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Biotinylated diaminopyridine: An approach to tagging oligosaccharides and exploring their biology

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ABSTRACT **Fluorescent tagging of free oligosaccharides by reductive amination permits sensitive detection and fractionation of these molecules. To expand the scope of this approach, we have synthesized a fluorescent reagent, 2-amino-(6-amidobiotinyl)pyridine. This reagent can tag oligosaccharides under nondegradative conditions with high efficiency. The resulting adducts show excellent fractionation by reverse-phase HPLC with sensitive detection in the low picomole range. When combined with sequential exoglycosidase digestion, stepwise sequencing of the sugar chains is possible. The biotinyl group can also be used to recover the sugar chain from reaction mixtures. The high-affinity interaction of the biotinyl group with multivalent avidin or streptavidin can be used to create the functional equivalent of neoglycoproteins carrying multiple copies of oligosaccharides of defined structure. These complexes allow the production of IgG antibodies directed against the oligosaccharide chain. They can also harness the power of (strept)avidin–biotin technology for the detection and isolation of oligosaccharide-specific receptors from native sources of recombinant libraries.**

Many important biological roles of oligosaccharides involve their interaction with specific receptors (1–4). Discovery and exploration of such interactions require comprehensive structural analyses of oligosaccharides, which necessitate their release and fractionation into structurally distinct species. Critical to the fractionation of oligosaccharides is their sensitive and specific detection. However, detection by UV absorption is neither sensitive nor specific, metabolic labeling (5) has limited applicability, and chemical labeling (6, 7) or pulsed-amperometric detection (8) exposes the sugar chains to strongly basic conditions. Also, the last method requires specialized equipment. Alternatively, free oligosaccharides can be tagged via reductive amination with neutral (9, 10) or acidic (11–13) fluorescent compounds or with amino-phospholipids (14).

Although these approaches are useful, fractionation of the tagged oligosaccharides has limitations. Separation of fluorescent adducts by gel electrophoresis (11–13) or capillary electrophoresis (15) requires technical proficiency and/or expensive quantitation methods. Fractionation of weakly hydrophobic 2-aminopyridine (AP) adducts by reverse-phase HPLC (RP-HPLC) requires careful adjustment of low concentrations of butanol (16). Phospholipid adducts are too hydrophobic for RP-HPLC and must be fractionated by TLC instead (14). Most such tagged and fractionated purified molecules are of limited use for anything other than structural analyses. The exception is phospholipid tagging, which allows probing of TLC plates with purified receptors known to recognize the oligosaccharides (14). However, these adducts cannot be used as soluble reagents to probe natural or recombinant sources for previously unknown receptors. For the latter, one needs neoglycoproteins made by chemical coupling of defined oligosaccharides to proteins (17). However, most methods for tagging and fractionating oligosaccharides eliminate the reducing terminus required for forming neoglycoproteins. Also, there is wide heterogeneity in the stoichiometry and arrangement of oligosaccharides on the protein.

An alternative is to couple oligosaccharides or glycopeptides to biotin, allowing formation of stable multivalent complexes with avidin or streptavidin (18). This can also harness the well-known power of avidin–biotin technology (19). However, as with neoglycoproteins, the underivatized oligosaccharide or glycopeptide must first be purified to homogeneity, prior to biotin coupling. Another approach to studying oligosaccharide biology is to obtain monospecific antibodies (20–23). However, immune responses to free sugar chains are T-cell-independent, and most antibodies are low-affinity IgMs (23), which are technically difficult to work with and likely to show nonspecific cross-reactivity (22). To obtain high-affinity IgG antibodies, the oligosaccharide must be covalently coupled to carrier proteins (20, 23), i.e., neoglycoproteins. Thus, each current approach to the tagging, fractionation, and biological study of oligosaccharides has its own advantages and limitations. Here, we report a reagent for making fluorescent oligosaccharide adducts that allows a more versatile approach to their subsequent study.

