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Recovery of sugar derivatives from 2-aminopyridine labeling mixtures for high-performance liquid chromatography using UV or fluorescence detection

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ABSTRACT

Labeling of reducing oligosaccharides with 2-aminopyridine by direct condensation to form a glycosylamine or by reductive amination to form a secondary amine has been used to allow the sensitive detection of oligosaccharides using both UV and fluorescence detection. For efficient labeling, a large excess of 2-aminopyridine is necessary in the derivatization mixture. The non-bound 2-aminopyridine absorbs and fluoresces near the wavelengths of the adducts and may obscure sample components. It must therefore be removed before the derivatives can be used for some chromatographic methods. It has been found that small cation-exchange columns (Extract-Clean columns from Alltech), if converted to the NH_4^+ form, bind free 2-aminopyridine but not labeled acidic oligosaccharides or neutral oligosaccharides of more than four residues. Thus, labeled oligomers can be obtained free enough of unbound 2-aminopyridine for fluorescence detection, by simple solid-phase extraction. As the 2-aminopyridine glycosylamine of oligosaccharides, and of galacturonic acid oligomers in particular, were found to be unstable, conditions for stable storage are presented. The conditions for complete hydrolysis of the 2-aminopyridine label from the glycosylamine are also presented if recovery of the native oligosaccharide after chromatographic purification is desired.

INTRODUCTION

Precolumn derivatization of reducing carbohydrates with 2-aminopyridine by direct condensation to form a glycosylamine or by reductive amination to form a secondary amine has allowed their rapid, reproducible and sensitive detection by high-performance liquid chromatography [1–3] and more recently by capillary zone electrophoresis [4]. The derivatives have also been used for the determination of oligosaccharide sequences [5–7]. For efficient labeling a large excess of 2-aminopyridine in the derivatization mixture is necessary. The non-bound 2-aminopyridine absorbs and fluoresces near the wavelengths of the adducts and may obscure sample components. We reported previously that precolumn derivatization of galacturonic acid oligomers by condensation reaction with 2-aminopyridine, and direct injection of an aliquot of the reaction mixture into a high-performance liquid chromatographic (HPLC) system, provides a method to determine oligogalacturonides differing in degree of polymerization [8]. Using UV detection, tailing of the 2-aminopyridine peak obscured the monomer and dimer of galacturonic acid, which eluted during the first 10–15 min of the chromatogram. We have since attempted to apply the technique using more sensitive fluorescence detection and found that interference from the 2-aminopyridine reagent front prevented detection of any labeled oligosaccharides. Additionally, 2-aminopyridine reagent is retained

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on some pellicular anion-exchange resin-based columns (PA-1; Dionex, Sunnyvale, CA, USA) and may co-elute with sample components.

In previous studies, a combination of ion-exchange and size-exclusion chromatography was used for the recovery of labeled sugars from 2-aminopyridine derivatization mixtures [9,10]. We report here that small cation-exchange columns (Extract-Clean columns from Alltech), if converted to the NH_4^+ form, bind free 2-aminopyridine but not labeled acidic oligosaccharides or neutral oligosaccharides of more than four sugar residues. Utilization of the procedure for both sample clean-up prior to chromatography and for removal of the 2-aminopyridine label following hydrolysis from glycosylamine adducts is discussed. Use of the procedure to recover the secondary sugar amines produced during reductive amination is also presented.

EXPERIMENTAL

Reagents and oligosaccharide standards

Pectic acid, sodium cyanoborohydride, 2-aminopyridine and curcumin were from Aldrich (Milwaukee, WI, USA) and lactose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, galacturonic acid and trigalacturonic acid from Sigma (St. Louis, MO, USA). Oligogalacturonides were prepared by autoclave hydrolysis of pectic acid as described by Robertsen [11]. The pH 2.0 supernatant was adjusted to 30 m*M* sodium acetate buffer (pH 5.2) and applied to a column of DEAE-Sephadex A-25 (90 mm × 50 mm I.D.) previously equilibrated using the same buffer. Oligogalacturonides were then eluted using stepwise increasing concentrations of potassium chloride in the same buffer. The fraction eluted with 375 m*M* potassium chloride (following a 350 m*M* potassium chloride elution), predominantly containing oligogalacturonides ranging from 8 to 16 sugar residues in length (determined as in ref. 8), was used. Purified tetra-, penta- and hexagalacturonic acid standards were prepared from an autoclave hydrolysate of pectic acid as described previously [12]. All other reagents were of analytical-reagent grade.

