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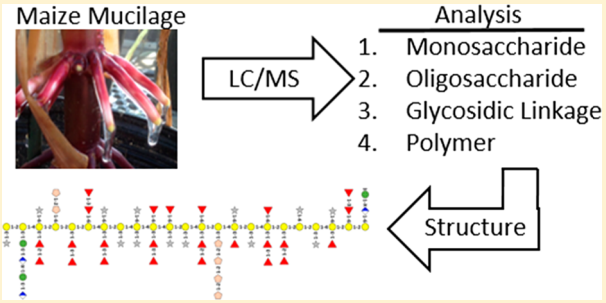
Strategy for Structural Elucidation of Polysaccharides: Elucidation of a Maize Mucilage that Harbors Diazotrophic Bacteria

Matthew J. Amicucci,[†,§] Ace G. Galermo,[†,§] Andres Guerrero,[†] Guy Treves,[†] Eshani Nandita,[†] Muchena J. Kailemia,[†] Shawn M. Higdon,[‡] Tania Pozzo,[‡] John M. Labavitch,[‡] Alan B. Bennett,[‡] and Carlito B. Lebrilla*[,†]

[†]Department of Chemistry, University of California—Davis, Davis, California 95616, United States

[‡]Department of Plant Sciences, University of California—Davis, Davis, California 95616, United States

ABSTRACT: The recruitment of a bacterial consortium by the host is a strategy not limited to animals but is also used in plants. A maize aerial root mucilage has been found that harbors nitrogen fixing bacteria that are attracted to the carbohydrate rich environment. This synbiotic relationship is facilitated by a polysaccharide, whose complicated structure has been previously unknown. In this report, we present the characterization of the maize polysaccharide by employing new analytical strategies combining chemical depolymerization, oligosaccharide sequencing, and monosaccharide and glycosidic linkage quantitation. The mucilage contains a single heterogeneous polysaccharide composed of a highly fucosylated and xylosylated galactose backbone with arabinan and mannoglucuronan branches. This unique polysaccharide structure may select for the diazotrophic community by containing monosaccharides and linkages that correspond to the glycosyl hydrolases associated with the microbial community. The elucidation of this complicated structure illustrates the power of the analytical methods, which may serve as a general platform for polysaccharide analysis in the future.



Polysaccharides are an important class of bioactive compounds that play large roles in host−microbe interactions.[1−4] However, the elucidation of their structures have remained difficult due to their large size and inherently complicated nature. Defining the structures of polysaccharides has become increasingly important as they dictate how organisms interact with their physical and biological environments. For example, pectin-derived oligogalacturonides have been found to mediate communication between plants and microorganisms that regulate plant growth and development.[5−7] Additionally, hemicellulose polysaccharides, such as xyloglucan, bind cellulose fibrils in plant primary cell walls and are responsible for cell growth and expansion.[8−10] In the context of mammalian nutrition, the nature of the monosaccharides, linkage positions, and stereochemistry of the glycosidic bonds dictate digestibility and modulate the gut-microbiome.[11−14]

This report presents a general liquid chromatography−mass spectrometry (LC-MS)-based workflow for the de novo characterization of structurally diverse polysaccharides. We demonstrate the approach by elucidating the structure of a mucilage polysaccharide secreted from the aerial roots of a landrace maize (*Zea mays Y.*) endogenous to Sierra Mixe, Mexico. The mucilage is a clear viscous gel found on the aerial roots at each node of the maize stalk and contains a diverse consortia of bacteria whose genomes are enriched in genes responsible for nitrogen fixation.[15] This variety of maize is of particular interest for its ability to fix as much as 82% of its nitrogen from the atmosphere.[16] Previous efforts in characterizing the mucilage composition revealed that it is rich in galactose, fucose, and arabinose; however, the nature of the glycosidic linkages and monomer arrangements were not elucidated.[16] This combination of monosaccharides is not commonly found in plant cell wall polysaccharides and may select for a specific mutualistic bacteria that are uniquely able to degrade and consume the mucilage polysaccharide in exchange for atmospheric nitrogen fixation. Recent work by Pozzo et al. demonstrated that the mucilage and its associated bacteria contain glycosyl hydrolases capable of depolymerizing the same monosaccharides described[17] and further supports the hypothesis that the associated rhizobiome can utilize the mucilage polysaccharide as its sole carbon source. Thus, elucidation of the mucilage polysaccharide structure has been proposed as the next-step for understanding the mechanism behind the maize host-microbe symbiosis.

The development of methods for polysaccharide characterization have lagged behind other macromolecules such as proteins and DNA. Unlike linear and template-derived proteins and DNA, polysaccharides do not consist of a single defined structure but instead represent a class of macromolecules that share similar but not exact monosaccharide and linkage composition, branching, and degree of polymerization (DP).[9,18,19] Due to these additional complications, no single

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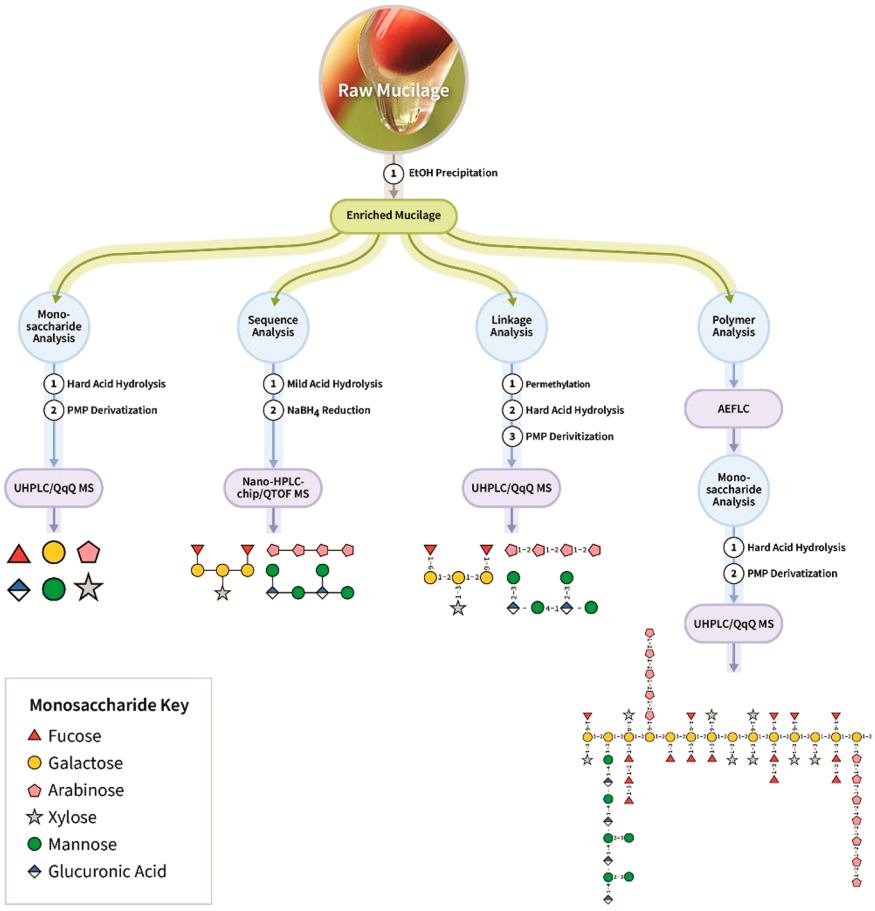

