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Structural features of water-soluble novel polysaccharide components from the leaves of *Tridax procumbens* Linn.

T. Shantha Raju [1], E.A. Davidson *

Department of Biochemistry and Molecular Biology, Georgetown University Medical School,
3900 Reservoir Road, N.W., Washington, D.C. 20007, USA

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Abstract

Two water-soluble polysaccharide fractions, WSTP-IA and WSTP-IB were purified from the leaves of *Tridax procumbens* Linn. with graded ethanol precipitation followed by mild delignification and size-exclusion chromatography. WSTP-IA contained L-Ara*f* and D-Gal*p* in ~1:3 molar proportions, and WSTP-IB contained only D-Gal*p* as the major sugar component. The results of methylation linkage analysis, and ^{1}H and ^{13}C NMR studies on the native and modified polysaccharides, indicated that WSTP-IA is an L-*arabino*-D-galactan with a β-(1 → 6)-D-galactan main chain in which at least one in every two D-Gal*p* residues carries single residues of either L-Ara*f* (α-/β-) or β-D-Gal*p* end-group as substituents at O-3. WSTP-IB is a linear β-(1 → 6)-D-galactan. This is the first report of polysaccharides containing a β-(1 → 6)-D-galactan main chain isolated from plant sources.

1. Introduction

Tridax procumbens Linn. is a common South American weed, with a woody base that sometimes roots at the nodes, and is now pantropical [1]. The leaves of the plant are used as a vegetable and as cattle feed. In Southern India, the leaves are employed in the treatment of bronchial catarrh, dysentery, diarrhea and for restoring hair [1]. The leaf juice possesses antiseptic, insecticidal, and parasiticidal properties, and is used to check haemorrhage from cuts, bruises, and wounds. The leaf juice is a known indigenous herbal drug which increases lysyl oxidase activity,

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* Corresponding author.

[1] Present address: Department of Cell Biology, Albert Einstein College of Medicine, Yeshiva University, 1300 Morris Park Avenue, Bronx. NY 10461, U.S.A. Tel: (718)430-3470; E-mail: raju@aecom.yu.edu.

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protein content, specific activity of lysyl oxidase, and breaking strength at the site of a wound, when applied [2–4]. An aqueous extract of the plant produced reflex tachycardia and shows a transient hypotensive effect on the normal blood pressure of dogs [1]. Although the leaves of *T. procumbens* contain ~ 39% soluble carbohydrates [1] (dry wt), no details are available about the nature of these carbohydrates. Because of the many medicinal applications of *T. procumbens* leaves [1–4], the structural elucidation of polysaccharide components present in these leaves was undertaken. The present paper describes the isolation, purification, and structure of novel water-soluble high molecular weight arabinogalactan and a linear low molecular weigt galactan from the leaves of *T. procumbens* Linn.

2. Results and discussion

Isolation, fractionation, and sugar composition.—The dried and powdered leaves, after pre-extraction with organic solvents, were extracted with hot water, giving an insoluble residue (72% yield) and an aqueous extract. The aqueous extract upon graded ethanol precipitation afforded two polysaccharide fractions, WSTP-I (1 vol, 10% yield) and WSTP-II (6 vol, 6% yield) which contained ~ 73% and 63% carbohydrates with 10.5 and 15.8% protein, respectively. The phosphate, sulfate, ash, lignin, and *O*-acetyl content of these fractions are shown in Table 1. From Table 1, it is evident that WSTP-II contains relatively more lignin and protein and less carbohydrate than WSTP-I. Further, the presence of very little phosphate, sulfate, and *O*-acetyl groups in these polysaccharide fractions (Table 1) indicates the absence of phosphated, sulfated, and *O*-acetylated sugars. Furthermore, WSTP-I, WSTP-II, and the crude extract of dry powdered leaves of *T. procumbens* were examined by SDS-PAGE electrophoresis [5] under both reducing and nonreducing conditions. When electrophoresis was carried out on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue for proteins and periodate–Schiff's reagent for carbohydrates [6], no low molecular weight bands were observed and

Table 1
Chemical composition of polysaccharide fractions isolated from the leaves of *Tridax procumbens* linn (% by wt)

Component	Powdered leaves	WSTP-I [a]	WSTP-IA [a]	WSTP-IB [a]	WSTP-II [a]
	(% by wt)				
Carbohydrate	39.5	73.0	89.3	86.2	63.0
Protein	28.5	10.5	2.2	3.3	15.8
Phosphate	5.0	0.5	0.4	0.2	0.6
Sulfate	3.0	0.3	0.2	0.1	2.8
Ash	12.0	5.0	4.6	5.5	4.1
Lignin	11.5	10.5	3.3	4.3	14.5
O-Acetyl	0.5	0.3	0.2	0.5	0.3

[a] Polysaccharide fractions isolated from the leaves (see Results and Discussion and Scheme 1).

