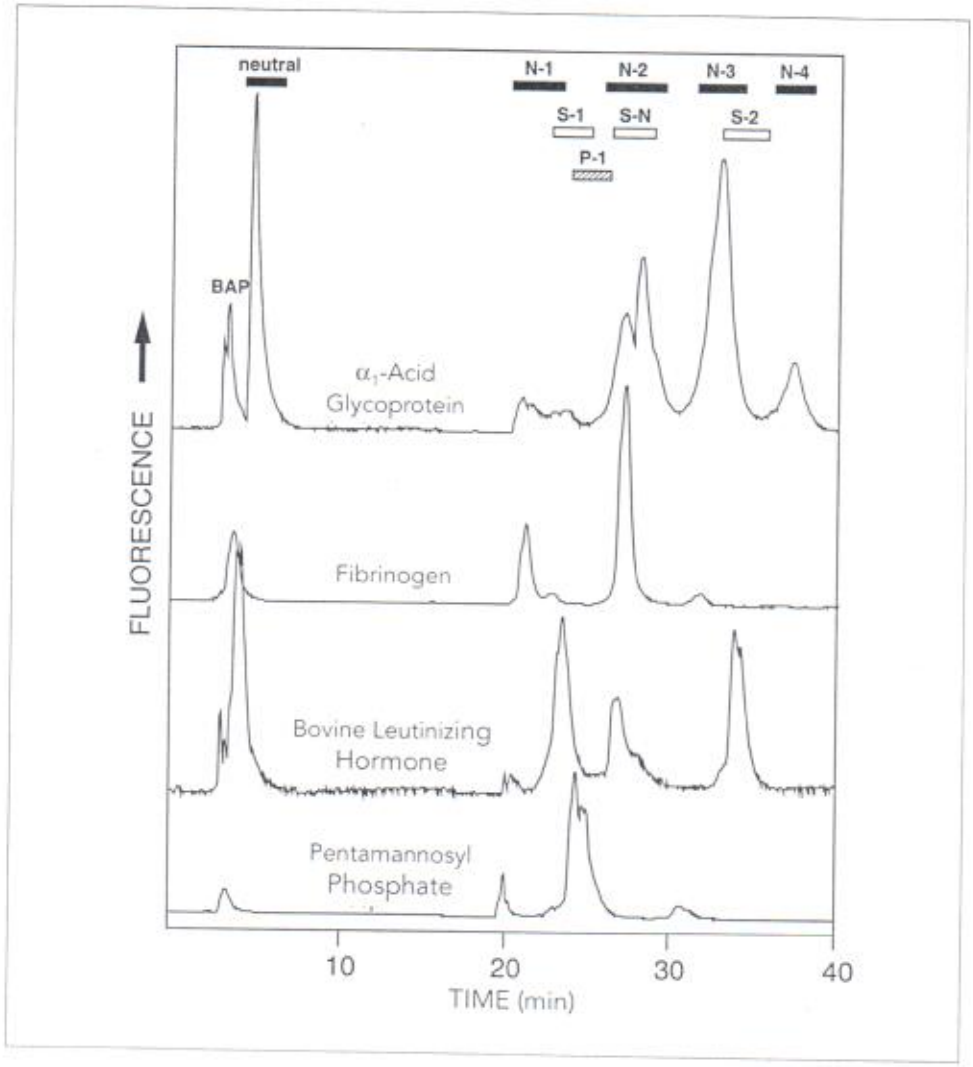


Figure 16.5 Anion-exchange HPLC of BAP-oligosaccharides on a TSK-DEAE-2SW column
N-linked oligosaccharides were released from the indicated glycoproteins with peptide-N-glycosidase F (PNGaseF), coupled to BAP and purified on a TSK-G3000PWxl column as in Figure 16.4. An aliquot of the Ω_0 eluting material was then analysed on a TSK-DEAE-2SW column eluted with an acetate gradient. The elution position of BAP oligosaccharides containing zero (neutral), one (N-1), two (N-2), three (N-3) and four (N-4) sialic acid residues is indicated with a solid box. BAP oligosaccharides containing one (S-1) or two (S-2) sulfate residues, or one sialic acid and one sulfate (S-N), are indicated with an open box. BAP oligosaccharides bearing one phosphate (P-1) are indicated with a hashed box. Typically, fibrinogen and bovine leutinizing hormone contain complex biantennary oligosaccharides, while α1-acid glycoprotein contains sialylated bi-, tri- and tetraantennary oligosaccharides. (From ref. 14, used with permission)

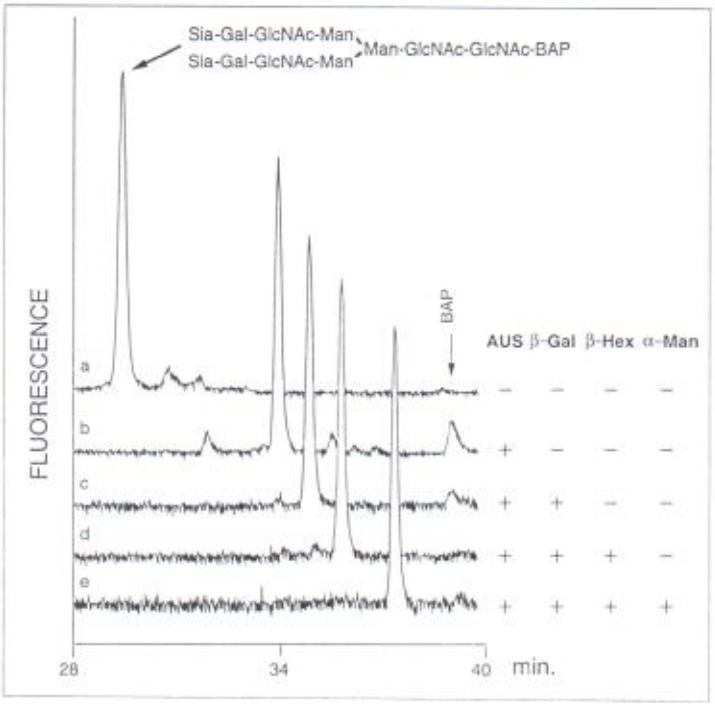


Figure 16. 6 Analysis of BAP adducts on RP-HPLC by sequential exoglycosidase digestions

