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Comparison of reduction methods for gas chromatographic–mass spectrometric identification and quantitation of uronic acids in acidic polysaccharides

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Summary

Four different reduction procedures were evaluated for their efficiency in dideuterating uronic acids in acidic polysaccharides. A method using 8 M urea to dissolve the polymers and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) to form uronide esters provided the best uronide dideuteration and uronic acid and neutral hexose sugar recoveries. Of the total uronic acid detected by a colorimetric assay, 20–23% was recovered as specific uronic acids from low-viscosity biopolymers by gas chromatography–mass spectrometry when this reduction procedure was employed. Lower and more variable recoveries of uronic acids were obtained from high-viscosity polysaccharides. The technique provided positive identification of the uronic acids in all five acidic polymers tested, even when the polymer also contained the corresponding neutral sugar analogue. Although quantitative recovery of uronic acids was not obtained, the technique nevertheless provides useful information on the relative contribution of uronic acids and neutral sugars in unpurified low-viscosity polymer preparations.

Key words: Acidic polysaccharide; Reduction; Uronic acid

Introduction

In a review of the literature, Fazio et al. [1] concluded that, among microorganisms, uronic acids (UA) are almost exclusively associated with exopolysaccharide material. UA have been reported as components of bacterial capsules [2–4] and as components of slime polymers released into the surrounding environment by some bacterial cells cultured under laboratory conditions [5–7]. Occasionally, UA are found in lipopoly-

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saccharides associated with the outer membrane of Gram-negative bacteria [8, 9]. UA also occur in eucaryotes as components of plant cell walls [10, 11].

The charged carboxylic acid residues of UA are a primary site of metal interactions with bacterial exopolymers. Complexes between the UA in exopolymers of *Pseudomonas aeruginosa* and Cu, Mo, Mg and Ba have been reported by Stojkovsky et al. [12]. Many other reported interactions between metals and bacterial exopolymers may also involve the participation of UA residues [13–18]. A better understanding of the role bacterial exopolymers play in the complexation of metals in aquatic environments could be achieved by assessing the types of uronic acids that occur in these capsular polymers. It would be useful, for example, to identify a method that could lead to identification and quantitative recovery of uronic acids from unpurified polysaccharides.

Identification of UA in polysaccharides requires hydrolysis of the polymer into its monomeric units. Quantitative recovery of UA from polysaccharides is hindered by the resistance of the glycosidic bond between uronic acids and other subunits to acid hydrolysis. Incomplete hydrolysis prevents efficient recovery of the monomeric units. In addition, some hydrolysis conditions lead to the conversion of UA to lactones which are difficult to identify. These problems can be minimized if the uronide residues are reduced to their neutral sugar analogue before acid hydrolysis. Reduction is achieved by first forming an ester with the carboxylic acid group on the UA, then adding a reductant such as sodium borohydride to yield the corresponding alcohol.

Different protocols have been presented to achieve UA reduction. Fazio et al. [1] formed a methyl ester while Taylor and Conrad [19], Anderson and Stone [20] and York et al. [11] used water-soluble carbodiimides for ester formation. Different solvents have also been used to solubilize and buffer the polysaccharides during reduction.

Identification of UA derived from biopolymers has been achieved by a number of different techniques. UA can be separated and detected by liquid chromatography (LC) [21–23] but conventional LC detectors (UV, RI and fluorescence) can not differentiate between UA that have been reduced prior to hydrolysis and their corresponding neutral sugars which may also be present in the polysaccharide.

In preliminary studies, we found that the reduction method of Fazio et al. [1] yielded varying UA recovery efficiencies for different polysaccharides when evaluated by gas chromatography–mass spectrometry (GC–MS). It was of interest, therefore, to determine whether other procedures yielded more consistent and higher recovery efficiencies. In this report, we compare sugar recovery efficiency from several different UA-containing polysaccharides using different reduction and hydrolysis techniques.

Materials and Methods

Materials

Reagent grade solvents and acids as well as dialysis membranes were obtained from Baxter Health Care, Los Angeles, California. Colorimetric assay reagents, derivitizing reagents, sugar standards, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) and polygalacturonic acid were purchased from Sigma Chemical, St Louis, Missouri. Commercial grade xanthan gum was acquired from Kelco, San Diego, California.

Commercial grade alginic acid was kindly provided by H. Ceri, University of Calgary, Calgary, Alberta. Sodium borodeuteride (98 atom % D_2) and deuterium oxide (99.8 atom % D_2) were purchased from Aldrich Chemical, Milwaukee, Wisconsin.