MATERIALS AND METHODS

Materials. Unless stated, most materials were obtained from Sigma. [6-^{3}H]Galβ1-4GlcNAcβ1-4GlcNAc was prepared as described (24). High-mannose-type N-linked oligosaccharides from bovine pancreatic RNase B (25) released with peptide:*N*-glycosidase F (26) were purified by passage through Biobeads SM-2 and Amberlite MB-3 resins. The following were gifts: isomaltoheptaose oligosaccharide (IM-7) (K. Granath, Pharmacia); synthetic sialyl Lewis^x (SLe^x) (C. Hummel and K. C. Nicolaou, University of California, San Diego); IgG monoclonal antibody against IM-7 (E. Kabat, Columbia University); yeast mannan pentamannose phosphate fragment (H. Freeze, La Jolla Cancer Research Foundation).

Synthesis, Purification, and Characterization of 2-Amino-(6-amidobiotinyl)pyridine (BAP). The active *N*-hydroxysulfosuccinimide (NHS) ester of biotin was formed *in situ* (27) and reacted with 2,6-diaminopyridine (DAP). NHS (5 mM; Pierce), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.1 M; Pierce), and DAP (0.3 M; Aldrich) were dissolved in 40 ml of 50 mM Mes (pH 6.5), and biotin (0.1 M, dissolved in 10 ml of dimethyl sulfoxide or dimethylforma-

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Abbreviations: BAP, 2-amino-(6-amidobiotinyl)pyridine; RP-HPLC, reverse-phase HPLC; AP, 2-aminopyridine; CI-MPR, cation-independent mannose-6-phosphate receptor; IM-7, isomaltoheptaose oligosaccharide; NHS, *N*-hydroxysulfosuccinimide; DAP, 2,6-diaminopyridine; LSIMS, liquid secondary ion mass spectrometry; 1D and 2D, one and two dimensional, respectively.

mide) was added. After stirring overnight at room temperature, the mixture was passed over a 20-ml Dowex AG 3x4a column (OH^- form, in water), and unbound material was lyophilized and dissolved in butanol with heating at 95°C. The solution was brought to 5% (wt/vol) trifluoroacetic acid and loaded onto a 500-g silica column (Silica gel grade 60, 230–400 mesh, 60 Å; Merck), equilibrated in 95% butanol/5% trifluoroacetic acid (vol/vol). When the leading fluorescent band (DAP, detected with UV) was half way down, the eluant was changed to 85% ethanol. Fractions containing BAP (analyzed by TLC) are pooled and concentrated at 45°C on a rotary evaporator with repeated ethanol additions to remove trifluoroacetic acid. The material was further purified by redissolving in 85% ethanol with heating and loading onto the original silica column, equilibrated with 85% ethanol (repeated if necessary). It was stored dry in a desiccator at room temperature or −20°C. BAP synthesis and purification were monitored on silica-gel TLC plates developed in 85% ethanol (BAP R_f, 0.75-0.80; DAP R_f 0.50—DAP has a stronger relative fluorescence at pH > 5.0). Iodine vapor visualized biotin, immediately below BAP, and other contaminants close by the origin.

Liquid Secondary Ion Mass Spectrometry (LSIMS) and ^1H NMR. BAP (1 μl dissolved in methanol at 5 μg/μl) was loaded onto the LSIMS target covered with nitrobenzyl alcohol, and 1 μl of trifluoroethanol was added. Analysis in the positive-ion mode used a VG analytical 70-SE mass spectrometer (La Jolla Cancer Research Foundation) with a cesium ion gun operated at 25 kV and an emission current of 2 μA. For ^1H NMR, BAP (1.7 mg in 0.5 ml of deuterated dimethyl sulfoxide) was studied with a 360-MHz Bruker instrument. One- and two-dimensional (1D and 2D) correlated spectroscopy (COSY) spectra were recorded at 25°C and referenced to an internal 3-trimethylsilylpropionate-2,2,3,3-d_4 standard.

