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Investigations on Biologically Active Carbohydrates from Natural Sources

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**INVESTIGATIONS ON BIOLOGICALLY ACTIVE CARBOHYDRATES
FROM NATURAL SOURCES**

**BY
JIADONG SUN**

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
PHARMACEUTICAL SCIENCES**

UNIVERSITY OF RHODE ISLAND

2016

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

Complex carbohydrates exist in almost all species of plants, animals and microorganisms. Because of their physiochemical properties, they have been traditionally applied as emulsifiers and stabilizers in food, cosmetics, textiles and as pharmaceutical ingredients. Complex carbohydrates generally exist as a layer of thick, sequential, hydrous polymers consisting of monosaccharides and non-carbohydrate substituents such as acetate, pyruvate, and phosphate. While complex carbohydrates are generally believed to serve as structural support or energy sources for the producing organisms, their potential biomedical properties have been less investigated. Our understanding of the biological activities of complex carbohydrates is further complicated since most have been tested after only rough purification and their structural characteristics need to be further elucidated. In order to establish structure-activity relationships for these molecules, careful structural studies need to be undertaken.

In **Manuscript I**, a group of oligosaccharides isolated from North American cranberries (*Vaccinium macrocarpon*) was investigated for their inhibition on the biofilm formation by uropathogenic bacterium *Escherichia coli* CFT073. Most urinary tract infections (UTIs) are associated with *E. coli* that could form biofilm along the urinary tract, especially on the bladder wall, to prevent eradication by the urinal and immune system. Intake of cranberry juice is traditionally believed to have preventive

effects against UTIs but the molecular mechanism is still unclear. In contrast to the role that phenolic secondary metabolites might play in preventing UTIs, carbohydrate constituents in cranberry have not been previously investigated. In this study, we identified certain oligosaccharides that are capable for reducing biofilm formation by uropathogenic *E. coli*.

In **Manuscript II**, a chemically characterized oligosaccharide-enriched fraction purified from the North American cranberries (*Vaccinium macrocarpon*) was evaluated for its capability of inhibiting the formation of advanced glycation end-products (AGEs) and antioxidant effects. AGEs are glycation adducts formed via non-enzymatic glycation occurred between carbohydrates and proteins. Long term *in vivo* accumulation of AGEs is involved in many chronic diseases. The oligosaccharide-enriched fraction inhibited AGE formation in with comparable activity to a synthetic anti-glycating agent. In the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay, Cranf1b-CL showed antioxidant activity superior to the synthetic commercial antioxidant, butylated hydroxytoluene.

In **Manuscript III**, a Rhamnogalacturonan-I type pectic polysaccharide was isolated from ginseng root aqueous extract and shown to prevent biofilm formation by *P. aeruginosa* both *in vivo* and *in vitro*. Mechanistic study on the polysaccharide exhibited its inhibitory effect on the intracellular cyclic di-GMP level of *P. aeruginosa*,

suggesting that this polysaccharide inhibits the biofilm formation by altering a core biochemical signaling process in *P. aeruginosa* to interrupt the biofilm formation and promote the biofilm dispersal.

In **Manuscript IV**, the polymeric substances from maple syrup were investigated. By ethanol precipitation and anion exchange chromatography, polysaccharides including inulin, rhamnogalacturonan, arabinogalactan and dextran were extracted and purified, among which inulin was isolated from maple syrup for the first time. The structures of these polysaccharides were analyzed by glycosyl composition analysis, glycosyl linkage analysis and NMR. Further investigations should be focused on the biological activities of maple syrup polysaccharides and their contributions to the overall health benefits of maple syrup.

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I would like to sincerely thank my major advisor, Dr. David Rowley, for offering me the opportunity and gracious support to study and work here for the past five years. I feel very fortunate that he has mentored, encouraged and challenged me to become a better scientist. More importantly, as I came here as an international student, I am very grateful that he has been providing great help, understanding and patience in my life thousands of miles away from home. It has been a great honor to work with him and I would be looking forward to collaborating with him in my future career.

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Lastly but not least, I would like to acknowledge my family. Special thanks to my wife, Fang Wang. Her love, support and sacrifices make my PhD journey much easier and

happier. For my parents, I would like to thank for their unconditional love and encouragement throughout my life.

PREFACE

This dissertation is written based on guidelines established by Graduate School of University of Rhode Island. The dissertation is written in manuscript format and composed of the following four manuscripts:

MANUSCRIPT I: Cranberry (*Vaccinium macrocarpon*) oligosaccharides decrease biofilm formation by uropathogenic *Escherichia coli*

This manuscript was published in “Journal of Functional Foods”, 2015, 17, 235-242.

MANUSCRIPT II: Cranberry (*Vaccinium macrocarpon*) Oligosaccharides Inhibit the Formation of Advanced Glycation End-Products

This manuscript was published in “Journal of Berry Research”, 2016, 6, 149-158.

MANUSCRIPT III: Pectic polysaccharide from *Panax ginseng* decreases *Pseudomonas aeruginosa* intracellular cyclic di-GMP level and disperses *in vivo* biofilm

This manuscript was prepared for submission to “Journal of Agricultural and Food Chemistry”.

MANUSCRIPT IV: Detection of inulin, a prebiotic polysaccharide in maple syrup

This manuscript was published in “Journal of Agricultural and Food Chemistry”, 2016,
DOI: 10.1021/acs.jafc.6b03139.

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LIST OF ABBREVIATIONS

UTI, urinary tract infection; AGEs, advanced glycation end-products; DPPH, 1,1-Diphenyl-2-picryl-hydrazyl; GMP, guanosine monophosphate; MALDI-TOF-MS, matrix assisted laser desorption/ionization-time of flight-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance; PACs, proanthocyanidines; LB, lysogeny broth; FPLC, fast protein liquid chromatography; HPSEC, high performance size exclusion chromatography; PMAAs, Partially methylated acetate alditols; BSA, bovine serum albumin; BHT, butylated hydroxytoluene; 2,5-DHB, 2,5-dihydroxy benzoic acid; TFA, trifluoroacetic acid; MGO, methylglyoxal; PD, 1,2-phenylenediamine; DQ, 2,3-dimethylquinoxaline; AG, aminoguanidine hydrochloride; SPE, solid phase extraction; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detector; CF, cystic fibrosis; GTP, guanosine-5'-triphosphate; DGC, diguanylyl cyclases; PDE, phosphodiesterases; pGpG, 5'-phosphoguanylyl-(3'→5')-guanosine phosphate; ELSD, evaporative light scattering detector; OD, optical rotation; CFU, colony-forming units; PBS, phosphate buffered saline; AUC, area under curve; MSD, mass selective detector; HG, homogalacturonan; RG, rhamnogalacturonan; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy.

MANUSCRIPT I

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Cranberry (*Vaccinium macrocarpon*) oligosaccharides decrease biofilm formation by uropathogenic *Escherichia coli*

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ABSTRACT

The preventive effects of the American cranberry (*Vaccinium macrocarpon*) against urinary tract infections are supported by extensive studies which have primarily focused on its phenolic constituents. Herein, a phenolic-free carbohydrate fraction (designated cranf1b-F2) was purified from cranberry fruit using ion exchange and size exclusion chromatography. MALDI-TOF-MS analysis revealed that the cranf1b-F2 constituents are predominantly oligosaccharides possessing various degrees of polymerisation and further structural analysis (by GC-MS and NMR) revealed mainly xyloglucan and arabinan residues. In antimicrobial assays, cranf1b-F2 (at 1.25 mg/mL concentration) reduced biofilm production by the uropathogenic *Escherichia coli* CFT073 strain by over 50% but did not inhibit bacterial growth. Cranf1b-F2 (ranging from 0.625 - 10 mg/mL) also inhibited biofilm formation of the non-pathogenic *E. coli* MG1655 strain up to 60% in a concentration-dependent manner. These results suggest that cranberry oligosaccharides, in addition to its phenolic constituents, may play a role in its preventive effects against urinary tract infections.

Keywords: American cranberry, *Vaccinium macrocarpon*, phenolic, oligosaccharide, biofilm, *Escherichia coli*

1. Introduction

Urinary tract infections (UTI) commonly occur anywhere from the kidney in the upper urinary tract to the bladder in the lower urinary tract. Although UTIs are generally easy to treat with antibiotics, acute infections can be dangerous for elderly, infant and immunocompromised patients (Jepson, Williams, & Craig, 2012). Some UTI patients can experience frequent recurrent infections and increased susceptibility to drug resistant uropathogens (Jepson et al., 2012; Reid et al., 2001). Over 80% of UTIs are associated with *Escherichia coli*, which may be transmitted from the bowel to urethra. Biofilms that form on the bladder wall help prevent the bacteria from being eradicated by the immune system and antibiotics (Anderson et al., 2003; Moreno et al., 2008). Evidence suggests that consumption of the American cranberry (*Vaccinium macrocarpon* Aiton) juice can inhibit the presence of bacteria in urine and reduce UTI symptoms associated with bacteriuria and pyuria (Avorn et al., 1994; Reid et al., 2001). Our group (LaPlante, Sarkisian, Woodmansee, Rowley, & Seeram, 2012), and others (Côté et al., 2011; Iswaldi et al., 2012; Lian, Maseko, Rhee, & Ng, 2012) have studied the antimicrobial effects of the phenolic constituents of cranberries. Some studies (Foo, Lu, Howell, & Vorsa, 2000a, 2000b; Gupta et al., 2012; Howell et al., 2005) have shown that cranberry proanthocyanidins (commonly known as PACs), with at least one A-type linkage, inhibit the adherence of type p-fimbriated *E. coli* to uroepithelial cells and human red blood cells. The chemistry of cranberry PACs (Lee, 2013) and their absorption and metabolism have been studied (Ou & Gu, 2014). However, the non-phenolic constituents in cranberry have been less investigated (Hotchkiss, Nunez, Khoo, & Strahan, 2013). Herein, we provide the first report describing the structural

characterization of a phenolic-free carbohydrate fraction purified from cranberry and its evaluation for inhibition of biofilm formation by both uropathogenic (*E. coli* CFT073) and non-pathogenic (*E. coli* MG1655) strains of *E. coli*.

2. Materials and methods

2.1. Bacterial strains and media

E. coli strains CFT073 and MG1655 were gifts from Dr. Paul Cohen (University of Rhode Island). Lysogeny Broth (LB) medium (BD, NJ, USA) was supplemented with 5 g/L dextrose. M63 medium (Bioworld, OH, USA) was supplemented with 1 mM MgSO₄, 2 g/L dextrose and 5 g/L casamino acid.

2.2. Fractionation of cranberry materials

2.2.1. Purification of crude cranberry hull extract (Cranf1)

Scheme S1 (see Supplementary data) shows the fractionation flow chart of cranberry materials with yields and their total phenolic contents. Briefly, a pectinase (Klerzyme 150, DSM Food Specialties, South Bend, IN, USA) degraded cranberry hull extract (Cranf1) was fractionated using an Agilent 971-FP flash purification system (Agilent Technologies, Santa Clara, CA, USA) with Biotage SNAP KP-C18-HS 120 g cartridges (Biotage, Charlotte, NC, USA). 50 mL of Cranf1 aqueous solution (100 mg/mL) was loaded onto the pre-conditioned C18 column cartridge and eluted sequentially with 500 mL of de-ionised H₂O, 500 mL of 15% methanol/water, and finally 500 mL of MeOH at 35 mL/min. Fractions eluted with 100% water were pooled as Cranf1W with a yield of 38.1% (w/w), fractions eluted with 15% methanol

were pooled as Cranf1b with a yield of 23.8%, and fractions eluted with 100% methanol were pooled as Cranf1M with a yield of 28.1% (see Scheme S1, Supplementary data).

2.2.2. Purification of oligosaccharide enriched fraction Cranf1b

Cranf1b was introduced onto an anion exchange column (Sephacryl S-100 HR 16/10, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and eluted with step-wise gradient of NaCl aqueous solution (0-1 M) at 5 mL/min on a ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences). Ten mL fractions were collected and assayed for total carbohydrate content assay. (Masuko et al., 2005) The pooled carbohydrate-containing fractions were freeze-dried and desalted (10×300 mm Bio-gel P2 column; BIO-RAD, Hercules, CA, USA). The constituents that eluted with 100% de-ionised H₂O and 0.1 M NaCl were combined and further purified by gel filtration (Sephacryl S-100 HR 16/60, GE Healthcare Life Sciences; elution with de-ionised H₂O at 0.25 mL/min), yielding two fractions designated as cranf1b-F1 and cranf1b-F2.

2.3. Biofilm assay

The antibiofilm property of the cranberry materials was measured against *E. coli* CFT073 and MG1655 using a modified crystal violet staining method in round bottom 96-well microtiter plates (George, 2011; Naves et al., 2008; Niu & Gilbert, 2004). Bacteria colonies from TSA plates were inoculated into LB broth and incubated at 37 °C with 175 rpm shaking for 24 h. The cultures were then diluted 100-fold in M63

medium, distributed in microtiter wells, and treated with a series of two-fold dilutions of test samples (10 - 0.019 mg/mL). The plates were incubated at 37 °C for 6 h or 48 h, gently washed with de-ionised water, and stained with 125 µL of 0.1% crystal violet solution for 15 min. The solution was removed and the wells were again gently washed with de-ionised water and dried for 1 h. 125 µL of 30% acetic acid solution was added to each well and incubated for 15 min. 100 µL from each well was transferred to a flat bottom microtiter plate and the OD₅₅₀ was measured (Spectramax M2, Molecular devices, Sunnyvale, CA, USA). Percent biofilm formation was calculated as the average OD₅₅₀ of three replicate treatment wells divided by average OD₅₅₀ of replicate control wells (30 wells/plate). Each experiment was conducted in duplicate.

2.4. High Performance Size Exclusion Chromatography (HPSEC)

HPSEC was carried out at 40 °C on a TSKgel G3000PW column [7.5 × 300 mm column, Tosoh Bioscience LLC, King of Prussia, PA, USA; Hitachi LaChrom Elite HPLC, Tokyo, Japan; 0.6 mL/min de-ionised water, refractive index (RI) detection]. The molecular weights of compounds were determined by comparison of retention times to a standard curve (Supplementary Fig. S1) generated with standard dextrans of molecular weights ranging from 1,000 to 50,000 Daltons.