Table 2
Comparison of sugar composition of the polysaccharide fractions isolated from the leaves of *Tridax procumbens* linn

Sugar	WSTP-I [a]	WSTP-IA [a]	WSTP-IA$_1$ [a]	WSTP-IB [a]	WSTP-II [a]
L-Rha	0.1	n.d.[b]	n.d.	n.d	0.3
L-Ara	0.3	0.4	n.d.	n.d.	0.4
D-Xyl	0.1	n.d.	n.d.	n.d.	0.3
D-Man	0.1	n.d.	n.d.	n.d.	0.3
D-Gal [c]	1.0	1.0	1.0 [d]	1.0 [d]	1.0
D-Glc	0.1	n.d.	n.d.	n.d.	1.3

[a] See Scheme 1 for details.
[b] n.d., Not detected.
[c] Compositions are normalized with respect to D-Gal and are based on GLC of alditol acetates.
[d] Only D-Gal was present.

all the bands remained at the top of the gel. When electrophoresis was carried out on either 7 or 5% SDS-polyacrylamide gel, the samples moved as a single polydisperse band (mol wt ~ 200 kDa) and stained with both Coomassie Blue and periodate–Schiff's reagent, indicating that most of the proteins are glycosylated.

The sugar compositions of WSTP-I and WSTP-II are shown in Table 2. Besides low proportions of L-Rha, D-Xyl, D-Man, and D-Glc, the WSTP-I fraction is mainly composed of L-Ara and D-Gal in ~ 1:3 proportions, indicating that this fraction contains mainly arabinogalactan type polysaccharides. WSTP-II contained mainly D-Glc with low proportions of L-Rha, L-Ara, D-Xyl, D-Man, and D-Gal. WSTP-I and WSTP-II did not contain uronic acids.

Delignification [7] of WSTP-I was achieved under mild conditions with no detectable loss of L-Ara residues. The delignified WSTP-I was separated into two fractions, WSTP-IA and WSTP-IB in ~ 4:1 proportions. WSTP-IA eluted as a single symmetric peak at the column void volume, whereas WSTP-IB eluted as a single symmetric peak at the included volume of the column, indicating different dp values for these fractions.

WSTP-IA eluted from a Sephadex G-200 column, calibrated with standard dextrans [8], as a single symmetric peak at an elution volume corresponding to molecular weight of ~ 72000. The sugar composition of the polysaccharide was unchanged after elution from Sephadex G-200 and Bio-Gel P-10 columns, and the polysaccharide gave a single symmetric peak on sedimentation analysis, indicating the absence of heterogeneity. The polysaccharide was composed of D-Gal and L-Ara in ~ 3:1 molar proportions (Table 2). Phosphate, sulfate, ash, lignin, and *O*-acetyl contents of WSTP-IA are shown in Table 1.

The WSTP-IB fraction was further purified on a column of Bio-Gel P-6. A major peak eluted at the column void volume and was used for further studies. Compositional analysis of the purified WSTP-IB showed that the polysaccharide contained D-Gal only, indicating that the polysaccharide is a homoglycan of D-Gal. Additional chemical analysis data of WSTP-IB are shown in Table 1. Purified WSTP-IB eluted from a Sephadex G-200 column, calibrated with standard dextrans, as a single symmetric peak at the column elution volume corresponding to a molecular weight of ~ 12000.

Structure of WSTP-IA.—Methylation linkage analysis of the permethylated [9] WSTP-IA by GLC and GLC–MS afforded 2,3,5-tri-*O*-methylarabinose, 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4-tri-*O*-methylgalactose, and 2,4-di-*O*-methylgalactose in ~ 2.00 : 1.00 : 2.83 : 3.32 proportions (Table 3). The formation of only 2,3,5-tri-*O*-methylarabinose derivative indicates that the L-Ara residues are present as terminal nonreducing furanosyl groups. The formation of 2,3,4,6-tetra-*O*-methylgalactose shows that a few D-Gal residues are also present as nonreducing pyranosyl end-groups. The formation of relatively high proportions of 2,3,4-tri-*O*-methylgalactose and 2,4-di-*O*-methylgalactose suggests the presence of (1 → 6)-linked and (1 → 3)-linked galactan chains, being either main chain or branching with frequent substitution at O-6 and/or O-3.