Synthesis and Purification of Oligosaccharide-BAP Adducts. Oligosaccharides were dissolved in reaction buffer [pyridine/glacial acetic acid, 2:1 (vol/vol)] in 200-μl tapered glass reactivials with final volumes of 11–100 μl. Some large neutral or acidic oligosaccharides required prior dissolution in minimal dimethyl sulfoxide or water, respectively. A 40- to 50-fold molar excess of BAP was added. After 1 h at 80°C, an equal volume of borane–dimethylamine complex (25 mg/200 μl of reaction buffer) was added and heated for another hour. Reactions were brought to 0.5 ml with 50% (vol/vol) acetonitrile and fractionated on a 1.5 cm × 48 cm Toyopearl HW40S column equilibrated with 50% acetonitrile/10 mM NaOAc, and 0.5- to 1-ml fractions were analyzed for fluorescence (see below). Fractions eluting ahead of unreacted BAP were pooled, concentrated, and redissolved in H_2O. To remove unreacted oligosaccharides, adducts were bound to a Sep-Pak C_{18} cartridge, washed with 10 ml of H_2O, and eluted with 50% acetonitrile.

Detection, Quantitation, and Fractionation. BAP and its adducts can be visualized with a hand-held UV lamp or quantitated in the picomole range by their intrinsic fluorescence at pH < 5, relative to known standards (see below). Biotinylated standards can be calibrated using the 4′-hydroxyazobenzene-2-carboxylic acid (HABA) dye-displacement method (28) (ImmunoPure HABA kit; Pierce). For fractionation of adducts by RP-HPLC, generic C_{18} HPLC columns (e.g., Microsorb MV C-18, Rainin, Woburn, MA) were equilibrated in 10 mM NH_4COOH (pH 4.1), and samples were loaded in the same buffer and eluted with linear gradients of acetonitrile against the same buffer, until unmodified BAP was eluted (usually ≈25% acetonitrile). Gradients can be adjusted to optimize separation of different compounds. Separations were monitored at an excitation wavelength of 345 nm and an emission wavelength of 400 nm.

Generation and Monitoring of Polyclonal Antibodies Against Oligosaccharide-BAP Adducts. HPLC-purified IM-7-BAP adducts (3.3 nmol) were coupled to streptavidin, and the complexes were mixed with complete Freund's adjuvant and injected i.p. into 16-week-old C3H/HeN female mice (29). One month later, three booster injections were given in incomplete Freund's adjuvant at 2-week intervals. Serial serum samples were screened for IgG antibody production by an ELISA assay against IM-7 coupled to bovine serum albumin via reductive amination (30). This assay would detect antibodies against the sugar chain but not against streptavidin or BAP.

Binding of Strepavidin–Pentamannose Phosphate–BAP Complexes to a Mannose-6-Phosphate Receptor (MPR) Column. Pentamannose phosphate (31) was coupled to BAP as described above, purified by TSK gel HPLC, and characterized by *Escherichia coli* alkaline phosphatase (D.T. and A.V., unpublished data). ^{35}S-labeled streptavidin (Amersham) was incubated with a 40-fold molar excess of adduct at room temperature for 5 min, and free adducts were removed by repeated ultrafiltration (five times) with a Centricon-10 (Amicon). The complex was added to 0.5 ml of 20 mM potassium phosphate/150 mM NaCl, pH 8.0, with 0.1% Triton X-100, loaded on a column of immobilized bovine cation-independent MPR (CI-MPR) (32) at 4°C, and washed in with 0.5 ml of buffer. After 15 min, the column was washed with 14 1-ml volumes of buffer, and bound sample was eluted with buffer containing 10 mM mannose 6-phosphate.