2.5. Glycosyl composition analysis

Sugar composition was determined by GC-MS analysis of monosaccharides (York, Darvill, McNeil, Stevenson, & Albersheim, 1986). Briefly, 100 µg of sample was

hydrolysed with 2 M TFA for 2 h at 121 °C. The hydrolyte was reduced with sodium borodeuteride (NaBD₄) at room temperature for 1.5 h. The reduced monosaccharides were O-acetylated with acetic anhydride at 50 °C for 20 min. The resulting product was extracted with dichloromethane and analysed by GC-MS (DB-1 column, GC Model 6890/MS Model 5973, Agilent Technologies, Santa Clara, CA, USA). The monosaccharide composition was determined by comparison with a GC-MS profile of monosaccharide standards.

2.6. Glycosyl linkage analysis

Partially methylated acetate alditols (PMAAs) of cranf1b-F2 were analysed by GC-MS (Ciucanu & Kerek, 1984; York et al., 1986). Briefly, 600 µg of sample was permethylated with iodomethane and concentrated sodium hydroxide in DMSO. The permethylated oligosaccharide was hydrolysed with 2 M TFA and reduced with NaBD₄. The sample was then acetylated with acetic anhydride and extracted with dichloromethane. GC-MS analysis was conducted using a Supelco SP2331 column (Sigma-Aldrich, St. Louis, MO, USA). The GC-MS profile was analysed by comparison of retention time and electron-impact fragmentation spectra with PMAA standards.

2.7. NMR analysis

The cranf1b-F2 was deuterium exchanged twice by D₂O shake and dissolved in D₂O with addition of 1 µL of DMSO as internal reference. ¹H, ¹³C, 2D COSY, TOCSY, NOESY, HSQC and HMBC spectra were obtained on a 500 MHz NMR spectrometer

(Varian VNMRS 500MHz, Agilent Technologies) at 25 °C.

2.8. MALDI mass spectrometry

Cranf1b-F2 (1 mg/mL in H₂O) was mixed with 2,3-dihydrobenzoic acid (DHB) matrix solution (v/v=1:1). Two µL of the mixture was analysed by MALDI-TOF-MS (Axima Performance, Shimadzu, Kyoto, Japan) in positive reflectron mode with power set at 80kV. 500 profiles were collected for each experiment.

3. Results and discussion

In this study, we investigated a carbohydrate fraction extracted from cranberry and evaluated its inhibitory effect on biofilm formation of two strains of *E. coli*. The ¹H NMR spectra of the original cranberry starting material (Cranf1) and its three major purified fractions namely, Cranf1W, Cranf1b and Cranf1M were obtained (see Supplementary data). The ¹H NMR spectrum of Cranf1b showed only trace resonances above 7.0 ppm, indicating that phenolics were mostly removed by C18 column chromatography. The crude cranberry extract cranf1b was purified by anion exchange chromatography and four fractions, cranf1b-F1 (64.0%), cranf1b-F2 (17.5%), cranf1b-F3 (2.5%) and cranf1b-F4 (<1%), were collected (Figure 1a). Due to the limited quantities of the latter fractions, only cranf1b-F1 and cranf1b-F2 were further studied. Cranf1b-F1 and cranf1b-F2 were next purified by gel filtration, resulting in only one peak for each sample (Figure 1b).

The homogeneity of cranf1b-F2 was further confirmed by HPSEC profile (Supplementary Fig. S6) and the average molecular size was predicted to be 1370 Da.

However, MALDI-TOF MS spectrometry of cranf1b-F2 produced a series of oligosaccharide sodium adduct ions (Supplementary Fig. S7), revealing it to be a mixture of oligomers within a close molecular weight range. The ions at approximately 1055, 1085, 1217, 1247, 1349, 1379, 1511, 1541 can be attributed to Hex₃Pen₄ (5 hexoses and 4 pentoses), Hex₄Pen₃, Hex₄Pen₄, Hex₅Pen₃, Hex₄Pen₅, Hex₅Pen₄, Hex₅Pen₅ and Hex₆Pen₄, respectively. Clusters of less abundant ions were observed above 1700 representing oligosaccharides with degrees of polymerisation (DP) larger than 11.

Figure 1a. Elution profile of Cranf1b on Sepharose Q XL 16/10 column, eluted by stepwise gradient of NaCl (0-1 M) (total sugars, -.-).

Figure 1b. Elution profile of Cranf1b-F2 on Sephacryl S-100 HR 16/60 column, eluted by de-ionised water (total sugars, -■-).

The GC-MS profile (Supplementary Fig. S8a) of the monosaccharide acetate alditols (Table 1) indicated that the cranf1b-F2 was primarily composed of arabinose (46%), glucose (40%), xylose (12%) and trace quantities of galactose (2%). The predominance of glucose, xylose and arabinose suggests that cranf1b-F2 is likely a xyloglucan (FRY, 1989; McNeil, Darvill, Fry, & Albersheim, 1984).

Glycosyl linkages of each monosaccharide are listed in Table 1 (GC-MS profile see Supplementary Fig. S8b). In addition to the common glycosyl linkages known for xyloglucan (Fry et al., 1993) 5- α -Arab, 3- α -Arab and 3,5- α -Arab were also found in cranf1b-F2. These additional linkages are consistent with arabinan side chains that are commonly present in cell-wall pectic substances (Caffall & Mohnen, 2009). In

xyloglucan nomenclature for side chain subunits (Fry et al., 1993) cranf1b-F2 glycosyl linkages belong to side chain subunits S, L, X and G. ^1H and ^{13}C NMR chemical shifts were assigned for the identified cranf1b-F2 subunits (Table 1) based on the recorded 1D NMR and 2D NMR spectra (see Supplementary data) and in consideration of previous reports (Busato et al., 2005; Hoffman et al., 2005; Jia, Cash, Darvill, & York, 2005; Shakhmatov, Toukach, Michailowa, & Makarova, 2014; Watt, Brasch, Larsen, & Melton, 1999).

Although commonly found as separate polymer components of plant cell walls, a portion of xyloglucan and pectic polysaccharides are proposed to be covalently bound (Femenia, Rigby, Selvendran, & Waldron, 1999; Popper & Fry, 2005, 2008; Thompson & Fry, 2000; Vidal, Williams, Doco, Moutounet, & Pellerin, 2003). The putative xyloglucan-pectin complex model was first introduced by Albersheim and coworkers in 1973 (Keegstra, Talmadge, Bauer, & Albersheim, 1973).

Table 1. ^{13}C NMR and ^1H NMR chemical shifts (δ in ppm) for cranf1b-F2.

Residue (Mol %)	Subunits	Linkages	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
Araf (56%)	Arabinan	t- α -Araf	107.69	81.56	77.17	84.58	61.75	-
			5.14	4.13	3.95	4.03	3.84	-
	Arabinan	3,5- α -Araf	108.11	79.83	82.90	82.11	67.14	-
			5.11	4.28	4.09	4.30	3.83/3.93	-
	Arabinan	5- α -Araf	108.15	81.53	77.25	83.02	66.89	-
			5.08	4.12	4.02	4.21	3.88/3.79	-
	Arabinan	3- α -Araf	107.72	80.26	84.33	83.09	-	-
			5.18	4.36	3.95	4.14	-	-
	S	t- α -Araf	109.87	81.66	77.05	84.44	61.81	-
			5.15	4.19	3.93	4.06	3.71	-
Xylp (14%)	S	2- α -Xylp	99.20	79.47	72.47	70.03	61.87	-
			5.08	3.56	3.85	3.65	3.55	-
	L	2- α -Xylp	98.98	81.14	-	-	-	-
			5.14	3.6	-	-	-	-
	X	t- α -Xylp	99.48	72.06	73.67	70.14	-	-
			4.94	3.54	3.71	3.61	-	-
Galp (2%)	L	t- β -Galp	105.10	-	-	-	-	-
			4.60-3.73	-	-	-	-	-
Glc p (27%)	_G	t- β -Glc p	105.24	-	-	-	-	-
			4.53	3.62	-	-	-	-
	<u>G</u>	4,6- β -Glc p	103.35	73.5	74.77	79.74	74.32	67.47
			4.53	3.38	3.66	3.67	3.82	3.87/3.80
	<u>G</u>	4,6- β -Glc p	103.20	73.5	74.77	79.64	74.32	67.04
			4.52	3.37	3.66	3.69	3.82	3.93/3.82
	G	4- β -Glc p	103.10	73.79	76.11	79.55	-	-
			4.51	3.3	3.49	3.54	-	-
	G_	α -Glc p	92.40	71.85	-	-	-	-
			5.21	3.57	3.82	3.64	3.94	3.86
	G_	β -Glc p	96.34	74.44	75.32	81.15	75.41	60.52
			4.65	3.28	3.63	3.62	3.59	3.80/3.94

G = -4)- β -D-Glc p-(1-

S = α -L-Araf-(1-2)- α -D-Xylp-(1-6)- β -D-Glc p-(1-

L = β -D-Galp-(1-2)- α -D-Xylp-(1-6)- β -D-Glc p-(1-

X = α -D-Xylp-(1-6)- β -D-Glc p-(1-

G: -4)- β -D-Glc p-(1-

G_: Reducing end glucose

_G: Non-reducing end glucose

Thompson and Fry (Thompson & Fry, 2000) observed xyloglucan that co-eluted with anionic pectin during anion exchange chromatography and remained part of the complex after treatment with 8 M urea, 6 M NaOH and proteinase. Treatment with arabinanase and/or galactanase converted a great portion of the complex into neutral compounds, suggesting that covalent bonding occurs between xyloglucan and the Ara/Gal-rich pectic domain, likely on the arabinan and/or arabinogalactan side chains of a Rhamnogalacturan I region (Abdel-Massih, Baydoun, & Brett, 2003; Popper & Fry, 2005; Thompson & Fry, 2000). However, no NMR spectroscopic evidence for a covalent linkage has yet been reported. In our study, co-elution of the xyloglucan and arabinan components of cranf1b-F2 in every chromatography step, coupled with its slight acidity, (Thompson & Fry, 2000) suggests the existence of a covalent linkage.

The original cranberry material (Cranf1) and its three major purified fractions, namely, Cranf1W, Cranf1b and Cranf1M were tested for the prevention of biofilm formation against *E. coli* MG1655, a non-uropathogenic strain, and *E. coli* CFT073, a well-studied uropathogenic strain (Welch et al., 2002) (see Table S1, Supplementary data). At equivalent concentrations (1.25 mg/mL), Cranf1b showed the most reduction in biofilm formation against the uropathogenic *E. coli* CFT073 strain, therefore its sub-fractions, Cranf1b-F1 and cranf1b-F2 were further tested against this strain. Although no activity was observed for cranf1b-F1, cranf1b-F2 reduced biofilm formation of *E. coli* CFT-073 by as much as 50 % at 1.25 mg/mL after 6 h of incubation (Figure 2a). The reductive effect on biofilm formation was maintained for at least 48 h (Figure 2a) with no growth inhibition, demonstrating that the reduced

biofilm after 6 h is not merely due to a delay in the initiation of biofilm production.

Figure 2a. Inhibition of *E. coli* CFT073 biofilm formation by Cranf1b-F2 at concentration from 0.019 mg/mL to 10 mg/mL.

Figure 2b. Inhibition of *E. coli* MG1655 biofilm formation by Cranf1b-F2 at concentration from 0.019 mg/mL to 10 mg/mL.

Interestingly, the highest inhibitory effect was not achieved at the highest concentration tested. While the reason for the declining prevention at higher concentration is not yet known, we hypothesise that aggregation of the cranf1b-F2 sample may be partially responsible. HPSEC analysis showed that large particles (>100,000 Da) formed at the higher concentration (Supplementary Fig. S6). Aggregation of oligosaccharides would lead to less concentration of active molecules in solution, hence having a potential impact on the overall activity. Biofilm formation by *E. coli* MG1655 was also sensitive to the effects of cranf1b-F2 (Figure 2b), but not to cranf1b-F1. A concentration-dependent reduction in biofilm formation was observed between 10 and 0.625 mg/mL; however, an increase in biofilm formation was consistently observed between 0.625 and 0.156 mg/mL of cranf1b-F2. The distinct dose-response patterns between CFT073 and MG1655 may derive from their different abilities to form and sustain biofilms. MG1655 naturally produces much lighter biofilm than CFT073, which likely makes it more vulnerable to biofilm modifying agents.

As previously discussed, the role of the polyphenols (including PACs) present in cranberries in its preventive effects against urinary tract infections has been extensively studied by several groups (LaPlante, Sarkisian, Woodmansee, Rowley, & Seeram, 2012; Gupta et al., 2012; Howell et al., 2005). Thus, it is possible that the multiple constituents, including polyphenols and oligosaccharides, present in the cranberry whole fruit act additively, complementarily, and/or synergistically in its overall biological effects. Interestingly, in the current study, we did not observe any

growth inhibitory and anti-biofilm effects of the Cranf1M fraction (which was enriched in polyphenol constituents) on both of the *E. coli* strains which was in agreement with our previous report (LaPlante, Sarkisian, Woodmansee, Rowley, & Seeram, 2012). Therefore, while it appears that the phenolic constituents did not contribute to the inhibition of biofilm formation by the uropathogenic *E. coli* CFT073 strain (based on our bioassays), their overall contribution to the prevention of urinary tract infections by the whole cranberry fruit should not be discounted.

4. Conclusion

In conclusion, our study demonstrates that a phenolic-free, oligosaccharide component of cranberry modifies the biofilm formation of *E. coli* strains CFT073 and MG1655. Thus, in addition to PACs and other polyphenols, certain carbohydrate components in cranberry may also contribute to its overall anti-infective properties. Further investigation to clarify the structure-activity relationships of these oligosaccharides is currently being pursued by our group.

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Supplementary data

Scheme of fractionation of cranberry materials. Detailed structural analysis data of cranberry materials including Cranf1, Cranf1W, Cranf1b, Cranf1M and cranf1b-F2. Complementary biofilm assay results of Cranf1, Cranf1W, Cranf1b and Cranf1M.

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Table S1. Biofilm assay results of Cranf1, Cranf1W, Cranf1b and Cranf1M.

Scheme S1

Sample	Total phenolic content (mg/100 g of gallic acid equivalents)*
Cranf1	5173 ± 12
Cranf1W	180 ± 4
Cranf1b	3833 ± 88
Cranf1M	15706 ± 457

Scheme S1. Fractionation flow chart of cranberry materials with yields and their total phenolic contents.

***Total phenolic contents were measured by the Folin-Ciocalteu method.**

Figure S1

Figure S1. Standard curve of dextrans (MW 1000 to 50000 Da) on TSKgel G3000PW column.