Preparation of oligosaccharide derivatives

Samples (10 μg–1 mg) were weighed on a Cahn 29 electrobalance and placed in 1-dram screw-capped vials or 1-ml Reacti-Vials (Pierce, Rockford, IL, USA). They were then derivatized by condensation reaction or by reductive amination with 2-aminopyridine. For condensation reaction, samples were dissolved in aqueous 2-aminopyridine labeling reagent (a minimum of 50 μl per mg sugar; prepared by dissolving 1 g of 2-aminopyridine in *ca.* 1 ml of water and adjusting the pH to 7.0 with glacial acetic acid, final volume 2.4 ml), sealed securely with Teflon-lined caps and incubated at 70°C overnight. For reductive amination, sodium cyanoborohydride was added to the 2-aminopyridine labeling reagent (1 mg per 50 μl) just prior to addition to samples, and reaction mixtures were incubated at 70°C overnight.

Solid-phase extraction of 2-aminopyridine from reaction mixtures

Cation-exchange Extract-Clean columns (100- and 500-mg packing sizes; exchange capacity 2–3 mequiv./g) were obtained from Alltech (Deerfield, IL, USA). Columns were converted to the ammonium form using concentrated ammonia solution (5 ml for 100-mg columns, 20 ml for 500-mg columns) and then rinsed with water (to pH 5–6) before the sample was applied. Aliquots of the 2-aminopyridine reaction mixtures (20 μl for 100-mg columns; 100 μl for 500-mg columns) were diluted 20-fold with 0.44 *M* acetic acid (final pH 4–5), applied to an Extract-Clean column and eluted with water. For evaluation of the percentage of adsorbed 2-aminopyridine, columns were eluted with 1 *M* sodim acetate buffer (pH 5.2) following the water elution. Appropriately sized aliquots were taken for sugar, 2-aminopyridine and borate assays. Columns were subsequently converted back to the ammonium form for re-use as described above.

Hydrolysis of 2-aminopyridine from glycosylamine derivatives

Glycosylamine derivatives were incubated in 100 m*M* acetic acid (200 μl per 0.1 mg of sugar) at 80°C for 1–23 h. Before cooling, the samples were diluted with 10% (v/v) acetic acid (100 ml per 0.1 mg of sugar) and applied to an Extract-Clean column. Sugars were eluted with two more volumes of water and then dried *in vacuo* at 40°C.

High-performance liquid chromatography

Liquid chromatographic separations were per-

formed using a Beckman (San Ramon, CA, USA) Model 334 gradient liquid chromatograph. The system consisted of a Model 421A system controller, two Model 110B high-pressure pumps, a Model 210A sample injector, a Model 163 variable-wavelength detector, a Model RF-535 variable excitation and emission wavelength fluorescence detector (Shimadzu, Kyoto, Japan) and a pulsed amperometric detector (PAD; Dionex) with a gold working electrode. Labeled sugars were detected by UV absorbance at 290 nm and by fluorescence with an excitation wavelength of 285 nm and an emission wavelength of 365 nm. Both labeled and non-labeled sugars were detected with PAD. Post-column delivery of 1 *M* sodium hydroxide solution through the PAD cell (total flow-rate 1.6 ml/min) was accomplished using a Dionex DQP-1 pump. The triple pulse sequence used for amperometric detection included the following potentials and durations: $E_1 = +0.05$ V ($t_1 = 480$ ms), $E_2 = +0.60$ V ($t_2 = 120$ ms) and $E_3 = -0.60$ V ($t_3 = 60$ ms). The integration sampling period was set at 200 ms and the response time at 3 s. Chromatographic data were integrated using an Apple IIe computer with chromatographic software. Eluents were prepared from analytical-reagent grade reagents and were filtered and degassed prior to use.