RESULTS AND DISCUSSION

Synthesis, Purification, and Characterization of BAP. The most useful and least expensive way to synthesize BAP forms the active NHS ester of biotin *in situ* and couples it to DAP (Fig. 1). DAP is used in a 3-fold excess, to maximize formation of the monobiotinylated product. Final yields as high as 50% are obtained, and the final product gives a single spot on TLC and a single peak with RP-HPLC (data not shown). Positive-mode LSIMS yields a major molecular ion signal at 336 map units, as expected (Fig. 2). The 1D ^1H NMR shows all expected signals from the two portions of the molecule, identified by comparison with the literature (33, 34). Exact assignment of the individual resonances was done by 2D correlated spectroscopy (COSY, spectra not shown): H-2β, 3.05 ppm ($J_{2\beta,3\beta}$ = 3.6 Hz); H-3β, 4.07 ppm ($J_{2\beta,\delta}$ = 7.2 Hz); H-4β, 4.24 ppm ($J_{3\beta,\delta}$ = 3.6 Hz); H-5α, 2.52 ppm ($J_{4\beta,5\beta}$ = 7.2 Hz); H-5β, 2.77 ppm ($J_{5\alpha,5\beta}$ = 10.8 Hz); H-α, 2.32 ppm ($J_{\alpha,\beta}$ = 7.2 Hz); H-β, 1.43 ppm; H-δ, 1.30 ppm; H-δ, 1.53 ppm ($J_{\delta gem}$ = 14.4 Hz; $J_{\delta vec}$ = 7.2 Hz); H′-δ, 1.39 ppm; H-3″,4″,5″,

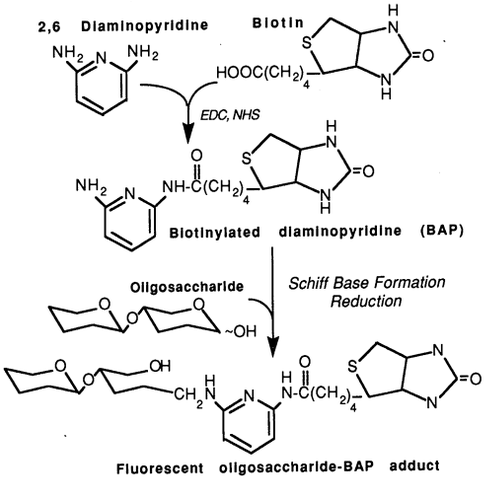

FIG. 1. Structure and synthesis of BAP and the chemistry of adduct formation with oligosaccharides. EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

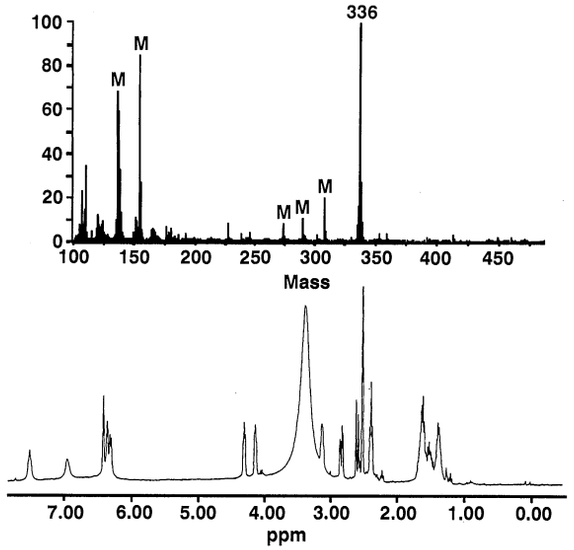

FIG. 2. Structural characterization of BAP. Purified BAP was analyzed by LSIMS (*Upper*) and 1D ^{1}H NMR (*Lower*). Ions marked M are also found in the matrix control.

6.28 ppm. UV/visual spectral analysis gave excitation and emission maxima of 341 nm and 387 nm, respectively (for BAP-oligosaccharide adducts, the corresponding values were 345 nm and 400 nm). Since fluorescence yields are markedly pH-dependent (Fig. 3), further studies utilized NaOAc or NH_4COOH buffers at pH ≈4. These do not degrade known oligosaccharide components, and the latter is volatile.