BAP adducts of fibrinogen oligosaccharides were collected from the N-2 region on a TSK-DEAE-2SW column (see Fig. 16.3) and treated with A. ureafaciens sialidase (AUS), jack bean β-galactosidase (β-Gal), S. pneumoniae β-N-acetylhexosaminidase (β-Hex) and a-mannosidase (α-Man) as indicated, and a fraction was analysed on Microsorb C_{18} column. Due 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. (From ref. 14, used with permission)

Materials-Methods-Equipment

- **Anion-exchange HPLC fractionation of BAP oligosaccharides**

  Column: TSK-DEAE-2SW column (TosoHass; 7.8 x 300 mm) with DEAE "guardgel" kit (TosoHass) and preguard column replaceable filter (A-101X; Upchurch Scientific)
  Solvent A: 0.5 mm pyridine acetate, pH 3.5, in 25% acetonitrile

Solvent B: 2 M pyridine acetate, pH 3.5, in 25% acetonitrile

Elution gradient: 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

- **Reverse-Phase HPLC fractionation of BAP oligosaccharides**

  Column: Microsorb MV C_{18} column (Rainin; 4.6 x 250 mm) and pre-column replaceable filter (A-101X; Upchurch Scientific).

This column can be replaced with other generic C_{18} columns; due to its inexpensive cost, no guard column is necessary.

