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The Acid Sensitivity of Gulose and Mannose in Chemically-Reduced Alginates Obtained from *Pseudomonas syringae*

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Abstract The chemical reduction of *Pseudomonas syringae* subsp. *phaseolicola* alginates produces neutral polymers of D-mannose and L-gulose in source specific ratios. L-Gulose was highly sensitive to degradation by 1N HCl at 100°C. As hydrolysis time increased, gulose recovery decreased to 22% after 4 hr, whereas 98% of the D-mannose was recovered under the same conditions. Thin layer chromatography showed the formation of a second product upon L-gulose acid hydrolysis. This new product had a rate of flow (Rf) value of 0.58, identical to that of 1,6 anhydro-β-D-mannopyranose and very close to that of 1,6 anhydro-β-D-glucopyranose (Rf=0.60). Because of the difference in acid sensitivity between L-gulose and D-mannose, normal acid hydrolytic techniques applied to reduced alginates produces erroneous mannuronic acid (M): guluronic acid (G) ratio's unless one accounts for the differential rates of destruction of each sugar.

Keywords: acid sensitivity, gulose, mannose, alginates

Introduction

Alginates are (1-4)-linked linear glycuronans composed of varying amounts of β-D-mannuronic acid (M) and its C-5 epimer α-guluronic acid (G) (1, 8, 13). Historically, alginates have been obtained from many of the brown seaweeds. Alginate-like exopolysaccharides are also produced by some species of bacteria, specifically *Azotobacter vinelandii,* many Pseudomonads including *Pseudomonas aeruginosa* (18), and numerous subspecies of *Pseudomonas syringae* (4). The mannuronic-guluronic ratios of the bacterial alginates are species specific, but they all contain O-acetyl groups (2, 5) associated with the C-2 and/or C-3 position of the D-mannuronic acid residues. The acetyl groups are believed to inhibit both epimerization of the C-5 carboxylate group during the formation of L-guluronic acid by mannuronan C-5 epimerase (2), and depolymerization by alginate lyases (21). The presence of the acetyl groups biosynthetically controls the proportion of the two uronic acids, which in turn determines the properties of the polysaccharide.

Acid hydrolysis has been investigated as a pretreatment for determining the physical compositions and structure of polysaccharides (19). Depolymerization is conducted using dilute sulfuric acid, hydrochloric acid, or trifluoroacetic acid at 100°C for varying lengths of time (7, 13, 14). This chemical reduction is necessary because the carboxyl group at C-5 confers resistance to the glycosyluronic acid linkage (6). In studies on the composition of alginates, it is common practice to first reduce the uronate polymer to the neutral polymer to facilitate acid hydrolysis and minimize thermal destruction of the component monosaccharides (4, 11, 22).

The demand for commercial applications for the use of alginates in food, phamaceutical and biotechnology industries has increased. Applications of alginates produced commercially include their use as immunostimulants using mannuronan, and in generating films or stabilizing, thickening and gelling agents (17, 18). The gelling property of alginates is dependent on the ratio of mannuronic acid (M) / guluronic acid (G), where higher ratios of M/G produce more elastic gels than lower ratios of M/G (18). The difference in acid sensitivity between L-gulose and D-mannose obtained by normal acid hydrolytic techniques applied to reduced alginates produces erroneous M:G ratio's unless one accounts for the differential rates of destruction of each sugar. This investigation documents the relative acid sensitivities of D-mannose to L-gulose under acid hydrolysis conditions as it applies to analysis of *P. syringae* subsp. *phaseolicola* alginates.

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Materials and Methods

Organism maintenance and bacterial alginate purification *P. syringae* subsp. *phaseolicola* ATCC 19304 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cultures were maintained at 4°C on Dworkin Foster (DF) agar (3) supplemented with 2%(w/v) gluconic acid. D-Mannose, L-gulose, and alginate from the brown seaweed, *Macrocystis pyrifera*, were purchased from Sigma Chemical Co., (St. Louis, MO, USA). The bacterial alginates were obtained from cultures grown either on DF agar or nutrient agar plates supplemented with 2%(w/v) gluconic acid. The bacterial alginates were scraped off the agar plates and resuspended in 150 mL of deionized water. The bacterial alginate was separated from the microbial cells by centrifugation at 27,000×g for 60 min. Three volumes of isopropanol were added to one volume of clarified supernatant to precipitate the polysaccharide. The precipitated alginate was removed by winding around a glass rod. The precipitate was then dried

first in acetone and then in air. Purity was determined by the carbazole assay (9) using sodium alginate from *M. pyrifera* as the standard. The purity of the bacterial alginate produced from *P. syringae* ATCC 19304 was more than 98%(w/v).