BAP Tagging of Oligosaccharides and Purification of Adducts. BAP is efficiently coupled to the reducing terminus of oligosaccharides via reductive amination (see Fig. 1), under nondegradative conditions. Conditions for coupling were optimized, based upon recent reports for AP (35, 36). In those studies, AP itself was used as the reaction buffer. We used pyridine acetate at pH 5.3 as buffer, and sufficient BAP (40- to 50-fold molar excess) to achieve maximum coupling (studied with [^{3}H]Galβ1-4GlcNAcβ1-4GlcNAc, data not shown). Under the conditions used, there was <5% hydrolysis of sialic acids, the most acid-labile sugar known in mammalian oligosaccharides (data not shown). Coupling efficiency varied with the oligosaccharide. Yields of 75–80% for small neutral chains such as [^{3}H]Galβ1-4GlcNAcβ1-4GlcNAc or IM-7 and 65–70% for a biantennary bisialylated N-linked oligosaccharide were obtained. The reasons for these differences are unknown. Initial purification and removal of excess reagents are by gel filtration on a HW40S column (BAP adducts give broad peaks with conventional Sephadex columns). Even adducts with small oligosaccharides separate from excess BAP, which elutes close to the total volume. Additional purification can be obtained with a C_{18} cartridge (binding in aqueous buffers and elution with 50% acetonitrile) or by amine adsorption via the oligosaccharide moiety on an LC-NH_2 cartridge (binding in >85% acetonitrile and elution with water for neutral oligosaccharides or with phosphate buffer for acidic compounds).

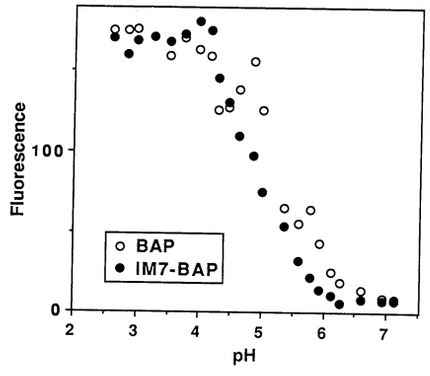

FIG. 3. pH dependence of the fluorescence of BAP and BAP-oligosaccharide adducts. Equal aliquots were studied for relative fluorescence in 100 mM NH_4COOH buffers covering a range of pH values.

Detection and Quantitation of BAP and Oligosaccharide-BAP Adducts. These compounds are readily detectable by fluorescence if pH is maintained at ≈4.0. Qualitative detection with a hand-held UV lamp or a UV light box is feasible. Quantitation is by fluorescence spectrophotometry (15 pmol is detectable in a 1-ml cuvette) or by UV absorbance spectrophotometry (ε = 7000 $M^{-1}\cdot cm^{-1}$ in water, practical limit ≈10 nmol in a 1-ml cuvette). The 4′-hydroxyazobenzene-2-carboxylic acid method can quantitate 1 nmol or more. With HPLC on-line detection, as little as 50 fmol can be detected with a fluorescence detector, and 50–100 pmol can be detected with UV detection (data not shown).

RP-HPLC Separation of Oligosaccharide-BAP Adducts. The amphipathic adducts allow versatile use of TLC, amine-adsorption HPLC, and RP-HPLC. The compounds show excellent separation in standard RP-HPLC with inexpensive C_{18} columns (see Fig. 4 for examples). The increased hydrophobicity of BAP relative to AP seems to achieve an excellent compromise with the hydrophilic nature of the oligosaccharide. Elution with simple gradients of acetonitrile and buffer gives sharp highly reproducible peaks. Unreacted BAP elutes last and is used as an internal marker. The sensitivity allows minor contaminants to be detected in samples thought to be homogenous by other criteria (data not shown).

