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Hydrodynamic properties of oxidized extracellular polysaccharides from *Erwinia chrysanthemi* spp

Byung Yun Yang, Qiong Ding, Rex Montgomery*

Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, IA 52242, USA

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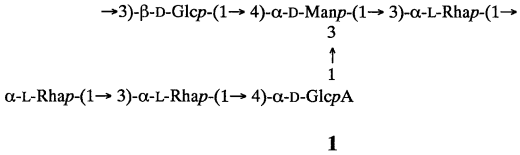
Abstract

The molecular weights of the native polysaccharides of *Erwinia chrysanthemi* strains range from 1.8 to 7.1×10^6 and their hydrodynamic properties are those of polydisperse, polyanionic biopolymers with pseudoplastic, non-thixotropic flow characteristics in aqueous solutions. The effect on the hydrodynamic properties of the polysaccharides by adding carboxyl groups to increase the charge density is studied, with particular reference to their molecular weight (MW), viscosity and conformation. In general, it is found that periodate oxidation of the extracellular polysaccharides of *E. chrysanthemi* strains, Ech9Sm6 and Ech6S+, introduces little change in the hydrodynamic properties of the resulting polyaldehydes. However, bromine oxidation at neutral pH of the polyaldehydes results in polycarboxylate biopolymers that show significant reduction in MW and viscosity, but they are still characteristic polyanions.

Keywords: *Erwinia chrysanthemi*; Polysaccharide; Periodate oxidation; Bromine oxidation; Hydrodynamics

1. Introduction

It has been clearly demonstrated from earlier studies of the extracellular polysaccharides (EPSs) from *Erwinia chrysanthemi* spp that the primary structure within each family is identical by the monosaccharide composition and 1D ^{1}H NMR spectrum analyses of the EPS and by the HPAEC-PAD analyses of the resulting oligosaccharides produced under the same condition of partial acid hydrolysis of each EPS family member.[1,2] The EPS9Sm6 and the EPS6S+ have the following defined structures **1** and **2**, respectively:



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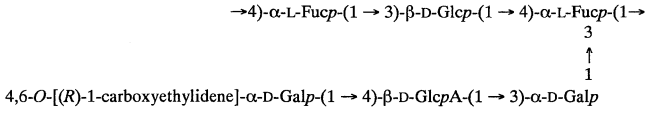

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Abbreviations: EPS, extracellular polysaccharide; RU, repeating unit; EPS **1**, EPS from Ech9Sm6; EPS **2**, EPS from Ech6S+; 1A1.6, polyaldehyde derivative of EPS **1** after 1.6 mol of periodate consumed per RU; 1A1.6/B, polycarboxyl derivative of 1A1.6 oxidiszed by bromine water; GLC, gas liquid chromatography; GLC-MS, gas chromatography with mass selective detector; HPAEC-PAD, high pH anion exchange chromatography with pulsed amperometric detection; LS, Light scattering; MWCO, molecular weight cutoff; SEC, size exclusive chromatography; dn/dc, specific refractive index increment; MW, weight average molecular weight; Mn, number average molecular weight; Rw, weight average root mean square radius; M, molecular weight; RMS, Root mean square radius; $[\eta]$, Intrinsic viscosity; $[\eta]_0$, viscosity at zero shear rate; I, Ionic strength; MALS, multi angle light scattering; RI, refractive index.

* Corresponding author. Tel.: +1-319-335-7897; fax: +1-319-335-9570.
E-mail address: rex-montgomery@uiowa.edu (R. Montgomery).

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The EPSs have the characteristics of pseudoplastic flow (shear-thinning) and of non-thixotropy, which are similar to xanthan.[1]

The molecular model of each EPS shows a systematic twist or helix in the backbone with the side chains branching from it.[1] Also, the side chains of adjacent repeating units (RU) would not overlay each other in the EPS9 family and the carboxyl groups would be well separated along the backbone chain. This is in contrast to the terminal pyruvate residue present in the side chain of the EPS6 family. It was proposed that these EPSs exist in solution as extended coil conformations that become less flexible with increase in the ionic strength of the solvent. Furthermore, the presence of the pyruvate unit would induce a stiffer conformation in the EPS6 family.[1]

It was of interest to examine the effects on these hydrodynamic properties by increasing the anionic character of the EPS, in particular to study the influence of the resulting polyanions on complex formation with calcium ions. In works with similar materials a number of metal chelating, water soluble or cross-linked polysaccharide derivatives from cellulose, dextran and starch have been applied to the recovery of transition and precious metals and to waste water treatment (for review, see Ref. 3). Also, oxidized maltodextrins with a degree of polymerization of ten or more were comparable for the complexation of calcium to those of tripolyphosphates and had potential applications as phosphate substitutes in detergent formulations.[4]

2. Experimental

2.1. Analytical and general methods

The methods used for gas liquid chromatography with flame ionization (GLC) or mass selective (GLC-MS) detection, and monosaccharide analysis by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) have been described previously.[2] The analyses of EPSs by light scattering (LS), and the determination of viscosity have been described elsewhere.[1]

2.2. Production of EPS

The *E. chrysanthemi* strains were either grown on a modified Scott's medium[5] supplemented with 1% glucose and solidified with 1.5% Difco agar (Difco Laboratories, Detroit, USA) or in a liquid culture medium[6] as described elsewhere. Crude polysaccharide in 4% NaCl solution was precipitated by the dropwise addition of EtOH (2 vol) and the polysaccharide was recovered by low-speed centrifugation. The precipitate was dissolved in distilled water, dialyzed extensively against distilled water and freeze dried. No further purification was applied.