Figure S2

Figure S2. ^1H NMR of Cranf1.

Figure S3

Figure S3. ^1H NMR of Cranf1W.

Figure S4

Figure S4. ^1H NMR of Cranf1b.

Figure S5

Figure S5. ^1H NMR of Cranf1M.

Figure S6

Figure S6. Normalized HPSEC profiles of 10 mg/mL (blue) and 50 mg/mL (red) of Cranf1b-F2.

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Figure S8a. GC-MS profile of Cranf1b-F2 glycosyl composition analysis.

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Figure S9

Figure S9. ^1H NMR of Cranf1b-F2.

Figure S10

Figure S10. ^{13}C NMR of Cranf1b-F2.

Figure S11

Figure S11. gCOSY NMR of Cranf1b-F2.

Figure S12

Figure S12. zTOCSY NMR of Cranf1b-F2.

Figure S13

Figure S13. gHSQC NMR of Cranf1b-F2.

Figure S14

Figure S14. gHMBC NMR of Cranf1b-F2.

Figure S15

Figure S15. NOESY NMR of Cranf1b-F2.

Table S1

Sample \ Strai	<i>E. Coli</i> CFT073	<i>E. Coli</i> MG1655
Cranf1	N.R.*.	N.R.
Cranf1W	9.1%	49.7%
Cranf1b	12.6%	43.4%
Cranf1M	N.R.	N.R.

Table S1. Percentage reduction in biofilm formation of *E. coli* strains CFT073 and MG1655 by Cranf1, Cranf1W, Cranf1b and Cranf1M at 1.25 mg/mL.

***N.R.: No observable reduction in biofilm formation.**

MANUSCRIPT II

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Effect of Cranberry (*Vaccinium macrocarpon*) Oligosaccharides on the Formation of Advanced Glycation End-Products

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ABSTRACT

BACKGROUND: The formation and accumulation of advanced glycation end-products (AGEs) are implicated in several chronic human illnesses including type-2 diabetes, renal failure, and neurodegenerative diseases. The cranberry (*Vaccinium macrocarpon*) fruit has been previously reported to show anti-AGEs effects, attributed primarily to its phenolic constituents. However, there is lack of similar data on the non-phenolic constituents found in the cranberry fruit, in particular, its carbohydrate constituents. Herein, a chemically characterized oligosaccharide-enriched fraction purified from the cranberry fruit was evaluated for its potential anti-AGEs and free radical scavenging effects.

OBJECTIVE: The aim of this study was to evaluate the *in vitro* anti-AGEs and free radical scavenging effects of a chemically characterized oligosaccharide-enriched fraction purified from the North American cranberry (*Vaccinium macrocarpon*) fruit.

METHOD: The cranberry oligosaccharide-enriched fraction was purified from cranberry hull powder and characterized based on spectroscopic and spectrometric (NMR, MALDI-TOF-MS, and HPAEC-PAD) data. The oligosaccharide-enriched fraction was evaluated for its anti-AGEs and free radical scavenging effects by the bovine serum albumin-fructose, and DPPH assays, respectively.

RESULTS: Fractionation of cranberry hull material yielded an oligosaccharide-enriched fraction named Cranf1b-CL. The ^1H NMR and

MALDI-TOF-MS revealed that Cranf1b-CL consists of oligosaccharides ranging primarily from 6-mers to 9-mers. The monosaccharide composition of Cranf1b-CL was arabinose (25%), galactose (5%), glucose (47%) and xylose (23%). In the bovine serum albumin-fructose assay, Cranf1b-CL inhibited AGEs formation in a concentration-dependent manner with comparable activity to the synthetic antiglycating agent, aminoguanidine, used as the positive control (57 vs. 75%; both at 500 µg/mL). In the DPPH free radical scavenging assay, Cranf1b-CL showed superior activity to the synthetic commercial antioxidant, butylated hydroxytoluene, used as the positive control (IC_{50} = 680 vs. 2200 µg/mL, respectively).

CONCLUSION: The *in vitro* anti-AGEs and free radical scavenging effects of cranberry oligosaccharides support previous data suggesting that these constituents may also contribute to biological effects of the whole fruit beyond its phenolic constituents alone. Also, this is the first study to evaluate a chemically characterized oligosaccharide fraction purified from the North American cranberry fruit for anti-AGEs and free radical scavenging properties.

Keywords: *Vaccinium macrocarpon*, cranberry, oligosaccharides, advanced glycation end-products, free radical scavenging

1. Introduction

The North American cranberry (*Vaccinium macrocarpon* Aiton) is widely consumed worldwide, both as a fruit and as processed products such as juice, sauces, and extracts. The cranberry fruit is a rich source of phytochemicals [1], in particular phenolic compounds belonging to the sub-classes flavonoids [2, 3], and proanthocyanidins (PACs) [4]. Moreover, phenolic-enriched cranberry extracts, and singly purified cranberry phenolic constituents have been extensively studied for a vast array of biological activities including antioxidant, antimicrobial, anti-inflammatory, anticancer, and anti-diabetic effects [5]. While the majority of these biological studies have focused on cranberry's phenolic constituents, emerging data, including recent studies from our group and others [6, 7], suggest that the non-phenolic constituents, especially oligosaccharides, may also play a role in its health benefits.

Glycation occurs spontaneously both *in vitro* and *in vivo* wherein protein side chains non-enzymatically react with carbohydrates to form glycation adducts, referred to as advanced glycation end-products (AGEs). The gradual accumulation of AGEs *in vivo* has been implicated in the pathology of many chronic diseases including diabetic complications and neurodegenerative disorders [8, 9]. The build-up of AGEs is deleterious for two reasons. First, AGEs bind specifically to proteins including the receptor for AGEs (known as RAGE) which upregulates oxidative stress and inflammation [8] and is implicated in several diseases. Second, AGEs can enhance the crosslinking of extracellular proteins, resulting in a loss of

functions [10]. Therefore, the reduction of AGE formation using synthetic pharmaceutical agents, such as aminoguanidine (AG), has been investigated in clinical studies [11]. Given the undesirable side effects of several synthetic agents, including AG [11], the utilization of natural products as dietary intervention strategies for the prevention and therapy of AGE-related diseases holds great promise [12].

While cranberry and its derived products have been extensively studied for a wide range of biological properties, to date, there is limited data on the effects of cranberry fruit constituents on the formation of AGEs [13, 14]. In these studies, a purified phenolic-enriched fraction, high in PAC content, was shown to inhibit protein glycation by scavenging reactive carbonyl species. However, the authors also reported that phenolic-free water fractions, purified from the cranberry fruit, also exerted antiglycative effects but the active constituents present therein were not identified [13, 14].

Our group has recently reported on the chemical characterization and antimicrobial effects of an oligosaccharide-enriched fraction purified from a water-methanol fraction of cranberry [6]. Therefore, we hypothesized that these oligosaccharide constituents could impart anti-AGEs effects to the fruit beyond its phenolic constituents alone. In light of the aforementioned points, and given our group's ongoing research interest in identifying natural products with antiglycating activities [15-17], the current study was designed to evaluate the inhibitory effects of cranberry oligosaccharides against the formation of AGEs.

Herein, an oligosaccharide-enriched fraction (named Cranf1b-CL), purified from pectinase treated whole cranberry fruit material, was evaluated for its anti-AGEs and free radical scavenging effects *in vitro*. Bovine serum albumin (BSA) and D-fructose were used as the model protein and glycation agent, respectively, for the AGEs assay. Inhibition of AGEs formation by Cranf1b-CL was comparable to AG at equivalent concentrations. Cranf1b-CL was also found to have free radical scavenging activity superior to the synthetic antioxidant, butylated hydroxytoluene (BHT) when evaluated in the DPPH assay.

2. Methods

2.1. Chemicals

Bovine serum albumin (BSA), D-fructose, 2,5-dihydroxy benzoic acid (2,5-DHB), trifluoroacetic acid (TFA), methylglyoxal (MGO), 1,2-phenylenediamine (PD), 2,3-dimethylquinoxaline (DQ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and aminoguanidine hydrochloride (AG) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All solvents used were HPLC grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Fractionation of Cranberry Material

Pectinase (Klerzyme 150, DSM Food Specialties, South Bend, IN, USA) treated cranberry hull powder was generously provided by Ocean Spray Cranberries, Inc. (Middleboro, MA, USA) and further fractionated as previously reported [6] with

modification (shown in **Scheme 1**) using a CombiFlash Rf purification system (Teledyne ISCO, Inc., Lincoln, NE, USA). Briefly, 20 mL of an aqueous solution of the pectinase treated cranberry powder (100 mg/mL) was injected onto a RediSep GOLD C18 Reverse-phase column (Teledyne ISCO, Inc., Lincoln, NE, USA) and eluted sequentially with 500 mL of de-ionized water, 500 mL of 15% methanol/water, and 500 mL of 100% methanol. Eluates from each gradient system were individually pooled and freeze-dried. Three major fractions, Cranf1W (761 mg, 38.1%) eluted with 100% de-ionized water, Cranf1b (476 mg, 23.8%) eluted with 15% methanol/water, and Cranf1M (562 mg, 28.1%) eluted with 100% methanol were obtained.

The oligosaccharide constituents of Cran1b were purified using a Hypersep Hypercarbon solid phase extraction (SPE) cartridge (1 gram, Thermo Scientific, Waltham, MA, USA). The SPE cartridge was first conditioned by eluting with 6 mL of acetonitrile/H₂O (50%, v/v) and then washed three times with 6 mL of deionized

Scheme 1. Fractionation and purification of cranberry materials.

H₂O. Two mL of an aqueous solution of Cran1b (5 mg/mL) was slowly eluted through the cartridge and washed three times with 6 mL of deionized water. The oligosaccharide-enriched fraction was then eluted with 6 mL of acetonitrile/ H₂O (30%) acidified with 0.1% trifluoroacetic acid (TFA). The resulting fraction was then finally purified using a C18 reverse-phase SPE cartridge (50 mg, Fisher Scientific, Waltham, MA, USA), by eluting with deionized H₂O, and dried *in vacuo* to yield Cranf1b-CL (7.5 mg).

2.3. Nuclear Magnetic Resonance (NMR) Spectroscopy

Five mg of Cranf1b-CL was dissolved in 0.5 mL D₂O (99.99%, Sigma-Aldrich, St. Louis, MO, USA) and transferred to a 5 mm NMR tube. ¹H NMR spectra was recorded at 25 °C on a Bruker 300 MHz spectrometer (Billerica, MA, USA).

2.4. Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS)

The molecular sizes of oligosaccharides in Cranf1b-CL were recorded by MALDI-TOF-MS (Axima Performance, Shimadzu, Kyoto, Japan) as previously reported using 2,5-dihydroxy benzoic acid (2,5-DHB) as the matrix [18]. Briefly, 2,5-DHB matrix solution was prepared as 50% acetonitrile/H₂O solution at 10 mg/mL. Two µL of Cranf1b-CL aqueous solution (5 mg/mL) was mixed well with 2 µL of the matrix solution then 2 µL of the mixture was spotted onto a MALDI plate, air dried, and analyzed in positive reflectron mode with the power set at 80 kV.

2.5. Monosaccharide Composition Analyses

Cranf1b-CL (200 µg) was hydrolyzed with 200 µL of 2M TFA in an ampoule for 2 h at 120 °C. The hydrolyte was dried under a stream of nitrogen. The residual acid was removed by adding 200 µL of isopropanol and dried *in vacuo*. The monosaccharide composition of Cranf1b-CL hydrolyte was analyzed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) [19] using a Hamilton RCX-30 250/4.6 column (Metrohm AG, Riverview, FL, USA) on a 940 Professional IC Vario system (Metrohm AG, Riverview, FL, USA) and eluted with isocratic 16 mM NaOH at 1 mL/min. Fucose, arabinose, rhamnose, galactose, glucose, xylose and fructose were used as monosaccharide standards. Unhydrolyzed aqueous solution of Cranf1b-CL was prepared (1 mg/mL) and its monosaccharide content was quantitatively analyzed by HPAEC-PAD eluting with 100 mM NaOH at 1 mL/min.

2.6. DPPH Free Radical Scavenging Assay

The free radical scavenging capacity of Cranf1b-CL was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay based on our previously reported method [20]. Briefly, 100 µL of test samples and 100 µL of a 0.20 mM DPPH in 50% aqueous methanol solution were mixed for 30 min at room temperature. The absorbance was read at 517 nm using a micro-plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA). The scavenging capacity of the

sample was calculated as follows: $[(\Delta A_{\text{control}} - \Delta A_{\text{sample}})/\Delta A_{\text{control}}] \times 100\%$.

2.7. Advanced Glycation End-Products (AGEs) Assay

The inhibitory effects of Cranf1b-CL of the formation of advanced glycation end-products (AGEs) was evaluated by the BSA-fructose assay wherein bovine serum albumin (BSA; 10 mg/mL) and D-fructose (100 mM) were used as the model protein and glycating agent, respectively as previously reported [17]. Treatments included varying concentrations of Cranf1b-CL ranging from 20 to 500 $\mu\text{g/mL}$ and a synthetic anti-AGEs agent, aminoguanidine (AG), at equivalent concentrations, which served as the positive control. Each sample was then incubated in the dark at 37 °C for 60 h and their intrinsic fluorescence was measured using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 340 nm and an emission wavelength of 435 nm. Statistical analysis was conducted using one-way factorial ANOVA with Tukey-Kramer post hoc comparisons. All results were expressed as means $\pm$ SD ($n = 3$) with significance (*) defined as $p \leq 0.05$.

2.8. Methylglyoxal (MGO) Trapping Assays

Cranf1b-CL was evaluated for methylglyoxal (MGO) trapping capacity based on our previously reported method [17]. A reaction solution consisting of MGO (5 mM), PD (derivatization reagent, 20 mM), and DQ (internal standard, 5 mM) were freshly prepared in 0.1 M phosphate buffer, pH 7.4. Cranf1b-CL (0.25 mL of 1000 $\mu\text{g/mL}$)

was added to MGO (0.25 mL of 5 mM). After incubating at 37 °C for 1 h, 0.125 mL of PD and DQ were each added to mixture. The reaction was kept at room temperature for 30 min. The amount of derivative of residual MGO (2-methylquinoxaline, 2-MQ) was quantified by HPLC-DAD. The percentage decrease of MGO was calculated using the following equation: % MGO decrease = $[1 - (\text{MGO amounts in solution with tested sample} / \text{MGO amounts in control solution})] \times 100\%$.