Oligosaccharide Sequencing by Exoglycosidase Digestions. A single monosaccharide difference is sufficient to cause a baseline shift in retention time of an adduct peak in RP-HPLC (Fig. 4). Single, combined, or sequential exoglycosidase treatment of purified adducts allows sensitive and rapid sequence analysis of the oligosaccharides (see example in Fig. 4 *Upper*). By using adducts of known structure, sensitive detection of contaminating enzymatic activities in exoglycosidase preparations is also possible. After endoglycosidase digestions, it should be possible to follow release of specific fragments after rederivatization as well as the shift in elution of the original adduct.

BAP Can also Be Used to Identify Released Monosaccharides. As with AP (37), BAP-adducts of monosaccharides can be separated by RP-HPLC (Fig. 5). Although some monosaccharides are not sufficiently separated (e.g., galactose and mannose), this should not be a problem when following

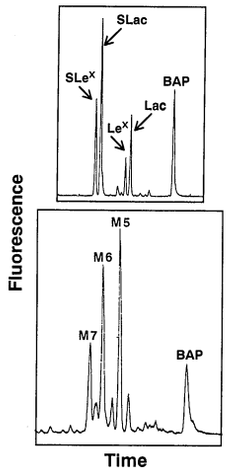

FIG. 4. Examples of fractionation and sequencing of oligosaccharide-BAP adducts by RP-HPLC. Free BAP (300 pmol) was added as an internal standard in each run. (*Upper*) BAP adducts of sialylactose (mixed isomers) or sialyl Lewis^x (SLe^x) tetrasaccharide were treated with *Arthrobacter ureafaciens* sialidase, boiled for 3 min, and centrifuged in a microcentrifuge, and the supernatant was directly studied. In the profile shown, a mixture of the two adducts was analyzed after partial digestion. (*Lower*) BAP adducts of N-linked oligosaccharides from RNase B. The major peaks represent adducts of $Man_5GlcNAc_2$ (M5), $Man_6GlcNAc_2$ (M6), and $Man_7GlcNAc_2$ (M7).

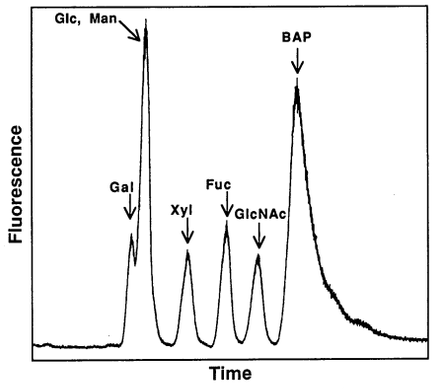

FIG. 5. Separation of monosaccharide-BAP adducts by RP-HPLC. Monosaccharide adducts of BAP were prepared with a 2-fold excess of BAP, and aliquots of each were studied by RP-HPLC. Shown here is a mixture of the monosaccharide adduct preparations (each at ≈100 pmol; without removal of excess BAP).

specific exoglycosidase digestion of an adduct after rederivatization with BAP. A two-component gradient on RP-HPLC should allow simultaneous detection of a shift in position of the original adduct (first part of the gradient) and the detection and quantitation of the released monosaccharide (second part of the gradient).

Complexes of Oligosaccharide-BAP Adducts with Avidin or Streptavidin. The most important advantage is that when a specific oligosaccharide adduct is purified and characterized from a mixture, a biotin group is already attached to it. Biotin is recognized with high affinity by avidin and streptavidin (dissociation constant $\approx 10^{-15}$ M). Monovalent avidin columns (Pierce) have a poorer binding constant (5×10^{-8} M), and the attachment can be disrupted with a volatile buffer at pH 2.8. This allows rapid repurification of adducts after various reactions, permitting removal of salts and reagents (data not shown).

The biotinyl group can be used to attach multiple copies of a purified and structurally defined oligosaccharide to avidin or streptavidin. Since both are tetravalent, each complex could theoretically have up to four oligosaccharides attached (although many commercial preparations have fewer available binding sites, see ref. 18). Although the attachment is noncovalent, it has a low off rate (18, 19), making the complex functionally equivalent to a neoglycoprotein (18), which can be used for several purposes.