Gradient separations of sugars were conducted using either a CarboPac PA1 pellicular anion-exchange resin-based column (250 mm × 4 mm I.D.) with a CarboPac PA1 precolumn (Dionex) or a TSK DEAE 2SW anion-exchange silica-based column (250 mm × 4.6 mm I.D.) (Supelco, Bellefonte, PA, USA) at a flow-rate of 1 ml/min. Separations involving the TSK DEAE 2SW column were conducted as described previously [8]. Separations involving the CarboPac PA1 column were conducted as follows. Eluent A was water and eluent B was 1 *M* sodium acetate (pH 5.2). Elution of short oligogalacturonides [degree of polymerization (DP) 1–4] and of neutral maltose oligomers was accomplished using a linear gradient from 20 to 200 m*M* acetate in 25 min. Oligogalacturonides ranging from DP 5 to 10 were eluted using a linear gradient from 200 to 600 m*M* acetate over the same time period. Longer oligogalacturonides (DP 10–17) were separated using a linear gradient from 400 to 700 m*M* acetate in 30 min. The initial buffer concentration was maintained for 2 min following injection and prior to the start of the gradient. The final gradient conditions were maintained for 5 min and then the column was washed with 850 m*M* acetate for 15 min. The initial gradient conditions were then maintained for at least 12 min prior to injection of subsequent samples.

Other procedures

Sugar contents of fractions taken from Extract-Clean columns were determined using the phenol–sulphuric acid assay [13]. All assay volumes were half those given in ref. 13. Results were compared with calibration graphs prepared from corresponding sugar standards. The 2-aminopyridine contents of fractions were determined by measuring the UV absorbance at 290 nm. Aliquots of fractions were diluted appropriately and then brought to 1 ml in 200 m*M* sodium acetate (pH 5.2) for spectrophotometric analyses. Results were quantified using a molar absorption coefficient of $11.4 \cdot 10^3$ l mol^{-1} cm^{-1} for 2-aminopyridine. Borate content of appropriately sized aliquots from Extract-Clean fractions was determined spectrophotometrically using the curcumin procedure [14] with boric acid as standard. Assay mixture volumes were one tenth those given in ref. 14 and were conducted in 1.5-ml plastic microcentrifuge tubes.

RESULTS AND DISCUSSION

Various parameters were tested to assess the effectiveness and convenience of Extract-Clean columns for recovering labeled oligomers from derivatization mixtures. We attempted to devise a protocol that would enable us to remove the maximum proportion of unreacted 2-aminopyridine from the derivatization mixture of a wide range of oligosaccharides, but keep the concentration of the labeled sugars as high as possible. We also wanted to avoid having to test column eluates to locate the desired sugars. Our aim was to be able to derivatize samples of as small as a few picomoles, pass the entire reaction mixture through the column, know where the desired oligomers would elute and then be able to use the entire purified sample for chromatography using fluorescence detection.

Elution behavior of labeled sugars

In initial experiments, 20-μl aliquots of the reac-

tion mixture were loaded onto 100-mg Extract-Clean columns whose only pretreatment had been extensive washing with water to remove any breakdown products of the beads. In this form (H^+), 2-aminopyridine-labeled neutral oligomers, short labeled acidic oligomers and free 2-aminopyridine all bound to the column. As 2-aminopyridine and labeled sugars have different pK_a values, we next tried to adjust the pH of the eluent to obtain differential adsorption. With the columns in the H^+ form, the eluate acidified during exchange of the 2-aminopyridine for H^+ ions. To avoid this acidification and promote differential adsorption, we converted the columns to the ammonium form. In addition, we substituted acetic acid for hydrochloric acid when adjusting the pH of the 2-aminopyridine derivatizing reagent. Samples were thus eluted from the columns in ammonium acetate, which is volatile, allowing concentration of the sample prior to chromatography, if necessary.