Solvent A: 10 mM ammonium formate, pH 4.0

Solvent B: acetonitrile (HPLC grade)

Elution gradient: Typically, after injection solvent B was increased linearly from 0 to 25% over 50 min at a flow rate of 1 ml/min

The optimal gradient conditions depend on both the column and the adducts that are desired to be best resolved.

- **NH_2-HPLC fractionation of neutral BAP oligosaccharides**

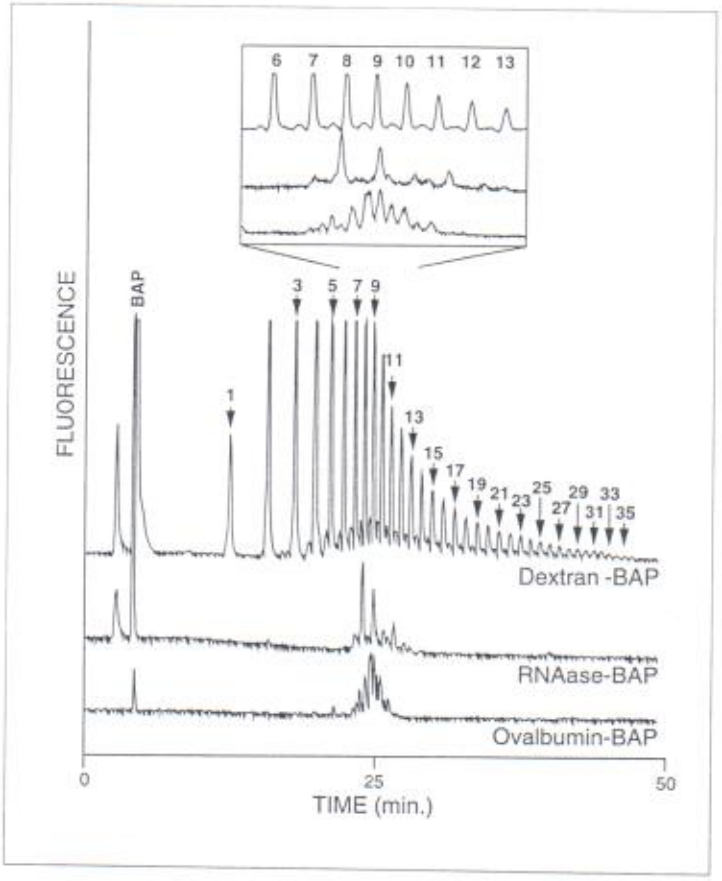
  Column: Microsorb MV NH_2 column (Rainin; 4.6 x 250 mm) and pre-column replaceable filter (A-101X; Upchurch Scientific).

This column can be replaced with other generic NH_2 columns; due to its inexpensive cost, no guard column is necessary.

Solvent A: 10 mM ammonium formate, pH 3.0,in 95% acetonitrile

Solvent B: 10 mM ammonium formate, pH 3.0, in water

Elution gradient: 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, at a flow rate of 1 ml/min



◀ *Figure 16.7*
Aliquots of
tran-BAP lac
rified on a C
B and ovalb
purified on a
ovalbumin a

Troublesh

(see remark

16.6 Pr gates wi

Once pure
plexes of t
16.2). Duc
tionally as
and permit
affinity im
which in tu
is itself a gl
the comple
cific recep
expression
that requir
Receptors
bound olig

These
avidin/biot
The forma
tails of var

rmate, pH 3.0, in

io of A : B was
: 40 over the first
aining 40 min, at

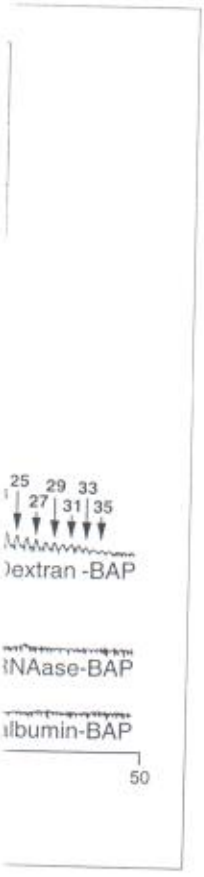
Troubleshooting guide

(see remarks on the purification of BAP oligosaccharides)