Production of IgG Antibodies Against Oligosaccharides Using the Complexes. Generation of high-affinity monospecific IgG antibodies to an oligosaccharide generally requires that the sugar chain be covalently attached to an immunogenic carrier protein (20, 23). We reasoned that the high-affinity streptavidin–BAP–adduct complex should behave functionally as a covalently attached neoglycoprotein (avidin should be avoided, because it is itself a glycoprotein). Thus, naive B cells recognizing the oligosaccharide via surface IgM should internalize the entire complex and be recognized and activated by T cells by presentation of foreign streptavidin peptides. Reimmunization should result in expansion of the clone, selection of high-affinity antibody producers, and a class switch to IgG production. To explore this, we used BAP adducts of IM-7, the molecule originally used by Matsuda and Kabat (23) for studies of antibody generation against oligosaccharides. In those studies, free chains generated a weak IgM response, whereas neoglycoprotein adducts gave IgG responses. When coupled to streptavidin, IM-7-BAP generated specific IgG responses directed against the oligosaccharide (Fig. 6). In principle, this is applicable to any oligosaccharide coupled to BAP and purified in low microgram quantities. Specific IgG monoclonal antibodies against the

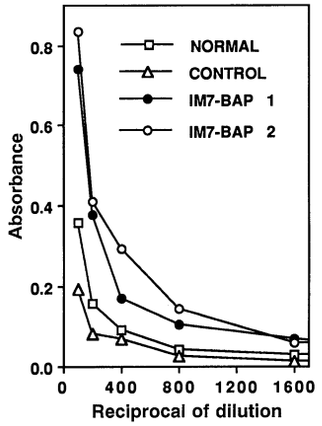

FIG. 6. Generation of specific IgG antibody responses against oligosaccharides. Mice were immunized with IM-7-BAP-streptavidin plus adjuvant or with adjuvant alone (control). The titer of IgG responses was evaluated by ELISA using IM-7-bovine serum albumin as the target reagent.

oligosaccharide should also be obtainable, using the spleen cells of immunized mice.

Complexes Can Be Used to Detect Oligosaccharide Receptors. The pentamannose phosphate fragment from yeast phosphomannan (31) can bind to the CI-MPR (38). However, oligosaccharides with a single phosphate group have a poor affinity (32, 38); multivalent ligands such as branched N-linked oligosaccharides or intact lysosomal enzymes are required for high-affinity binding (32, 38). As shown in Fig. 7, multivalent complexes of streptavidin with pentamannose phosphate-BAP adducts bound to immobilized CI-MPR and required mannose 6-phosphate for elution. Another subpopulation of the complexes was selectively retarded in elution compared with the control and presumably bound with lower affinity. The incomplete binding and heterogeneous behavior of the complexes are likely due to preexisting occupation of some binding sites by biotin in the commercial strepavidin. Alternatively, the chemical modification involved in preparing ^{35}S-labeled streptavidin may have inactivated some binding sites. A similar experiment with RNase B oligosaccharides and Con A indicated that only 27% of the labeled molecules are functional (D.T. and A.V., unpublished data). Of course, the precise positioning of the oligosaccharides in the potential binding sites (which are not all symmetrical, see refs. 39–41) may also affect the affinity. Regardless, these data indicate that multivalent complexes of oligosaccharide-

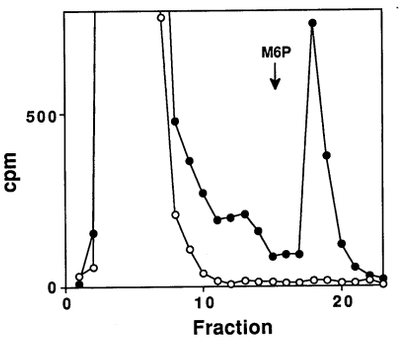

FIG. 7. Binding of streptavidin–pentamannose phosphate-BAP complexes to the MPR. ^{35}S-labeled strepavidin–pentamannose phosphate-BAP complexes were chromatographed on immobilized CI-MPR (●). The arrow indicates where 10 mM mannose 6-phosphate was added to the elution buffer. To control for nonspecific binding, ^{35}S-labeled strepavidin (○) was passed over the column in an identical manner.