Labeling mixtures (20 μl) were diluted with 0.44 *M* acetic acid to 400 μl, passed through 100-mg Extract-Clean columns and rinsed with four 400-μl aliquots of water. Bound components were subsequently eluted with 1 *M* sodium acetate buffer (pH 5.2). Each 400 μl of eluate was tested for the presence of sugar, 2-aminopyridine (bound + free) and borate. Borate is formed in the reductive amination reaction by breakdown of the cyanoborohydride.

Fig. 1A shows the elution behavior of a series of malto-oligomers linked non-reductively to 2-aminopyridine. Oligomers containing five or more sugar residues gave satisfactory recoveries (85% or more), whereas shorter oligomers adsorbed more firmly to the column. By analogy with the behavior (late elution) of aromatic amino acids on polystyrene-based cation-exchange columns, we expect that 2-aminopyridine interacts with the Extract-Clean column matrix by a hydrophobic effect as well as by an ionic effect with the sulphonate ion-exchange groups. We suspect that the hydrophobic interaction with the 2-aminopyridine label is weakened by the hydrophilic nature of sugars. For the neutral sugar oligomers with more than four sugars, their hydrophilicity overcomes the adsorptive interaction of the 2-aminopyridine label with the column matrix. The labeled oligogalacturonides did not bind to the columns under these conditions (Fig. 1B). Sugar recoveries for the secondary amine derivatives were

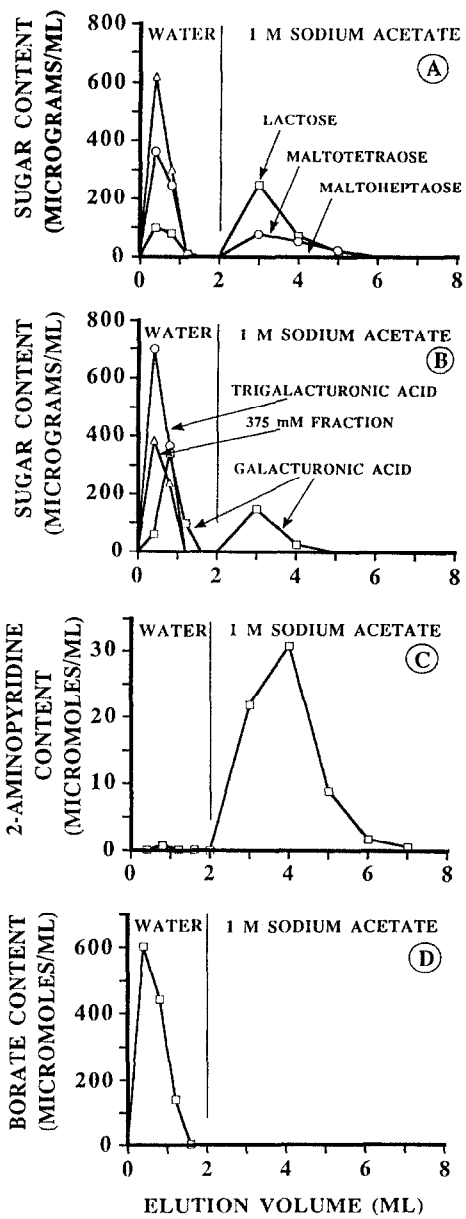

Fig. 1. Passage of various (A) neutral and (B) charged oligosaccharides, (C) 2-aminopyridine and (D) borate through Extract-Clean columns. Samples for A, B and C were prepared by non-reductive amination and sample for D was prepared by reductive amination. Sugar derivatives (20 μl) were diluted 20-fold with 0.44 *M* acetic acid, applied to 100-mg columns and eluted with 2 ml of water. Bound sample components were then eluted with 5 ml of 1 *M* sodium acetate buffer (pH 5.2). Fractions were analyzed for sugar, 2-aminopyridine and borate contents as described under Experimental.

generally lower than those for the corresponding glycosylamine derivatives. Almost all of the non-bound sugar eluted in the first 1.2 ml of eluate. Fig. 1C illustrates that essentially all of the 2-aminopyridine adsorbed to the column (at least 99%). However, none of the borate bound (Fig. 1D). As borate can interfere with chromatography by forming complexes with sugars, it may be necessary to remove borate by some other means. At least 70% of the borate can be removed by repeated evaporation with acidified methanol [15].