3. Results

3.1. Purification of Cranberry Oligosaccharides

Pectinase treatment is a common process in cranberry juice production and is used to help extract, clarify, and modify the juice [21]. Therefore, starting from an pectinase enzyme treated cranberry powder, an oligosaccharide-enriched fraction, Cranf1b (23.8%; the 15% methanol:H₂O eluate) was obtained from C18 reverse phase flash chromatography (**Scheme 1**). The oligosaccharides of Cranf1b were further purified by porous graphitized carbon solid phase extraction (SPE) and C18 SPE to remove the residual monosaccharides, salts, and other trace contaminants (proteins, lipids, and pigments). The ¹H NMR spectrum (**Figure 1**) of Cranf1b-CL showed proton resonances between 3.0 ppm and 4.5 ppm, and a group of resonances between 4.5 ppm and 5.3 ppm that are consistent with characteristic signals for anomeric protons in carbohydrates. Notably, there are no visible aromatic protons between 6.5 ppm and 8.0 ppm in the ¹H NMR spectrum of Cranf1b-CL indicating

that no phenolic components are present in this fraction. This confirmed that Cranf1b-CL was a “phenolic-free” and “oligosaccharide-enriched” fraction purified from cranberry fruit.

3.2. Analysis of Cranberry Oligosaccharides by MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry (**Figure 2**) of Cranf1b-CL revealed several oligosaccharide sodium adduct ions $[M+Na]^+$ that were composed of hexoses (Hex) and pentoses (Pen). Major adduct ions at m/z 923, 953, 1055, 1085, 1217, 1247 and 1379 were consistent with oligosaccharides with compositions of Hex₃Pen₃, Hex₄Pen₂, Hex₃Pen₄, Hex₄Pen₃, Hex₄Pen₄, Hex₅Pen₃ and Hex₅Pen₄, respectively.

Figure 1. ^1H NMR spectrum (300 MHz, D_2O) of Cranf1b-CL.

Figure 2. MALDI-TOF-MS of Cranf1b-CL. H3P3: [Hexose₃Pentose₃+Na]⁺, *m/z* 923; H4P2: [Hexose₄Pentose₂+Na]⁺, *m/z* 953; H3P4: [Hexose₃Pentose₄+Na]⁺, *m/z* 1055; H4P3: [Hexose₄Pentose₃+Na]⁺, *m/z* 1085; H4P4: [Hexose₄Pentose₄+Na]⁺, *m/z* 1217; H5P3: [Hexose₅Pentose₃+Na]⁺, *m/z* 1247; H5P4: [Hexose₅Pentose₄+Na]⁺, *m/z* 1379.

3.3. Monosaccharide Composition Analysis of Cranberry Oligosaccharides by HPAEC-PAD

The HPAEC-PAD chromatogram (**Figure 3**) of hydrolyzed Cranf1b-CL indicated that Cranf1b-CL was composed of four major monosaccharides arabinose (25%), galactose (5%), glucose (47%) and xylose (23%). The monosaccharide content of unhydrolyzed Cranf1b-CL was also evaluated by HPAEC-PAD (**Figure 4**) which showed only a trace amount (0.7%) of glucose present in Cranf1b-CL and no other measurable levels of monosaccharides (**Figure 4**).

3.4. Free Radical Scavenging Activity of Cranberry Oligosaccharides

As shown in **Figure 5**, Cranf1b-CL showed free radical scavenging capacity in a concentration-dependent manner. At concentrations ranging from 1250 – 5000 µg/mL, the oligosaccharides scavenged more than 50% of free radicals (59 - 84%, respectively). Also, Cranf1b-CL had an IC₅₀ value of 680 µg/mL, superior to the positive control, butylated hydroxytoulene (BHT), a synthetic commercial antioxidant, which had an IC₅₀ value of 2200 µg/mL. Notably, Cranf1b-CL (at 1000 µg/mL) did not show any methylglyoxal (MGO) trapping capacity (data not shown).

3.5. Anti-AGEs activity of Cranberry Oligosaccharides

For the anti-AGEs assay (**Figure 6**), the glycation levels of a BSA-fructose

Figure 3. HPAEC-PAD chromatogram of monosaccharide components in hydrolyzed Cranf1b-CL.

Figure 4. HPAEC-PAD chromatogram of monosaccharide components present in unhydrolyzed Cranf1b-CL.

Figure 5. Cranf1b-CL showed free radical scavenging activity in the DPPH assay. Each experiment was conducted in triplicate and all data were expressed in mean $\pm$ SD ($n=3$). BHT (butylated hydroxytoluene) served as the positive control.

Figure 6. Advanced glycation end-product (AGEs) levels as characterized by intrinsic fluorescence (FU) in BSA-fructose solutions. Each experiment was conducted in triplicate and all data were expressed in mean $\pm$ SD ($n=3$) with significance (*) defined as $p \leq 0.05$. Aminoguanidine, a synthetic antiglycating agent, served as the positive control.

solution (negative control), or BSA-fructose solutions co-treated with Cranf1b-CL (treatment) or with AG (positive control) were evaluated. After an incubation period of 60 h, all of the BSA-fructose solutions generated fluorescence detectable at 340 nm/435 nm confirming the formation of AGEs. However, solutions treated with Cranf1b-CL and AG yielded fluorescence significantly lower than that of negative control ($p \leq 0.05$). Among the Cranf1b-CL treated samples, the highest inhibitory effects were observed at 200 and 500 $\mu\text{g/mL}$ with reduction in AGEs formation by 53.3% and 56.8%, respectively. At equivalent concentrations, the inhibitory levels of AG were comparable to Cranf1b-CL with reduction in AGEs formation by 73.1% and 75.2%, respectively. However, at concentrations equal to or lower than 100 $\mu\text{g/mL}$, Cranf1b-CL showed comparable or more potent anti-AGEs activities than AG. For example, at 50 $\mu\text{g/mL}$, Cranf1b-CL reduced AGEs formation by 34.8% while AG reduced AGEs formation by 28.8% at an equivalent concentration of 50 $\mu\text{g/mL}$.

4. DISCUSSION

Oligosaccharides show numerous biological activities including antioxidant [22, 23], anti-inflammatory [24], and immuno-stimulating effects [25] but those purified from cranberry have only been previously evaluated for antimicrobial properties [6, 7]. Therefore, in the current study, purified cranberry oligosaccharides, namely, Cranf1b-CL, was tested for its *in vitro* antiglycative and free radical scavenging effects.

Overall, Cranf1b-CL inhibited the formation of AGEs in a concentration-dependent manner which is likely to be related to its oligosaccharide content. Notably, it has been reported that the antiglycating effects of the polyphenolic constituents, namely PACs, in cranberries are mediated by their reactive carbonyl species scavenging capacity [13]. In the current study, we evaluated the ability of Cranf1b-CL to trap the reactive carbonyl species, methylglyoxal (MGO), using methods previously reported by our group [17]. Interestingly, Cranf1b-CL did not show any MGO scavenging capacity suggesting that its mechanisms of antiglycative effects are different from the polyphenolic constituents of cranberries. Previous studies have shown that oligosaccharides purified from wheat bran show antiglycating effects mediated through metal chelating activities [26]. Therefore, further studies are warranted to evaluate the mechanisms of antiglycating activities of oligosaccharides purified from cranberry.

The limitations of our current study are common to *in vitro* studies which do not account for physiological relevant considerations such as bioavailability and metabolism. While there is limited data on the bioavailability and metabolism of oligosaccharides, recent studies have underscored the role of intestinal microbiota in their biotransformation [27-29]. Given the relatively large size of these molecules, it is unlikely that they would be absorbed directly as their intact forms. Thus, whether these compounds would be present in tissues in their bioactive (intact and/or metabolized) forms to exert potential anti-AGEs effects would require further *in vivo* studies. Nevertheless, the current study provides initial data to support the

anti-AGEs and free radical scavenging potential of oligosaccharides purified from the North American cranberry fruit.

5. CONCLUSION

In summary, a chemically characterized oligosaccharide-enriched fraction purified from cranberry fruit was shown to exert anti-AGEs and free radical scavenging effects. The current study adds to emerging data supporting the biological effects of cranberry oligosaccharides suggesting that these constituents may impart potential health benefits to the whole cranberry fruit beyond its phenolic constituents alone. Also, this is the first study to evaluate a chemically characterized oligosaccharide fraction purified from the North American cranberry fruit for anti-AGEs and free radical scavenging properties.

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MANUSCRIPT III

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Pectic polysaccharide from *Panax ginseng* decreases *Pseudomonas aeruginosa* intracellular cyclic di-GMP level and disperses *in vivo* biofilm

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Abstract

Chronic *Pseudomonas aeruginosa* infections are often associated with biofilm formation that protects the bacteria from antibiotic treatment and host immune defense. Previously, ginseng aqueous extract were shown to significantly inhibit the biofilm formation by *P. aeruginosa*. One of the key factor in controlling the biofilm formation and dispersal is a secondary messenger molecule cyclic di-GMP. Biofilm-associated bacterial physiologies are closely regulated by the intracellular cyclic di-GMP level, therefore molecules that decrease intracellular cyclic di-GMP level would potentially prevent biofilm formation or cause mature biofilm dispersal. In the present study, activity-directed fractionation of ginseng polysaccharides identified an acidic pectic polysaccharide CGP-6-f2 that exhibited a concentration-dependent reduction of the intracellular cyclic di-GMP level to as low as 30% at 5 mg/mL. In addition, intraperitoneal injection of CGP-6-f2 in mice successfully dispersed pre-formed biofilm on a silicon implant within 24 h. Our study suggests that ginseng pectic polysaccharide may help disrupt *P. aeruginosa* biofilm and treat biofilm associated chronic infections.

Keywords: *Pseudomonas aeruginosa*, biofilm, ginseng, cyclic di-GMP, pectic polysaccharide

1. Introduction

Pseudomonas aeruginosa is a common Gram-negative, opportunistic human pathogen that causes varieties of chronic infections with significant morbidity and mortality rates especially in immune-compromised patients.^{1,2} *P. aeruginosa* is the leading pathogenic bacterium associated with the chronic lung infection in cystic fibrosis (CF) patients. It develops thick biofilm in the respiratory tract, which may ultimately lead to lung failure. Furthermore, *P. aeruginosa* infections account for 10%-15% of nosocomial infections worldwide, which are often associated with medical devices and include eye infections by contact lenses, urinary tract infections by urinal catheter, and pneumonia by mechanic ventilator.³ Together with innate high resistance to a great number of antibiotics,⁴ biofilm associated *P. aeruginosa* chronic infection is the foremost factor responsible for repeated hospitalizations, declined organ functions and early mortality of infected patients.

Biofilm is an aggregation of bacterial cells embedded in a polymeric matrix consisting of polysaccharides, proteins and DNA.^{5,6} In fact, biofilm is the predominant living style for most bacteria and is associated with over 80 percent of microbial infections.⁷ Living in biofilm as a multicellular community provides not only significant protection to the bacterial cells from environmental stresses, such as host immune defense system or antibiotic treatment (up to 1,000-fold more resistant), but also an ideal platform for exchanging genes that would benefit the bacterial community, thus facilitating the emergence and spread of drug resistance.⁸ Because

of the development of biofilm formation, pathogens associated with chronic infections are remarkably difficult to eradicate with traditional antibiotics, causing huge global economic burden. In fact, it is estimated that 17 million people in United States are suffering biofilm related chronic infections.⁹ During the past three decades, only a few new antibiotics have reached the market while multi-drug resistant strains of human pathogens are emerging at a much faster rate.^{10,11} Thus, rather than new molecules in the same traditional antimicrobial categories, therapeutics with novel mechanisms of action are in urgent need. Drugs that prevent or eradicate existing biofilm infections are especially important. Such drugs would not necessarily involve bactericidal effect, hence lowering the possibility of establishing drug resistance.

The secondary messenger molecule cyclic dimeric guanosine monophosphate (c-di-GMP) has numerous effects on *P. aeruginosa* physiology (see **Figure 1**). Studies have shown that c-di-GMP plays an essential role in regulating the formation and dispersal of biofilm.^{7,12,13} Intracellular level of c-di-GMP is dynamically synthesized from two guanosine-5'-triphosphates (GTPs) by diguanylyl cyclases (DGCs) and is degraded into 5'-phosphoguanylyl-(3'→5')-guanosine phosphate (pGpG) by phosphodiesterases (PDEs). DGCs and PDEs are often associated with sensor domains that are stimulated by environmental input signals such as quorum sensing molecules, oxygen level, nitric oxide level, temperature and chemical stresses. Environmental input signals induce fluctuations of intracellular level of

c-di-GMP that are then sensed by a group of c-di-GMP binding proteins (effectors) or RNA aptamers. These effectors or RNA aptamers will directly or indirectly effect immediate downstream cellular transcriptional, translational or post-translational functions. For *P. aeruginosa*, these c-di-GMP binding receptors include PilZ that downregulates fimbriae involved twitching motility; Alg44 and PelD that upregulate the production of extracellular polysaccharide alginate and pel, which are primary components for *P. aeruginosa* biofilm matrix; and FleQ that downregulates the synthesis of flagella and upregulates the pel production. Therefore, modulating intracellular levels of c-di-GMP could represent a new target for shifting the bacterial living state from sessile to mobile.

Figure 1. Cyclic di-GMP and regulation of biofilm dispersal

Panax ginseng C.A. Meyer has been traditionally used as an herbal medicine in the East Asia, which is believed to strengthen immune system and boost energy. Because ginseng is often prepared by boiling in hot water, water-soluble components from ginseng have been extensively investigated. As one of the major water-soluble components, ginseng polysaccharides have been reported as having immunostimulating,¹⁴ anti-tumor,¹⁵ anti-oxidant,¹⁶ anti-adhesive¹⁷ and antirotavirus¹⁸ properties. Previously, ginseng hot water extract has been reported to prevent *P. aeruginosa* biofilm formation as well as to disperse mature biofilm without having bactericidal effects. In the present study, we identified and characterized a pectic polysaccharide CGP-6-f2 that inhibits the cyclic di-GMP production in *P. aeruginosa* and exhibits *in vivo* biofilm dispersing effect.