Bacterial exopolymers

Two variants of *Pseudomonas atlantica* strain T_6C (T_6C-1, T_6C-2) were cultured with agitation at 25 °C in 2 l broth medium containing 2.5% (wt/vol) Rila salts (Rila Products, Teaneck, New Jersey), 0.5% (wt/vol) proteose peptone (Difco) and 1.0% (wt/vol) galactose, pH 7.5. Stationary phase cultures were harvested by centrifugation (16000 × *g*) and the extracellular polymers were recovered from the culture menstruum by alcohol precipitation (3:1 vol/vol isopropyl alcohol:culture menstruum). The precipitated polymers were redissolved in double-distilled H_2O (dd-H_2O), dialyzed against dd-H_2O overnight at 4 °C using dialysis tubing (M_r cutoff, 12000) and then lyophilized to dryness.

Reduction of uronic acid in polymers

Four different procedures were employed to reduce the uronide residues present in the exopolymer samples.

Method 1 followed the procedure described by Fazio et al. [1]: 10–15 mg polymer were dissolved in 1–2 ml methanolic HCl (10 ml reagent grade methanol, 1 ml chloroform and 1 ml concentrated HCl) and stirred overnight at room temperature. The solution was brought to a pH of 5–6 with a 1 M sodium bicarbonate solution, dialyzed against dd-H_2O at room temperature overnight using dialysis tubing (2000 M_r cutoff) and lyophilized. The dry material was then reduced with 1–2 ml sodium borodeuteride solution (0.5 $mg \cdot ml^{-1}$ dd-H_2O), with stirring at 4 °C overnight. The reduced polymer solution was brought to a pH of 5–6 with 10% acetic acid, dialyzed overnight against dd-H_2O and lyophilized until dry.

Method 2 followed the procedure of Taylor and Conrad [19]. In this method, 10–15 mg polymer were dissolved in 10 ml dd-H_2O. 1 mmol solid EDC was added to the solution for every microequivalent UA in the sample (as determined by the colorimetric assay). The pH of the reaction was maintained at 4.75 by adding 0.1 or 0.5 N HCl to the reaction solution. The reaction was allowed to proceed for 2 h until H^+ ion uptake had ceased. Sodium borodeuteride (15–25 ml 2 M solution) was slowly added over a 30-min period to reduce the carbodiimide-activated UA while the pH was maintained at 7.0 with 0.5 N HCl. After a 60-min reduction period, the solution was transferred to dialysis tubing and dialyzed against dd-H_2O at 4 °C for 24 h and then lyophilized.

Method 3 followed a procedure described by Anderson and Stone [20]. Polymer (10–15 mg) was dissolved in 3–5 ml dd-H_2O and mixed with an equal volume 0.05-M pyridine-pyridinium chloride buffer, pH 4.75. Solid EDC was added directly to the reaction mixture (95 μmol $EDC \cdot \mu Eq^{-1}$ UA) while the pH was maintained at 4.75 with 0.1 N HCl. After stirring the solution for 1 h, the pH was increased to 7.0 with 3 M Tris buffer and sodium borodeuteride (2 M), prepared in 0.05 M NaOH, was slowly added to the reaction mixture to achieve a final reductant concentration of 15 $mg \cdot \mu Eq^{-1}$ UA. The pH was maintained at 7.0 with 2 N HCl during $NaBD_4$ addition. A few drops of octanol were added to prevent excessive foaming. After 2 h, the pH

of the solution was adjusted to 5–6 with 1 M $NaHCO_3$, dialyzed against dd-H_2O at 4 °C overnight and then lyophilized to dryness.

Method 4 followed the procedure of York et al. [11]. Polymer (10–15 mg) was dissolved in 2 ml 8 M urea, prepared with D_2O and the pH adjusted to 4.75 using 0.025 M HCl (prepared in D_2O). Solid CMC was slowly added to the solution to achieve a CMC: polymer ratio of 10: 1 (wt: wt). The solution pH was maintained at 4.75 with 0.025 M HCl. 1 h after completing the addition of CMC, the pH was adjusted to 7.0 with 2 M HCl and $NaBD_4$ (250 mg $NaBD_4$ in 3.0 ml D_2O) was added over a 30-min period using a peristaltic pump at a rate of 0.1 $ml \cdot min^{-1}$. During addition of reductant, the pH was maintained at 7.0 using 1, 2 or 5 M HCl as required (all solutions prepared in D_2O). 10–25 μl *n*-octanol were added during the reductant addition to control excessive foaming. The reaction was allowed to continue for 30 min after the $NaBD_4$ delivery was completed. The solution pH was reduced to 5–6 with 2 M HCl, dialyzed against dd-H_2O at 4 °C for 24 h and lyophilized to dryness. The entire procedure was then repeated a second time. Traces of boric acid were removed from the dried polymer by dissolving in 0.5 ml water, adding 0.5 ml acetic acid–methanol solution (1 : 9) and evaporating the methyl borate esters under a stream of N. After repeating the boric acid extraction three times, the residue was extracted three times in 1 ml methanol.

