Structural Elements of an Antioxidative Pectic Arabinogalactan from *Solanum virginianum*

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Abstract

The water-soluble polysaccharide-containing fraction from *Solanum virginianum* leaves upon anion exchange chromatography yielded a highly branched pectic arabinogalactan-containing fraction (F2). Herein, direct evidences for the (i) presence of a chain having 1,6-linked Gal units substituted at O-3, (ii) coexistence of Ara and Gal residues in the same molecule, (iii) existence of a chain containing 1,3-linked Gal*p* residues substituted at C-6, and (iv) occurrence of 1,5-linked Ara*f* residues substituted at O-3 were presented. This polysaccharide that showed a dose-dependent antioxidative property formed a water-soluble complex with bovine serum albumin. Thus, F2 could be used as a natural ingredient in functional food and pharmaceutical products to mollify oxidative stress.

Key words
Solanum virginianum · Solanaceae · polysaccharide structure · antioxidative effect · BSA-polysaccharide complex

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The importance of free radicals as an exacerbating factor in cellular injury and the aging process has attracted increasing attention over the years [1,2]. Recently, reactive oxygen species are evidenced to be closely related to degenerative diseases such as Alzheimer's, neuronal death including ischemic stroke, and acute and chronic degenerative cardiac myocyte death [1,3,4].

Solanum virginianum L. (Solanaceae) is being utilized for the management of a number of diseases throughout the Indian subcontinent since ancient times [5,6]. Indeed, a range of pharmacological activities, such as antiurolithiatic [7], antihyperglycemic, and antioxidative [8], was observed from extracts and pure compounds from this herb. So far, only secondary metabolites, namely, glycosides [9] and steroid alkaloids [10], have been chemically characterized. Incidentally, polysaccharides from many medicinal plants stimulate a range of biological activities [11–14], but a report on the antioxidative activity of polysaccharides from S. virginianum leaves has not yet been explored.

Herein, we report the isolation and structural analysis of a water-extracted polysaccharide from *S. virginianum* leaves. Moreover, we have evaluated the antioxidative property of this polysaccharide using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. Additionally, the interaction of this polysaccharide with bovine serum albumin has been investigated.

Results and Discussion

Extraction of the leaves of *S. virginianum* with water yielded a crude polysaccharide-containing fraction having antitussive activity [15]. This crude extract upon anion exchange chromatography (AEC) yielded two fractions: F1 and F2 eluted with 0.05 and 0.5 M NaOAc (pH 5.5), respectively. The major fraction F2, which consisted of 60% of the total material loaded onto the column, contained Ara, Gal, Rha, and Glc residues in the molar ratio of 44 : 40 : 2:trace. F2 contained 71% (w/w) polysaccharide including 8% uronic acid. TLC analysis of the acid generated monosaccharides indicates the presence of *inter alia*, an uronic acid with R*f* values similar to that of GalA. GC analysis of the per-O-trimethyl-silylated methyl glycoside derivatives confirmed this result. This purified macromolecule, a pectic arabinogalactan, was subjected to structural and biological analyses.

Size exclusion chromatography of F2 shows that this polymer is homogeneous. Based on the calibration with standard pullulans, the molecular mass of F2 was estimated to be 320 kDa. Methylation analysis (**Table 1**) indicated that F2 contained, *inter alia*, terminal-, (1,3)-, (1,6)- and (1,3,6)-linked Gal*p* residues together with terminal-, (1,5)- and (1,3,5)-linked units of Ara*f*. Moreover, the presence of 1,2- and 1,2,4-linked Rha*p* residues, characteristics of rhamnogalacturonan type I, was also revealed. The ^{1}H NMR spectrum (**Fig. 1 S**, Supporting Information) of F2 contained signals from 5.02 to 5.08 ppm originating from anomeric protons (H1) of the nonreducing terminal α-Ara*f* units [16,17]. The signals at 5.15 and 5.22 ppm were assigned to H1 of 1,5- and 1,3,5-linked α-Ara*f* residues, respectively. Another group of H1 signals from δ 4.44 to 4.59 originating from different β-Gal*p* residues were also observed. Additionally, it contained resonances be-

Methylation products[a]	Linkages	Peak area[b]		
		F2	PRF2	A1O
2,3,5-Me_3-Ara	Terminal-	15	5	46
3,5-Me_2-Ara	1,2-	1		
2,3-Me_2-Ara	1,5-/1,4-	24	12	23
2-Me-Ara	1,3,5-/1,3,4-	9		
3,4,6-Me_3-Rha	1,2-	1		
3,6-Me_2-Rha	1,2,4-	2		
2,3,6-Me_3-Glc	1,4-	2		
2,3,4,6-Me_4-Gal	Terminal-	6	16	15
2,4,6-Me_3-Gal	1,3-	18	31	
2,3,4-Me_3-Gal	1,6-	8	29	16
2,4-Me_2-Gal	1,3,6-	14	7	

Table 1 Methylation analysis data of the water-extracted polysaccharide (F2) isolated from *S. virginianum* leaves and the ethanol-precipitated (PRF2) and soluble (A1O) fractions derived from F2 by Smith degradation.