2. Materials and methods

2.1. Materials and chemicals

The ginseng (*Panax ginseng* C.A. Meyer) root powder (ginseng age: 5-6 years, Jilin, China) was kindly provided by University of Copenhagen (Copenhagen, Denmark). The Q Sepharose, Sepharose CL-6B, Sephadex G-25 resins were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.2. Bacterial culture

P. aeruginosa strains were inoculated on ABTG (2g/L (NH₄)₂SO₄, 6g/L Na₂HPO₄, 3

g/L KH₂PO₄, 3g/L NaCl, 11 mg/L CaCl₂, 95 mg/L MgCl₂, 0.5 mg/L FeCl₃, 2.5 mg/L thiamine, 5 g/L glucose) agar plate supplemented with gentamicin (60 mg/L). For measurement of intracellular c-di-GMP level, *P. aeruginosa* strains were cultured in ABTG supplemented with casamino acids (5 g/L) and gentamicin (60 mg/L). For *in vivo* biofilm dispersal study, *P. aeruginosa* strains were cultured in Lysogeny Broth (LB) medium.

2.3. General methods

Total carbohydrate content was determined by phenol-sulfuric acid method¹⁹, using glucose as a standard. Uronic acid content was determined by *m*-hydroxydiphenyl method²⁰ using galacturonic acid as a standard. Ion exchange and gel permeation chromatographies were performed on a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare Life Sciences) and detected by total carbohydrate and uronic acid content assays.

2.4. Extraction

Raw ginseng powder was washed with 95% ethanol three times and then dissolved in distilled water. With the addition of 95% ethanol (4 volumes), the crude polysaccharide was precipitated overnight at 4 °C. After centrifugation, the precipitate was re-dissolved in distilled water and treated with 1/4 volume of Sevag reagent²¹ (4:1 Chloroform: n-Butanol, v/v) to remove any remaining protein. After centrifugation, the water layer was dialyzed (MWCO 12,000-14,000 Da, Spectrum

Laboratories, Inc., Rancho Dominguez, CA, USA) against distilled water with stirring for 48 h. The retained materials were dried by lyophilization to yield a de-proteined crude ginseng polysaccharide (CGP). The extraction and fractionation of ginseng polysaccharide are described in **Figure 2**.

Figure 2. Ginseng polysaccharide extraction and purification flow chart.

2.5. Polysaccharide purification

2.5.1 Anion exchange chromatography on Q Sepharose

CGP (1 gram) was dissolved in 50 mL of distilled water and loaded onto a semi-preparative anion exchange column (Hiprep Q XL 16×100 mm, GE Healthcare Life Sciences) connected to the FPLC system. The column was pre-equilibrated with distilled water and eluted by an increasing step-wise gradient of sodium chloride (0 to 1 M) at 5 mL/min for 300 min (see **Figure 3**). The fractions were collected every 15 mL and pooled based on total carbohydrate and uronic acid content assays. The

pooled fractions were then reduced in volume by lyophilization and subjected to a G-25 desalting column (Hiprep desalting 26×100 mm, GE Healthcare Life Sciences). The desalting process was monitored using a conductivity detector and total carbohydrate content assay. The salt free fractions were then freeze-dried to obtain seven fractions, CGP-1 (32.1 mg), CGP-2 (32.2 mg), CGP-4 (42.3 mg), CGP-5 (73.2 mg), CGP-6 (57.8 mg) and CGP-7 (46.2 mg).

Figure 3. The elution profile of CGP on Q Sepharose XL column.

2.5.2 Gel permeation chromatography on Sepharose CL-6B

CGP-6 (100mg) was dissolved in 10 mL of distilled water and applied to a semi-preparative Sepharose CL-6B gel permeation column (16×400 mm, GE Healthcare Life Sciences) and eluted with 0.15 M NaCl at 0.5 mL/min for 500 min (see **Figure 4**). The eluate was collected every 5 mL and pooled based on the total carbohydrate and uronic acid assays. The pooled fractions of interest were desalted as described above and then lyophilized to obtain two major fractions, CGP-6-f1

(7.9 mg) and CGP-6-f2 (70.4 mg).

Figure 4. The elution profile of CGP-6 on Sepharose CL-6B column

2.6. High performance size-exclusion chromatography

A 1 mg/mL CGP-6-f2 solution was prepared and high performance size-exclusion chromatography was carried out by Complex Carbohydrate Research Center (CCRC, University of Georgia, Athens, GA) using a Superose 12 column (GE Healthcare Life Sciences) connected to an Agilent 1200 HPLC system (Agilent, Santa Clara, CA, USA). The column was pre-calibrated with dextran standards. 75 μ L of sample was injected, eluted with 50 mM ammonium acetate and monitored using an evaporative light scattering detector (ELSD).

2.7. Glycosyl composition analysis

Glycosyl composition analysis was performed by CCRC. Gas Chromatography/Mass spectrometry (GC/MS) analysis of per-*O*-trimethylsilyl (TMS) derivatives of the methyl glycosides was carried out as previously described.²²⁻²⁴ Briefly, 300 µg CGP-6-f2 was mixed with 20 µg inositol as internal standard and subjected to methanolysis using 1 M methanolic HCl at 80 °C for 17 h, followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol. The sample was then per-*O*-trimethylsilylated by treatment with Tri-Sil reagent (Thermo Scientific, Waltham, MA, USA) at 80 °C for 0.5 h. GC/MS analysis of the TMS methyl glycosides was performed on an Agilent 7890A GC interfaced to a 5975C Mass Selective Detector (MSD, Agilent) using an Agilent DB-1 fused silica capillary column (30 m × 0.25 mm I.D., Agilent). The absolute configuration of each monosaccharide was determined by comparing TMS (+)-2-butyl derivatives to authentic monosaccharide derivatives on GC/MS as previously described.^{25,26} The GC oven temperature was held at 80 °C for 2 min, ramped linearly to 140 °C at 20 °C/min, then to 200 °C at 2 °C/min, and to 250 °C at 30 °C/min. The glycosyl composition was determined by comparison to the retention time of monosaccharide standard TMS derivatives.

2.8. Glycosyl linkage analysis

Glycosyl linkage analysis was performed by CCRC as previously described.²⁴ Briefly, CGP-6-f2 was dissolved in 200 µL DMSO and stirred overnight. The sample was

initially permethylated by treatment with potassium methylsulfinylmethyllide and methyl iodide.²² The permethylated sample was hydrolyzed by 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h, reduced by sodium borodeuteride, and acetylated by acetic anhydride and TFA. The resulting partially methylated alditol acetates (PMAAs) were analyzed by GC/MS using a Supelco sp-2330 fused silica capillary column (30 m × 0.25 mm I.D., Sigma-Aldrich). The oven temperature was held at 80 °C for 2 min and raised linearly to 170 °C at 30 °C/min, then to 240 °C at 4 °C/min, and held for 5 min. The electro-impact (EI) fragmentation patterns of PMAAs were compared to the PMAA standards EI fragmentation database.

2.8. De-esterification

The de-esterification of CGP-6-f2 was performed as previously described.²⁷ Briefly, 20 mg of CGP-6-f2 was dissolved in 1 mL distilled water. The pH was titrated to 12.0 by 0.1 M NaOH with constant agitation. The vial was sealed and stirred at 0 °C for 24 h. The pH of solution was then adjusted to 4.0 by drop-wise addition of 0.1 M HCl. The sample was then desalted and lyophilized.

2.9. Nuclear magnetic resonance spectroscopy

The samples were deuterium exchanged three times by D₂O shake and dissolved in D₂O containing 1 µL DMSO as reference (δ 2.71/39.4 ppm). One dimensional ¹H and ¹³C NMR spectra, as well as two-dimensional COSY, TOCSY, NOESY, HSQC and

HMBC NMR spectra were obtained on a Varian 500 MHz NMR spectrometer (Agilent) with 5 mm OneNMR probe at 50 °C.

2.10. Measurement of intracellular c-di-GMP level of *P. aeruginosa*

The effects of ginseng polysaccharide samples on the intracellular c-di-GMP level of *P. aeruginosa* were analyzed by University of Copenhagen as described previously by Rybtke *et al.*²⁸ Briefly, genes of *pelA*, *pslBCD* with or without *wspF* were deleted from wide-type *P. aeruginosa* PAO1 to get the background strains of Δpel and $\Delta pel\Delta psl$ in relation to different cellular concentrations of c-di-GMP. Deletion of *wspF* causes continuous activation of DGC WSpR, resulting elevated levels of c-di-GMP. Deletions of *pel* and *psl* disable *P. aeruginosa* to produce extracellular polysaccharides *pel* and *psl* that cause bacterial cells to aggregate and lead to an inaccurate cell density measurement. The reporter strains monitoring c-di-GMP were constructed based on these two background strains. The plasmid of $p_{cdrA}-gfp$, a cyclic-di-GMP-responsive promoter, was introduced by transcriptional fusion into the background strains and the intracellular level of c-di-GMP would be readout according to the presented fluorescent intensity. The *P. aeruginosa* PAO1 reporter strains were grown at 37 °C with shaking at 185 rpm. 200 μ l of culture were transferred into each well of a 96-well microtiter plate. With addition of testing samples, the plates were incubated for approximately 22 h in a Tecan Infinite Pro2000 (Männedorf, Switzerland) plate reader at 37 °C and measured optical density (OD) at 600 nm and

fluorescence (490 nm excitation, 515 nm emission) every 30 min. Fluorescence intensity units (FIU) were normalized to OD₆₀₀ in order to offset the effects from alteration of bacterial growth.

2.11. *In vivo* biofilm dispersal effects of ginseng polysaccharides

Silicone implants were prepared as described previously by Christensen *et al*²⁹ with modifications. A centrifuged precipitate of *P. aeruginosa* cells from overnight culture was re-suspended in 0.9 % NaCl to an OD₆₀₀ of 0.1 (~10⁸ CFU/ml). The prepared silicone tubes (half of tube, length, 3 mm; I.D., 4 mm; O.D., 6 mm) were placed into 24-well plate containing 1 ml of bacterial suspension (OD₆₀₀ = 0.1) per well, and were shaken at 37 °C at 200 rpm overnight to allow bacterial cells attach the surface of silicon tubes. The silicon tubes were washed three times with 0.9 % saline. Female BALB/c mice, aged at 9 weeks (Taconic M&B A/S, Denmark), were placed ventral side up after subcutaneous anaesthesia and made an incision of approximate 5 mm in left groin area to expose peritoneal cavity after disinfection. The silicon implant was placed into the peritoneal cavity. The incision was closed with a suture, and healed without complications.

After 24 h inoculation, mice were treated intraperitoneally with testing samples (0.5% CGP/CGP-6 or 0.9% NaCl) at three time points, 0, 4 and 8 h. Mice were sacrificed at 0, 4, 8 and 24 h after treatments. Silicon tubes and spleens were

collected for analyzing bacteriology. The spleens were homogenized individually in 3 ml phosphate-buffered saline (PBS). The silicone implants were sonicated (Bransonic model 2510, Branson Ultrasonic Corporation, USA) for 5 min twice with 5 min degas interval to detach bacteria from the silicon tubes. 100 μ L bacterial suspensions were serially diluted and plated on blue agar plates for the determination of CFU after incubation at 37 °C overnight.

3. Results

3.1. Bioassay-guided fractionation of ginseng polysaccharides

The crude ginseng polysaccharide (CGP) at five concentrations (serial two times dilutions from 0.25% to 0.15%) were incubated with *P. aeruginosa* reporter strain in microtiter plates and the intracellular levels of c-di-GMP were measured by recording fluorescent readout every 30 min and normalized to OD₆₀₀. **Figure 5** showed that CGP had a concentration-dependent inhibitory effect on the c-di-GMP level, among which at 15 h 0.25 % of CGP treated group had approximate 40% lower c-di-GMP level compared to the untreated group.

Figure 5. Effect of various concentrations of CGP on the intracellular c-di-GMP level of *P. aeruginosa* c-di-GMP reporter strain.

Therefore, CGP was further fractionated and screened for molecules having inhibitory effects of c-di-GMP level. CGP was firstly separated using step-gradient anion exchange chromatography (**Figure 3**). Seven major fractions were generated and 1 mg/mL of each fraction was tested for their inhibition on c-di-GMP level. **Figure 6** showed that at 1 mg/mL, CGP-4, CGP-5 and CGP-6 showed comparable or better activity than CGP tested at 5 mg/mL and 2.5 mg/mL, among which CGP-6 showed the most reduction in c-di-GMP level by over 50%. Therefore, CGP-6, obtained from the 0.4 M NaCl elution, was further investigated and accounted for 5.8% of the CGP dried weight.

After further gel permeation chromatography (**Figure 4**), two fractions, one neutral fraction CGP-6-f1 (7.9%) with relatively high molecular weight and one acidic fraction CGP-6-f2 (70%) with relatively low molecular weight, were obtained. As the primary component of CGP-6, CGP-6-f2 at six concentrations (serial two times dilutions from 5.0 mg/mL to 0.15 mg/mL) were evaluated for their effects on the *P. aeruginosa* c-di-GMP level (**Figure 7**). Within the range of concentrations tested, CGP-6-f2 showed concentration-dependent activity. 5.0 mg/mL CGP-6-f2 decreased c-di-GMP level to 30% at 15 h and 50% reduction of c-di-GMP level was achieved at as low as 0.6 mg/mL.

Figure 6. Effect of CGP (5 mg/mL), CGP (2.5 mg/mL), CGP-1 (1 mg/mL), CGP-2 (1 mg/mL), CGP-3 (1 mg/mL), CGP-4 (1 mg/mL), CGP-5 (1 mg/mL),

CGP-6 (1 mg/mL) and CGP-7 (1 mg/mL) on the intracellular c-di-GMP level of *P. aeruginosa* c-di-GMP monitor strain.

Figure 7. Effect of various concentrations of CGP-6-f2 on the intracellular c-di-GMP level of *P. aeruginosa* c-di-GMP monitor strain.

3.2. Effect of ginseng polysaccharides on *P. aeruginosa* *in vivo*

Because one of major consequences of decreased level of c-di-GMP is causing bacterial biofilm dispersal, the *in vivo* effects of ginseng polysaccharides on mature

bacterial biofilm were analyzed using mice with intraperitoneal insertion of silicone tubes attached with *P.aeruginosa* PAO1. After 24 h formation of biofilm on the silicon tube, testing samples were injected into the intraperitoneal cavity. Molecules decreasing c-di-GMP level would then cause bacterial cells within biofilm to disperse and be transferred to spleen for clearing out the body therefore a lower bacterial cell count from the silicon tube but a higher bacterial cell count from the spleen. In this assay, due to limited quantity of CGP-6-f2, only CGP and CGP-6 were tested. In **Figure 8**, CGP-6 treated groups showed significantly lower bacterial cell counts from silicon tubes at 4, 8, and 24 h after treatment and significantly higher bacterial cell counts in spleens at 4, and 8 h after treatment when compared to the groups treated with 0.9% NaCl. At 24 h, there is no significant difference in bacterial cell counts from spleen between CGP-6 group and control, suggesting that the bacterial cells have been efficiently cleared out within 24 h. In CGP treated group, there were fewer bacteria from silicon tubes at 8 and 24 h and more bacteria in spleens at 4 and 8 h compared to the control group. These data suggested that both of CGP and CGP-6 showed *in vivo* dispersal effect. In addition, compared to CGP, CGP-6 treatment showed significantly faster bacterial dispersal on silicone tubes as early as at 4 h and lower bacterial cell number on the silicon after 24 h treatment.