Figure 1. Workflow for the structural characterization of the maize polysaccharide using a combination of LC and LC-MS/MS methods. The workflow provides monosaccharide composition, linkage composition, and oligosaccharide structures.

method can characterize an entire polysaccharide structure. Rather, a series of orthogonal tools must be employed to probe specific defining features such as monosaccharide composition, linkage positions, and monomer arrangements. For this reason, it may take several lab groups over several years to elucidate a single polysaccharide structure using outdated analytical methodologies.[20−23]

Polysaccharide characterization requires analysis of the monosaccharide composition. The current standard method of analysis employs gas chromatography coupled to mass spectrometry (GC-MS) or flame ionization detection (GC-FID).[20,23−26] However, these methods have difficulty analyzing a broad range of monosaccharides and suffer from tedious derivatization, low sensitivity, and extensive analysis times. Methods involving high-performance anion exchange chromatography paired with pulsed amperometric detection (HPAEC-PAD) avoid chemical derivatization but also suffer in terms of sensitivity and speed.[27−30] Furthermore, extensive analysis times of 45−90 min are typically required for isomer separation by GC-MS, GC-FID, and HPAEC-PAD, rendering it difficult to obtain adequate experimental replicates. Previous methods for linkage analysis also rely on GC-MS and similarly suffer from low sensitivity and unfavorable chromatographic conditions.[21,31] For these reasons, we have recently developed methods for rapid-throughput monosaccharide and linkage analysis that employ 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization in combination with ultrahigh performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC/QqQ MS).[32,33]

A separate but necessary approach for characterizing the monosaccharide arrangements involves depolymerization of the polysaccharide into smaller oligosaccharides by either partial acid or enzymatic hydrolysis followed by analysis of the oligosaccharides.[34−40] Unlike the proteolytic enzymes employed in proteomics, a trypsin-equivalent enzyme for polysaccharides does not exist, resulting in the need for multiple enzymes to be employed. Partial acid hydrolysis has been shown to be an attractive method for generating oligosaccharides from polysaccharides.[40,41] However, commercial standards are limited and structural databases for the generated oligosaccharides do not exist, resulting in the need for characterization de novo. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques have been highlighted by our lab and elsewhere as an effective platform for de novo oligosaccharide analysis.[42,43]

In this report, a workflow integrating a series of modern LC-MS/MS techniques was developed to structurally characterize a unique maize mucilage polysaccharide (Figure 1). Partial acid hydrolysis involving trifluoroacetic acid (TFA) was performed to depolymerize polysaccharides into oligosaccharides. Nano-chip high-performance liquid chromatography paired with quadrupole time-of-flight mass spectrometry (nano-HPLC-chip-QTOF MS) was employed to characterize the oligosaccharide structures and construct a library. Semipreparative anion exchange flash liquid chromatography (AEFLC) paired with

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Table 1. Monosaccharide Composition of Enriched Mucilage Polysaccharides from the UC Davis Greenhouse (n = 3) and Linkage Compositions of Enriched Mucilage Polysaccharides from Both the UC Davis Greenhouse (n = 3) and Sierra Mixe, Mexico (n = 3)[a]

monosaccharide	monosaccharide composition (%)	linkage residue	greenhouse mucilage linkage composition (%)	Sierra Mixe mucilage linkage composition (%)
fucose	28.6 ± 0.8	T-fucose	70.3 ± 1.9	70.8 ± 4.1
		2-fucose	18.2 ± 0.9	17.4 ± 2.0
		2,X-fucose	6.2 ± 0.8	6.1 ± 1.4
		X-fucose (I)	2.9 ± 0.3	3.8 ± 0.7
		X-fucose (II)	2.5 ± 0.2	2.0 ± 0.2
galactose	22.4 ± 0.3	2,X-galactose	45.9 ± 2.5	51.7 ± 2.2
		2-galactose	27.0 ± 4.2	25.8 ± 1.2
		T-galactose	25.0 ± 2.1	17.0 ± 1.4
		3,4,6-galactose	0.9 ± 0.2	2.9 ± 0.4
		6-galactose	0.7 ± 0.0	0.9 ± 0.3
		2,X,X-galactose (II)	0.2 ± 0.0	1.2 ± 0.4
		4-galactose	0.2 ± 0.0	0.4 ± 0.1
		2,X,X-galactose (I)	0.1 ± 0.0	0.1 ± 0.0
arabinose	15.3 ± 0.2	2-f-arabinose	61.0 ± 3.7	48.8 ± 1.2
		T-f-arabinose	31.8 ± 4.1	40.5 ± 0.6
		3-f-arabinose	3.1 ± 0.8	2.5 ± 0.2
		2,X-f-arabinose	2.2 ± 1.3	2.7 ± 0.9
		5-f-arabinose	1.8 ± 0.2	5.4 ± 0.4
glucuronic acid	11.3 ± 0.2	*	*	*
xylose	11.0 ± 0.5	T-*p*-xylose	94.5 ± 1.0	80.2 ± 3.8
		3,4-*p*-xylose	4.1 ± 1.0	9.1 ± 1.9
		4-*p*-xylose	1.5 ± 0.2	10.7 ± 1.9
mannose	7.9 ± 0.2	3-mannose	55.5 ± 4.3	57.8 ± 4.3
		4-mannose	17.3 ± 4.8	23.0 ± 3.3
		2,X-mannose	15.5 ± 7.8	14.6 ± 1.4
		T-mannose	11.3 ± 2.6	0.8 ± 0.0
		2-mannose	0.4 ± 0.1	3.9 ± 0.3
glucose	1.3 ± 0.3	X,X-glucose (II)	81.0 ± 3.7	84.5 ± 1.3
		T-glucose	8.9 ± 6.0	1.1 ± 0.2
		4-glucose	8.6 ± 2.4	6.7 ± 1.4
		X,X-glucose (I)	1.5 ± 0.3	6.3 ± 0.7
		2-glucose	ND	1.4 ± 0.0
galacturonic acid	1.0 ± 0.1	*	*	*
rhamnose	0.8 ± 0.1	*	*	*
allose	0.4 ± 0.1	*	*	*

[a]Monosaccharide abundances are expressed in molar percent. Linkage compositions are reported as relative abundance. *Not available.

monosaccharide analysis was performed to determine if the mucilage is comprised of a single polysaccharide or multiple coexisting polysaccharides. The linkage compositions of maize mucilage grown in both a controlled greenhouse and the native environment were compared. The workflow provides unprecedented characterization of an important root mucilage and provides new insight into the mechanism by which the maize supports and selects for the community of diazotrophic bacteria that it relies on. This workflow is not limited to elucidating mucilage polysaccharides but will provide faster and more thorough polysaccharide characterizations that will also enhance our ability to monitor diet-microbiome interactions.

RESULTS

The mucilage was obtained from maize aerial roots grown either in Sierra Mixe, Mexico or the University of California—Davis greenhouse. The mucilage productions at the two sites were compared with the eventual goal of understanding the role that variations in local conditions and potentially local microbiomes play in mucilage biosynthesis. To better understand those effects, it was first necessary to develop a workflow for elucidating the structures of the mucilage carbohydrates (Figure 1). We used UHPLC/QqQ MS for both monosaccharide and linkage analyses. Nano-HPLC-chip-QTOF MS was used for oligosaccharide arrangement analysis. Anion-exchange flash-LC was used to determine whether the mucilage comprised of a single polysaccharide structure or a mixture of structurally different polysaccharides. Furthermore, unique to this effort was the integration of newly developed LC-MS methods for monosaccharide analysis,[32] linkage analysis,[33] and oligosaccharide sequencing that makes the structural elucidation of complicated polysaccharide structures faster and more feasible.