2.3. Periodate oxidation of EPS

Each EPS (0.1 mmol of RU) was dissolved in 0.2 M NaOAc (50 mL, pH 4.5) and cooled in an ice-water bath. To this solution was slowly added with stirring 0.12, 0.24, 0.48 or 1.0 mmol $NaIO_4$ (50 mL) and kept at room temperature for 16 h. At intervals, an aliquot was analyzed by spectroscopy (260 nm) for the periodate consumption. Unreacted periodate was destroyed with ethylene glycol (2 mL, 2 h, rt). The resulting polyaldehyde was extensively dialysed (MWCO 6–8 K, Spectrum, CA, USA) against distilled water, and appropriately concentrated for further experiments using a centrifugal filter device (MWCO 50K, Millipore, MA, USA). For some experiments, the polyaldehyde was precipitated with EtOH (2 vol) and recovered by low-speed centrifugation. The least oxidized material, if dried, was only sparingly soluble in water and Me_2SO, and the more oxidized materials were not soluble in either solvent.

2.4. Bromine oxidation of polyaldehyde from 1 and 2 in water

The polyaldehyde solution ($\sim$ 50 mg of dry weight; 0.1–0.15 mmol of aldehyde as determined by periodate consumption) in water (30 mL containing 50 mmol $CaCO_3$) was stirred. A 3-fold mole excess (0.3–0.45 mmol) of aq bromine soln was slowly added with stirring and the resulting soln ($\sim$ 4 mM bromine) kept at room temperature for 16 h. The final soln was adjusted to pH 3.5 with glacial AcOH. The resulting clear soln was extensively dialyzed (MWCO 6–8 K) against distilled water. The polycarboxylate derivative was concentrated as described above, but with a smaller pore size centrifugal filter device (MWCO 3 K).

2.5. GLC and GLC-MS analysis of the oxidized material

The polycarboxylate derivative (100–150 μg) was hydrolyzed (200 μl of 2 M TFA; 120 °C;1 h) and reduced ($NaBD_4$). Excess of reducing agent was destroyed with AcOH and borate was removed as methyl borate by coevaporation with anhyd MeOH. Following removal of the derived AcONa by cation exchange chromatography (spin column of 250 μl, AG50W-X8, Bio-Rad), the resulting alditols were converted to trimethylsilyl ether or ester derivatives[7,8] and analyzed on a capillary column of DB5 (J&W, CA, USA) with splitless injection using temperature program: 60 °C (3 min) to 280 °C at 5 °C min^{-1}.

2.6. HPAEC-PAD analysis of the oxidized material

Acid hydrolyzates of the reduced polyaldehyde or the polycarboxyl derivative were prepared as described above and analyzed on an anion-exchange PA1 column[1] using a two-step isocratic elution: 20 mM NaOH for the first 16 min, then 20 mM NaOH in the presence of 200 mM NaOAc.

2.7. Size exclusion chromatography/light scattering (SEC/LS)

Because of the difficulty of redissolving dried samples of these derivatives in water, which is particularly true for the polyaldehydes, their preparations concluded with concentrated solutions, thus a portion of the resulting soln ($\sim$10 mg) was dried under diminished pressure (80 °C, 24 h) to determine the concentration of the derivative. To an aliquot of the concentrated EPS solution, now of known solute content, was added an equal vol of 0.3 M Na_2SO_4 containing 0.03 M EDTA, and the soln was dialyzed against 0.15 M Na_2SO_4 containing 0.015 M EDTA for 3 days. It was then filtered (0.45 μm; Millipore Co., Bedford, MA, USA) prior to LS measurements. The dn/dc determination of EPS and the SEC/LS measurement was carried out as previously reported.[1,9,10] The data from the LS photometers were processed together with the signal from the RI detector by Wyatt Technology's ASTRA V4.73.04 software. The software cuts the elution peak into around 1000 slices, then calculates the concentration of each slice by specific refractive index increment (dn/dc) combined with a detector constant of the RI detector. ASTRA takes each slice, which is considered as a single polysaccharide, calculates the RMS and M from a Zimm plot of the light scattering and the concentration data. From this, RMS is plotted against M. The RMS is a so-called z-average (the mean square radius of the molecule having a z-average molecular weight) from the Zimm plot that is different from the Rw, which is obtained as a weight average over all the slices of the peak obtained in the SEC/LS. The MW, Rw, polydispersity (MW/Mn) and the relation between RMS and M across the entire elution peak are reported.

The Flory–Fox theory was used to calculate the intrinsic viscosity from the RMS and MW data, using a phi value of 2.87×10^{23}.[11,12]

2.8. Determination of viscosity

For a qualitative comparison, the intrinsic viscosity [η] of each EPS soln in 0.5 M NaCl was measured at 25 °C using Ostwald capillary viscometers and determined by the Huggins and Kraemer plots.[13,14]

The viscosity of each EPS soln at different shear rates was determined at 25 °C with a plate-and-cone viscometer (DV-III V3.3 RV, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA). The viscosity at zero shear rate (η_0) was obtained by fitting of the Cross-equation.[15–21] The intrinsic viscosity was then calculated from the Huggins and Kraemer plots from a set of $[\eta]_0$ data at different concentrations that ranged from 0.2 to 2 mg mL^{-1}.