Thin layer chromatography (TLC) Standards of D-mannose and L-gulose (Sigma Chemical Co.) were prepared at a concentration of 6.66 mg/mL (w/v) in deionized water. One mL of acid (1 N HCl or 1 N H_2SO_4) and 1 mL of standard sugar solution were mixed and the solution was heated at 100°C for 0.5, 1, 2, 3, and 4 hr. Each solution was then neutralized with 1 mL of 1 N NaOH giving a final sugar concentration of 1.67 mg/mL (w/v) and a final pH of between 6.0 and 7.0. Thin layer chromatography was performed on these hydrolysates on 0.25 mm plates coated with silica gel 150A (Whatman Co., Maidstone, England), or Kieselgel 60 F_{254} (E. Merck Co., Darmstadt, Germany). The hydrolyzed sugar solutions were spotted at a concentration of 50 μg sugar/spot. The running solvent was *n*-propanol and water at a ratio of 85:15 (v/v). The plates were developed by spraying with 20%(v/v) H_2SO_4 in methanol and charring at 100°C for 15-20 min. TLC was used to help identify the acid hydrolysis product of L-gulose. L-Gulose (Sigma Chemical Co.) was acid hydrolyzed and separated on a TLC plate as described above. At the end of each run, the TLC plate was air dried. The silica gel in the region corresponding to the rate of flow (Rf) value of the spot of interest was scraped from the plate and eluted in deionized water for 10 min. The silica gel was removed by micro-centrifugation in a Sorval Microspin 24S microcentrifuge (Du Pont Co., Wilmington, DE, USA) for 3 min. The supernatant was freeze dried in a Flexi-Dry freeze dry apparatus (FTS Systems, Stone Ridge, NY, USA). Freeze dried samples were resuspended in 0.2 mL of deionized water and again spotted on a 0.25 mm TLC plate coated with Kieselgel 60 F_{254} (E. Merck Co.) as described previously. The sample was run in *n*-propanol and water at a ratio of 85:15 (v/v) with samples of 1,6 anhydro-β-D-mannopyranose, and 1,6 anhydro-β-D-glucopyranose (Sigma Chemical Co.). The migration of the unknown was compared to the known standards.

Ion chromatography Standards of D-mannose and L-gulose (Sigma Chemical Co.) were prepared at a concentration of 1 mg/mL (w/v) in deionized water. Composition measurements of the alginates from *M. pyrifera* and *P. syringae* ATCC 19304 were made by ion chromatography of the acid hydrolyzed, reduced polymers. The reduced samples were mixed at a concentration of 1 mg/mL and 1 mL of solution was mixed with 1 mL of acid (1 N HCl or 1 N H_2SO_4). The samples were then hydrolyzed at 100°C for 0.5, 1, 2, 3, and 4 hr. After hydrolysis, ion chromatography was performed on each hydrolyzed standard, using a Dionex ion chromatography system (Dionex Corp., Sunnyvale, CA, USA) fitted with a carbopac PA1 column (4×250 mm). A 100 mM solution of NaOH was used as eluent and pumped at 0.5 mL/min by a gradient pump. Fifty μL of each sample was injected into the Dionex ion chromatography system. Signal was detected by a pulsed amperometric detector. Integration was carried out by a Dionex 4400 integrator.

Alginate reductions The method of Taylor *et al.* (20) was used for the reduction of uronic acids in alginates. An aqueous solution of alginate containing 100 micro-equivalents of carboxylic acid in 10 mL of deionized water was adjusted to pH 4.75 with 0.1 M NaOH. One millimole of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added to the alginate solution to convert the uronides to esters. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 M HCl. The reaction was allowed to continue until hydrogen ion uptake ceased (45-60 min), and then 25 mL of a 3 M $NaBH_4$ solution was added dropwise over a 1 hr period to reduce the uronides to the neutral polymers. The pH was maintained at 7.0 by titration with 4 M HCl. 1-Propanol was added dropwise as necessary to minimize foaming. The reaction mixture was then made slightly acidic to destroy any remaining sodium borohydride, and the solution was dialyzed exhaustively against deionized water. Each reduced alginate sample was then concentrated by evaporation under vacuum and precipitated by the addition of three volumes of iso-propanol. The precipitate was dried by washing with acetone.