Mucilage Polysaccharide Is Comprised of a Unique Set of Monosaccharides. The most basic description of a polysaccharide's structure is the monosaccharide composition. Monosaccharide analysis was performed by using a combination of acid hydrolysis and PMP-derivatization followed by UHPLC/QqQ MS analysis. The method provided broad monosaccharide coverage that included both neutral and acidic monosaccharides and high reproducibility (≤0.8% standard deviation). The major

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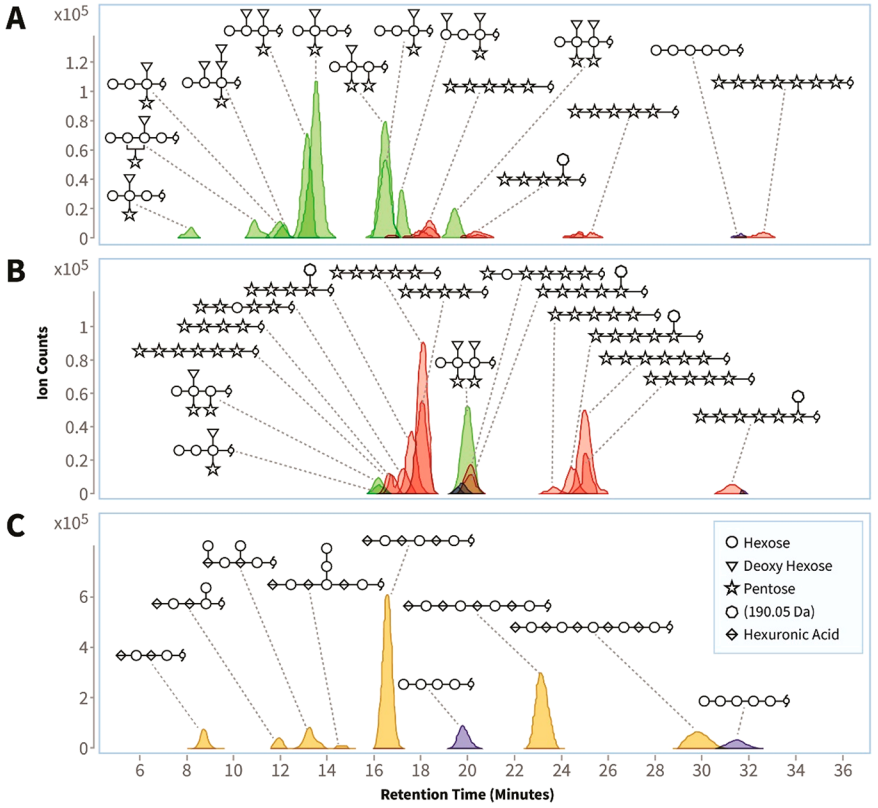

Figure 2. Extracted compound chromatograms of oligosaccharides generated from 0.5, 2, and 12 h of partial acid hydrolysis. Four classes of oligosaccharides were produced: hexose-deoxyhexose-pentose oligosaccharides (green), pentose and modified pentose oligosaccharides (red), hexose oligosaccharides (blue), and alternating hexose-hexuronic acid oligosaccharides (orange).

monosaccharides found in the enriched greenhouse grown maize mucilage includes fucose (28.6 ± 0.8%), galactose (22.4 ± 0.3%), arabinose (15.3 ± 0.2%), glucuronic acid (11.3 ± 0.2%), xylose (11.0 ± 0.5%), and mannose (7.9 ± 0.2%), while minor components include allose (0.4 ± 0.1%), glucose (1.3 ± 0.3%), rhamnose (0.8 ± 0.1%), and galacturonic acid (1.0 ± 0.1%; Table 1). The data indicates that the mucilage contains a diverse mixture of monosaccharides that result from either a highly heterogeneous polysaccharide or from several coexisting polysaccharides.

Glycosidic Linkages in the Mucilage Polysaccharide Are Highly Diverse. The aerial root mucilage polysaccharides obtained from the UC Davis greenhouse and Sierra Mixe, Mexico were characterized for their glycosidic linkages. The enriched polysaccharides were permethylated, hydrolyzed with TFA, derivatized with PMP, and analyzed using UHPLC/QqQ MS. The short-hand annotation system developed by Galermo, et al. was used to describe the identified linkage residues.[33] Rather than using (1 → 2)-galactose, (1 → 2,3)-galactose, or (1 → 2,3,4)-galactose to describe linear, bisecting, or trisecting galactose residues, the short-hand annotation, 2-galactose, 2,3-galactose, or 2,3,4-galactose is used. The designation "X" was used when a linkage isomer was monitored but the precise position was not able to be determined. Additionally, pyranose and furanose designations for pentoses were annotated with the letter "p" or "f". Roman numerals were assigned to isomers based on their elution order. A total of 31 linkage residues were identified for the two mucilage samples (Table 1). The top three most abundant linkage residues (based on relative peak area%) observed for each monosaccharide were terminal (T)-fucose (70.3 ± 1.9% UC Davis; 70.8 ± 4.1% Sierra Mixe), 2-fucose (18.2 ± 0.9%; 17.4 ± 2.0%), 2,X-fucose (6.2 ± 0.8%; 6.1 ± 1.4%), 2,X-galactose (45.9 ± 2.5%; 51.7 ± 2.2%), 2-galactose (27.0 ± 4.2%; 25.8 ± 1.2%), T-galactose (25.0 ± 2.1%; 17.0 ± 1.4%), 2-f-arabinose (61.0 ± 3.7%; 48.8 ± 1.2%), T-f-arabinose (31.8 ± 4.1%; 40.5 ± 0.6%), 5-f- arabinose (1.8 ± 0.2%; 5.4 ± 0.4%), T-*p*-xylose (94.5 ± 1.0%; 80.2 ± 3.8%), 3,4-*p*-xylose (4.1 ± 1.0%; 9.1 ± 1.9%), 4-*p*-xylose (1.5 ± 0.2%; 10.7 ± 1.9%), 3-mannose (55.5 ± 4.3%; 57.8 ± 4.3%), 4-mannose (17.3 ± 4.8; 23.0 ± 3.3), 2,X-mannose (15.5 ± 7.8%; 14.6 ± 1.4%), X,X-glucose (II) (81.0 ± 3.7%; 84.5 ± 1.3%), T-glucose (8.9 ± 6.0%; 1.1 ± 0.2%), and 4-glucose (8.6 ± 2.4%; 6.7 ± 1.4%). Standard deviations ≤7.8% were obtained for the linkage measurements. The results agree with the monosaccharide compositions data and indicate that the two mucilage samples contain polysaccharides that are nearly identical.