Degree of labeling of oligosaccharides with 2-aminopyridine

As non-labeled sugars would pass through the Extract-Clean columns without binding and thereby cause overestimation of the relative proportion of 2-aminopyridine-labeled sugar washing through the columns, we checked to see what proportion of the oligomers became labeled during the labeling reaction.

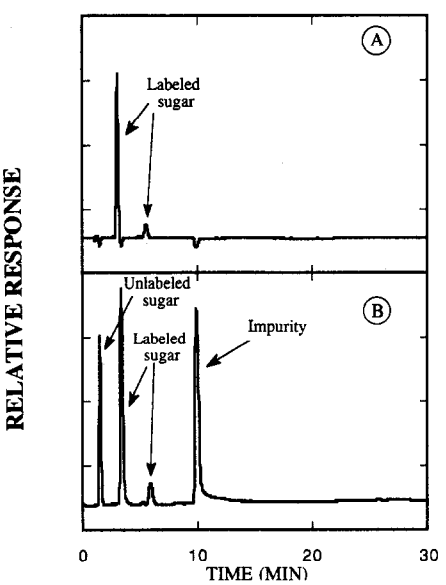

Fig. 2. Anion-exchange HPLC of labeled maltopentaose. Maltopentaose was derivatized by condensation, purified from the derivatization mixture with an Extract-Clean column and an aliquot (containing 9–10 nmol of oligosaccharide) was injected directly onto a Dionex PA-1 column (250 mm × 4 mm I.D.). The column effluent was monitored first (A) by UV absorbance at 290 nm and then (B) by pulsed amperometric detection. Non-labeled sugar eluted at about 2 min and labeled sugar at 3.5 and 6 min.

Non-reductively labeled maltopentaose was separated from the excess of 2-aminopyridine using Extract-Clean columns and aliquots were injected onto a PA-1 ion-exchange column. The effluent from the column passed first through a UV detector for detection of 2-aminopyridine and then through a pulsed amperometric electrochemical cell for detection of sugars. Fig. 2 shows the elution of labeled sugar in the UV trace (A) and the same plus non-labeled sugar in the electrochemical trace (B). By injection of the 2-aminopyridine reaction mixture alone, a peak eluting at 23 min was shown to be 2-aminopyridine. As the non-bound 2-aminopyridine was removed from the sample in Fig. 2 using an Extract-Clean column prior to injection, no free 2-aminopyridine peak was observed. The late elution of 2-aminopyridine from the polystyrene-based anion-exchange column supports our suggestion that 2-aminopyridine adsorbs to the Extract-Clean columns by more than just an ion-exchange interaction. Assuming a similar molar absorption coefficient for free and bound 2-aminopyridine and from a large number of independent injections, we can estimate that >95% of the 2-aminopyridine coming through the Extract-Clean column is bound to sugar. From the electrochemical trace (assuming that the molar responses are similar for labeled and non-labeled sugar), the peak area associated with the 2-aminopyridine sugar adduct represents 70–75% of the total peak area. We suspect that the actual proportion of sugar adduct to free sugar is greater than the 70–75% of peak area indicated. Preliminary evidence indicates that the 2-aminopyridine adduct of sugars exhibits a much lower electrochemical response than free sugars.

While investigating the degree of labeling for glycosylamine derivatives, we noticed that the degree of labeling of the purified oligomers decreased with time. After 1 day at room temperature these derivatives retained only *ca.* 75% of their original label. Hence it appeared that once the excess of 2-aminopyridine was removed, the glycosylamine came apart slowly. We investigated the lability of glycosylamines using maltopentaose and the pentagalacturonide at various pH values. As was pointed out by Her *et al.* [3], reversibility of the labeling could be useful if the oligosaccharide was needed in its original form after chromatography for further chemical characterization or testing of potential