Modifications to Method 4

Several modifications of Method 4 were tested using exopolymer obtained from *P. atlantica* T_6C-2. In Method 4b, the polymer was reduced only one time. In Method 4c, a single reduction was performed using 800 mg $NaBD_4 \cdot 10-15\ mg^{-1}$ polymer. In Method 4d, two reductions were performed using 800 mg $NaBD_4 \cdot 10-15\ mg^{-1}$ polymer. In Method 4e, water was substituted for D_2O to make up the urea, carbodiimide and sodium borodeuteride solutions.

Hydrolysis of reduced polysaccharides

Several acids were tested to determine which provided the highest recovery of hexoses (neutral sugars and uronic acids) from exopolymer of *P. atlantica* T_6C-1. Exopolymer reduced by Method 1 was hydrolyzed with either 3 N trifluoroacetic, 2 N sulfuric acid or 2 N hydrochloric acid for 2 h at 100 °C. Further tests were performed to determine which concentration of HCl and times of hydrolysis yielded the highest recoveries of uronic acids from alginic acid and polygalacturonic acid preparations reduced by Method 4. All preparations of reduced polysaccharide were dissolved in 1–2 ml of the appropriate concentration of acid. Studies that compared different methods of polysaccharide reduction hydrolyzed the reduced polymers using 2 N HCl for 2 h at 100 °C. After hydrolysis, the solutions were neutralized to a pH of 5–6 with 1 M $NaHCO_3$ and then lyophilized to dryness.

Derivatization of sugar subunits

The monomeric sugars were extracted three times with 3 ml methanol at 60 °C. All three fractions were combined and the methanol was evaporated under a stream of N at 40 °C. An internal standard of 100 μl 20 mM solution of myo-inositol (2 μmol) was added to each hydrolyzed sample. Each sample was treated first with 1 ml hy-

droxylamine hydrochloride solution (150 mg · 10 ml^{-1} pyridine) at 60 °C for 1 h, then with 1 ml acetic anhydride at 60 °C for an additional hour. The reaction was terminated by the addition of 3 ml chloroform. The sample was extracted three times with 3 ml 15% tartaric acid solution to remove unreacted reagents. The chloroform phase containing the peracetylated aldononitrile sugar derivatives was refrigerated overnight at 4 °C to allow separation of residual water, then transferred to a clean test tube and evaporated under N. The derivatives were resuspended in 1 ml chloroform.

Preparation of sugar standards

Pentose and hexose standards were prepared as 20 mM solutions and derivatized as described above.

Gas chromatography

Gas chromatography was performed with a Varian model 3700 gas chromatograph with a flame-ionization detector (GC – FID) and Varian CDS 111 data system. Samples (1 μl) were injected into a polar 30-m fused silica capillary column, 0.25 mm id (SP-2330, Supelco, Bellefonte, Pennsylvania), using splitless injection. The temperature was programmed to rise from 160 to 210 °C at 2 °C · min^{-1} after which an isothermal period was held for 10 min, followed by a temperature ramp to 225 °C at a rate of 5 °C · min^{-1} and this temperature held for 3 min. H was used as the carrier gas at a flow rate of 30 cc · min^{-1}. The injection port and the detector were held at 250 °C.

Gas chromatography – mass spectrometry

Gas chromatography – mass spectrometry (GC – MS) was performed with a Hewlett-Packard 5890 gas chromatograph and 5970 mass selective detector (MSD). Temperature programs, MSD parameters and data analysis were controlled with a Hewlett-Packard 59970 MS chemstation. Samples (1 μl) were injected into a polar 30-m fused silica capillary column (DB225 – 30N, J & W Scientific, Rancho Cordova, California), with splitless injection and a 0.75-min venting time. The temperature was set to rise from 160 to 210 °C at 2 °C · min^{-1}, followed by an 18-min isothermal period, then the temperature was raised to 225 °C at 5 °C · min^{-1} and held at this temperature for 4 min. The He carrier gas was operated at a head pressure of 5 lb · in^{-2} and a column flow rate of 30 cm · s^{-1}. The injection port was held at 250 °C and the detector at 280 °C. The mass spectrometer was autotuned with perfluorotributilamine (PFTBA), the electron multiplier voltage was 2000 V. The instrument was used in the selective ion monitoring (SIM) mode to attain the highest sensitivity. Only the abundances of four ions (M/Z 115, 117, 145, and 147) were saved. Data acquisition was carried out at 2.0 cycles · s^{-1} and a dwell time of 100 ms · ion^{-1}.