Characterization of Polysaccharide-Derived Oligosaccharides Reveals Three Unique Classes of Oligosaccharides. To gain further insight into the arrangement of the observed monosaccharides, the mucilage polysaccharide was incubated with TFA at an elevated temperature to produce oligosaccharides that are representative of the parent structure. The resulting oligosaccharides were analyzed using nano-HPLC/QTOF MS and structurally characterized by interpreting their CID fragmentation spectra. Incubation for 0.5, 2, and 12 h produced three unique classes of oligosaccharides, respectively: (1) deoxyhexose and pentose decorated hexose polymers, (2) pentose polymers, and (3) alternating hexose and hexuronic acid polymers (Figure 2). The three distinct groups of oligosaccharides suggested that the mucilage consisted of either a single polysaccharide with heterogeneous domains or multiple coexisting polysaccharides.

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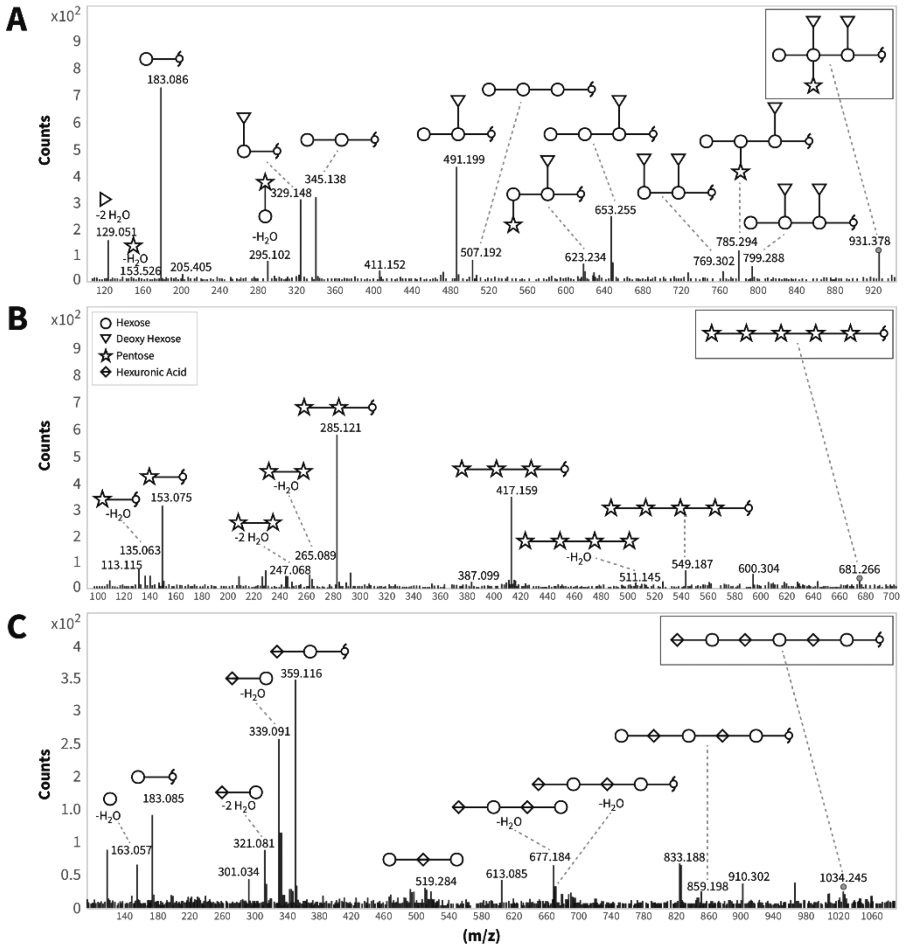

Figure 3. Representative tandem MS spectra of oligosaccharides produced by partial acid hydrolysis. (A) Structure composed of hexoses, deoxyhexoses, and pentoses produced from 0.5 h of hydrolysis at retention time 13.0 min and precursor m/z 931.38. (B) Structure composed of pentoses produced from 2 h of hydrolysis at retention time 17.6 min and precursor m/z 681.27. (C) Structure composed of alternating hexose and hexuronic acid structure from 12 h of hydrolysis at retention time 16.1 min and precursor m/z 1035.25.

In total, over 40 oligosaccharides were observed >1% relative abundance and were structurally characterized by manual interpretation of their fragmentation spectra. Examples of annotated tandem mass spectra are shown in Figure 3. Mucilage hydrolyzed for 0.5 h produced a group of oligosaccharides that mostly comprised of structures with hexose backbones and frequent, but short, branches containing one or two pentose or deoxyhexose residues. The 2 h hydrolysis condition produced a group of pentose polymers ranging from 3 to 6 pentoses in length. Occasionally, a monomer with a neutral mass loss of 190.05 Da was found to be linked within the pentose chains (Table 2). This monomer may be attributed to a singly methylated hexuronic acid and will be putatively labeled as so; this moiety is commonly found in xylans.[44,45] Hexoses were also observed to be occasionally interspersed within the pentose backbone. When the mucilage was hydrolyzed for 12 h, alternating hexose and hexuronic acid oligosaccharides, containing up to five disaccharide units, were observed. Lesser abundant species were found with terminal branching from both the hexose and hexuronic acids in the backbone. Hexose oligomers up to seven DP were also observed when the mucilage was hydrolyzed for 12 h. The characterized oligosaccharides were used to construct a library containing oligosaccharide mass, retention time, structure, peak area, and composition (Table 2). To our knowledge, this is the first reporting of partial acid hydrolysis paired with LC-MS/MS for the structural characterization of polysaccharides.

Maize Aerial Root Mucilage Is Composed of a Single Polysaccharide Structure. Semipreparative AEFLC fractionation paired with monosaccharide analysis of the enriched greenhouse polysaccharides was performed to investigate whether the oligosaccharides produced by partial acid hydrolysis represented domains of the same polysaccharide or were derived from independent polysaccharides. Anion-exchange media was utilized for the charge-based separation of neutral polysaccharides and acidic polysaccharides. A total of 84 collected fractions were analyzed for their monosaccharide composition to reconstruct the polysaccharide elution profile. A single large peak composed of fucose (39.4%), galactose (25.4%), mannose (13.2%), arabinose (10.6%), glucuronic acid (6.7%), and xylose (3.8%), was observed to elute between fractions 33 and 46 (Figure 4). This composition of monosaccharides matched that of the mucilage polysaccharide and indicates that the mucilage is comprised of a single heterogeneous polysaccharide (Table 1). Additionally, fraction 23 was found to be rich in glucose, which may be attributed to β-glucan polysaccharides commonly found in maize cell walls.[46,47]

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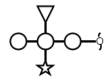
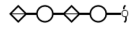
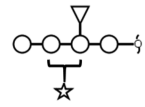
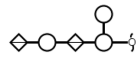
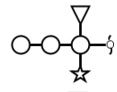
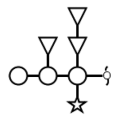
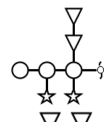
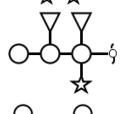
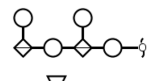
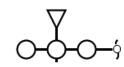
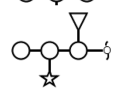
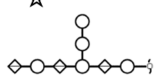
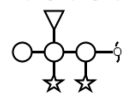
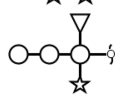
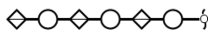
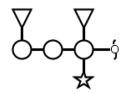
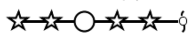
Table 2. Oligosaccharides Produced by Partial Acid Hydrolysis of the Mucilage[a]