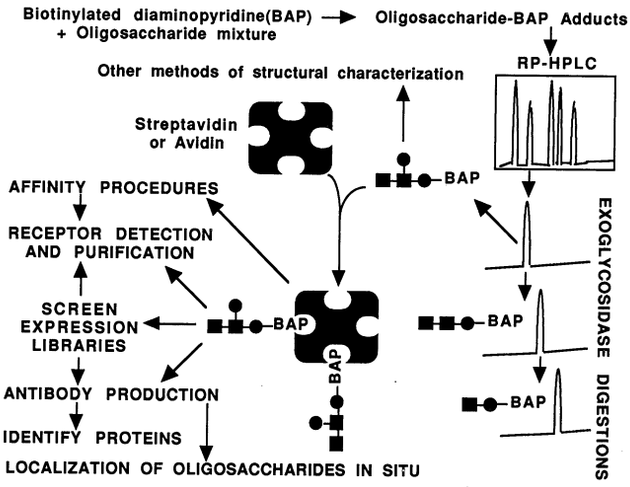

FIG. 8. Versatility of use of BAP-coupled oligosaccharides.

BAP adducts can be used to detect animal lectins that require multivalency for binding.

CONCLUSIONS AND FUTURE DIRECTIONS

BAP allows the rapid, efficient, and sensitive tagging, fractionation, and quantitation of heterogeneous mixtures of oligosaccharides using relatively inexpensive reagents and commonly available instruments and separation technologies. These purified adducts can be used to sequence and characterize the oligosaccharide chains and to explore their biology (Fig. 8). Release of monosaccharides or oligosaccharide fragments after glycosidase digestions can also be monitored. It is also likely that attaching the hydrophobic tag will enhance sensitivity in certain mass spectrometric analyses (10). Once well-characterized purified oligosaccharide-BAP adducts are obtained, stable multivalent complexes of the oligosaccharides with avidin or streptavidin can be prepared. Small quantities of such complexes should be sufficient to produce monospecific high-affinity IgG antibodies (polyclonal or monoclonal) directed against the sugar chains, which in turn could be used to study their expression and tissue distribution. The complexes should also be useful to detect and study 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). If an oligosaccharide-specific receptor is discovered in natural or recombinant sources, the adducts could then be used with immobilized streptavidin to affinity-purify it. Thus, this simple reagent provides a versatile approach to studying oligosaccharides and their biology that combines many advantages of previously described techniques.

Further work is needed to optimize this system. (*i*) The variability in coupling efficiency of different oligosaccharides needs to be addressed (this is a general feature of all procedures for reductive amination of oligosaccharides, a fact not acknowledged in most studies). (*ii*) The binding constants of the oligosaccharide-BAP adducts for avidin and streptavidin must be examined, to see whether they are different from those for native biotin (18). (*iii*) If all commercial preparations of streptavidin and avidin are indeed partially saturated with contaminating biotin (18), conditions need to be found to restore full tetravalent binding. This should allow production of much better defined complexes with oligosaccharide-BAP adducts. Separation into individual defined complexes (monovalent through tetravalent) should be possible based on the properties of the oligosaccharide (e.g., complexes with negatively charged adducts could be separated by ion-exchange chromatography or by isoelectric focusing). Once formed and fractionated, the complexes should be quite stable, assuming that the adducts share the low off rate of the biotin–avidin interaction (18). Since the crystal structures of both avidin and streptavidin have been elucidated (39–41), the nature of these complexes can be investigated to see whether oligosaccharide spacing is important for recognition by specific receptors.

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