The effect of ionic strength (I) on the viscosity of the polysaccharide soln was determined by adding a saturated sodium chloride solution to the polysaccharide soln. The viscosity of the polysaccharide solutions remained nearly unchanged at salt concentrations above 0.2 M, thus the subsequent experiments of pH and calcium complexation were performed in the presence of 0.5 M NaCl. The pH of the polysaccharide dissolved in 0.5 M NaCl was adjusted by adding hydrochloric acid or sodium hydroxide solutions (0.01–2 M) and the viscosity of the resulting soln was determined at each pH. Calcium chloride, solid or in soln, was added to the polysaccharide soln in 0.5 M NaCl. The B value was determined by the relationship of the intrinsic viscosity and the ionic strength ($I^{0.5}$).[22–30]

3. Results

The EPSs obtained by ethanol precipitation alone from the culture fluid were somewhat different from those that had been further purified by the anion exchange chromatographic procedure of the resulting ethanol precipitate.[6] However, the ethanol precipitates are more representative of the products that would be prepared on a large scale and spray-dried. The EPS **2** showed two peaks on a SEC column of the same monosaccharide composition. The larger peak comprised 70% of the sample and had a molecular weight of 7.1×10^6 and the small peak had 1.8×10^6. Upon oxidation by minimum amounts of periodate (1A1.1 in Fig. 1) the two peaks collapsed into one peak with a molecule weight of 1.4×10^6. This suggests that **2** is present to a significant extent as an aggregate in solution. EPS **1** was twice the molecular size of that reported earlier, but of the same composition. The other physical properties of the EPSs were the same as reported earlier.[1]

3.1. Abbreviation codes for the oxidized derivatives

Abbreviated codes were used to designate the oxidized derivatives. E.g. the digit 1 is for those from EPS **1** and 2 for EPS **2**. The extent of periodate oxidation is indicated, e.g. such as 1A1.6 representing the EPS **1** after 1.6 moles of periodate have been consumed per RU. The letter B indicates bromine oxidation of the periodate oxidized derivative, so that 1A1.6/B is the

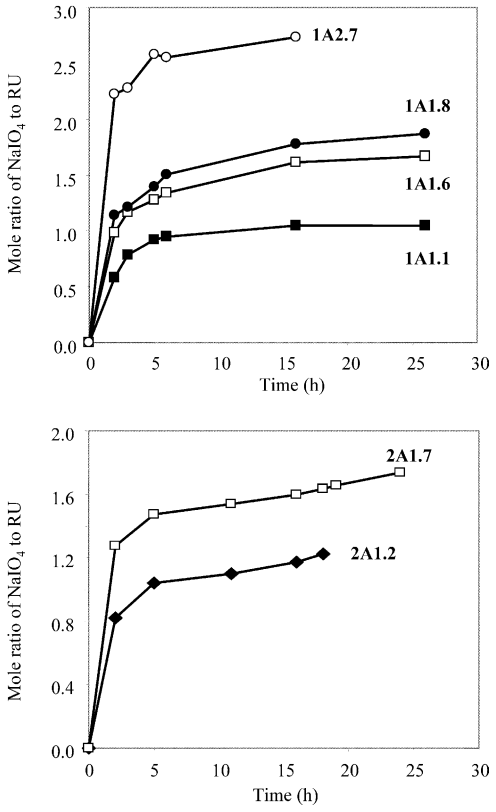

Fig. 1. Periodate oxidation of EPS **1** and **2**. Sample codes: 1A2.7, polyaldehyde derivative of EPS **1** after 2.7 mol of periodate consumed per RU etc (see text).

bromine oxidation product of EPS **1** that has been oxidized to an extent of 1.6 moles of periodate per RU.

3.2. Periodate oxidation of EPS 1 and 2

Periodate oxidation of carbohydrates has been studied in many systems and has been shown to cleave each vicinal hydroxyl group with the formation of a dialdehyde. Exceptions are found where the adjacent hydroxyl groups are present in a "fixed-*trans*" configuration due to some steric constraints. The resulting aldehyde groups formed in the oxidation of polysaccharides may condense with free hydroxyl groups inter- or intra-molecularly. Reduction of the polyaldehyde derivative generates acid-sensitive acetal linkages that may be selectively hydrolyzed by mild acid treatment without the cleavage of existing glycosidic linkages—the Smith degradation. Alternatively, the polyaldehyde may be further oxidized by bromine to generate a polycarboxylate derivative, the properties of which are significantly different from the parent polysaccharide and which also contains acid-sensitive acetal linkages. The polyaldehyde and polycarboxylate derivatives should offer alternate routes to polysaccharide derivatives, such as conjugation with amines and proteins.[31,32]

Oxidation of EPSs was examined with two different extracellular polysaccharides of *E. chrysanthemi*, the structures for which **1** and **2** were known to have three vicinal glycol groups in their repeating units. Polyaldehyde EPSs with various degrees of oxidation were obtained by reacting the EPS in 0.1 M NaOAc (pH 4.5, rt) with sodium periodate at 2.4:1.0, 4.8:1.0 or 10.0:1.0 ($NaIO_4$/RU) mole ratios. The reactions were near to completion within 16 h (Fig. 1) and pyruvate was still present in the resulting polyaldehyde of EPS **2**.

EPS **1** has a branched hexasaccharide RU, composed of one residue each of 3-substituted Glc*p*, 4-substitued Glc*p*A, 3,4-disubstituted Man*p*, terminal Rha*p*, and two residues of 3-substitued Rha*p*. The side chain of **1** has three potential cis-vicinal hydroxyl groups oxidizable to polyaldehyde EPS by periodate oxidation: two from a terminal α-L-Rha and one from an α-D-Glc*p*A residue. EPS **2** also has a branched hexasaccharide RU, composed of one residue each of 3-substituted Glc*p*, 4-substitued Glc*p*A, 4-substituted Fuc*p*, 3,4-disubstituted Fuc*p*, 3-substitued Gal*p* and terminal 4,6-pyruvated Gal*p*. Thus, **2** has also three potential oxidizable vicinal hydroxyl groups, one each from the terminal pyruvated Gal*p*, the 4-substituted Glc*p*A and the 4-substituted Fuc*p*.