To establish the linkage type of nonreducing terminal L-Ara*f* residues to D-Gal*p*, WSTP-IA was subjected to partial hydrolysis. Treatment of WSTP-IA with dilute acetic acid followed by gel-filtration chromatography on a column of Bio-Gel P-4 afforded two fractions. One of these eluted at the column void volume (WSTP-IA$_1$) and the other at the monosaccharide elution volume. Analysis of the latter fraction showed that it contained free L-Ara*f* only. Compositional analysis of the WSTP-IA$_1$ gave D-Gal*p* only, indicating that partial hydrolysis of WSTP-IA removed all the L-Ara*f* residues. Methylation linkage analysis of WSTP-IA$_1$ gave 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4-tri-*O*-methylgalactose, and 2,4-di-*O*-methylgalactose in a ratio of ~ 1.00 : 5.00 : 1.00 (Table 3). The increase in 2,3,4-tri-*O*-methylgalactose and the concomitant decrease in 2,4-di-*O*-methylgalactose in the methylation analysis suggests the presence of a (1 → 3)-linkage for nonreducing L-Ara*f* residues to D-Gal*p* in WSTP-IA. Further, the data suggest that the polysaccharide is composed of a (1 → 6)-D-galactan main chain which, on average, has a nonreducing terminal D-Gal*p* residue linked to every fifth residue.

Table 3
Comparison of methylation linkage analysis data of polysaccharide fractions isolated from the leaves of *Tridax procumbens* linn

Partially methylated alditol acetates	WSTP-IA	WSTP-IA$_1$	Smith-degraded WSTP-IA	WSTP-IB
2,3,5-Me$_3$-arabinose [L-Ara*f*-(1 →]	2.00	n.d. [a]	n.d.	n.d.
2,3,4,6-Me$_4$-galactose [b] [D-Gal*p*-(1 →]	1.00	1.00	n.d.	1.00
2,3,4-Me$_3$-galactose [→ 6)-D-Gal*p*-(1 →]	2.83	5.00	n.d.	25.00
2,4-Me$_2$-galactose [→ 3,6)-D-Gal*p*-(1 →]	3.32	1.01	[c]	n.d.

[a] n.d., Not detected.

[b] Compositions are normalized with respect to 2,3,4,6-Me$_4$-galactose and are based on GLC analysis of partially methylated alditol acetates.

[c] Only 2,3,4-Me$_3$-D-galactose was observed.

Supporting evidence for the presence of a (1 → 6)-linked D-galactan main chain was obtained from Smith degradation [10] studies. Periodate oxidation and sodium borohydride reduction of WSTP-IA followed by purification of the product by gel-filtration chromatography afforded a modified polysaccharide (polyalcohol). Methylation linkage analysis of the Smith-degraded WSTP-IA showed the formation of 2,4-di-*O*-methylgalactose only (Table 3), thus showing the presence of a (1 → 6)-D-galactan main chain in the polysaccharide. Further, periodate oxidation and sodium borohydride reduction followed by methylation linkage analysis of WSTP-IA$_1$ afforded only small amounts of 2,4-di-*O*-methylgalactose, confirming the presence of a (1 → 6)-D-galactan main chain in the polysaccharide. Mild acid hydrolysis of the Smith-degraded WSTP-IA and WSTP-IA$_1$ afforded D-Gal*p* only, further confirming the presence of a (1 → 6)-D-galactan main chain for the polysaccharide. The results also indicated that in WSTP-IA the nonreducing terminal residues of L-Ara and D-Gal are attached to the (1 → 6)-D-galactan main chain at O-3 alternatively as substituents.

The anomeric configuration of sugar residues was established by chromium trioxide oxidation [11], and ^{1}H and ^{13}C NMR spectroscopy. Peracetylation of WSTP-IA$_1$ with trifluoroacetic anhydride and acetic acid [12] followed by oxidation with chromium trioxide afforded a product which, upon compositional analysis, showed that ~ 90% of D-Gal*p* residues underwent oxidation, indicating that these have the β configuration.