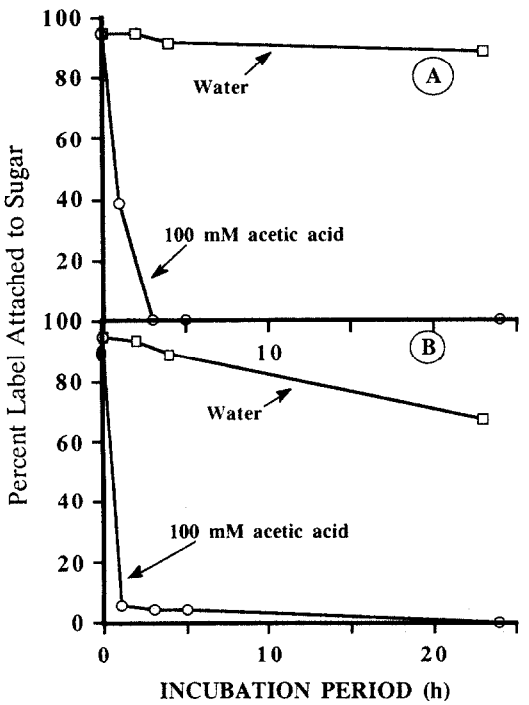

Fig. 3. Time course for hydrolysis of the glycosylamine derivatives of (A) maltopentaose and (B) trigalacturonic acid using 100 m*M* acetic acid at 80°C or water at room temperature. Samples were chromatographed as described in Fig. 2 and detected by UV absorbance at 290 nm. Values represent the percentage of 2-aminopyridine label remaining attached to sugar.

biological activity. Very mild acid hydrolysis was used to remove the 2-aminopyridine from the non-reductively labeled sugars. The time course for loss of label from both neutral and acidic oligomers after standing in the effluent from the Extract-Clean column (*ca.* pH 5) at room temperature, or after heating in 100 m*M* acetic acid at 80°C, is shown in Fig. 3. It is apparent that the neutral sugar glycosylamines are more stable than those of acidic oligomers, but that both types can be hydrolyzed rapidly in 100 m*M* acetic acid at 80°C. In addition to investigating the conditions for removal of the 2-aminopyridine label from glycosylamines, we also evaluated the conditions for retention of the label. After testing various conditions for the stable storage of the derivatives, we found that if the samples are frozen, especially at slightly elevated pH (6–8), they are more stable and should be useful for quantitative analysis.

Examples of the use of 2-aminopyridine-labeled oligosaccharides after elution from Extract-Clean columns

To determine if the method is useful for minute samples, it was applied to the preparation of 100-pmol samples of oligogalacturonides. Samples of pentagalacturonic acid were non-reductively aminated using 10-μl reaction mixtures. After the reaction they were diluted to 200 μl with 0.44 *M* acetic acid and then passed through 100-mg Extract-Clean columns. Only the first 600 μl of effluent were collected and kept in an ice-bath until used. Aliquots of 100 μl were injected onto a TSK DEAE 2SW column, eluted as described previously [8] and monitored with a fluorescence detector. The peak corresponding to free 2-aminopyridine at the elution volume (not retained by this column) was on scale at the same sensitivity setting needed to see the *ca.* 16 pmol of pentagalacturonide, which gave a peak about one quarter of full scale. The signal-to-noise ratio was about 25:1 for the pentagalacturonide.

Larger samples of non-reductively aminated oligogalacturonides and malto-oligomers recovered from Extrsct-Clean columns could be used directly for mass spectrometry after concentration by freeze-drying. However, additional sample clean-up was necessary before the reductively aminated oligomers gave good mass spectra owing to co-elution of borate with labeled sugars. Direct fractionation by reversed-phase chromatography of reductively aminated fragments of xyloglucans after removal of excess of 2-aminopyridine using an Extract-Clean column was possible, and fractions from the effluent were used directly for mass spectral identification [16].

CONCLUSIONS

Cation-exchange Extract-Clean columns in the ammonium form can be used efficiently to remove unreacted 2-aminopyridine from labeled sugars. However, borate co-elutes with the labeled sugars, and neutral oligosaccharides of less than five residues do not elute quantitatively. A sufficient amount of the excess of 2-aminopyridine is removed to allow the use of any kind of chromatography even with high-sensitivity fluorescence detection.

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