The monosaccharide composition of reduced polyaldehyde EPS (Table 1) indicated that the terminal Rha*p* residue in **1** was completely oxidized at the 2.4:1.0 mole ratio of periodate to the RU (1A1.6 in Table 1) while the oxidation of the 4-substituted Glc*p*A was small (10%). The resistance of Glc*p*A in **1** to oxidation was persistent even with a 10-fold excess of periodate, as seen by only 30% oxidation of Glc*p*A (1A2.7 in Table 1).

Preferential oxidation of a neutral sugar residue over Glc*p*A was also observed in EPS **2**. At the ratio of 1.2:1.0 ($NaIO_4$/RU) the 4-substituted Fuc*p* residue was near to complete oxidation (2A1.2 in Table 1). However, oxidation of the terminal pyruvated Gal*p* residue, present as a (4,6)-*O*-(1-carboxyethylidene) derivative,[33,34] was slower than that of the 4-substituted Glc*p*A residue.

The oxidation of the β-D-configured Glc*p*A residue in **2** under similar conditions, i.e. at 2.4:1.0 and 4.8:1.0 mole ratios ($NaIO_4$/RU), was more extensive than of the α-D-configured Glc*p*A residue of **1**, which showed only 30% oxidation. Steric or conformational hindrance of complex formation of periodate with the 2,3-diol of the α-D-Glc*p*A residue, which is directly linked to the branching point of the backbone, may cause the slower oxidation. This was more significant with the terminal pyruvated Gal in **2**, where oxidation of the residue was minimal, even with 4.8-fold excess of periodate. One reason may be the fixed conformation of the Gal imposed by the pyruvate ketal ring that restricted the conformation of the pyranose ring, resulting in resis-

Table 1
Monosaccharide and hydroxy acid analyses of periodate-oxidized **1** and **2**, and their subsequent products by bromine water

Sugar and acid	**1**	Oxidized* **1** (mole ratio)					
		1A1.6	1A1.6/B	1A1.8	1A1.8/B	1A2.7	1A2.7/B
Rha	3.0	2.1	1.8	2.0	1.8	2.0	1.9
Glc	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Man	1.0	1.0	1.0	1.0	1.1	1.0	1.1
GlcA	1.0	0.9	1.0	0.9	0.9	0.6	0.7
Lactic (a)**			0.6		0.7		0.8
Glycolic (b)			0.5		0.6		0.8
Tetraric[1](c)			0.1		0.2		0.5
$NaIO_4$ consumption		1.6		1.8		2.7	
	2	Oxidized* **2** (mole ratio)					
		2A1.2	2A1.2/B	2A1.7	2A1.7/B		
Fuc	2.0	1.1	1.1	1.0	1.0		
Gal	2.0	1.7	1.7	1.6	1.6		
Glc	1.0	1.0	1.0	1.0	1.0		
GlcA	1.0	0.5	0.5	0.3	0.3		
Pyruvic acid (a)***			0.3		0.4		
Glycolic (b)			0.4		0.8		
4-Deoxytetronic[2] (c)			0.4		0.5		
Erythronic (d)			<0.1		<0.1		
Tetraric (e)			0.2		0.7		
$NaIO_4$ consumption		1.2		1.7			

Monosaccharide composition was determined by HPAEC-PAD and hydroxy acids by GLC-MS.
[1] Erythronic acid equivalent.
[2] Lactic acid equivalent.
* 1A1.6, Polyaldehyde derivative of EPS **1** after 1.6 mol of periodate consumed per RU, and etc; 1A1.6/B, Polycarboxyl derivative of 1A1.6 by bromine water etc.
** a–e corresponding peak in Fig. 3 of the GLC chromatogram.
*** Pyruvate was detected as 1,2-bis-*O*-$(CH_3)_3$Si-2-deuteriopropanoic acid.

tance of the oxidation of the diol between C-2 and C-3 to an even greater extent than for the glucuronosyl residue.

3.3. Bromine oxidation of polyaldehydes of 1 and 2

Analysis of the polycarboxylate EPSs revealed that the monosaccharide composition was the same before and after bromine oxidation of the polyaldehyde EPS (Table 1). Additional new peaks appeared, resulting from the formation of mono- or di-carboxylic acids (Fig. 2) that proportionally increased with the degree of oxidation. This indicated that the bromine had reacted principally in the oxidation of polyaldehyde into polycarboxylic acid with minimum oxidation of other sugar residues. The use of dilute bromine water ($\sim$4 mM) minimized undesirable oxidation of sugar residues. This is consistent with earlier studies[35,36] in which it was shown that bromine oxidation at neutrality is slow, oxidation of primary hydroxyl groups is minimal and oxidation at ring positions is hindered where the hydrogen is axial to bulky substituents in 1,3-diaxial configurations. These situations apply to the residues in the polyaldehydes under study.

The expected products derived from oxidation of EPS by periodate and subsequent bromine reaction of the resulting polyaldehyde are summarized in Table 2. The acids are found in the compositional analyses (Table 1 and Fig. 2). They are released by hydrolysis and were reduced with $NaBD_4$ before being derivatized for GLC and GLC/MS analysis (Fig. 2). In the analysis of oxidized **2**, the pyruvic acid derived from the terminal 4,6-pyruvated Gal*p* residue was reduced to 2-deuteriolactic acid and detected as 1,2-bis-*O*-$(CH_3)_3$Si-2-deuterio-propanoic acid. The recovery of the acids was in proportion to the extent of periodate oxidation seen in Table 1.