Colorimetric assays

Neutral hexose content of the exopolymer preparations was determined following the phenol – sulfuric acid procedure of Dubois et al. [24]. Total UA content was measured using the meta-hydroxydiphenyl reagent of Blumenkrantz & Asboe-Hansen [25].

Determination of uronic acid and neutral hexose recovery efficiencies

Sugar standards were used to calculate the response factors (RF) for pentoses and

hexoses (RF = area inositol·area^{-1} sugar) using GC–FID. The concentrations of individual hexoses ($\mu g \cdot mg^{-1}$ polymer) in the samples were calculated as follows: (area sugar·area^{-1} inositol) × RF sugar × mol internal standard added × M_r sugar (μg) × $1 \cdot mg^{-1}$ polymer reduced. All these areas were obtained with GC–FID. The contribution of UA to each hexose peak was obtained from the ion abundance ratio $M/Z_{147}:(M/Z_{145} + M/Z_{147})$ based on GC–MS analysis of the derivatized polymer hydrolysis products (a percentage unit was substracted to correct for the natural ^{13}C contribution to M/Z_{147}). The concentration of each UA was obtained by multiplying the ratio obtained above by the total concentration of the corresponding sugar as determined by GC–FID. The neutral hexose sugar concentration was taken as the difference between the total concentration of a particular sugar and the portion of the total concentration contributed by the corresponding UA. Uronic acid recovery efficiency was obtained by dividing the sum of all the UA concentrations based on GC/MS analysis by the UA concentration obtained by the UA colorimetric assay. Total hexose recovery efficiency was obtained by dividing the sum of all neutral hexose concentrations (GC–FID analysis) by the hexose sugar concentration obtained by the phenol–sulfuric acid colorimetric assay.

Justification for using concentrations obtained from colorimetric assays as a basis for determining neutral hexose and UA recoveries

Since commercial preparations of alginic acid, polygalacturonic acid and xantham gum were not 100% pure, neutral sugar and UA recovery efficiency could not be expressed on a dry weight basis. Neutral hexose recoveries were based on the color reaction of the phenol sulfuric acid assay after correction for interference by UA and pentose sugars (11% of the total UA present and 64% of the total pentose sugars present contribute to the neutral hexose color reaction). No correction factors were needed for the uronic acid assay employing the meta-hydroxydiphenyl reagent since only 1% of the total neutral hexose sugar present was found to contribute to the uronic acid color reaction. Nevertheless, samples were first assayed for hexose sugars to make sure that hexose sugar concentrations did not exceed 200 $\mu g \cdot ml^{-1}$ before uronic acids were assayed. No interference was observed in the uronic acid assay when performed in the presence of DNA at concentrations as high as 300 $\mu g \cdot ml^{-1}$ or protein (as bovine serum albumin) at concentrations as high as 500 $\mu g \cdot ml^{-1}$. Humic acids which possess carboxylated ring structures like UA did not interfere with the UA color reaction when present at concentrations as high as 100 $\mu g \cdot ml^{-1}$. While the use of these colorimetric assays for neutral hexoses and UA may not provide absolute concentrations of these components in the polymer preparations, they do provide a useful basis for evaluating recovery efficiencies of these classes of sugars using GC–MS when working with unpurified polymer preparations.

Results

Of the three acids tested, hydrochloric acid yielded the highest average recoveries of hexose sugars (neutral + acidic) from *P. atlantica* exopolymer (Table 1). Recovery of galacturonic acid from polygalacturonic acid was highest when the reduced polymer was hydrolyzed with 0.25 N HCl for 5 h or 2 N HCl for 2 h (Table 2). Recovery of

TABLE 1

EFFECTS OF DIFFERENT HYDROLYSIS CONDITIONS ON RECOVERY OF HEXOSE SUGARS FROM *P. ATLANTICA* T_6C-1 EXOPOLYMER

Hexose	3 N Trifluoroacetic acid (under N)	2 N Hydrochloric acid (in air)	2 N Sulfuric acid (under N)	2 N Sulfuric acid (in air)
Mannose	156 ± 5[b]	223 ± 12	240 ± 12	185 ± 24
Glucose	605 ± 22	737 ± 14	468 ± 26	661 ± 17
Galactose	388 ± 32	476 ± 18	323 ± 29	393 ± 9

[a] nmol neutral + acidic sugars (as determined by GC – FID)·mg^{-1} dry wt exopolymer. Reduction and derivatization protocol followed that of Fazio et al. (1982).
[b] Mean value of three determinations ± 1 SD.

mannuronic acid from alginic acid was highest when the reduced polymer was hydrolyzed with 1 N HCl for 5 h whereas guluronic acid recovery was highest at 0.25 N HCl for 5 h. The highest combined recovery of mannuronic and guluronic acids from alginic acid was obtained with the same hydrolysis conditions that yielded the highest mannuronic acid recoveries. Nevertheless, the recovery efficiency for UA obtained as a result of alginic acid hydrolysis with 1 N HCl for 5 h (22.6%) was only slightly higher than that obtained when the polymer was hydrolyzed with 2 N HCl for 2 h (20.2%). The latter hydrolysis conditions where therefore used to compare the efficiency of uronic acid recovery from polysaccharides using different reduction procedures.