The ^{1}H NMR spectrum of WSTP-IA revealed three anomeric signals, two singlets at δ 5.19 and 5.03 ppm, and a pair of doublets between δ 4.41–4.49 ppm, whereas the WSTP-IA$_1$ spectrum contained only one anomeric signal, a doublet at δ 4.42 ppm (J 7.8 Hz). The presence of two singlets (at δ 5.19 and 5.03 ppm) in WSTP-IA and their absence in WSTP-IA$_1$ (Fig. 1) suggests that they are derived from L-Ara*f* residues [13], one due to α-residues (δ 5.19), and the other due to α/β-residues (δ 5.03). The pair of doublets (at δ 4.41–4.49 ppm) in WSTP-IA was resolved as a single doublet (at δ 4.42 ppm, J 7.8 Hz) in WSTP-A$_1$, indicating that all D-Gal*p* residues have the β configuration. Further, the ^{13}C NMR spectrum of WSTP-IA contained three signals in the anomeric region at δ 109.4, 107.6, and 103.6 ppm. The signals at δ 109.4 and 107.6 ppm could be assigned to the α- and α/β-carbon of L-Ara*f* residues [14], respectively. The absence of these signals in ^{13}C NMR spectra of WSTP-IA$_1$ confirmed the assignment. The signal at δ 103.6 ppm was assigned to the anomeric carbon of β-D-Gal*p* residues. Due to the complexity of the spectra, the other signals were difficult to assign. The ^{13}C NMR spectrum of WSTP-IA$_1$ contained one signal in the anomeric region at δ 103.57 ppm which confirms the β configuration of D-Gal*p* residues. Furthermore, DEPT sequence-studies [14] indicated that the signal at δ 68.83 ppm had a negative amplitude and could be assigned to a glycosidically linked secondary carbon atom, $-CH_2$ (i.e., C-6 of D-Gal*p*. The other signals at δ 73.91, 72.81, 70.91, and 69.63 ppm were assigned to C-3, C-2, C-4, and C-5 of D-Gal*p* [14], respectively. These data suggest that β-(1 → 6)-linked D-Gal*p* residues form the main chain in the polysaccharide.

The foregoing results suggest that WSTP-IA is a novel arabinogalactan with

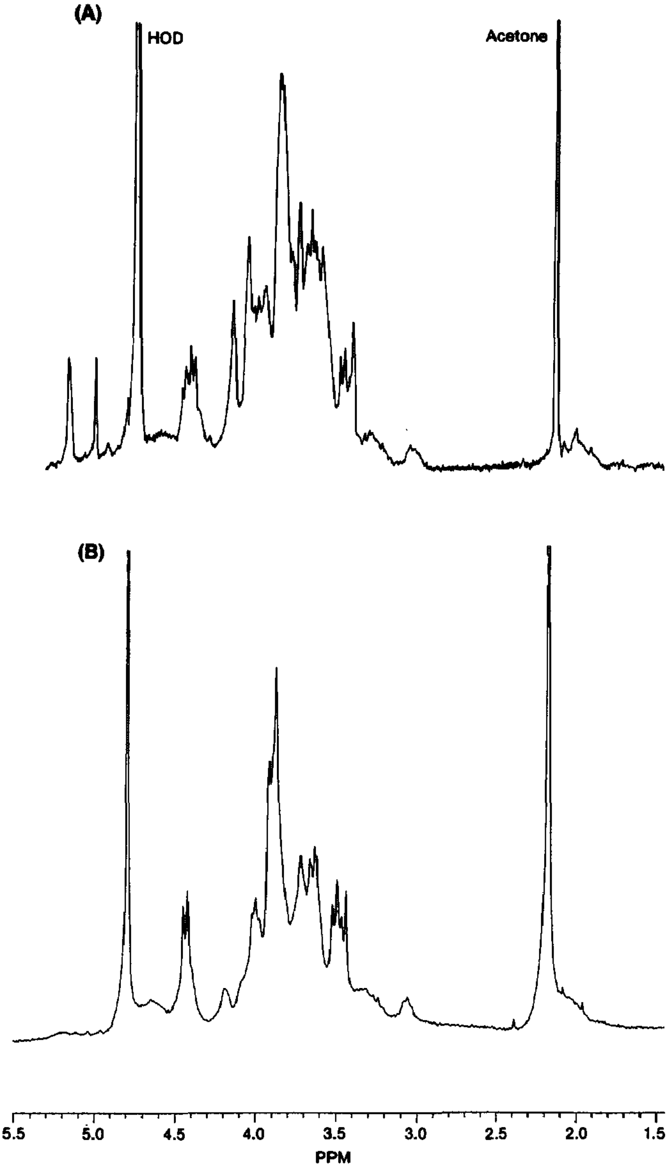


Fig. 1. 300 MHz ^{1}H NMR spectra of (A) WSTP-IA and (B) WSTP-IA$_1$ measured at 25°C.