3.4. Molecular parameters of EPSs

The least oxidized material (1A1.1 and 1A1.6), when dried, was only sparingly soluble in water and dimethyl sulfoxide, and the more oxidized materials (1A1.8 and 1A2.7) were not soluble in either solvent. To improve

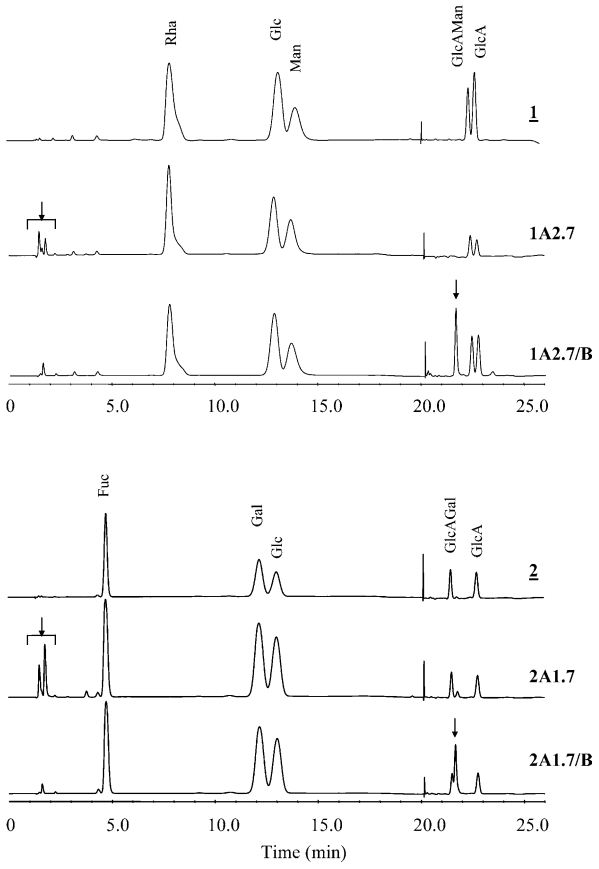

Fig. 2. HPAEC-PAD analysis of acid hydrolyzates (2 M TFA, 120 °C, 1 h) of oxidized EPS **1** and **2**. The peaks of the derived polyols at 2 min and hydroxy acids at 22 min are indicated as an arrow. Sample codes are the same as in Fig. 1 and the polycarboxyl derivative of the polyaldehyde by bromine water is indicated by adding '/B' (see text).

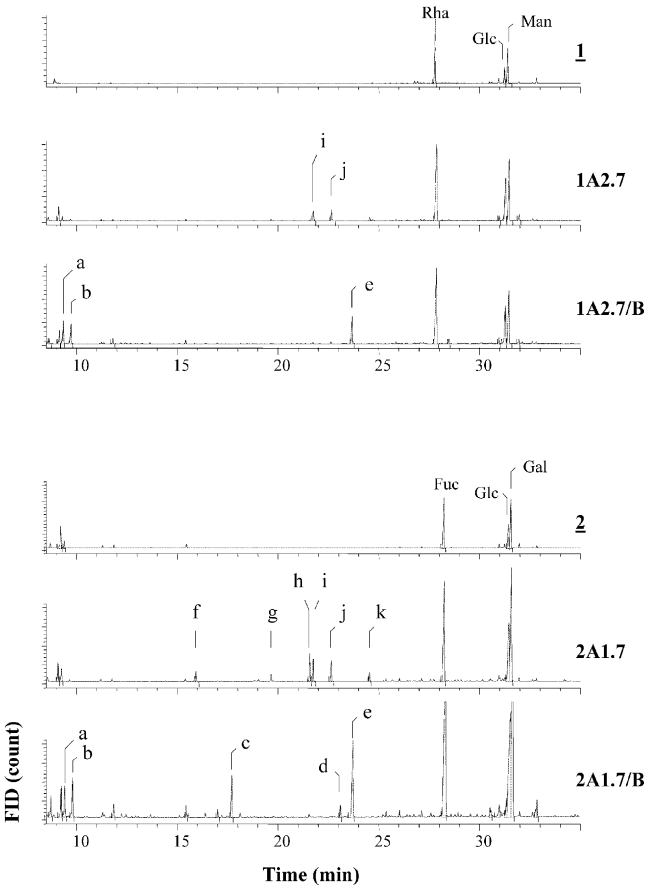

Fig. 3. GLC analysis of the $(CH_3)_3Si$ derivatives of oxidized products derived from **1** and **2**. The compounds were identified by GLC-MS: a, lactic acid; b, glycolic acid; c, 4-deoxytetronic acid; d, tetronic acid, e, tetraric acid; f. 4-deoxytetritol; g, 1,4-lactone of j; h, tetritol; i, tetritol isomer; j, tetronic acid; k, unknown. Sample codes are the same as in Fig. 2.

the solubility of the oxidized sample in water, the reaction product was dried in the presence of sodium bisulfite (0.1 M) to stabilize the carbonyl group. It was interesting to note that the viscosity of a freshly dissolved bisulfite derivative in water was high (256 cP), but in 3 days it reduced to 14 cP, a value close to the polyaldehyde from which it was derived.

The molecular weights of the EPSs were examined by SEC chromatography, using MALS and RI detectors. Periodate oxidized derivatives of **1** and **2** showed no or little changes in molecular weight, Rw values and polydispersity (Table 3). The general conformation of the polyaldehydes, as seen by the slope of the RMS/M plots, are similar to that of the native structure. This implies that intermolecular condensations are minimal and the peripheral change of the resulting oxidized side chain, in particular with **1**, did not affect the conformation of the backbone.

All bromine oxidized derivatives of **1** and **2** showed significant decrease in molecular weight (around 1/100 for **1** series and 1/5 for **2** series), but little change in their polydispersities.