Number	Composition	RT	Mass (exp)	Mass (cal)	Error (Da)	Abundance (0.5 hour)	Abundance (2 hours)	Abundance (12 hours)	Structure
1	31100a	7.90	784.284	784.285	0.001	196254	N.D.	N.D.	
2	20020a	8.74	696.196	696.196	0.000	N.D.	N.D.	2015952	
3	41100a	10.63	946.341	946.338	-0.003	358524	N.D.	N.D.	
4	30020a	11.68	858.250	858.249	-0.002	N.D.	N.D.	3554492	
5	31100b	11.73	784.285	784.285	-0.001	386710	N.D.	N.D.	
6	33100a	11.88	1076.400	1076.401	0.001	251888	N.D.	N.D.	
7	33200a	12.72	1208.444	1208.443	-0.001	593610	N.D.	N.D.	
8	32100a	12.89	930.345	930.343	-0.002	1891237	N.D.	N.D.	
9	40020a	13.23	1020.303	1020.302	-0.002	N.D.	N.D.	2827774	
10	31100c	13.29	784.286	784.285	-0.001	3593052	57261	N.D.	
11	31100d	13.34	784.284	784.285	0.000	N.D.	124166	N.D.	
12	50030b	14.21	1358.384	1358.387	0.003	N.D.	N.D.	2251452	
13	31200a	16.26	916.325	916.327	0.002	2598270	300511	N.D.	
14	31100e	16.27	784.284	784.285	0.001	1708464	169726	N.D.	
15	30030a	16.41	1034.275	1034.281	0.006	N.D.	N.D.	364279	
16	00600a	16.60	812.280	812.280	0.000	N.D.	344608	N.D.	
17	00400a	16.61	548.196	548.195	-0.001	24886	336961	N.D.	
18	10500a	16.78	842.292	842.291	-0.001	N.D.	307344	N.D.	
19	32100b	16.97	930.341	930.343	0.002	752914	N.D.	N.D.	
20	10400a	17.24	710.248	710.248	0.000	32549	484775	N.D.	
21	00401a	17.60	738.244	548.195	-	188614	1165884	N.D.	

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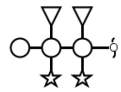
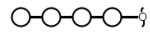
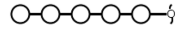
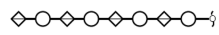
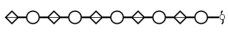
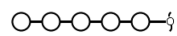
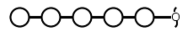
Anal. Chem. 2019, 91, 7254−7265

Table 2. continued

Number	Composition	RT	Mass (exp)	Mass (cal)	Error (Da)	Abundance (0.5 hour)	Abundance (2 hours)	Abundance (12 hours)	Structure
22	00400b	18.05	548.195	548.195	0.000	N.D.	1874697	N.D.	
23	00500a	18.05	680.237	680.238	0.001	N.D.	2993277	N.D.	
24	00400c	18.17	548.196	548.195	-0.001	261539	N.D.	N.D.	
25	00500b	18.18	680.239	680.238	-0.001	380945	N.D.	N.D.	
26	32200a	19.25	1062.383	1062.385	0.002	603591	N.D.	N.D.	
27	40000a	19.63	668.239	668.237	-0.001	35222	56402	3066616	
28	50000b	19.76	830.289	830.290	0.001	N.D.	202115	N.D.	
29	10500b	20.13	842.290	842.291	0.001	72053	371613	N.D.	
30	00501a	20.13	870.285	680.238	-	88805	523860	N.D.	
31	00401b	20.24	738.243	548.195	-	187266	1924420	N.D.	
32	00500c	20.77	680.237	680.238	0.000	116612	N.D.	N.D.	
33	40040a	23.16	1372.367	1372.366	-0.001	N.D.	N.D.	11746379	
34	00500d	23.71	680.238	680.238	0.000	N.D.	134512	N.D.	
35	00501a	24.64	870.288	680.238	-	75338	N.D.	N.D.	
36	00600b	24.99	812.279	812.280	0.001	N.D.	1881101	N.D.	
37	00500a	25.19	680.237	680.238	0.001	108730	613376	N.D.	
38	50050a	29.83	1710.447	1710.451	0.004	N.D.	N.D.	4498951	
39	00601a	31.30	1002.328	812.280	-	51189	261857	N.D.	
40	50000c	31.49	830.290	830.290	0.000	37939	N.D.	1910534	
41	00700a	32.41	944.324	944.322	-0.001	32.575	894286	N.D.	
42	50000a	34.51	830.291	830.290	-0.001	N.D.	137610	N.D.	

[a]Circles represent hexoses, stars represent pentoses, triangles represent deoxyhexoses, diamonds represent hexuronic acids, and heptagons represent the putatively labeled O-methyl hexuronic acid. A shorthand nomenclature is used to describe composition where each digit represents a monosaccharide (in the order of hexose, deoxyhexose, pentose, hexuronic acid, and the putatively labeled O-methyl hexuronic acid). The value of the digit represents the amount of each specific monosaccharide. For compounds with the same composition, the letter labels the specific isomer.

DISCUSSION

Combining information obtained from the oligosaccharide, linkage, monosaccharide, and AEFLC analysis of the mucilage enabled us to propose a polysaccharide structure (Figure 5). The two most abundant monosaccharides, galactose (22.4% mol) and fucose (28.6%), both likely comprise the observed hexose and deoxyhexose monomers in the oligosaccharides generated from the 0.5 h partial hydrolysis treatment. The primary galactose linkages observed were 2-linked (27.0%) and 2,X-linked branching (45.9%), which indicates that the backbone is comprised of a 2-linked galactose polymer with frequent fucose branches. These oligosaccharides were also observed to contain pentose branches. The monosaccharide composition showed that two pentoses, arabinose (15.3%) and xylose (11.0%), were both highly abundant. The nature of the linkages allowed us to assign xylose to the short branches because it was found to be almost exclusively terminal (94.5%). On the contrary, arabinose was primarily 2-linked (61.0%), which implies that it was

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Anal. Chem. 2019, 91, 7254−7265

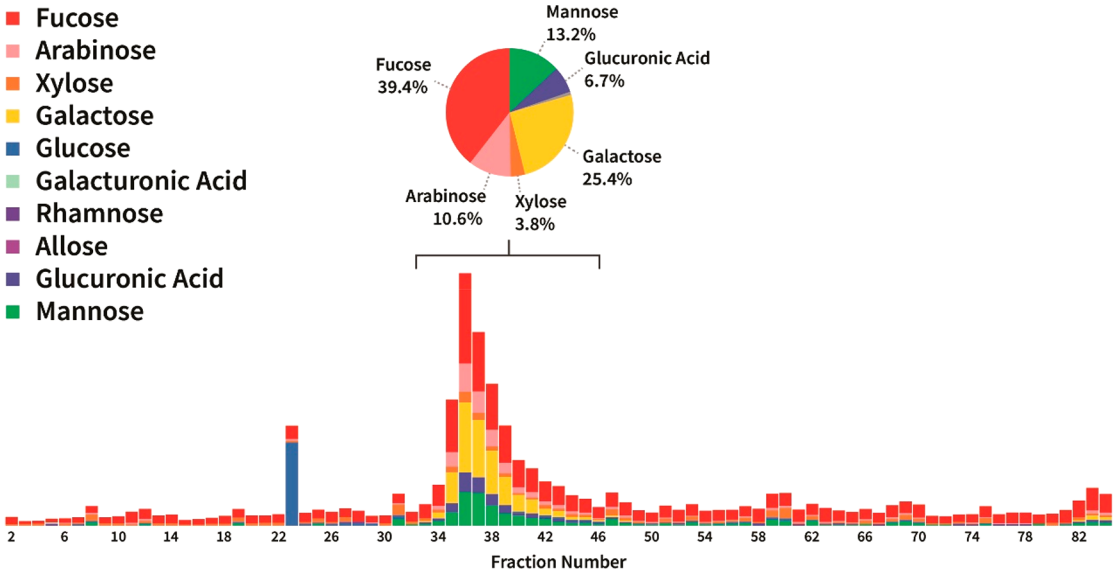

Figure 4. Mucilage polysaccharide subjected to anion exchange flash liquid chromatography. Each fraction was subjected to monosaccharide analysis. The monosaccharide compositions are stacked at their respective retention times. Pie chart shows the relative abundance of monosaccharides from the combined fractions 33 to 46.