β-(1 → 6)-linked D-Gal*p* residues forming the main chain in which at least one in every two residues carries single residues of either α/β-L-Ara*f* or β-D-Gal*p* as terminal nonreducing end-groups at O-3.

Structure of WSTP-IB.—Methylation linkage analysis [9] of permethylated WSTP-I yielded 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methylgalactose in

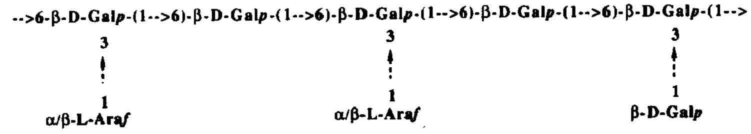


Fig. 2. Average structure proposed for the novel water-soluble arabinogalactan isolated from *T. procumbens* linn.

~ 1:25 proportions, along with minor amounts of other derivatives (see Table 3). The formation of a large amount of 2,3,4-tri-*O*-methylgalactose suggests that WSTP-IB is a linear galactan with (1 → 6)-linkage. Smith degradation was carried out on WSTP-IB, in which O-6 linked D-Gal*p* residues undergo oxidation. Compositional and methylation linkage analysis of the product obtained from the oxidation–reduction followed by gel-filtration chromatography did not yield sugar derivatives. These results are in accordance with methylation linkage analysis data. The ^{1}H NMR spectrum of WSTP-IB contained a single doublet at δ 4.43 ppm (J 7.7 Hz) which was assigned to the anomeric proton of β-D-Gal*p* residues. The ^{13}C NMR spectra of WSTP-IB contained signals with chemical shifts at δ 103.57, 73.91, 72.81, 70.91, 69.63, and 68.83 ppm, which were assigned to C-1, C-3, C-2, C-4, C-5, and C-6 of β-(1 → 6)-linked D-Gal*p* residues, respectively [13]. These results suggests that WSTP-IB is a linear β-(1 → 6)-D-galactan.

3. Conclusions

Arabinogalactans containing (1 → 4)-linked β-D-galactans (Type-I) in which the L-Ara*f* constituents vary from 25 to 43% and are linked to D-Gal*p* at O-3 have been reported [15]. Further, polysaccharides containing (1 → 3)-linked β-D-galactans (Type-II) which carry L-Ara*f* substituents at O-6 as nonreducing terminal end-groups were also reported [15]. The present paper describes the results that demonstrate the presence of (1 → 6)-linked β-D-galactans in *T. procumbens* leaves to which single nonreducing L-Ara*f* (α-/β-) and some β-D-Gal*p* residues are substituted at O-3. The results also suggest that the low molecular weight (1 → 6)-linked β-D-galactan (WSTP-IB fraction) is a biosynthetically incomplete version of WSTP-IA. This observation is not surprising since leaves of different ages were used to extract the polysaccharide. However, from the present results, it is possible to propose an average structure of the polysaccharide (Fig. 2).

4. Experimental

General.—Plant leaves were collected during April–May from locally available plants (Manasagangotri campus, Mysore, India). The leaves were dried at ambient temperature for a week, then at 45°C for 24 h, and powdered using a mortar and pestle.

All evaporations were performed under reduced pressure below 40°C. Dialysis was carried out against three changes of glass distilled water with continuous stirring at 4°C. Ultrafiltration was carried out at 4°C using an Amicon series 8000 10-mL stirred cell with a membrane having a mol wt cutoff of 500. All chemicals used were of analytical grade.

Analytical procedures.—Quantitative colorimetric methods used were the phenol–H_2SO_4 method [16] for neutral glycoses, the carbazole reagent assay [17] for hexuronic acids, the method of Chen et al. [18] for phosphate, the method of Lowry et al. [19] for protein, and the method of Hestrin [20] for *O*-acetyl determination. Sulfate [21], ash [22], and lignin [23] contents were determined according to procedures previously reported.