3.5. Viscometric behavior of EPSs

Generally, the viscometric behaviors of **1** and **2** polyaldehyde derivatives were similar to those of the native EPS.[1] The EPSs demonstrate characteristics of pseudoplastic flow (shear-thinning), the relative decrease in viscosity being more pronounced in the range of 7–20 s^{-1} shear rate than at values larger than 20 s^{-1}. The EPSs are not thixotropic (Fig. 4A). The viscosity at zero shear rate (η_0) was obtained by fitting of the Cross-equation[15–21] using the data obtained by a Brookfield viscometer. The fitting gives curves close to the measured data points with the native EPSs **1** and **2**, but not with the polyaldehyde and polycarboxyl derivatives (1A1.6, 1A1.6/B and 2A1.7 and 2A1.7/B) because of small shear thinning effects. However the derivatives gave a similar result to that determined by a capillary viscometer. The capillary viscometer is used only for

Table 2
The expected degradation products derived from EPS **1** and **2** oxidized by $NaIO_4$ and the subsequent bromine water of the resulting polyaldehyde derivative

Oxidizable residue by $NaIO_4$	$NaIO_4$-oxidized and reduced EPS	Br_2 oxidation of polyaldehyde EPS
t-Rha	propylene glycol	lactic acid
	4-deoxyerythritol	4-deoxyerythronic acid
	ethylene glycol	glycolic acid
4-GlcA	ethylene glycol	glycolic acid
	erythronic acid	erythraric acid
4-Fuc	ethylene glycol	glycolic acid
	4-deoxythreitol	4-deoxytetronic acid
Py(4,6)Gal	ethylene glycol	glycolic acid
	Py (2,4) threitol	Py (2,4) threonic acid

qualitative comparisons of the pseudoplastic EPSs. The Flory–Fox method to calculate the intrinsic viscosity from the light scattering data gave results close to those obtained by fitting of the Cross-equation from the Brookfield viscometer measurement.

The viscosity $[\eta]_0$ of **1**, **2** and their derivatives is almost independent of pH between 4 and 9, and decreases over the pH range of 1–4 or 9–13 partly due to degradation and also at the lower pH to the suppressed ionization of the carboxyl groups and at higher pH to the formation of stable carboxylate ion.[1,9,10]

The intrinsic viscosity $[\eta]$ of the polysaccharides decreases in the range of 0.05–0.5 M NaCl, but when the sodium chloride concentration reaches 0.2 M the viscosity shows an asymptote, a typical viscometric behavior of polyelectrolytes.[25] The B values for the EPSs and some oxidized derivatives are listed in Table 3. This value approximately reflects the flexibility of polysaccharide chains in solution although it is an experimental parameter for which there does not appear to be a physical meaning. The lower the B value, the stiffer the molecular chains. EPS **1** and **2** and their derivatives are in the same range of intermediate flexibilities, as found for pectin, villain, alginate, chitosan, λ-carrageenan, carboxymethylcellulose and hyaluronate.[1] Thus, 2A1.7/B, similar to rhamsan,[38] is stiffer than **2** and 2A1.7 while **1** and 1A1.6 are similar in their flexibility term.

The addition of $CaCl_2$ increases the intrinsic viscosity $[\eta]$ of **1**, **2** and their derivatives (Fig. 4B). The $[\eta]$ of **1** increases significantly with increase in $CaCl_2$ concentration commencing at 0.1% (w/w), possibly due to the aggregation of molecules. This is in contrast with the

Table 3
Molecular parameters, intrinsic viscosities $[\eta]$ and B values of EPSs in 0.15 M Na_2SO_4 containing 0.015 M EDTA at 25 °C by LS and in 0.5 M NaCl by viscometry

Sample[a]	dn/dc	SEC/LS					$[\eta]_0$ (mL g^{-1})	B
		MW ($\times 10^6$)	Rw (nm)	Slope of RMS vs. M	Polydisp. (MW/Mn)	$[\eta]$[b] (mL g^{-1})		
1	0.159	4.11 ± 0.07	166.8 ± 2.1	0.52 ± 0.06	2.0	1256	1224[h]	0.034
1A1.1	0.144	4.47 ± 0.08	164.0 ± 6.4	0.56 ± 0.03	1.9	1300		
1A1.6	0.157	3.72 ± 0.17	161.9 ± 2.1	0.55 ± 0.02	1.9	1215	986[c]	0.040
1A1.8	0.147	3.80 ± 0.12	156.6 ± 0.8	0.50 ± 0.04	2.0	1239		
1A2.7	0.157	4.33 ± 0.09	159.0 ± 2.1	0.56 ± 0.02	1.7	1317		
1A1.6/B	0.163	0.05[d]	nd[e]	nd[e]	1.3	nd[e]	34[c]	nd[f]
1A1.8/B	0.164	0.06[d]	nd[e]	nd[e]	1.4	nd[e]	23[c]	
1A2.7/B	0.162	0.04[d]	nd[e]	nd[e]	1.3	nd[e]	22[c]	
2	0.155	7.06 ± 0.39	310.8 ± 1.7	0.51 ± 0.02	1.7	4430[g]	4365[h]	0.010
		1.75 ± 0.39	162.2 ± 2.1		1.2			
2A1.2	0.147	1.41 ± 0.09	72.2 ± 6.6	0.62 ± 0.04	1.8	378		
2A1.7	0.151	1.34 ± 0.05	72.8 ± 0.3	0.58 ± 0.07	1.7	388	416[c]	0.080
2A1.2/B	0.160	0.27 ± 0.01	31.7 ± 3.3	0.58 ± 0.07	1.6	201		
2A1.7/B	0.162	0.13 ± 0.01	21.5 ± 0.2	0.55 ± 0.03	1.5	158	201[c]	0.005

[a] Sample codes described in Table 1.
[b] The intrinsic viscosity calculated by Astra software using Flory–Fox equation.
[c] The viscosity determined by capillary viscometer.
[d] The errors were less than 0.01×10^6.
[e] The size of the molecules is so small (less than 10 nm) that they showed no angle dependence of the LS signal and thus no Rw value, no RMS/M relation and it was not possible to determine the viscosity using the Flory–Fox equation.
[f] Undetectable because of the low viscosity.
[g] The average of the two SEC peaks.
[h] The viscosity at zero shear rate fitted by the Cross equation.