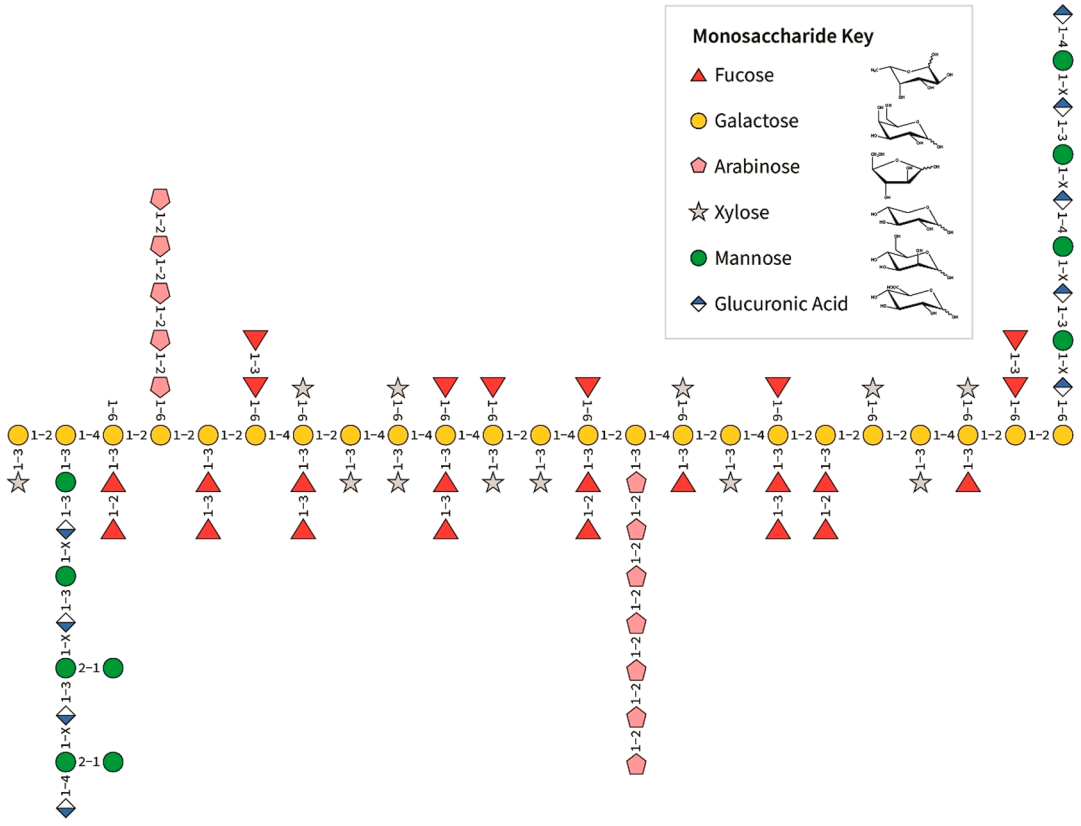

Figure 5. Proposed structure of the mucilage polysaccharide based on the monosaccharide, linkage, and oligosaccharide analyses. The polysaccharide is composed of a 2-linked galactose backbone with radiating fucose and xylose groups. Long chains of 2-linked arabinose branch from the galactose backbone. An acidic component contains alternating 4-and 3-linked mannose and glucuronic acid. The appearances of the monosaccharides and the linkages are based on their relative abundances in their respective analyses.

conjugated as a larger polymer. These results agree with the production of pentose polymers when the mucilage was hydrolyzed for 2 h and suggest that linear 2-linked arabinose chains comprise part of the polysaccharide structure. Lastly, we propose that the observed alternating hexose-hexuronic acid oligosaccharides produced by 12 h of hydrolysis, are composed on glucuronic acid (11.3%) and mannose (7.9%), based of their near equal proportions. The most abundant mannose linkages were observed to be 3-linked (55.5%), 4-linked (17.3%) and, 2,X-linked (branched, 15.5%). Several details of the mucilage structure will require further characterization, including the α/β stereochemistry of the bonds, the linkage of the glucuronic acid

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Anal. Chem. 2019, 91, 7254−7265

residues, and whether the three polymers are distributed as branches from the main backbone or as unique domains.

In the first report of the mucilage polysaccharide, Van Deynze et al. analyzed the monosaccharide composition of the maize aerial root mucilage by GC-MS, and showed a similar but not exact monosaccharide composition to our work.[16] In both analyses, fucose and galactose were the most abundant monosaccharides. In Van Dezyne's work, these two monosaccharides made up 78% of the total carbohydrate. In our work, they contributed to only 51%. Additionally, our results showed similar amounts of arabinose, but higher amounts of xylose, glucuronic acid, and mannose. Several other minor components were also found in our analysis that were not identified in the earlier work. Differences may be attributed to the analytical methods, such as LC as opposed to GC analysis, different stages of plant growth, and modulation by different bacterial communities.

Work by Pozzo et al. probed the enzymatic activity of glycosyl hydrolases from several enzyme classes that were found in the mucilage metagenome.[17] The characterized enzymes acted on the major mucilage components: galactose, mannose, fucose, xylose, and arabinose. For example, one GH 39 family enzyme was found to depolymerize $\beta(1 \rightarrow 4)$ xylose, which was found to be composed of 10% of the xylose observed in the Sierra Mixe mucilage. Another interesting enzyme from the GH 2 family can depolymerize both mannose and galactose polymers, both of which make up large components of the structure. The elucidation of the mucilage structure combined with the observed enzymatic activity from several bacteria living in the mucilage further support the hypothesis that the mucilage polysaccharide and the bacterial glycolytic machinery may have coevolved to provide a specific and mutualistic relationship. Furthermore, the high level of structural variation in the mucilage polysaccharide structure may allow the maize to selectively modulate the populations of specific communities of bacteria.