Recovery efficiency of UA from five different acidic polysaccharides of microbial and plant origin by GC – MS was <25% of that based on the colorimetric method using metahydroxydiphenyl reagent. A comparison of four different reduction proce-

TABLE 2

EFFECTS OF DIFFERENT HYDROLYSIS CONDITIONS ON RECOVERY OF URONIC ACIDS FROM ALGINIC ACID AND POLYGALACTURONIC ACID[a]

Conditions	Alginic acid			Polygalacturonic acid
	Mannuronic acid	Guluronic acid	Total	Galacturonic acid
0.25 N HCl				
5 h	514	144	658	725
10 h	588	132	720	334
1.0 N HCl				
5 h	710	128	838	662
10 h	452	39	491	407
2.0 N HCl				
2 h	649	90	739	727

[a] nmol uronic acid (as determined by GC – MS)·mg^{-1} dry wt polymer.

TABLE 3

EFFICIENCY OF URONIC ACID RECOVERY (%)[a]

Sample	Method of reduction			
	1	2	3	4
Alginic acid	< 1	9	13	20
Polygalacturonic acid	< 1	ND	ND	23
P. atlantica T_6C-1 exopolymer	< 1	6	< 1	21
P. atlantica T_6C-2 exopolymer	ND	ND	ND	13
Xanthan gum	< 1	< 1	< 1	5

[a] $\frac{\text{Conc. UA (GC-FID, GC-MS)}}{\text{Conc. UA (colorimetric assay)}} \times 100.$

ND, not determined.

dures used in the GC–MS analysis of UA revealed that Method 1 yielded the lowest (1%) UA recoveries from four different polysaccharide preparations while Method 4 provided the highest (5–23%) recoveries (Table 3). The lowest recoveries occurred with *P. atlantica* T_6C-2 exopolymer and xanthan gum both of which exhibited high viscosities.

Dideuteration efficiency was found to vary depending on the type of UA present when either Methods 1 or 3 were used. Mannuronic acid was dideuterated more efficiently than guluronic acid when alginic acid was reduced by these two methods. Methods 2 and 4, when applied to alginic acid, yielded dideuteration efficiencies that were the same (69%) for both polymer subunits. Method 4 yielded a similar (68%) dideuteration efficiency for galacturonic acid during reduction of polygalacturonic acid (Table 4). Guluronic and galacturonic acid were dideuterated at low efficiency when alginic acid and polygalacturonic acid, respectively, were reduced using Method 1. Of the four methods evaluated, Method 4 and possibly Method 2 appeared to be most efficient in reducing UA in the polymers that were tested.

TABLE 4

EFFICIENCY OF DIDEUTERATION (%)[a]

Sample	Method of reduction			
	1	2	3	4
Alginic acid				
Mannuronic acid	59	69	68	69
Guluronic acid	8	69	25	69
Polygalacturonic acid	11	ND	ND	68

[a] $\frac{M/Z_{147}}{M/Z_{145} + M/Z_{147}} \times 100.$

ND, not determined.

TABLE 5

FRACTION OF HEXOSE SUGAR CONTRIBUTED BY URONIC ACID (%)[a]

Sample	Method of reduction			
	1	2	3	4
P. atlantica T_6C-1				
Mannose[b]	<5	<5	<5	<5
Glucose	<5	7	<5	19
Galactose	<5	6	<5	9
P. atlantica T_6C-2				
Mannose[b]	ND	ND	ND	<5
Glucose[b]	ND	ND	ND	<5
Galactose	ND	ND	ND	25
Xanthan gum				
Mannose[b]	<5	<5	<5	<5
Glucose	<5	<5	<5	<5

[a] $\frac{M/Z_{147}}{M/Z_{145} + M/Z_{147}} \times 100.$

[b] No corresponding uronic acid present.

ND, not determined.

The four reduction methods were compared for their ability to dideuterate specific UA in polysaccharides that also contained the corresponding neutral sugar. Reduction of *P. atlantica* T_6C-1 exopolymer by Method 4 yielded a higher percentage of glucuronic and galacturonic acid relative to glucose and galactose than the other three methods (Table 5). None of the reduction methods tested provided good recoveries of glucuronic acid from xanthan gum. Recovery of neutral sugars from the acidic polysaccharides (xanthan gum and exopolymer from *P. atlantica* T_6C-1) varied widely depending on the reduction method employed. Of the methods evaluated, Method 4 provided the highest recoveries for both polysaccharide preparations (Table 6).