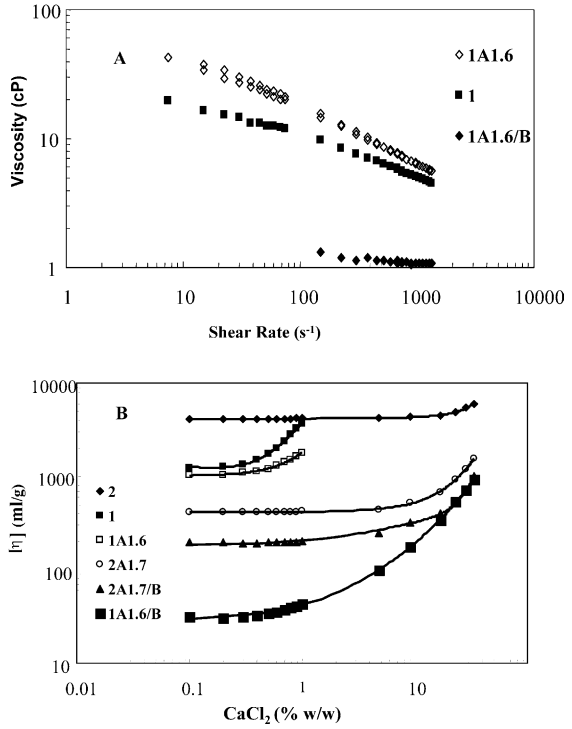

Fig. 4. Viscometric properties of EPSs. A: The effect of shear rates on the viscosity of **1** and its derivatives in water at 25 °C, as determined by a Brookfield viscometer. Measurement was made by increasing, then decreasing the shear rate. B: The effect of $CaCl_2$ concentration on the intrinsic viscosity of EPSs at 25 °C, as determined by capillary viscometer. $CaCl_2$ was added to the polysaccharide solution dissolved in 0.5 M NaCl. Sample code is the same as in Fig. 2.

behavior of **2** where the first additions of calcium associates with carboxyl groups on the same chain with little influence on the $[\eta]$ up to 1% (w/w) $CaCl_2$.[1] The periodate oxidation derivatives, 1A1.6 and 2A1.7, showed similar $[\eta]$ as their native EPSs. The bromine oxidation derivatives, 1A1.6/B and 2A1.7/B, followed a similar response to the $CaCl_2$ as the parent molecule, but the intrinsic viscosity of 1A1.6/B increased at higher concentration of $CaCl_2$ due to the greater number of carboxyl groups available for intermolecular complexation.

4. Discussion

The original objective of this study was to examine the change in the hydrodynamic properties of two well characterized EPSs after the introduction of additional carboxyl groups by periodate oxidation of the EPS and bromine oxidation of the resulting aldehydes. The extensive degradation of the polysaccharides in the course of the bromine oxidations clearly defeated the objective but added to our knowledge of the side reactions.

Reactions of polysaccharides with hypohalite reagents are complex and not fully understood, as exemplified by the oxidations of starch[35] and nigeran,[37] the products from which are not fully described. However it is clear from the present studies that the sugar residues resistant to periodate oxidation are present in the bromine-oxidized products in the same proportion. This is consistent with earlier studies.[35,36] The principal effects of the bromine at neutral pH are therefore oxidation of the aldehyde groups to acids and depolymerization to yield polyanionic derivatives of MW $1.3–2.7 \times 10^5$ for **2** and MW $4–6 \times 10^4$ for **1**. The latter shows no angular dependence in light scattering and can thus be predicted to have Rw of < 10 nm, consistent with low viscosities in dilute salt solutions.

The polyacid polymer from **2** permits a more detailed analysis of its conformation compared to the native EPS and its polyaldehyde. Similarities are seen for the dependence of RMS on M and the *B*-values. It is also noted that the calculated values of the intrinsic viscosities from the Rw and MW are close to the experimental values.

The physical properties of the polyaldehydes showed that the molecular weights, Rw values and polydispersity are little different from the native EPS (Table 3). This indicates that intermolecular condensations are minimal and from the slope of the RMS/M graph the general conformation of the polyaldehydes are similar to that of the native polysaccharide. Interestingly, however, the viscosity of the polyaldehyde from **2** is around 10% of the native EPS, whereas the polyaldehyde from **1** is similar to that of the native polysaccharide. These differences may reflect that the oxidation of **2** occurs principally in the backbone, in contrast to that of the side chain from **1**. In addition, it is seen that the **2** is a mixture of the monomer EPS, MW 1.75×10^6, and an aggregate, MW 7.06×10^6, the latter being approximately 70% of the mixture. The derived polyaldehyde shows one product, MW 1.4×10^6, reflecting the monomer structure and therefore without the viscometric contribution of an aggregate.

No explanation is offered for the much greater (100-fold) reduction of the MW of the polycarboxylate derivatives of EPS **1**, in which the initial periodate oxidation should occur exclusively in the side chain. The loss of these residues from the polycarboxylate by hydrolysis could not account for such a change in MW, $0.04–0.06 \times 10^6$.