As with many biological processes, environmental and growth conditions may affect the biosynthetic pathways and resulting structure(s) of the mucilage polysaccharide. Linkage analysis was performed to determine if the maize grown in the UC Davis greenhouse produced mucilage of a similar polysaccharide composition to that of the native Sierra Mixe maize. There was very little variation between the two mucilage sources, but some variations in the ratios of the components were observed (Table 1). For example, fucose, mannose, and galactose linkages were highly similar, while some variation was observed in the arabinose and xylose constituents. Terminal arabinose was higher in the Sierra Mixe mucilage, while the 2-linked arabinose was higher in the greenhouse mucilage, which suggests that the arabinose chains in the native Sierra Mixe mucilage are shorter than those in the greenhouse. Additionally, there appeared to be more terminal xylose in the greenhouse mucilage, while the native mucilage had higher amounts of 3,4-bisecting and 4-linked xylose, which suggests that the greenhouse-grown mucilage has more frequent and shorter xylose branches. The homogeneity between the monosaccharide and linkage compositions of the two mucilages suggest that the same polysaccharide is being secreted at both sources. Small variations in the structures may have been caused by the different growing conditions, different stages of development, biological variation, or different microbial communities that have distinct carbohydrate consumption patterns.

Several researchers have studied the polysaccharide composition of maize underground root mucilage.[48−50] At the monosaccharide composition level, our results for the aerial root mucilage show some similarity to those reported for maize underground roots. One study, by Osborne et al., found that the polysaccharide fraction of the ground root mucilage contained mainly fucose (61.0%) and glucose (31.4%), while another study by Chaboud found the mucilage to contain mostly galactose (30.7%), with high amounts of fucose (19.3%), glucose (18.5%), xylose (15.2%), and arabinose (13.4%).[48,49] Both of these studies on underground root mucilage showed high amounts of fucose, xylose, arabinose, and galactose, which is similar to our analysis of the aerial root mucilage. The large amounts of glucose found in underground root mucilage differ from our aerial root analysis and may reflect the presence of different polysaccharides from different plant organs. We also found differences in the glycosidic linkages between the aerial root mucilage and the maize underground root mucilage studied by Bacic et al. For example, Bacic et al. report the presence of only 2,3-bisecting mannose residues, whereas our analysis reveals 3-, 4-linked and terminal-linked mannose structures.[50] However, Bacic reported terminal fucose as being the major linkage component, which agrees with our analysis of the aerial root mucilage. Also, galactose was reported to be predominantly 2-linked, 2,3-bisecting, and 3,6-bisecting in ground root mucilage. Similarly, a linear, bisecting, and two trisecting 2-linked galactose structures were found in our analysis. The comparison of the monosaccharide and linkage profiles of both underground and aerial root mucilage suggests that similar but not identical polysaccharides are produced in both root types.

This report lays out a series of analytical methods capable of determining the in-depth structure(s) of previously uncharacterized polysaccharides by employing a purely LC-MS based platform. We report the first structural elucidation of a polysaccharide found in maize aerial root mucilage. An oligosaccharide library was created and will be used in the future to monitor changes in distinct portions of the polysaccharide that are due, presumably, to consumption by aerial root-associated bacteria. The structural characterization of the polysaccharide will allow a more targeted approach when mining the maize genome for carbohydrate-active enzymes that may play a role in the biosynthesis, degradation, and utilization of the aerial root polysaccharide. Additionally, by laying out a platform for the fast and deep structural characterization and analysis of polysaccharides, we hope to better understand the role polysaccharides play in host-microbe interactions and aspects of plant cell wall development.

METHODS

Experimental Materials. Nanopure water was used for all experiments. Absolute ethanol (EtOH) was purchased from VWR (Radnor, PA). Solid load cartridges (25 g, 20 μm frit pore-size) were purchased from Luknova (Mansfield, MA). QAE Sephadex A-25 anion exchange media was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Nonporous graphitized carbon cartridges (GCC) (150 mg) were purchased from Grace Alltech (Columbia, MD). D-Glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, D-arabinose, D-xylose, D-ribose, D-glucuronic acid, D-galacturonic acid, ammonium acetate (NH_4Ac), TFA, iodomethane (contains copper stabilizer, 99.5%), sodium hydroxide pellets (NaOH) (semiconductor grade, 99.99% trace metals basis), ammonium hydroxide solution (NH_4OH) (28−30%, NH_3 basis), PMP, dichloro-

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Anal. Chem. 2019, 91, 7254−7265

methane (DCM), chloroform, methanol (MeOH; HPLC grade), stachyose, anhydrous dimethyl sulfoxide (DMSO), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium borohydride, and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (FA; 99.5%+ optima LC-MS grade) was purchased from Fisher Scientific (Hampton, NH). Acetonitrile (ACN; LC-MS grade) was purchased from Honeywell (Muskegon, MI). 2-*O*-(a-D-Manopyranosyl)-D-mannopyranose, 1,4-D-xylobiose, 1,5-a-L-arabinotriose, 1,3-a-1,6-a-D-mannotriose, isomaltotriose, 4-*O*-(b-D-galactopyranosyl)-D-galactopyranose, lactose, 2′-fucosyllactose (synthetic), nigerose, 3-*O*-(b-D-galactopyranosyl)-D-galactopyranose, 3-*O*-(a-D-mannopyranosyl)-D-mannopyranose, 1,4-b-D-mannotriose, maltohexaose, and 1,6-a-D-mannotriose were obtained from Carbosynth (Compton, U.K.). Sophorose and 3^3-α-L-arabinofuranosyl-xylotetraose were acquired from Megazyme (Chicago, IL). Formic acid (FA) (99.5% + optima LC-MS grade) was purchased from Fisher Scientific (Hampton, NH). Mucilage was harvested from maize grown in both the UC Davis greenhouse (Davis, CA) and Sierra Mixe region of Oaxaca, Mexico.

Establishment of the Mucilage Factory. The development of a reliable source of mucilage for structural characterization and further experiments was achieved by creating a greenhouse-based mucilage factory that simulated the environmental conditions of the Sierra Mixe region in Oaxaca, Mexico, where the maize natively grows. Plants were grown in groups of 60, and mucilage production was optimized so that mucilage could be collected from individual plants for a combined total volume of at least 20 mL per day. A 70-PSI sprinkler system was used to maintain high humidity (≥90%). Pipettes were used to collect the mucilage from the aerial roots and stored in presterilized 10 to 50 mL tubes. Samples were then either processed immediately for analysis or stored at −20 °C.

Polysaccharide Enrichment. Raw mucilage produced by the ariel roots of maize grown at the UC Davis greenhouse was collected and pooled. Mucilage from Sierra Mixe, Mexico was gifted by Alan B. Bennett. The raw mucilages were precipitated by adding absolute EtOH to make a final 80% (v/v) EtOH/mucilage (v/v) solution. The solution was homogenized by vigorous manual shaking and placed in a −80 °C freezer overnight. Next, the samples were centrifuged at 4k rpm for 30 min at 4 °C, and the supernatant was discarded. The pellet was washed with 2 × 2 mL 80% (v/v) EtOH/water and centrifuged. The supernatant was discarded and the pellet containing enriched polysaccharides was collected and stored at −20 °C until the time of analysis. All experiments were performed using enriched greenhouse mucilage polysaccharides unless otherwise noted.

Monosaccharide Composition of the Mucilage Polysaccharides. Enriched mucilage polysaccharide was prepared in a 4 M TFA solution and hydrolyzed at 100 °C for 2 h, dried by vacuum centrifugation, and subjected to PMP-derivatization and subsequent chloroform extraction described by Xu et al.[32] The AEFLC fractions were further purified using Discovery DSC-18 cartridges (Supelco, 500 mg) to remove excess salts. The DSC-18 cartridges were conditioned in the following order: 4 mL of water, 4 mL of ACN, then 4 mL. A 250 μL aliquot containing the sample was loaded onto the cartridge, desalted with 10 mL of water and then eluted with 4 mL of ACN and collected. The samples were dried by vacuum centrifugation and reconstituted to the original volume in water. The samples were analyzed by UHPLC/QqQ MS using the method developed by Xu et al.