The extent of polymer subunit degradation varied depending on the reduction

TABLE 6

TOTAL NEUTRAL SUGAR RECOVERY (%)[a]

Sample	Method of reduction			
	1	2	3	4
Xanthan gum	43	ND	16	38
P. atlantica T_6C-1 exopolymer	42	8	13	77
P. atlantica T_6C-2 exopolymer	ND	ND	ND	58

[a] $\frac{\Sigma \text{ hexoses (GC-FID)}}{\Sigma \text{ hexoses (colorimetric assay)}} \times 100.$

ND, not determined.

procedure used. The combined area of unidentified peaks as well as those corresponding to pentose sugars and lactones were compared to the combined area of hexose sugars (including hexuronic acids) in chromatograms of alginic acid and polygalacturonic acid to evaluate polymer subunit destruction. Only 56–57% of the UAs in these polyuronides were recovered as hexose sugars when Method 1 was used (Table 7). The detection of significant quantities of pentose sugars suggested that decarboxylation of the UAs occurred during treatment of these polymers by this reduction procedure. Methods 3 and 4 yielded the least amount of subunit destruction in these polymers: 80–86% of the total peak area was assigned to peaks corresponding to hexose sugars. Minimal hexose destruction occurred when Method 1 was applied to *P. atlantica* T_6C exopolymer and xanthan gum. All reduction methods except Method 2 yielded chromatograms of *P. atlantica* exopolymer and xanthan gum in which 82–89% of their total peak areas corresponded to hexose sugars (Table 7).

In view of the evidence indicating that Method 4 generally provided the highest recoveries of neutral and acidic sugars and minimal polymer subunit degradation of the four reduction procedures tested, a series of studies were conducted to determine whether additional improvements to this method could be made that would further increase subunit recovery. When the reduction step was carried out only once instead of two times on exopolymer from *P. atlantica* T_6C-2, no significant improvement in uronic acid or neutral hexose recovery was achieved and dideuteration efficiency decreased from 25 to 21%. This 4% decrease was not significant since dideuteration efficiency commonly varies from 2–3% among sample replicates. Colorimetric UA assays on polygalacturonic acid reduced once and two times indicated that 17 and 15%, respectively, of the UA in the polyuronide remained unreduced after reaction with $NaBD_4$. A similar comparison was attempted with *P. atlantica* T_6C-2 exopolymer reduced one or two times. However, an intense color reaction, not representative of UA and not observed with the unreduced polymer, prevented the determination of the UA content in the $NaBD_4$-treated polymer. Thus, on the basis of the results obtained from polygalacturonic acid, there appears to be little benefit obtained from repeating

TABLE 7

PERCENTAGE OF TOTAL AREA FROM ALL PEAKS OF CHROMATOGRAM CONTRIBUTED BY HEXOSE SUGARS (INCLUDING HEXURONIC ACIDS)[a]

	Method of reduction			
	1	2	3	4
Alginic acid	57	70	85	80
Polygalacturonic acid	56	ND	ND	86
P. atlantica T_6C-1 exopolymer	89	50	82	89
P. atlantica T_6C-2 exopolymer	ND	ND	ND	82
Xanthan gum	96	ND	81	94

[a] $\frac{\text{Total hexose current}}{\text{Total ion current}} \times 100.$

ND, not determined.

the reduction step in Method 4. In fact, there was a loss of accountable material following each reduction step. In the case of exopolymer from *P. atlantica* T_6C-2 and polygalacturonic acid, 18 and 13% of the initial weight of the respective polymer was lost after each reduction cycle.

Increasing the concentration of reductant (sodium borodeuteride) from 250 to 800 mg·10–15 mg^{-1} dry weight polymer had little effect on neutral and acidic sugar recovery when the reduction step was carried out only one time. However, repeated reduction with the higher concentration of sodium borodeuteride reduced the recovery efficiency of the neutral sugars and UA to 30 and 7%, respectively.

Substitution of H_2O for D_2O for preparation of the 8 M urea and HCl solutions to dissolve the biopolymers and control the pH of the reaction during esterification and reduction, respectively, resulted in no significant difference in dideuteration efficiency or UA recovery efficiency in alginic acid.