[a] 2,3,5-Me_3-Ara denotes 1,4-di-O-acetyl-2,3,5-tri-O-methylarabinitol, etc.; [b] Percentage of total area of the identified peaks

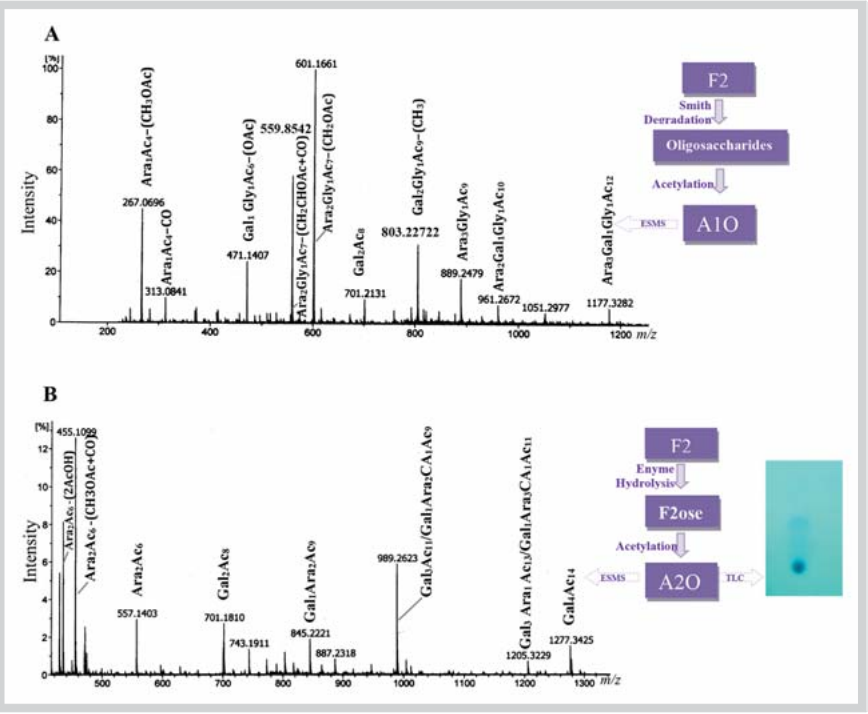

Fig. 1 Positive ion mode ESMS spectra of acetylated oligosaccharides generated by (**A**) Smith degradation and (**B**) positive ion mode ESMS spectra and TLC analysis of acetylated oligomeric fragments (A1O and A2O) generated by enzyme hydrolysis of the polysaccharide (F2) from *S. virginianum* leaves.

tween 3.27 and 4.01 ppm, characteristic of ring protons (H2–H5). The other distinctive feature of F2 was the presence of esterified phenolic acid residues. Indeed, the total phenolic content of F2 was 10 mg GAE/g of sample and it contained *p*-coumaric acid, sinapic acid, ferulic acid, and 8,5′-diferulic acid (diFA) in a molar ratio of 82 : 2 : 6 : 10.

The polysaccharide F2, on Smith degradation using the standard protocol, yielded a periodate resistant polymeric fraction (PRF2) together with an aqueous 80% ethanol soluble fraction (PRF2 S) containing oligomeric fragments. As shown in **Fig. 1 A** "A1O", the acetylated derivative of PRF2S comprises three series of oligomeric fragments, many of which contained a glycerol label (O-Gly). The O-Gly tag at the reducing end of oligomeric fragments such as $Ara_2Gly_1Ac_7$-[CH_2OAc], $Ara_2Gly_1Ac_7$-[CH_2CHOAc +CO] (molecular rearrangement products), $Ara_3Gly_1Ac_9$, and others were generated from the oxidative cleavage of 1,6-linked Gal units by periodate. These oligomeric fragments together with the presence of $Ara_2Gal_1Gly_1Ac_{10}$ and $Ara_3Gal_1Gly_1Ac_{12}$ imparted evidences for the existence of Ara and Gal residues in the same molecule, indicating that the polymer was an arabinogalactan and not a mixture of arabinan and galactan. Moreover, the presence of a segment containing at least three consecutive 1,3,5-linked Ara in accord with $Ara_3Gly_1Ac_9$ was revealed. Incidentally, methylation analysis of PRF2S revealed the presence of terminal- and 1,6-linked Gal*p* units (**Table 1**). Fragments such as Gal_2Ac_8, Ara_1Ac_4-[CO], and Ara_1Ac_4-[CH_3OAc], probably arisen by the removal of O-Gly tag with CF_3CO_2H during Smith degradation, were also present. In addition, electrospray mass spectrometry (ESMS) analysis coupled with glycosidic linkage analysis confirmed the existence of two consecutive 1,6-linked Gal units each substituted at O-3 in the parental F2. The periodate-resistant polymeric product (PRF2) contained Gal and Ara in a molar ratio of 74 : 26. Indeed, large quantities of Ara residues were destroyed during Smith degradation. Certainly F2 possesses a large amount of terminal- and 1,5-linked Ara*f* residues (**Table 1**), both of which were destroyed by periodate. Remarkably, the proportion of 1,6-linked residues increased significantly after Smith degradation (**Table 1**). Hence, the presence of a chain containing 1,6-linked Gal*p* residues substituted at O-3 was ascertained. Moreover, the existence of a chain containing 1,3-linked Gal*p* residues substituted at C-6 was also established. Notably, a major part of the Ara residues was either degraded or present in the ethanol-soluble part (PRF2S) as a monomer or oligomer.