Linkage Analysis of the Mucilage Polysaccharides. Samples were placed through the linkage analysis workflow and subjected to UHPLC/QqQ-MS analysis as described by Galermo et al.[33] Samples containing 50 μg of enriched polysaccharide were permethylated under argon atmosphere using iodomethane in a solution of DMSO containing concentrated NaOH. The samples were reacted on a shaker for 50 min at room temperature then quenched using ice-cold water. Permethylated products were extracted by liquid−liquid extraction using DCM and subsequent washes with ice-cold water. The upper aqueous layer was discarded, and the bottom organic layer was collected and dried. Acid hydrolysis and PMP-labeling was then performed using the procedure above with exception of the liquid−liquid extraction. Samples were reconstituted in 70% MeOH/water and 1 μL was subjected to UHPLC/QqQ MS analysis following the method developed by Galermo et al.

Partial Acid Hydrolysis of the Mucilage Polysaccharides. The isolated polysaccharide was reconstituted in water and subjected to hydrolysis with 50 mM TFA at 100 °C for 0.5, 1, 2, 4, 8, 12, and 16 h. Samples were then neutralized by adding a half volume of 0.1 M cold NaOH and dried under evaporative centrifugation to completion. The samples were reduced with 1 M sodium borohydride for 2 h at 60 °C. The reduced oligosaccharides were desalted and further purified using GCC.[51] Briefly, cartridges were conditioned with two volumes of water, two volumes of 80% (v/v) ACN/water, and again two times with water. Samples were loaded and desalted with five volumes of water before being eluted with two volumes of 20% (v/v) ACN/water and two volumes of 40% (v/v) ACN/water. The eluent was dried to completion by evaporative centrifugation and reconstituted in water for analysis.

Fractionation of the Mucilage Polysaccharides. A total of 5 mg of enriched mucilage polysaccharides was fractionated using anion-exchange flash liquid chromatography (AEFLC). Fractionation was performed on a Teledyne-Isco CombiFlash Rf 200 (Lincoln, NE) equipped with a column wet-packed with QAE SEPHADEX A-25 anion-exchange media (14.1 cm × 2.8 cm i.d., 40−120 μm particle size) using a 61 min binary gradient with a constant flow rate of 5 mL/min and a collection rate of collect 2 mL/fraction. Mobile phase A: 2 mM carbonate (pH 7); mobile phase B: 2 mM carbonate (pH 7) with 1 M NaCl. The following elution gradient was used: 0.00−5.00 min, 0.00% B; 5.00−33.00 min, 0.0−100.00% B; 33.00−61.00 min, 100.00% B.

Mass Spectrometry Analysis. Monosaccharide composition analysis was performed using an Agilent 6495 triple quadrupole (QqQ) equipped with an Agilent 1290 infinity II UHPLC system. Separation was performed on an Agilent ZORBAX C18 UHPLC column (2.1 × 100 mm, 1.7 μm particle size) using a 10 min binary gradient with a constant flow rate of 0.5 mL/min. Mobile phase A: 25 mM NH_4Ac (pH adjusted to 8.2 using NH_4OH) in 5% (v/v) ACN/water. Mobile phase B: 95% (v/v) ACN/water. The following elution gradient was used: 0.00−7.00 min, 12.00−15.00% B, 7.00−7.10 min, 15.00−99.00% B, 7.10−8.50 min, 99.00% B, 8.60−8.61 min, 99.00−12.00% B, 8.61−10.00 min, 12.00% B. The electrospray ionization (ESI) source was operated in positive ion mode. The QqQ was operated in dynamic multiple reaction monitoring (DMRM) mode and utilized both quantifier ion (m/z 175.0) and qualifier ions (m/z 217.1 and m/z 216.1) to monitor the PMP-labeled monosaccharides.

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Anal. Chem. 2019, 91, 7254−7265

Samples for linkage analysis were analyzed following the method developed by Galermo et al. using the UHPLC/QqQ MS system operated in multiple reaction monitoring (MRM) mode. Separation was performed on an Agilent ZORBAX RRHD ECLIPSE Plus UHPLC C18 column (2.1 × 150 mm, 1.8 μm particle size) using a 15 min binary gradient with a constant flow rate of 0.22 mL/min. The mobile phase system for monosaccharide analysis was used. The following binary gradient was used: 0.00−5.00 min, 21.00% B; 5.00−9.00 min, 21.00−22.00% B; 9.00−11.00 min, 22.00% B; 11.00−13.60 min, 22.00−24.50% B; 13.60−13.61 min, 24.50−99.00% B; 13.61−13.80 min, 99.00% B; 13.80−13.81 min, 99.00−21.00% B; 13.81−15.00 min, 21.00% B. The quantifier ion mass monitored was m/z 175.1, while the qualifier ion masses were m/z 231.2 and m/z 217.2. Specific monitoring for 2-linked residues was performed using the m/z 217.2 fragment ion. Comparison of observed linkage residue peak retention times in the mucilage polysaccharide samples were compared with the oligosaccharide standards to identify isomeric peaks. For the linkages where standards were unavailable, calculated precursor and corresponding product ion masses were used to track the peaks for future identification.

The partial acid-derived oligosaccharides were analyzed on an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 6520 Accurate Mass Q-TOF mass spectrometer equipped with a microfluidic chip cube interface (nano-HPLC-chip-QTOF MS). The microfluidic chip is composed of a porous graphitized carbon (PGC)-based enrichment column (40 nL) and analytical column (75 × 43 mm). Separation was performed using a 60 min binary gradient system with a constant flow rate of 0.4 μL/min. Mobile phase A: 3% (v/v) ACN/water in 0.1% FA. Mobile phase B: 90% (v/v) ACN/water in 0.1% FA. The following gradient was used: 0.00−2.00 min, 1.00−5.00% B, 2.00−35.00 min, 5.00−30.00% B, 35.00−40.00 min, 30.00−99.00% B, 40.00−50.00 min, 99.00−99.00% B, 50.00−51.00 min, 99.00−1.00% B, 51.00−60.00 min, 1.00% B. The ESI source was operated in the positive ion mode and data dependent fragmentation was performed on ions with an m/z of 450−3000. Data analysis was performed using Agilent Mass Hunter software. Fragmentation spectra obtain by CID was interpreted to determine the oligosaccharide composition and arrangement.

AUTHOR INFORMATION

Corresponding Author

*E-mail: cblebrilla@ucdavis.edu.

ORCID

Matthew J. Amicucci: 0000-0002-1392-9252
Ace G. Galermo: 0000-0002-5662-1987
Muchena J. Kailemia: 0000-0001-9359-1038
Carlito B. Lebrilla: 0000-0001-7190-5323

Author Contributions

M.J.A., A.G.G., and E.N. drafted the initial manuscript. M.J.A., A.G.G., E.N., A.G., G.T., J.M.L., T.P., S.H., and J.K.M. conceived and performed the experiments for the elucidation of the mucilage structure. A.B.B. and C.B.L. planned the experimental work and reviewed the manuscript

Author Contributions

§M.J.A. and A.G.G. contributed equally

Notes

The authors declare no competing financial interest.

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