Discussion

The results of this study show that the efficiency of recovery of total hexoses (neutral sugars and uronic acids) from polysaccharides varies with different reduction procedures. Reduction Method 4 provided the highest recoveries of UA and neutral sugars from the biopolymers that were tested. The main differences between Method 4 and those of Methods 2 and 3 were: (1) that the former used 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) whereas the latter two methods used 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to form a carboxylic acid ester prior to reduction; and (2) in Method 4, the polymers were suspended in 8 M urea rather than water or buffer during esterification and reduction. Anderson and Stone [20] found that esterification with CMC resulted in the reduction of ≈1.5 times more carboxyl groups than with EDC. The use of urea likely promote greater polymer solubility during the esterification and reduction steps. Use of a solvent that permits maximum solubility of the biopolymer during esterification and reduction appears to optimize subunit dideuteration (in the case of UA) and recovery of the neutral and acidic sugar subunits. The low UA recoveries and poor efficiency of dideuteration in polymers treated by Method 1 were, in part, due to low solubility of the polymers in methanolic HCl.

Method 3 also produced a large unidentified peak which eluted between mannose and glucose (mass spectra of this peak clearly showed that it is not related to carbohydrates). Since this unknown compound has a retention time similar to talose and altrose, it could mask their detection and quantitation.

Other investigators have reported efficiencies of UA recovery from other biopolymers using methods similar to those tested in this study that are substantially higher than the recovery efficiencies we obtained with Method 4. Karamanos et al. [26] reported 90–95% efficiency of UA recovery from the glycoaminoglycans, dermatan sulfate, heparin sulfate and chondroitin sulfate. Unfortunately, determination of recovery efficiency in their study was not presented in sufficient detail to permit an examination and explanation of what accounted for the differences in our results.

The reasonable efficiency of dideuteration of UA in alginic acid and polygalacturonic acid achieved by Method 4 suggests that the low UA recoveries were not due

to inefficient reduction. The colorimetric assay for UA provided comparable results when performed on once-reduced and twice-reduced polygalacturonic acids and indicated that only 15 – 17% of the subunits are inaccessible to the esterification reagent and/or reductant.

The low (21 – 25%) dideuteration efficiency of *P. atlantica* T_6C-2 exopolymer was at least partially due to the rheological properties of this polymer. The high viscosities created by both *P. atlantica* T_6C-2 exopolymer and xanthan gum resulted in poor mixing and diffusion of the reagents in the solution. This was evident by the delays between the time of addition of the $NaBD_4$ or the HCl solutions and the time of detection of a pH change in the solution, even when the solution was stirred vigorously. This delay in pH meter response was also observed during the addition of the carbodiimide reagent. Furthermore, viscous samples always produced excessive foaming which could not be controlled with the addition of *n*-octanol. The persistent foam also appeared to affect the distribution of the reductant and HCl solutions. Since most bacterial exopolymers do not produce the high viscosities displayed by *P. atlantica* T_6C-2 exopolymer and xanthan gum, low efficiency of dideuteration is not anticipated to be a common problem when Method 4 is used for polymer reduction.

The low UA recovery efficiencies obtained in this study may have been due to incomplete hydrolysis of the reduced polymers. This is supported by an evaluation of the hydrolysis products from *P. atlantica* T_6C-2 exopolymer by HPLC. Chromatograms revealed the presence of incompletely-hydrolyzed oligosaccharides as well as the monosaccharide subunits (data not shown). Although different acids, acid concentrations and hydrolysis times yielded different subunit recoveries from the various polymers, the differences resulted in UA and neutral sugar recovery efficiencies that varied by only a few percent. These results suggest that the use of one set of hydrolysis conditions which provides maximum recovery of UA and neutral sugars from one type of polysaccharide (such as 2 N HCl for 2 h at 100 °C in the case of polygalacturonic acid) will provide recovery efficiencies for subunits from other polymers that are within 5% of the values obtained under the optimal hydrolysis conditions for these polymers.

Although none of the reduction and hydrolysis methods used in this study for GC – MS identification of polymeric UA and neutral sugars provided high recovery efficiencies for these polymeric subunits, Reduction Method 4, when used in conjunction with hydrolysis conditions of 2 N HCl for 2 h at 100 °C, yielded comparable (20 – 23%) UA recovery efficiencies from three of the five polysaccharides tested. Only those polysaccharides with high viscosities yielded recovery efficiencies that were significantly lower. It should be possible, therefore, to use this procedure to identify polymeric forms of specific neutral and acidic sugars and, after correcting for 20% recovery efficiency, obtain an estimation of their relative abundance in a sample. While this estimation is only likely to be accurate to within an order of magnitude of the true sugar subunit concentration, it provides a means to obtain a general understanding of the abundance of polymeric forms of specific uronic acids in samples that have not been purified.