Results and Discussion

Thin layer chromatography (TLC) Acid hydrolyzed (HCl) D-mannose produced a single spot on TLC with an Rf value of 0.40. This spot corresponded with the unhydrolyzed D-mannose standard. Its intensity remained constant over a 4 hr hydrolysis time. TLC of acid hydrolyzed L-gulose produced results that were much different than those obtained with D-mannose. L-gulose was stable to hydrolysis for only 1 hr after which a second spot having an Rf value of 0.56 (Fig. 1) was detected. The intensity of this spot increased with time of HCl-hydrolysis as the intensity of the gulose spot (Rf= 0.37) decreased proportionally. The patterns described above were seen with both HCl and sulfuric acid treatments (Table 1).

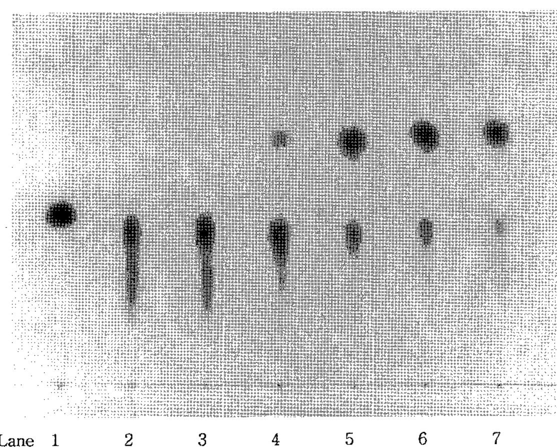

Fig. 1. Thin layer chromatograph of HCl hydrolyzed L-gulose. Lane 1, unhydrolyzed L-gulose in H_2O; lane 2, unhydrolyzed L-gulose in HCl; lane 3-7, hydrolyzed L-gulose in HCl for 0.5, 1, 2, 3, and 4 hr, respectively, at 100°C.

Ion chromatography The relative peak areas were determined and extrapolated back to time zero to determine the percentage of mannose and gulose originally present in the reduced polymer. Calculation of ion chromatogram peak areas showed that after 4 hr of HCl or H_2SO_4 hydrolysis, 98% of the D-mannose was recovered (Fig. 2) whereas only 22% of the L-gulose was recovered. TLC of the isolated gulose degradation product showed it had a migration equal to 1,6 anhydro-β-D-mannopyranose (Rf=0.58), and very close to 1,6 anhydro-β-D-glucopyranose (Rf=0.60, Fig. 3).

The behavior of the uronate-reduced alginates on acid hydrolysis paralleled the results seen with the D-mannose and L-gulose standards. After correcting for gulose destruction by extrapolating back to zero hydrolysis time, a composition of 60% mannuronic acid and 40% guluronic acid was obtained for *M. pyrifera* alginate, and 82% mannuronic acid and 18% guluronic for *P. syringae* ATCC 19304 alginate (Fig. 4). However, if there is no correction for gulose destruction, values of 15% gulose for seaweed and 5% gulose for *P. syringae* alginates would be obtained. These results correlated well with the reported composition of *M. alginate* (60% mannuronic acid and 40 % guluronic acid), and *P. aeruginosa* alginate (80% mannuronic acid and 20% guluronic acid). Sugars of the gulo, ido, and altro configurations undergo spontaneous conversion to 1,6 anhydrides in acids. Sugars of the gluco, manno, and galacto configurations produce very little 1,6 anhydride at equilibrium and exist almost completely as the free aldose under acid conditions (10). Axially oriented hydroxyl groups, particularly at C-3, destabilize anhydride formation. Equatorially oriented hydroxyl groups favor the formation of 1,6 anhydrides (15).

D-Mannose, when free energy favors the $^4C_1$ chair conformation, does not form the 1,6 anhydride bond. Although the $^1C_4$ conformtion brings C-6 and the anomeric hydroxyl group into axial positions around the ring and into proximity, this conformation also brings the hydroxyl groups at C-3 and C-4 into axial positions destabilizing anhydride formation. In the $^1C_4$ conformation, D-mannose, D-glucose, and D-galactose all have axial hydroxyl groups at C-3 and therefore do not readily form 1,6 anhydrides.