16.6 Preparation of BAP-Oligosaccharide Neoglycoconjugates with Streptavidin or Avidin and Their Applications

Once pure BAP-oligosaccharide adducts are obtained, stable multivalent complexes of the oligosaccharides with streptavidin or avidin can be prepared (Fig. 16.2). Due to the high affinity of the interaction, these complexes behave functionally as covalently attached neoglycoproteins (14). The protein acts as a carrier and permits small quantities of such complexes to produce monospecific high-affinity immunoglobin G (IgG) antibodies directed against the sugar chains (13), which in turn can be used to study their expression and tissue distribution (avidin is itself a glycoprotein and should be avoided during immunization). Furthermore, the complexes can potentially be used to detect and study the expression of specific receptors for the oligosaccharide in cells and tissues, or to screen recombinant expression libraries for cDNAs encoding such receptor proteins – including those that require multivalent presentation of natural ligands for proper recognition (1). Receptors could also be affinity-purified on immobilized streptavidin containing bound oligosaccharide adducts.

These examples serve to highlight numerous opportunities available in using avidin/biotin technology for characterizing the functional role of oligosaccharides. The formation of the complexes is described below, but due to limited space, details of various functional applications can be found elsewhere (13).



◀ *Figure 16.7 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. RNAase 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 RNAase B adducts. (From ref. 14, used with permission)

Materials-Methods-Equipment

- Avidin and streptavidin (Scripps Laboratories)
- Centricon-10 ultrafiltration tubes (Amicon)

Protocol 16.6 Formation of BAP-oligosaccharide neoglycoproteins with streptavidin or avidin

1. Purified BAP oligosaccharides are incubated for ~10 min at 4°C with either avidin or streptavidin in water or buffer.
2. Unbound BAP adducts are removed by repeated ultrafiltration with multiple washings (e.g. four times) on a Centricon-10 ultrafiltration tube (10,000 MWCO), per the manufacturer's instructions. This also permits concentration of the neoglycoprotein. The volume of the final retentate is typically ~50 µl.

- The molar ratio of the BAP oligosaccharides to streptavidin or avidin will depend on the desired applications. If neoglycoproteins containing multivalent glycans are desired, then excess BAP adducts should be used, recalling that up to four biotinylated adducts may be bound per molecule of streptavidin or avidin. If maximal incorporation of the adducts into neoglycoproteins is desired, such as when preparing complexes for the generation of antibodies, then the use of excess streptavidin or avidin may be desired. Similarly, neoglycoprotein complexes can be formed with labelled (radioactively or enzymatically) or immobilized streptavidin or avidin.
- The neoglycoprotein complex is stable at ambient temperatures but should be stored at 4°C until used.

Troubleshooting guide

Low valency of BAP adducts on neoglyococonjugates

Although up to four biotin binding sites are theoretically available on tetrameric streptavidin or avidin, many commercial preparations have fewer available bind-

ing sites (e.g. <3) (see ref. 11). Thus, only commercial sources of high purity should be used, and the number of binding sites measured if needed.

16.7 Comparison of BAP With Other Currently Available Fluorescent Tags

While many fluorescent tags are commercially available, including 2-aminopyridine, various naphthalene derivatives (e.g. 8-aminonaphtalene-1,3,6-trisulphonic acid (ANTS)), 4-aminobenzoic acid and 2-aminobenzamide, there is no "ideal" fluorescent tag for oligosaccharides. The ideal tag should meet the following criteria:

(1) Inexpensive and readily available
(2) Efficient and quantitative coupling to oligosaccharides
(3) Large Stokes shift and high quantum yield
(4) Compatible with various methods for fractionation and sensitive detection
(5) Useful in biological application

While many of these tags meet some of these criteria, none to our knowledge adequately meets all of them. For instance, some of the most fluorescent tags tend to have bulky multiaromatic rings that tend to decrease coupling efficiency and can complicate fractionation schemes. Of course, the ideal tag may depend on the question being addressed and the equipment available. For instance, tags which are compatible for quick fluophore-assisted carbohydrate electrophoresis (FACE) profiling may differ from those best suited for HPLC applications, and vice-versa.