Afterwards, oligosaccharide subunits generated from F2 using a commercial enzyme preparation possessing endogalactanase activity [12] were then acetylated. ESMS spectrum (**Fig. 1 B**) of acetylated oligomers (A2O) revealed the presence of numerous fragments. On the basis of their molecular weight, ions at *m/z* 413, 557, 701, 845, 989, 1205, and 1277 could be assigned to $[M + Na]^+$ of Hex_1Ac_5 (Hex_1 = one hexose residue, Ac_5 = five acetyl substituent), $Pent_2Ac_6$ ($Pent_2$ = two pentose residues), Hex_2Ac_8, $Hex_1Pent_2Ac_9$, Hex_3Ac_{11}, $Hex_3Pent_1Ac_{13}$, and Hex_4Ac_{14}, respectively. Fragment ions at *m/z* 437 and 455 could have arisen from $Pent_2Ac_6$-[2AcOH] and $Pent_2Ac_6$-[CH_3OAc + CO] units. Sugar compositional analysis indicated that A2O contained galactose and arabinose residues as neutral sugars. Taken together, Hex_1Ac_5, $Pent_2Ac_6$, Hex_2Ac_8, $Hex_1Pent_2Ac_9$, Hex_3Ac_{11}, $Hex_3Pent_1Ac_{13}$, and Hex_4Ac_{14} could be assigned to Gal_1Ac_5, Ara_2Ac_6, Gal_2Ac_8, $Gal_1Ara_2Ac_9$, Gal_3Ac_{11}, $Gal_3Ara_1Ac_{13}$, and Gal_4Ac_{14}, respectively. Remarkably, A2O showed UV absorption bands at 230 and 277 nm, characteristic of phenolic acids. The presence of two phenolic acids containing oligosaccharides in A2O was also indicated by the presence of fluorescent spots on TLC. Especially p-coumaric acid was the predominant phenolic acid of F2. Taken together, ions at *m/z* 989 and 1205 may also be due to $[M + Na]^+$ of $Gal_1Ara_2CA_1Ac_9$ (CA = coumaric acid) and $Gal_1Ara_3CA_1Ac_{11}$, respectively. Notably, the presence of $Gal_1Ara_2CA_1Ac_9$, $Gal_1Ara_3CA_1Ac_{11}$, $Gal_1Ara_2Ac_9$, and $Gal_3Ara_1Ac_{13}$ suggested that galactose, arabinose, and phenolic acid residues were an integral part of the same polysaccharide. Therefore, it may be concluded that the studied biomacromolecule is a pectic arabinogalactan esterified with phenolic acid.

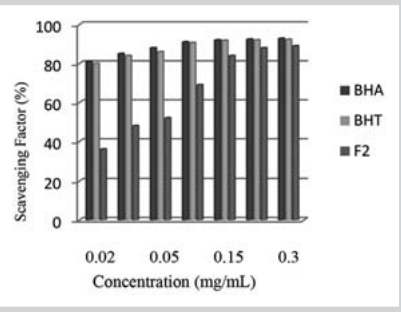

Fig. 2 Scavenging effect on DPPH radicals of the water-extracted polysaccharide (F2) from *S. virginianum* leaves along with standard antioxidants butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT).

F2 showed a dose-dependent DPPH-radical scavenging activity up to 200 µg/mL (**Fig. 2 S**, Supporting Information). Interestingly, at the 200 µg/mL concentration, F2 scavenged 88 ± 0.3% DPPH radicals, where as for butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), the values were 92.8 ± 0.3% and 92.3 ± 0.3%, respectively (**Fig. 2**).