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References

1 Fazio, S. A., Uhlinger, D. J., Parker, J. H. and White, D. C. (1982) Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. Appl. Environ. Microbiol. 43, 1151–1159.
2 Smith, E. J. (1968) Purification and properties of an acidic polysaccharide isolated from *Achromobacter georgiopolitanum*. J. Biol. Chem. 243, 5139–5144.
3 Sutherland, I. W. (1980) Polysaccharides in the adhesion of marine and freshwater bacteria. In: Microbial Adhesion to Surfaces (Berkeley R. C. W., Lynch, J. M., Melling, J., Rutter, P. R. and Vincent, B., eds.), pp. 329–338, Ellis Horwood, London.
4 Costerton, J. W. and Irvin, R. T. (1981) The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. 35, 299–324.
5 Hacking, A. J., Taylor, I. W. F., Jarman, T. R. and Govan, J. R. W. (1983) Alginate biosynthesis by *Pseudomonas mendocina*. J. Gen. Microbiol. 129, 3473–3480.
6 Nagahama, T., Suganuma, T., Ichikawa, N., Fujimoto, S. and Sato, H. (1985) Determination of uronic acid in the polysaccharides from the slime-producing coryneform bacteria and their immunological study. Mem. Fac. Agr. Kagoshima Univ. 21, 157–165.
7 Hejzlar, J. and Chudoba, J. (1986) Microbial polymers in the aquatic environment. Water Res. 20, 1209–1216.
8 Ray, T. C., Smith, A. R. W., Carter, K. J. and Hignett, R. C. (1986) Composition of lipopolysaccharides from four strains of *Erwinia amylovora*. J. Gen. Microbiol. 132, 3159–3167.
9 Puvanesarajah, V., Schell, F. M., Gerhold, D. and Stacey, G. (1987) Cell surface polysaccharides from *Bradyrhizobium japonicum* and a nonnodulating mutant. J. Bacteriol. 169, 137–141.
10 Jones, T. M. and Albersheim, P. (1972) A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. Plant Physiol. 49, 926–936.
11 York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T. and Albersheim, P. (1985) Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. 118, 3–40.
12 Stojkovski, S., Magee, R. J. and Liesegang, J. (1986) Molybdenum binding by *Pseudomonas aeruginosa*. Aust. J. Chem. 39, 1205–1212.
13 Bitton, G. and Freihofer, V. (1978) Influence of extracellular polysaccharides on the toxicity of copper and cadmium toward *Klebsiella aerogenes*. Microb. Ecol. 4, 119–125.
14 Brown, M. J. and Lester, J. N. (1979) Metal removal in activated sludge: the role of bacterial extracellular polymers. Water Res. 13, 817–837.
15 Ridgway, H. F., Means, E. G. and Olson, B. H. (1981) Iron bacteria in drinking water distribution systems: elemental analysis of *Gallionella* stalks, using X-ray energy-dispersive microanalysis. Appl. Environ. Microbiol. 41, 288–297.
16 Houba, C. and Remacle, J. (1984) Removal of cadmium by microorganisms in a two-stage chemostat. Appl. Environ. Microbiol. 47, 1158–1160.
17 Mittelman, M. W. and Geesey, G. G. (1985) Copper-binding characteristics of exopolymers from a freshwater sediment bacterium. Appl. Environ. Microbiol. 49, 846–851.
18 Cowen, J. P. and Bruland, J. W. (1985) Metal deposits associated with bacteria: implications for Fe and Mn marine biogeochemistry. Deep Sea Res. 32, 253–272.
19 Taylor, R. L. and Conrad, H. E. (1972) Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. Biochem. 11, 1383–1388.
20 Anderson, M. A. and Stone, B. A. (1985) A radiochemical approach to the determination of carboxylic acid groups in polysaccharides. Carbohydr. Polym. 5, 115–129.
21 Annison, G., Cheetam, N. W. H. and Couperwhite, I. (1983) Determination of the uronic acid composi-

tion of alginates by high-performance liquid chromatography. J. Chromatogr. 264, 137–143.

22 Hicks, K.B., Lim, P.C. and Haas, M.J. (1985) Analysis of uronic and aldonic acids, their lactones, and related compounds by high-performance liquid chromatography on cation-exchange resins. J. Chromatogr. 319, 159–171.

23 Kennedy, A.F.D. and Sutherland, I.W. (1987) Analysis of bacterial exopolysaccharides. Biotechnol. Appl. Biochem. 9, 12–19.

24 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 349–356.

25 Blumenkrantz, N. and Asboe-Hansen, G. (1973) New method for the quantitative determination of uronic acids. Anal. Biochem. 54, 484–489.

26 Karamanos, N.K., Hjerpe, A., Tsegenidis, T., Enfeldt, B. and Antonopoulos, C.A. (1988) Determination of iduronic acid and glucuronic acid in glycosaminoglycans after stoichiometric reduction and depolymerization using high-performance liquid chromatography and ultraviolet detection. Anal. Biochem. 172, 410–419.