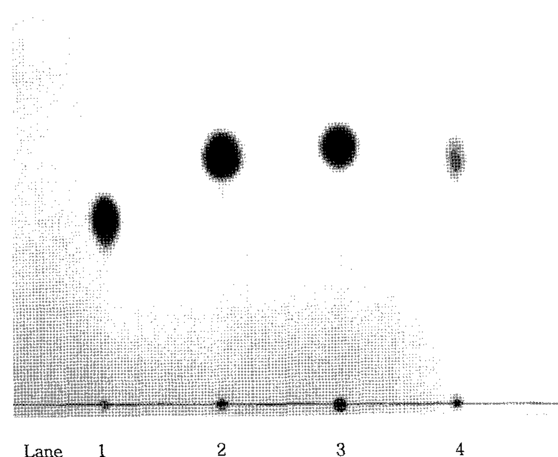

Fig. 3. Thin layer chromatograph of the acid hydrolyzed product of L-gulose. Lane 1, unhydrolyzed L-gulose in H_2O; lane 2, standard 1,6 β-D-Mannopyranose; lane 3, standard 1,6 β-D-glucopyranose; lane 4, acid hydrolysis product of L-gulose.

Table 1. Rf values of acid hydrolyzed D-mannose and L-gulose standards derived from thin layer chromatographs

Sample[1)]	Spot A (Rf)	Spot B (Rf)
D-mannose in HCl	0.40	N/A[2)]
L-gulose in HCl	0.37	0.56
D-mannose in H_2SO_4	0.37	N/A
L-gulose in H_2SO_4	0.34	0.50

[1)]Each sample was hydrolyzed in an equal volume of 1 N acid for 4 hr at 100°C prior to application on TLC, [2)]N/A= Not applicable.

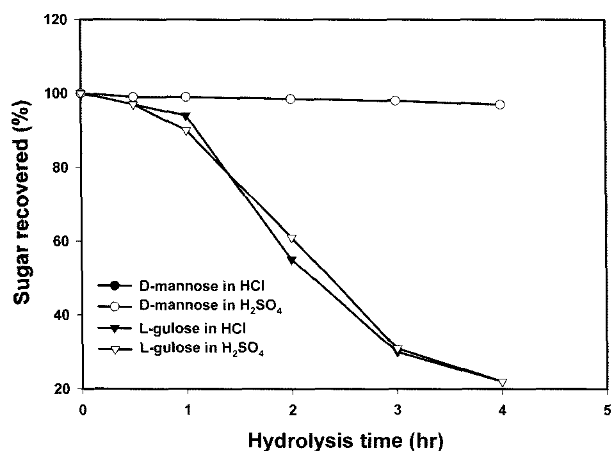

Fig. 2. The stability of monomeric D-mannose and L-gulose in HCl and H_2SO_4 under hydrolysis conditions at 100°C, as determined by ion chromatography. Two lines representing D-mannose in HCl or H_2SO_4 are overlapped.

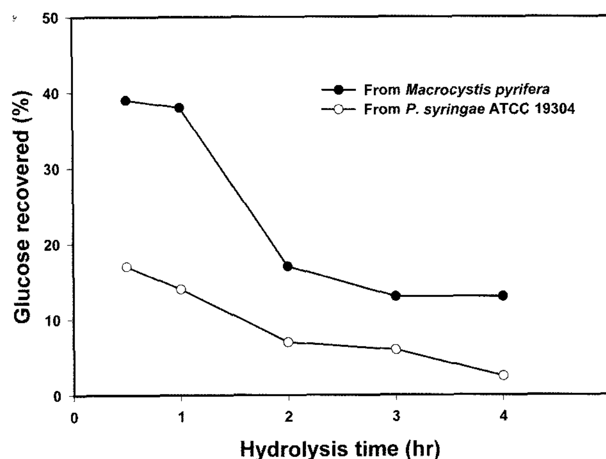

Fig. 4. Gulose recovered, expressed as a percent of the total sugar present in the HCl hydrolyzed reduced alginates, from *Macrocystis pyrifera* (●) and *Pseudomonas syringae* ATCC 19304 (○), as determined by ion chromatography.

Published methods for the determination of alginate composition recommends hydrolysis of the reduced neutral polymer in 1 M H_2SO_4 for 90 min at 100°C (11, 21). However, since L-gulose is more susceptible to conformational modification than is D-mannose under these conditions, one must account for gulose destruction. When correction is made for gulose destruction by extrapolation back to time zero, the results of the analysis of seaweed alginate correlate exactly with the published results of 60% mannuronic acid and 40% guluronic acid as determined by the reductive cleavage method of Zeller and Gray (22). It has been reported that the alginates from *P. syringae* contain trace amounts of polyguluronate as determined by ^{1}H-NMR (11). The reported composition of the *P. syringae* ATCC 19304 alginate was greater than 95 % D-mannuronic acid and less than 5% L-guluronic acid (4). It was difficult to explain this observation from the analytical data which suggested that almost no L-guluronate was present in these alginates. Since the amount of L-guluronate in the bacterial alginates is actually significantly higher than previously reported, the presence of polyguluronate regions are readily explained. The actual composition of the *P. syringae* ATCC 19304 alginate was 82% D-mannuronic acid and 18% L-guluronic acid in this study after correcting for the relative acid sensitivities of each corresponding neutral sugar. Our study showed high correlation to the published composition of *P. aeruginosa* alginate of 80% D-mannuronic acid and 20% L-guluronic acid (22).