Bacteriology of silicone implants following different periods of treatments

Bacteriology of spleens following different periods of treatments

Figure 8. Bacteriology at different times after treatments for silicone tubes (up-panel) and spleens (down-panel): Inocula (⊙); Group treated with 0.9% Sodium Chloride (○); Group treated with CGP (◇); Group treated with CGP-6 (△).

3.3. Structural characterization of CGP-6-f2

3.3.1 Homogeneity and molecular weight

The homogeneity of CGP-6-f2 was analyzed by HPSEC on a Superose 12 column and eluted with 50 mM ammonium acetate. The elution profile (**Figure 9**) showed two peaks. The major peak (CGP-6-f2) eluted around 10 minutes was estimated to be 70,000 Da. The second peak eluted around 20 minutes was estimated to be very small and very likely to be salt.

Figure 9. HPSEC elution profile of CGP-6-f2 on a Superose 12 column.

3.3.2 Glycosyl composition analysis

The glycosyl composition and absolute configuration analysis showed that CGP-6-f2 was composed primarily of D-galacturonic acid (D-GalA, 67.0%), L-Arabinose (L-Ara, 13.3%), L-Rhamnose (L-Rha, 9.2%), D-Galactose (D-Gal, 9.3%) and some minor components (Fucose 0.4%, Xylose 0.2%, Glucuronic acid 0.4% and Glucose 0.2%).

3.3.3. Glycosyl linkage analysis

For the glycosyl linkage analysis, CGP-6-f2 was permethylated, hydrolyzed and acetylated to have a mixture of partially methylated alditol acetates (PMAAs). In GC/MS analysis, each PMAA was identified by comparison of retention time and fragmentation pattern with standards. The glycosyl linkages present in CGP-6-f2 were listed in **Table 1**.

Major Glycosyl Linkages
Terminal arabinofuranosyl residue (t-Araf)
5 linked arabinofuranosyl residue (5-Araf)
3,5 linked arabinofuranosyl residue (3,5-Araf)
2 linked rhamnopyranosyl residue (2-Rhap)
2,4 linked rhamnopyranosyl residue (2,4-Rhap)
4 linked galactopyranosyl residue (4-Galp)
4 linked galacturonic acid residue (4-GalpA)
2,4 linked galacturonic acid residue (2,4-GalpA)
3,4 linked galacturonic acid residue (3,4-GalpA)

Table 1. Major glycosyl linkages presented in CGP-6-f2

Glycosyl composition and linkage analyses suggested that CGP-6-f2 belongs to pectins that are important structural components of plant primary cell wall.³⁰ Unlike polymers with single repeating unit, pectins are heteropolysaccharides that often contain a combination of various backbones and side chains.³¹⁻³⁵ The heterogeneous backbone was composed of a combination of homogalacturonan (HG) with repeating 4)- α -GalpA-(1, and rhamnogalacturonan (RG) with alternate disaccharide unit 4)- α -GalpA-(1,2)- α -L-Rhap-(1,.³⁶ Galactan, arabinan and arabinogalactan are the three

most common side chains that are exclusively branched at C-4 of rhamnosyl residues.

30,37

3.3.4. NMR analysis

^1H and ^{13}C NMR resonances allowed assignment of nine different sugar residues and are represented as residues A-I (see **Table 2**).

The ^1H NMR spectrum of CGP-6-f2 (**Figure 10**) revealed four major anomeric proton signals, which were assigned as, H-F1 (δ 5.11), H-E1 (δ 5.07), H-G1 (δ 4.95) and H-H1 (δ 4.90). According to HSQC (**Figure S3**), anomeric carbons for residues E, F, G and H were assigned as C-F1 (δ 99.81), C-E1 (δ 99.50), C-G1 (δ 100.74) and C-H1 (δ 100.49). The chemical shift of a pyranosyl anomeric carbon in an α -linkage ($\sim\delta$ 97-101) is generally more upfield than β -linkage ($\sim\delta$ 97-105).³⁸ The chemical shifts of a furanosyl anomeric carbon are generally more downfield ($\sim\delta$ 106-109) than pyranosyl anomeric carbons.³⁹ Therefore, residues E, F, G, H were assigned to α -linked GalpA, which is the most abundant glycosyl residue. The intense resonance at δ 3.80 in the ^1H NMR spectrum and δ 53.38 in the ^{13}C NMR spectrum (**Figure 11**) were assigned to the $-\text{CH}_3$ in the methyl esterified GalpA (GalpAMe), which was further confirmed by HMBC correlations at δ 3.80/171.25 and δ 3.80/171.27 (**Figure S4**). The carbonyl carbons at δ 171.25 and δ 171.27 were assigned to GalpAMe, which are slightly more upfield than the carbonyl carbons of GalpA at δ 175.39 and δ

175.67. The two HSQC resonances at δ 2.17/21.08 and δ 2.08/20.76 suggested that some residues were acetylated.

For residue F, the COSY (**Figure S2**) correlations at δ 5.11/3.74, δ 3.74/3.99, δ 3.99/4.46, δ 4.46/5.11, and a spin system in the TOCSY spectrum along the sugar ring were observed. Accordingly, the carbon chemical shifts of C-F2, C-F3, C-F4 and C-F5 were assigned by HSQC correlations at δ 3.74/68.60, δ 3.99/68.69, δ 4.46/78.83 and δ 5.11/71.02, respectively. The HMBC correlation at δ 5.11/171.27 suggested that residue F was methyl esterified. The downfield chemical shift of C-F4 at δ 78.83 indicated its linkage at position 4. In total, the evidence points to residue F as a 4- α -D-GalpAMe. Similarly, for residue G, ^1H resonances of residue protons H-G2, H-G3, H-G4 and H-G5 were assigned as δ 3.72, δ 3.98, δ 4.45 and δ 5.05, respectively. Accordingly, the carbon chemical shifts of C-G2, C-G3, C-G4 and C-G5 were assigned by HSQC correlations at δ 3.72/68.64, δ 3.98/69.25, δ 4.45/79.30 and δ 5.05/71.04, respectively. The HMBC correlation at δ 5.05/171.25 indicated that residue G was also methyl esterified, and the C-G4 resonance at δ 79.30 indicated residue G was linked at position 4. All the evidences above suggested that residue G was also 4- α -D-GalpAMe. However, slight difference in chemical shifts of residue F and G suggested their different chemical environments within the polymeric structure.

For residue E, the COSY correlations at δ 5.07/3.75, δ 3.75/3.98, δ 3.98/4.40, δ 4.40/4.70, and a spin system along the sugar ring from TOCSY were detected.

Accordingly, C-E2, C-E3, C-E4 and C-E5 were assigned by HSQC correlations at δ 3.75/68.72, δ 3.98/69.47, δ 4.40/78.54, and δ 4.70/71.88, respectively. The HMBC correlation at δ 4.70/175.67 indicated that residue E was a galacturonic acid (GalpA). The chemical shift of C-E4 at δ 78.54 indicated residue E was linked at position 4. All together residue E was assigned as 4- α -D-GalpA. Similarly, for residue H, ^1H resonances of residue protons H-H2, H-H3, H-H4 and H-H5 were assigned as δ 3.73, δ 3.96, δ 4.39 and δ 4.68, respectively. Accordingly, C-H1, C-H2, C-H3, C-H4 and C-H5 were assigned by HSQC correlations at δ 3.73/68.64, δ 3.96/69.25, δ 4.39/79.15 and δ 4.68/71.90, respectively. The HMBC correlation at δ 4.68/175.39 indicated that residue H was also GalpA. The chemical shift of C-H4 at δ 79.15 indicated residue H was linked at position 4. Together, the evidence above is consistent with residue H as 4- α -D-GalpA.

Figure 10. ^1H NMR spectrum of CGP-6-f2. (D_2O , 500 MHz)

Figure 11. ^{13}C NMR spectrum of CGP-6-f2. (D_2O , 125 MHz)

Table 2. ^1H and ^{13}C NMR chemical shifts assignment of CGP-6-f2 (δ in ppm). (*p*: pyranosyl; *f*: furanosyl)

	Sugar residues	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6	-OMe	Key correlations across glycosidic bonds	
									NOESY	HMBC
A	t- α -L-Araf	107.69 5.15	81.63 4.13	77.03 3.95	84.41 4.03	61.48 3.81				
B	3,5- α -L-Araf	108.05 5.10	79.66 4.28	82.83 4.09	82.03 4.30	67.14 3.83/3.93				C-B3/H-A1, C-B5/H-C1
C	5- α -L-Araf	108.05 5.08	81.63 4.12	77.18 4.02	82.69 4.21	66.89 3.88/3.79				C-C5/H-B1
D	4- β -D-Galp	104.85 4.62	72.43 3.68	73.77 3.76	78.00 4.15	74.96 3.70	60.00 3.74		H-D1/H-D4	
E	4- α -D-GalpA (I)	99.50 5.07	68.72 3.75	69.47 3.98	78.54 4.40	71.88 4.70	175.67		H-E1/H-E4	H-E1/C-E4
F	4- α -D-GalpAMe (I)	99.81 5.11	68.60 3.74	68.69 3.99	78.83 4.46	71.02 5.11	171.27	53.38 3.80	H-F1/H-H4	C-F6/H-F(-OMe), C-F4/H-H1
G	4- α -D-GalpAMe (II)	100.74 4.95	68.64 3.72	69.25 3.98	79.30 4.45	71.04 5.05	171.25	53.38 3.80	H-G1/H-G4	C-G6/H-G(-OMe), C-G4/H-G1
H	4- α -D-GalpA (II)	100.49 4.90	68.64 3.73	69.25 3.96	79.15 4.39	71.90 4.68	175.39	– –	H-H1/H-F4	C-H4/H-F1
I	2- β -L-Rhap	101.08 4.77	79.73 4.57	69 3.95	– –	70.54 3.77	17.12 1.24			

The TOCSY spectrum (**Figure S1**) further revealed four additional spin systems with anomeric protons assigned as H-A1 (δ 5.15), H-B1 (δ 5.10), H-C1 (δ 5.08) and H-D1 (δ 4.62). In the case of residue D, COSY correlations at δ 4.62/3.68, δ 3.68/3.76, δ 3.76/4.15, δ 4.15/3.70 and δ 3.70/3.74 were detected. Accordingly, HSQC spectrum revealed carbon resonances at δ 104.85, δ 72.43, δ 73.77, δ 78.00, δ 74.96 and δ 69.00. The chemical shift of C-D4 at δ 78.00 suggested that residue D was linked at 4 position, and the C-D1 resonance at δ 104.85 indicated that residue D was β -linked. Therefore the residue D was assigned as 4- β -D-Galp.

For residues A, B and C, the HSQC correlations at δ 5.15/107.69, 5.10/108.05 and δ 5.08/108.05 were assigned to the correlation between anomeric proton and carbon of A, B and C, respectively. Further downfield carbon chemical shifts indicated that they were all furanosyl residues and α -linked.³² From COSY and TOCSY spectra, residue A protons were assigned as H-A1 (δ 5.15), H-A2 (δ 4.13), H-A3 (δ 3.95), H-A4 (δ 4.03), and H-A5 (δ 3.81). Residue B protons were assigned as H-B1 (δ 5.10), H-B2 (δ 4.28), H-B3 (δ 4.09), H-B4 (δ 4.30) and H-B5 (H^a : δ 3.83, H^b : δ 3.93). Residue C protons were assigned as H-C1 (δ 5.08), H-C2 (δ 4.12), H-C3 (δ 4.02), H-C4 (δ 4.21), H-C5 (H^a : δ 3.79, H^b : δ 3.88). Furanosyl residue A, B and C can be attributed to the arabinofuranosyl residues. From HSQC spectrum, the rest of carbon signals of residue A, B and C were assigned as C-A2 (δ 81.63), δ 77.03, δ 84.41, and δ 61.48; C-B2 (δ 79.66), C-B3 (δ 82.83), C-B4 (δ 82.03), and C-B5 (δ 67.14); C-C2 (δ 81.63), C-C3 (δ 77.18), C-C4 (δ 82.69), and C-C5 (δ 66.89). C-A5 at δ 61.48 indicated residue A was

a terminal arabinofuranosyl residue (t-Araf). The more downfield C-B3, C-B5 and C-C5 suggested that residue B was 3,5-linked arabinofuranosyl residue (3,5-Araf) and residue C was 5-linked arabinofuranosyl residue (5-Araf).

Although anomeric proton or carbon was not initially detected for the Rhap, they were later identified from NMR spectra of de-esterified CGP-6-f2 and assigned as H-I1 (δ 4.77) and C-I1 (δ 101.08). In addition, ^1H resonance at δ 1.24 and its corresponding ^{13}C resonance at δ 17.12 were characteristic to the methyl group of Rhap. For residue I, from the COSY spectrum of de-esterified CGP-6-f2 (**Figure S7**), the H-I2, H-I3, H-I5 and H-I6 were assigned as δ 4.57, δ 3.95, δ 3.77 and δ 1.24, respectively. Accordingly, the carbons of residue I were assigned by the HSQC spectrum of de-esterified CGP-6-f2 (**Figure S8**) at δ 4.77/101.08, δ 4.57/79.73, δ 3.95/69.00, δ 3.77/70.54 and δ 1.24/17.12. The chemical shift of C-I2 at δ 79.73 indicated that residue I was linked at position 2. The anomeric chemical shifts of residue I1 at δ 4.77/101.08 indicated that residue I was β -linked.^{33,40} Thus, residue I was assigned as 2- β -L-Rhap.