BAP has the following advantages: (i) it is inexpensive and easy to synthesize; (ii) it has a moderately good quantum yield and Stokes shift; (iii) it provides sensitive detection (low picomole) and good fractionation of oligosaccharides; and (iv) it is the only oligosaccharide tag which is already biotinylated for direct use in functional studies. The limitations of BAP as a tagging system include: (i) the tag can bind or smear on certain supports; (ii) it can undergo partial degradation under highly acidic conditions; (iii) the fluorescence is pH sensitive; and (iv) although the coupling for model oligosaccharides is good, the coupling efficiency for very large or highly charged oligosaccharides is lower. Although most of these caveats can be circumvented by an adequate choice of support and operating parameters, the variability of coupling efficiency is a general problem seen with most fluorescent tags. One notable exception may be 2-aminobenzamide, which was recently shown to

give nearly quantitative coupling to a host of standard oligosaccharides, as well as good chromatographic properties (20). However, lack of a biotin moiety limits use of these tagged oligosaccharides purely to structural studies. The prospect of creating biotinylated versions of 2-aminobenzamide is an alternative we are pursuing.

References

1 Lee YC (1992) *FASEB J.* **6**: 3193.

2 Kornfeld S (1992) *Annu. Rev. Biochem.* **61**: 307.

3 Bevilacqua MP and Nelson RM (1993) *J. Clin. Invest.* **91**: 379.

4 Varki A (1993) *Glycobiology* **3**: 97.

5 Hase S (1994) *Methods Enzymol.* **230**: 225.

6 Lee KB, Al-Hakim A, Loganathan D and Linhardt RJ (1991) *Carbohydr. Res.* **214**: 155.

7 Stack RJ and Sullivan MT (1992) *Glycobiology* **2**: 85.

8 Jackson P (1991) *Anal. Biochem.* **196**: 238.

9 Tomiya N, Lee YC, Yoshida T, Wada Y, Awaya J, Kurono M and Takahashi N (1991) *Anal. Biochem.* **193**: 90.

10 Shao MC, Sokolik CW and Wold F (1994) Noncovalent Neoglycoproteins. *In:* YC Lee and RT Lee (eds) *Neoglycoconjugates: Preparation and Applications*, p.225, Academic Press, San Diego.

11 Shao MC, Chen LM and Wold F (1990) *Methods Enzymol.* **184**: 653.

12 Hase S (1992) *J. Biochem. (Tokyo)* **112**: 266.

13 Rothenberg BE, Hayes BK, Toomre D, Manzi AE and Varki A (1993) *Proc. Natl. Acad. Sci. USA* **90**: 11939.

14 Toomre DK and Varki A (1994) *Glycobiology* **4**: 653.

15 Shao MC (1992) *Anal. Biochem.* **205**: 77.

16 Wilchek M and Bayer EA (1988) *Anal. Biochem.* **171**: 1.

17 Honda S and Suzuki S (1984) *Anal. Biochem.* **142**: 167.

18 Savage D, Mattson G, Desai S, Nielander G, Morgansen S and Conklin E (eds), *Avidin-Biotin Chemistry: A Handbook* (1992) Pierce Chemical Co., Rockford, IL.

19 Green NM (1965) *Biochem. J.* **94**: 23c.

20 Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM and Parekh R (1995) *Anal. Biochem.* **230**: 229.