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References

1. Ding, Q.; LaBelle, M.; Yang, B. Y.; Montgomery, R. *Carbohydr. Polym.* **2003**, *51*, 331–346 (and references cited therein).
2. Gray, J. S. S.; Yang, B. Y.; Montgomery, R. *Carbohydr. Res.* **2000**, *324*, 255–267.
3. Yalpani, M.; Sanford, P. In *Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications*; Yalpani, M., Ed.; Elsevier: Amsterdam, 1987; pp 311–335.
4. Nieuwenhuizen, M. S.; Kieboom, A. P. G.; van Bekkum, H. In *Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications*; Yalpani, M., Ed.; Elsevier: Amsterdam, 1987; pp 157–161.
5. Gray, J. S. S.; Brand, J. M.; Koerner, T. A. W.; Montgomery, R. *Carbohydr. Res.* **1993**, *245*, 271–287.
6. Yang, B. Y.; Brand, J. M.; Gray, J. S. S.; Montgomery, R. *Carbohydr. Res.* **2001**, *333*, 295–302.
7. Gates, S. G.; Dendramis, N.; Sweely, C. C. *Clin. Chem.* **1978**, *24*, 1674–1679.
8. Yang, B. Y.; Montgomery, R. *Carbohydr. Res.* **1996**, *280*, 27–45.
9. Yang, B. Y.; Ding, Q.; Montgomery, R. *Carbohydr. Res.* **2002**, *337*, 731–742.
10. Yang, B. Y.; Ding, Q.; Montgomery, R. *Carbohydr. Res.* **2002**, *337*, 2469–2480.
11. Flory, P. J.; Fox, T. G., Jr. *J. Am. Chem. Soc.* **1951**, *73*, 1904–1908.
12. Ptitsyn, O. B.; Eizner, Y. E. *Vysokomol. Soedin.* **1959**, *1*, 1200–1206.
13. Huggins, M. L. *J. Am. Chem. Soc.* **1942**, *64*, 2716–2718.
14. Kraemer, E. O. *Ind. Eng. Chem.* **1938**, *30*, 1200–1203.
15. Cross, M. M. *J. Col. Sci.* **1965**, *20*, 417–437.
16. Morris, G. A.; Li, P.; Paund, M.; Liu, Z.; Mitchell, J. R.; Harding, S. E. *Carbohydr. Polym.* **2001**, *44*, 261–268.
17. Rodd, A. B.; Dunstan, D. E.; Boger, D. V. *Carbohydr. Polym.* **2000**, *42*, 159–174.
18. Manca, S.; Lapasin, R.; Partal, P.; Gallegos, C. *Rheol. Acta* **2001**, *40*, 128–134.
19. Rayment, P.; Ross-murphy, S. B.; Ellis, P. R. *Carbohydr. Polym.* **1998**, *35*, 55–63.
20. Grassi, M.; Lapasin, R.; Pricl, S. *Carbohydr. Polym.* **1996**, *29*, 169–181.
21. Wang, Q.; Ellis, P.; Ross-Murphy, S. B.; Burchard, W. *Carbohydr. Polym.* **1997**, *33*, 115–124.
22. Smidsrød, O. *Carbohydr. Res.* **1970**, *13*, 359–372.
23. Smidsrød, O.; Haug, A. *Biopolymers* **1971**, *10*, 1213–1227.
24. Tobitani, A.; Ross-Murphy, S. B. *Polym. Int.* **1997**, *44*, 338–347.
25. Lapsin, R.; Pricl, S. *Rheology of Industrial Polysaccharides: Theory and Applications*; Blackie Academic: London, 1995; pp 269–281.
26. Higashimura, M.; Mulder-Bosman, B. W.; Reich, R.; Iwasaki, T.; Robijn, G. W. *Biopolymers* **2000**, *54*, 143–158.
27. Jampen, S.; Britt, I. J.; Tung, M. A. *Food Res. Int.* **2000**, *33*, 579–586.
28. Lai, L. S.; Tung, J.; Lin, P. S. *Food Hydrocol.* **2000**, *14*, 287–294.
29. Cescutti, P.; Paoletti, S.; Navarini, L.; Flaibani, A. *Int. J. Biol. Macromol.* **1993**, *15*, 201–207.
30. Flaibani, A.; Leonhartsberger, S.; Navarini, L.; Cescutti, P.; Paoletti, S. *Int. J. Biol. Macromol.* **1994**, *16*, 65–70.
31. Wessels, M. R.; Paoletti, L. C.; Kasper, D. L.; DiFabio, J.; Michon, F.; Holme, K.; Jennings, H. J. *J. Clin. Invest.* **1990**, *86*, 1428–1433.
32. Robbins, J. B.; Schneerson, R.; Szu, S. C.; Pozsgay, V. *Pure Appl. Chem.* **1999**, *71*, 745–754.
33. Yang, B. Y.; Brand, J. M.; Montgomery, R. *Carbohydr. Res.* **2001**, *331*, 59–67.
34. Yang, B. Y.; Gray, J. S. S.; Montgomery, R. *Int. J. Biol. Macromol.* **1994**, *16*, 306–312.
35. Salomonsson, A.-C. B.; Andersson, R. E.; Torneport, L. J.; Theander, O. *Carbohydr. Res.* **1991**, *217*, 221–225.
36. Larm, O.; Scholander, E.; Theander, O. *Carbohydr. Res.* **1976**, *49*, 69–77.
37. Peplow, P. V.; Somers, P. J. *Carbohydr. Res.* **1972**, *22*, 281–291.
38. Kwon, B.-D.; Foss, P. A.; Rha, C.-K. In *Industrial Polysaccharides: Genetic Engineering Structure/Property Relations and Applications*; Yalpani, M., Ed.; Elsevier: Amsterdam, 1987; pp 253–266.