The sequences of the repeating units were determined by NOESY (**Figure S5**) and HMBC experiments. The correlations at δ 5.07/4.40 (H-E1/H-E4) in NOESY and δ 5.07/78.54 (H-E1/C-E4) in HMBC were detected, which suggested that residue E was mostly connected to identical residues at position 4. The correlations at δ 4.90/4.46 (H-H1/H-F4) and δ 5.11/4.39 (H-F1/H-H4) in NOESY, together with δ 4.90/78.83

(H-H1/C-F4) and δ 5.11/79.15 (H-F1/C-H4) in HMBC were detected, suggesting that residue F and H were mostly linked alternately through connections at position 4. The correlations at δ 4.95/4.45 (H-G1/H-G4) in NOESY and δ 4.95/79.30 (H-G1/C-G4) in HMBC were also observed, which is consistent with G residues G being connected to other G residues at position 4. The similarity in chemical shifts among the 1,4- α -D-GalpA and its methyl esters in addition with their alternate pattern suggested that these four residues are lined in the main chain with slightly different chemical environment from esterifications and acetylations. It could also be supported by the ^1H spectrum of the de-esterified CGP-6-f2 (**Figure S6**), since there was only one GalpA-type residue following de-esterification. Due to the complexity in the original NMR spectra, the following correlations can only be observed in de-esterified CGP-6-f2 NMR spectra. The correlations at δ 4.62/4.15 (H-D1/H-D4) in NOESY (**Figure S10**) indicated that residue D was connected to other D residues as a galactan side chain. The correlations at δ 5.15/82.83 (H-A1/C-B3), δ 5.10/66.89 (H-B1/C-C5) and δ 5.08/67.14 (H-C1/C-B5) from HMBC (**Figure S9**) suggested that residue A connected to B residues at position 3 and residues B and C connected to each other at position 5.

Thus, the backbone structure of CGP-6-f2 contains a combination of two repeating units, namely -4)- α -D-GalpA-(1- and -4)- α -D-GalpA-(1,2)- α -L-Rhap-(1-. Side chains of CGP-6-f2 are partially branched at position 4 on Rhap by galactan and arabinan.

The combined structural features of CGP-6-f2 are consistent with a RG-I type pectic polysaccharide^{30,41} and a tentative structure is proposed as in **Figure 12**.

4. Discussion

The Asian ginseng (*Panax ginseng* Meyer) is one of the most popular herbal medicines and the root has been traditionally consumed in East Asia to improve human immune system.^{42,43} Recent studies on ginseng have suggested its multiple health benefits including physical performance improvement⁴⁴, immunomodulatory⁴⁵, anti-cancer⁴⁶ and anti-hypertension activities⁴⁷. Today, over 200 molecules have been identified from *Panax ginseng* including peptides, amino acids, ginsenosides and polysaccharides.⁴⁸ Ginsenosides are a unique group of triterpene saponins isolated from ginseng, among which a ginsenoside compound K has exhibited anti-allergic⁴⁹, anti-diabetic⁵⁰, anti-aging⁵¹ and anti-inflammatory⁵² effects. Because ginseng root is traditionally processed by boiling with hot water, components from its aqueous extract such as polysaccharides have attracted great interests. Many kinds of polysaccharides have been isolated from *Panax ginseng* such as Ginsan⁵³, PGP2a⁵⁴, and PGPW1⁵⁵ but very few of the intact structures were characterized.

Due to the key role of biofilm in persistent bacterial infections, increasing attention has been drawn to the nature of biofilm and searching for molecules that inhibit the biofilm formation. Interestingly, certain polysaccharides have been found to inhibit *P. aeruginosa* biofilm formation. Examples include capsular polysaccharide K2 from the *E. coli* CFT073⁵⁶, r-EPS from bacterium *Lactobacillus acidophilus* A4⁵⁷, PI80 EPS from *Streptococcus phocae*⁵⁸ and A101 from *Vibrio* sp. QY101⁵⁹. In addition, previous

study⁶⁰ on *Panax ginseng* aqueous extract has exhibited biofilm prevention and dispersal effects.

However, the mechanism of these anti-biofilm complex carbohydrates is still unknown. In this study, a pectic polysaccharide derived from *Panax ginseng* was found to decrease *P. aeruginosa* intracellular c-di-GMP level. Inhibition of c-di-GMP production will cause downregulation of biosynthesis of biofilm matrix polysaccharides as well as upregulation of production of motility-involved appendages. Therefore, the anti-biofilm effects of ginseng pectic polysaccharide are very likely through inhibition of c-di-GMP production. Further *in vivo* investigation of its effect in dispersing preformed biofilm suggests its efficacy in potential application in treating biofilm-associated infections.

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Figure S1. TOCSY spectrum of CGP-6-f2. (D₂O, 500 MHz)

Figure S2. COSY spectrum of CGP-6-f2. (D₂O, 500 MHz)

Figure S3. HSQC spectrum of CGP-6-f2. (D₂O, 500 MHz)

Figure S4. HMBC spectrum of CGP-6-f2. (D₂O, 500 MHz)

Figure S5. NOESY spectrum of CGP-6-f2. (D₂O, 500 MHz)

Figure S6. ^1H NMR spectrum of de-esterified CGP-6-f2. (D_2O , 500 MHz)

Figure S7. COSY spectrum of de-esterified CGP-6-f2. (D₂O, 500 MHz)

Figure S8. HSQC spectrum of de-esterified CGP-6-f2. (D₂O, 500 MHz)

Figure S9. HMBC spectrum of de-esterified CGP-6-f2. (D₂O, 500 MHz)

Figure S10. NOESY spectrum of de-esterified CGP-6-f2. (D₂O, 500 MHz)

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Detection of Inulin, a Prebiotic Polysaccharide in Maple Syrup

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Abstract

Maple syrup is a widely consumed plant-derived natural sweetener produced by concentrating xylem sap collected from certain maple (*Acer*) species. During thermal evaporation of water, natural phytochemical components are concentrated in maple syrup. The polymeric components from maple syrup were isolated by ethanol precipitation, dialysis, and anion exchange chromatography and structurally characterized by glycosyl composition analysis, glycosyl linkage analysis, and nuclear magnetic resonance spectroscopy. Among the maple syrup polysaccharides, one neutral polysaccharide was characterized as inulin with a broad molecular weight distribution, representing the first isolation of this prebiotic carbohydrate from a xylem sap. In addition, two acidic polysaccharides with structural similarity were identified as arabinogalactans derived from rhamnogalacturonan type I pectic polysaccharides.

Keywords: Maple syrup, polysaccharide, inulin, arabinogalactan.

Introduction

Maple syrup is a natural sweetener produced from concentrated xylem sap collected from certain maple (genus *Acer*) species, primarily the sugar maple (*Acer saccharum* Marsh).^{1,2} The sugar maple is widely distributed in the northeastern region of North America with the majority of maple syrup being commercially produced in this region, primarily in Quebec, Canada.² Maple syrup is of great economical importance to the eastern North American region since it is the largest commercially produced and consumed tree-sap derived food product worldwide.³ As a commercial product that requires minimal processing (thermal evaporation), maple syrup contains several phytochemicals, primarily (poly)phenolics, which have been reported to impart a variety of biological effects. While sucrose is the major component in maple syrup, this plant-derived natural sweetener also contains simple sugars (glucose and fructose), amino acids, minerals (e.g. potassium, calcium, and magnesium), vitamins (e.g. B2 and niacin), organic acids (e.g. succinic and fumaric acid), and complex carbohydrates.¹ Among the various chemical constituents reported from maple syrup, biological evaluation has primarily focused on its diverse phenolic components. For instance, phenolic-enriched extracts of maple syrup have been reported to show anti-oxidant, anti-mutagenic, anti-cancer, anti-inflammatory and anti-neurodegenerative effects.³⁻⁷

In contrast to the phenolic components of maple syrup, molecules of larger molecular weight, such as oligosaccharides and polysaccharides, have been less

investigated. Previous reports have identified dextran, arabinogalactan and rhamnogalacturonan in maple syrup.^{8,9} These biopolymers are derived from primary cell wall components of maple trees.

In this study, an inulin type fructan has been isolated for the first time from maple syrup. Inulins are commercially extracted from chicory root and used as dietary fiber in the food industry.^{10,11} As one type of poly-fructan, the inulin structure features a polydisperse molecular size and is a non-branched polymer consisting exclusively of β -1,2-fructosyl linkages between fructosyl residues and a terminal glucose.^{12,13} Inulin is generally believed to be synthesized from sucrose by fructosyltransferases and to serve as energy storage.¹⁰ Because the linkage type within inulin is resistant to hydrolysis by human digestive enzymes, inulin is considered a non-digestive polysaccharide, which makes its biological effects reside in its interactions with the human lower gastrointestinal tract, primarily in the colon.^{10,14,15} Inulin-type fructans have been reported to benefit immune systems by interacting with gut lymphoid tissues,^{16,17} colon health by selectively promoting the growth of bifidobacteria and lactobacilli probiotic bacteria,^{18,19} and cardiovascular systems by decreasing cholesterol and triglyceride levels in serum.^{10,20}

Materials and methods

Materials

Maple syrup (grade C, 40 L) was shipped frozen to our laboratory by the Federation

of Maple Syrup Producers of Quebec (Longueuil, Quebec, Canada) and stored at -20 °C. Solvents for nuclear magnetic resonance (NMR) spectroscopy were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Total carbohydrate content determination

Total carbohydrate content assay was performed in microtiter plates using a colorimetric assay as previously described.²¹ Briefly, in each well of a 96-well microtiter plate, 30 µL of each fraction was added to 100 µL concentrated sulfuric acid and 20 µL 5% phenol solution. The microtiter plates were then incubated at 90 °C for 5 min and recorded for absorbance at 490 nm using a Spectramax M2 microplate reader (Molecular Devices, Sunnyville, CA). Glucose was used as the standard for the total carbohydrate content assay.

Extraction of maple syrup polysaccharides

Maple syrup (300 mL, 412 g) was first diluted with 450 mL distilled water followed by 95% ethanol to achieve a 60% ethanol/water solution. Crude polysaccharides were precipitated at -20 °C overnight. Precipitates were centrifuged and dried by sequential solvent exchanges with 100% ethanol and ether. The supernatants were combined and diluted with 95% ethanol to create an 80% ethanol/water solution, resulting in additional precipitation of polysaccharides as described above. Dry

precipitates were re-dissolved in 50 mL distilled water and dialyzed through a membrane with molecular weight cut-off (MWCO) of 12,000-14,000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA) against distilled water with stirring for 48 h. The retained polysaccharides were lyophilized to obtain Fr.1 (30 mg) from the 60% ethanol precipitation and Fr.2 (23 mg) from the 80% ethanol precipitation, respectively.

Purification of polysaccharides

Polysaccharides were fractionated on a Hiprep Q Sepharose anion exchange column (100 mm × 16 mm i.d., GE Healthcare Life Sciences, Pittsburgh, PA). 20 mg of Fr.1 were firstly eluted with 65 mL distilled water and then a linear gradient of NaCl (0-1 M) for 200 mL at 5 mL/min (**Figure 1**). 20 mg of Fr.2 were subjected to the same anion exchange chromatography. The eluate was collected in 5 mL fractions and pooled by total carbohydrate content. All fractions were dialyzed (MWCO 12,000-14,000) against distilled water with stirring for 48 h and lyophilized to obtain Fr.1-1 (10.5 mg, 0.004%, w/w), Fr.1-2 (5.5 mg, 0.002%, w/w), Fr.2-1 (1.1 mg, 0.0003%, w/w) and Fr.2-2 (8.3 mg, 0.002%, w/w).

Homogeneity and molecular size

The homogeneity and molecular sizes of polysaccharides were analyzed using a high performance size exclusion column TSK-gel G3000PW (300 mm × 7.5 mm i.d., TOSOH, Tokyo, Japan) at 40 °C on a Hitachi LaChrom Elite HPLC (Hitachi, Tokyo,

Japan) equipped with a refractive index (RI) detector. Standard curves for molecular size determinations were generated using dextran standards (1000, 5000, 12,000, 25,000, 50,000, 80,000 and 150,000 Da).

Glycosyl composition analysis

Glycosyl composition analysis was accomplished with polysaccharide acid hydrolysates using high performance anion exchange chromatograph with pulsed amperometric detection (HPAEC-PAD) as previously described.²² Briefly, 500 µg of Fr.1-1, Fr.1-2, Fr.2-1 and Fr.2-2 were hydrolyzed with 200 µL of 2M trifluoroacetic acid (TFA) in sealed ampoules at 120 °C for 2 h. The resulting hydrolysates were dried with a stream of nitrogen gas. 200 µL of isopropanol was added and dried by nitrogen gas. This process was repeated twice to remove residual TFA. Due to the fragility of fructose under acidic conditions, an alternate mild acid hydrolysis for Fr.1-1 was performed.²³ 200 µg Fr.1-1 were hydrolyzed with 200 µL 0.2% TFA in sealed ampoules at 90 °C for 1 h.

Hydrolysates from both hydrolytic reactions were separated on a Hamilton RCX-30 column (250 mm × 4.6 mm i.d., Metrohm, Herisau, Switzerland) on a 9600 Professional IC Vario ion chromatograph with a 945 Professional Vario IC pulsed amperometric detector (Metrohm, Herisau, Switzerland). Monosaccharide standard curves for fucose, arabinose, xylose, rhamnose, galactose, glucose, fructose, galacturonic acid and glucuronic acid were created by HPAEC-PAD on the same

column. Each monosaccharide and uronic acid was dissolved in distilled water at six concentrations (10, 20, 50, 100, 500, and 1000 ppm) and an aliquot of 10 μ L was injected. Standard curves were created by plotting area under curves (AUCs) with concentrations. For analyzing fucose, arabinose, xylose, rhamnose, galactose and glucose, the column was eluted with isocratic 16 mM NaOH over 40 min. For analyzing fructose, the column was eluted with isocratic 100 mM NaOH over 20 min. For analyzing glucuronic and galacturonic acids, the column was eluted with isocratic 20 mM sodium acetate in 100 mM NaOH over 20 min.