This biopolymer interacts with BSA. Indeed, with the gradual addition of F2 to the BSA solution (pH 7.4), the intensity of the peak at 215 nm in the UV spectrum of BSA decreased (**Fig. 3 A**). Moreover, the λ_{max} (maximum wavelength) for the particles in the solution shifted toward a longer wavelength. The spectral changes around λ_{max} (215 nm) might occur from the disturbance of the microenvironment around the polypeptide caused by the binding of F2 with BSA.

Moreover, the maximum fluorescence emission wavelength (λ_{em}) of BSA also showed a red shift (24 nm at pH 7.4; **Fig. 3 B**). This phenomenon was likely due to the complexation of F2 with BSA, resulting in the latter's conformational changes. According to a modified Stern-Volmer equation, the binding constant (K) for the F2-BSA complexation at pH 7.4 was 2.68×10^5/M. This value was analogous to other strong ligand-protein complexes with binding constants ranging from 10^6 to 10^8/M [18].

Thus, a polysaccharide containing esterified phenolic acids in monomeric and dimeric types was isolated from *S. virginianum*. ESMS analysis of a spectrum of per acetylated oligomeric fragments generated by Smith degradation and enzyme hydrolysis imparts finer structural details of this polymer. Remarkably, F2 formed a water-soluble complex with BSA and, hence, was biocompatible with the transport protein. Furthermore, antioxidative activity of the studied biomacromolecule provides a scientific basis for the use of this herb in traditional medicine.

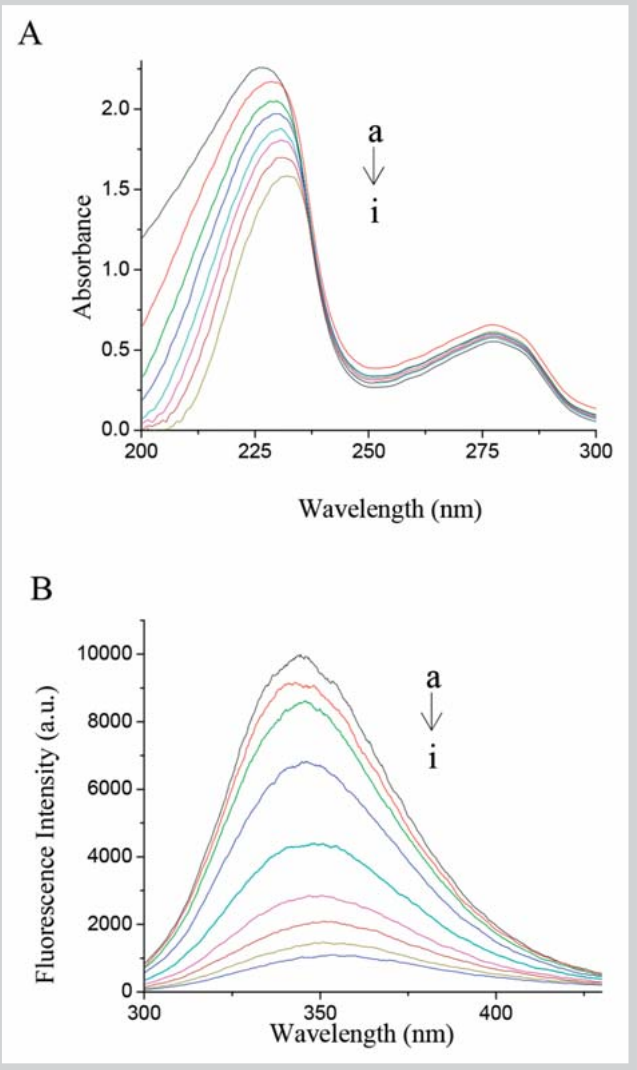

Fig. 3 Effect of the polysaccharide (F2) from *S. virginianum* leaves on (**A**) UV absorption spectra and (**B**) fluorescence emission spectra of BSA in 10 mM phosphate buffer (pH 7.4). The black line in **A** is the UV spectrum of native BSA (1 mg/mL). The colored lines correspond to the spectra of BSA induced by various concentrations (0.2–1.0 mg/mL) of F2. In the fluorescence emission spectra (**B**), line "a" comes from 0.75 µM BSA alone, whereas lines "b–i" originate from 0.75 µM BSA with 0.16, 0.31, 0.63, 1.25, 1.88, 2.50, 3.13, and 3.75 µM F2, respectively.

Materials and Methods

This material is available as Supporting Information.

Supporting information

The general experimental procedures, extraction, and purification of the pectic arabinogalactan followed by analytical methods as well as copies of the ^{1}H NMR spectrum and scavenging effect on DPPH radicals of this polysaccharide are available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no competing financial interest.

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