References

1. Baekkevig KH, Sletta M, Gimmestad R, Aune H, Ertesvag K, Degnes BE, Christensen TE, Ellingsen SV. Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular enviroment. J. Bacteriol. 187: 8375-8384 (2005)
2. Davidson JW, Sutherland IW, Lawson CI. An alginate lysate from *Azotobacter vinelandii* phage. J. Gen. Microbiol. 98: 603-606 (1977)
3. Dworkin M, Foster W. Experiments with some microorganisms which utilize ethane and hydrogen. J. Bacteriol. 75: 592-603 (1958)
4. Fett WF, Osman SF, Fishman ML, Siebles TS. Alginate production by plant-pathogenic *Pseudomonas*. Appl. Environ. Microbiol. 52: 466-473 (1986)
5. Franklin MJ, Ohman DE. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. J. Bacteriol. 184: 3000-3007 (2002)
6. Gorin PAT, Spencer JFT. Exocellular alginic acid from *Azotobacter vinelandii*. Can. J. Chem. 44: 993-998 (1966)
7. Ilic IJ, Jeremic K, Jovanovic S. Kinetics of pullulan depolymerization in hydrochloric acid. Eur. Polym. J. 27: 1227-1229 (1991)
8. Kim EJ, Kim BY, Rhim JW. Enhancement of the water-resistance and physical properties of sodium alginate film. Food Sci. Biotechnol. 14: 108-111 (2005).
9. Knutson CA, Jeanes A. Determination of the composition of uronic acid mixtures. Anal. Biochem. 24: 482-490 (1968)
10. Miller JA. The stereochemistry of cyclic derivatives of carbohydrates. Adv. Carbohyd. Chem. 10: 1-53 (1955)
11. Osman SF, Fett WF, Fishman ML. Exopolysaccharides of the phytopathogen *Pseudomonas* syringae pv. *Glycinea*. J. Bacteriol. 166: 66-71 (1986)
12. Pena C, Miranda L, Segura D, Nunez C, Espin G, Galindo E. Alginate production by *Azotobacter vinelandii* mutants altered in poly-β-hydroxybutyrate and alginate biosynthesis. J. Ind. Microbiol. Biot. 29: 209-213 (2005)
13. Penner MH, Hashimoto AG, Esteghlalian A, Fenske JJ. Acid-catalyzed hydrolysis of lignocellulosic materials. ACS Sym. Ser. 647: 11-31 (1996)
14. Prasanna V, Prabha TN, Tharanathan RN. Pectic polysaccharides of mango: structural studies. J. Sci. Food Agric. 84: 1731-1735 (2004)
15. Reeves RE. Chemistry of the carbohydrates. Ann. Rev. Biochem. 27: 15-34 (1958)
16. Rehm BHA, Valla S. Bacterial alginates: biosynthesis and applications. Appl. Microbiol. Biot. 48: 281-288 (1997)
17. Rhim JW, Lee, JH. Effect of $CaCl_2$ treatment on mechanical and moisture barrier properties of sodium alginate and soy protein-based films. Food Sci. Biotechnol. 13: 728-732 (2004)
18. Skjak-Braek G, Grasdalem H, Larsen L. Monomer sequence and acetylation pattern in some bacterial alginates. Carbohyd. Res. 154: 239-250 (1986)
19. Synnove H, Qiujin Z, Wenche IS, Gudmund SB. Characterization of the hydrolysis mechanism of polyalternating alginate in weak acid and assignment of the resulting MG-oligosaccharides by NMR spectroscopy and ESI-mass spectrometry. Biomacromolecules 7: 2108-2121 (2006)
20. Taylor RI, Shively JE, Conrad HE. Stoichiometric reduction of uronic acid carboxyl groups in polysaccharides. Method Carbohyd. Chem. 7: 148-151 (1976)
21. Yamasaki M, Ogura K, Mikami W, Murata K. A structural basis for depolymerization of alginate by polysaccharide lyase family-7. J. Mol. Biol. 352: 11-21 (2005)
22. Zeller SG, Gray GR. Analysis of *macrocytis pyrifera and Pseudomonas aeruginosa* alginic acids by the reductive-cleavage method. Carbohyd. Res. 226: 313-326 (1992)