Glycosyl Linkage Analysis

Glycosyl linkage analysis was performed as previously described.²⁴ 800 μ g Fr.1-1, Fr.1-2, Fr.2-1 and Fr.2-2 were stirred overnight in 300 μ L dimethyl sulfoxide (DMSO). Intact polysaccharides were thrice permethylated in anhydrous DMSO solution of NaOH and methyl iodide for 20 min. The permethylated polysaccharides were extracted with methylene chloride and then hydrolyzed with 200 μ L of 2M TFA at 120 °C for 2 h. Permethylated Fr.1-1 was hydrolyzed with 200 μ L 0.2% TFA at 90 °C for 1 h. Hydrolysates were dried with N₂ gas and residual TFA was removed by repeatedly drying with isopropanol. The partially methylated hydrolysates were reduced with NaBD₄ and then acetylated with acetic anhydride. The resulting partially methylated acetic acetates (PMAAs) were analyzed on a Series 6890 gas chromatography (Agilent Technologies, Santa Clara, CA) coupled with a mass selective detector (MSD) on a Supelco SP-2330 capillary column (30 m

× 0.25 mm i.d., Sigma-Aldrich). The oven temperature was held at 80 °C for 2 min and increased to 170 °C at 30 °C/min, then to 240 °C at 4 °C/min, and held for 5 min.

NMR

The ¹H and ¹³C NMR spectra and two-dimensional COSY, TOCSY, HSQC and HMBC data were recorded on a Varian 500 MHz NMR spectrometer equipped with a 5 mm OneNMR probe (Agilent Technologies). The samples were D₂O exchanged twice and dissolved in D₂O (0.5 mL, 99.96%). All spectra were recorded at 25 °C.

RESULTS AND DISCUSSION

Crude polysaccharides Fr.1 and Fr.2 were fractionated using a semi-preparative Q Sepharose anion exchange column and polysaccharide- enriched fractions were pooled based on a total carbohydrate content assay.²¹ While subject to few interferences, this colorimetric assay is highly sensitive and has wide applicability for estimating the carbohydrate contents of plant natural products.²² The elution profiles (**Figure 1**) showed that Fr.1 contained a neutral polysaccharide fraction Fr.1-1 and an acidic polysaccharide fraction Fr.1-2. Similarly, Fr.2 also contained a neutral polysaccharide fraction Fr.2-1 and an acidic polysaccharide fraction Fr.2-2.

Figure 1. The elution profile of Fr. 1 by anion exchange chromatography.

The molecular sizes of the purified polysaccharide components were analyzed by HPSEC-RI. The dispersal of Fr.1-1 across a broad region of elution volume indicated that Fr.1-1 was comprised of polydisperse and large molecular weight (>12,000 Da) polymers. Elution profiles of Fr.1-2 and Fr.2-2 showed symmetric peaks, indicating the molecular weights of Fr.1-2 and Fr.2-2 to be approximately 140,000 Da and 70,000 Da, respectively. Due to the limited quantity of Fr.2-1 and sensitivity of the RI detector, the molecular size of Fr.2-1 was not successfully measured.

The glycosyl and linkage compositions of the purified polysaccharides were evaluated as follows. Because fructose is easily degraded, Fr.1-1 was hydrolyzed with two hydrolytic conditions. Hydrolysis with 2M TFA at 120 °C for 2 h totally degraded fructose, leaving only glucose detectable in the HPAEC profile. Mild acid

hydrolysis of Fr.1-1 using 0.2% TFA at 90 °C for 1 h was performed to hydrolyze the glycosidic bonds between fructose residues and preserve the resulting monosaccharides. By comparison to the standard curves, the glycosyl composition of Fr.1-1 (**Table 1**) contained primarily fructosyl (96%) and glucosyl (4%) residues, which suggested that Fr.1-1 is a poly-fructan with minor inclusion of glucose.

Glycosyl residue	Fr.1-1	Fr.1-2	Fr.2-1	Fr.2-2
Mole %				
Rhamnosyl	0	9	0	7
Arabinosyl	0	35	25	42
Galactosyl	0	47	35	40
Glucosyl	4	1	40	2
Mannosyl	0	1	0	2
Fructosyl	96	0	0	0
Galacturonic acid	0	7	0	7

Table 1. Glycosyl compositions of Fr. 1-1, Fr.1-2, Fr.2-1 and Fr.2-2.

The presence of 2-fructosyl residues indicated that the fructosyl residues were linked at position 2, which is characteristic for inulin. Inulin contains a non-branched 1,2-linked fructosyl backbone and terminates with a glucosyl residue (GF_n) (**Figure 2**).¹² The neutral monosaccharide compositions of Fr.1-2, Fr.2-1 and Fr.2-2 were analyzed on HPAEC-PAD and eluted with isocratic 16 mM NaOH for improved resolution. Neutral polysaccharide Fr.2-1 was composed of arabinosyl (25%), galactosyl (35%) and glucosyl (40%). Together with the presence of a terminal

arabinosyl, 3- and 3,6-galactosyl, and 6-glucosyl linkages, Fr.2-1 may be a mixture of arabinogalactan and dextran, which has been speculated to be a microbial derived polysaccharide.⁸ Acidic polysaccharides Fr.1-2 and Fr.2-2 hydrolysates were also analyzed for their uronic acids (**Table 1**). Together with their neutral monosaccharide compositions, Fr.1-2 and Fr.2-2 appear to share structural similarity. The two polysaccharides were primarily composed of terminal and 5-arabinosyl and 3- and 3,6-galactosyl with a lower percentage of 2,4-rhamnosyl and 4-galacturonic acid residues. These results suggest that Fr.1-2 and Fr.2-2 are arabinogalactans derived from degradation of acidic rhamnogalacturonan type I (RG-I) pectic polysaccharides. These molecules are important plant cell wall components and often contain alternate rhamnosyl and galacturonic acid backbones and arabinogalactan side chains.²⁵

Figure 2. General chemical structure of inulin.

Figure 3. ^1H NMR spectrum of Fr. 1-1 (500MHz, D_2O).

The structures of the polysaccharides were characterized by NMR analyses. The ^1H NMR spectrum of Fr.1-1 (**Figure 3**) revealed seven major protons between 3.3 to 4.2 ppm and a very small anomeric proton signal at 4.8 ppm. The ^{13}C NMR and HSQC spectra showed one quaternary carbon at 104.1 ppm, three CH and two CH_2 , which suggested characteristic structural features of the ketonic monosaccharide fructose, which lacks an anomeric proton but contains two methylenes at positions 1 and 6. The chemical shifts of Fr.1-1 were assigned using two-dimensional NMR experiments (**Table 2**). Briefly, with the correlations in COSY, the chemical shifts of proton 3-6 in the 2-Fructosyl were assigned as H-3 at δ 4.02, H-4 at δ 3.93, H-5 at δ 3.79 and H-6 at δ 3.40/3.74. The chemical shifts of carbon 3-6 in the 2-Fructosyl were assigned accordingly with correlations in HSQC spectra as C-3 at δ 76.4, C-4

at δ 75.3, C-5 at δ 80.2 and C-6 at δ 63.3. The two doublets at δ 3.52 and δ 3.60 correlate to one carbon at δ 60.0, which were assigned as the proton and carbon chemical shifts of the H-1/C-1 methylene in 2-Fructosyl. A correlation was observed between C2 and H1 in the HMBC spectrum, which further confirmed that the fructosyl residues were linked at position 2. The small proton signal at 4.8 ppm was attributed to the anomeric proton of glucose. The ^1H NMR spectra of Fr.1-2, Fr.2-1 and Fr.2-2 showed only proton resonances belonging to carbohydrates (anomeric protons, 4.4-5.5 ppm; clustered resonances between 3.4 ppm and 4.0 ppm)²⁶, suggesting that only polysaccharides were present in the samples. The proton resonances appearing near 1.1 ppm for Fr.1-2 and Fr.2-2 are characteristic for the rhamnose methyl group at position 5. However, further investigations on these molecules by 2D NMR analyses are needed to better define their complete structural features.

Residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-5
2-Fructosyl	3.52/3.60	-	4.02	3.93	3.79	3.40/3.74
	60.0	104.1	76.4	75.3	80.2	63.3
Terminal Glucosyl	4.82	3.42	3.73	-	-	-
	-	-	-	-	-	-

Table 2. ^1H and ^{13}C chemical shift assignments of Fr. 1-1 in parts per million (ppm).

Maple syrup is widely consumed as a functional food in large part because of its phenolic constituents which are believed to impart health benefits. For example,

quebecol is a phenolic compound from maple syrup²⁷ that possesses anti-inflammatory²⁸ and anti-proliferative properties.²⁹ A variety of phenolic derivatives from maple syrup demonstrate free radical scavenging activity superior to that of vitamin C.³ Several polyphenolic molecules present in maple syrup show anti-proliferative effects through arresting cell cycles, suggesting potential effects in cancer prevention.³⁰ In addition, *in vivo* studies on phenolic-enriched maple syrup extracts have shown promising therapeutic potential for liver protection,³¹ anti-inflammation³² and anti-diabetes.^{33,34}

The polysaccharides dextran, arabinogalactan and rhamnogalacturonan have been previously identified from maple syrup.^{8,9} Dextran is a common polysaccharide synthesized from sucrose and prominently features repeating units of α -6-linked glucose.³⁵ Previous studies^{8,9} have attributed the glycosyl compositions and glycosydic linkages of arabinogalactans and rhamnogalacturonans to partially degraded pectins. Pectins are heteropolysaccharides and are one of the major structural components of plant cell walls.³⁶ The heterogeneous backbone of pectin is composed of homogalacturonan (HG) with repeating 4)- α -GalpA-(1, and rhamnogalacturonan (RG) with the alternating disaccharide 4)- α -GalpA-(1,2)- α -L-Rhap-(1,.³⁷ Arabinogalactan, a major side chain branched at C-4 of rhamnosyl residues, contains a linear 6-linked galactan substituted at C-3 with arabinoses.³⁸

Herein, inulin is being reported from maple syrup for the first time. Inulins are widely present in several plant families including Liliaceae, Amaryllidaceae,

Gramineae, and Compositae. Many edible fruits and vegetables such as banana, onion, artichoke, chicory roots, garlic, and leek contain inulin.³⁹ However, this study is the first report of inulin-type fructooligosaccharides isolated from xylem sap of higher plants. The identification of inulin in maple syrup, along with its presence in other natural plant-derived sweeteners including agave⁴⁰ and stevia,⁴¹ raises the possibility of this prebiotic polysaccharide contributing to the overall health benefits reported for these foods.

Ecologically, besides serving as an energy storage carbohydrate, plant cells regulate osmotic potential by quickly altering the degree of polymerization of inulin. This function allows plants to withstand cold temperatures and dehydration.^{42,43} This phenomenon also encourages future investigations on the quantity of inulin present in maple sap collected at various times during the tapping season to potentially maximize its inclusion in maple-derived food products.

In addition to its low caloric value, the prebiotic effects of inulin have been reported.^{19,44-46} Because inulin passes through the human upper gastrointestinal tract (GIT) intact, it interacts primarily with human gut microbiota. Prebiotic molecules such as inulin^{47,48} have been shown to selectively enhance the growth of beneficial bacteria, such as species of *Bifidobacterium* and *Lactobacillus*, while suppressing the growth of pathogenic strains.

In this study, the presence of inulin in maple syrup potentially supports an investigation by Cochu *et al.*⁴⁹ who compared the growth of probiotic *Lactobacillus* sp. when using raw maple sap and sucrose as carbon sources. Results showed that

maple sap based media increased the viable cell counts of two strains of lactobacilli, *Lactobacillus helveticus* R0052 and *Lactobacillus acidophilus* AC-10, and enhanced their lactic acid production. This study suggested that molecules in the raw maple sap possess prebiotic effects and potentially provide health benefits to the GIT. Evidence^{44,46} shows that bifidobacteria and lactobacilli have different preferences for utilizing inulin-type fructooligosaccharides of different chain lengths. This suggests that despite having the same repeating unit, maple syrup-derived inulin could potentially have unique effects on GIT bacteria. Thus, while our finding that maple syrup contains inulin adds to the potential health benefits of this food, the overall contribution of this inulin polysaccharide remains unclear given the lack of supporting in vivo data. Further investigation to clarify the contributions of inulin to the overall benefits of maple syrup is currently being pursued by our group.

ASSOCIATED CONTENT

Supporting Information

Figure S1 anion exchange chromatographic profile of Fr.2; **Figure S2-S5** size exclusion chromatographic profiles of Fr.1-1, Fr.1-2 and Fr.2-2 and standard calibration curve; **Figure S6-S12** HPAEC-PAD profiles of Fr.1-1, Fr.1-2, Fr.2-1 and Fr.2-2 acid hydrolysates; **Figure S13-S19** 1D and 2D NMR spectra; **Table S1** Glycosyl linkages present in the four polysaccharides.

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Notes

The authors declare no conflicts of interest

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Table S1. Glycosyl linkages present in Fr.1-1, Fr.1-2, Fr.2-1 and Fr.2-2.

Figure S1. Anion exchange chromatographic profile of Fr.2.

Figure S2. HPSEC profile of Fr.1-1.

Figure S3. HPSEC profile of Fr.1-2.

Figure S4. HPSEC profile of Fr.2-2.

Figure S5. Standard calibration curve for HPSEC profiles.

Figure S6. HPAEC-PAD profile of neutral monosaccharide standards fucose, rhamnose, arabinose, galactose, glucose, mannose and fructose by isocratic 16 mM NaOH elution.

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Figure S13. ^{13}C NMR spectrum of Fr.1-1 (125 MHz, D_2O).

Figure S14. COSY NMR spectrum of Fr.1-1 (500 MHz, D₂O).

Figure S15. HSQC NMR spectrum of Fr.1-1 (500 MHz, D₂O).

Figure S17. ^1H NMR spectrum of Fr.1-2 (500 MHz, D_2O).

Figure S18. ^1H NMR spectrum of Fr.2-1 (500 MHz, D_2O).

Figure S19. ^1H NMR spectrum of Fr.2-2 (500 MHz, D_2O).

Glycosyl residue	Linkage	Fragmentation pattern	Maple syrup polysaccharide(s)
Rhamnosyl	2, 4	190, 203	Fr.1-2, Fr.2-2
Arabinosyl	Terminal	118, 161, 162	Fr.1-2, Fr.2-2, Fr.2-1
	5	118, 189	Fr.1-2, Fr.2-2,
Galactosyl	3	118, 161, 234, 277	Fr.1-2, Fr.2-2 Fr.2-2
	3, 6	118, 189, 234, 305	Fr.1-2, Fr.2-2 Fr.2-1
Fructosyl	2	129, 87, 161, 190	Fr.1-1
Glucosyl	6	118, 129, 162, 189	Fr.2-1
Galacturonic acid	4	118, 234	Fr.1-2, Fr.2-2

Table S1. Glycosyl linkages present in Fr.1-1, Fr.1-2, Fr.2-1 and Fr.2-2. Primary glycosyl linkages of each maple syrup polysaccharide and their characteristic fragmentation patterns in GC/MS.