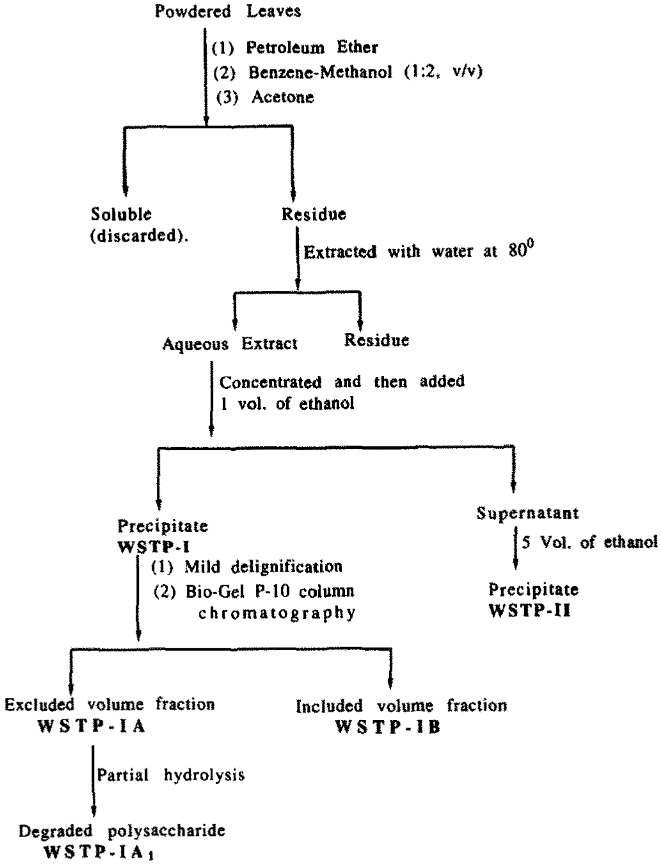
Analytical and preparative PC was performed in the descending mode on Whatman No. 1 and 3MM papers, respectively, using *A*, 4:1:3:3 1-butanol–benzene–pyridine–water (upper layer); *B*, 8:2:5 EtOAc–pyridine–water; *C*, 6:4:3 1-butanol–pyridine–water; *D*, 4:1:5 1-butanol–EtOH–water (upper layer); *E*, 4:1:5 1-butanol–AcOH–water (upper layer); *F*, 5:5:1:3 EtOAc–pyridine–AcOH–water; and *G*, 4::1:3:3 1-butanol–AcOH–pyridine–water. Solvents *A–D* were used for neutral sugars and solvents *E–G* for acidic sugars. Sugars were detected with *p*-anisidine hydrochloride [24] or alkaline silver nitrate [25].

Gas–liquid chromatography (GLC) was conducted on a Hewlett–Packard model 5890 chromatograph. Separations were carried out using capillary columns and temperature programming as follows: (A) DB-23 (15 m × 0.5 mm or 30 m × 0.25 mm) isothermally at 220°C; (B) DB-23 (30 m × 0.25 mm) isothermally at 190°C; (C) DB-23 (30 m × 0.25 mm), 180°C (10 min), 180 → 230°C at 2°C/min, 230°C (10 min). GLC–MS analyses were carried out on a VG Micromass 16F instrument with electron impact ionization at 70 eV, equipped with a Pye Unicam series 204 chromatograph using capillary columns as above [26].

^1H and ^{13}C Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM 300 spectrometer. The polysaccharide samples were exchanged (× 3) with D_2O (99.8%), lyophilized, and dissolved in D_2O (99.9%). ^1H NMR spectra were recorded using a 5-mm C/H probe at 25 and/or 40°C, and chemical shifts were measured relative to internal acetone (δ 2.17 ppm). ^{13}C NMR spectra were broad-band decoupled and recorded at 25°C using a 5-mm C/H probe, and chemical shifts were measured relative to internal acetone (δ 30.4 ppm). ^{13}C NMR DEPT experiments were carried out using the manufacturer supplied software [14].

Isolation of polysaccharides.—The dried and powdered leaves (30 g) were successively extracted with light petroleum ether (bp 60–80°C), 1:2 benzene–MeOH, and acetone, under reflex in a Soxhlet apparatus until each extract was colorless, to give a leaves powder (27.2 g) and organic extractable matter (2.8 g) see Scheme 1).

The leaves powder (25 g) was allowed to swell overnight in water (500 mL), stirred at room temperature for 4 h, followed by stirring for 30 min at 80°C, and then filtered through a linen cloth. The insoluble residue was washed with water (3 × 500 mL), followed by 95% EtOH (2 × 250 mL), and dried (18 g).



Scheme 1. Flow diagram of the isolation of the polysaccharides from leaves of *T. procumbens* linn.

The filtrate and the washings of the insoluble residue were combined and concentrated (~ 250 mL) by rotary evaporation. The solution was cooled to 4°C and 1 vol of 95% EtOH was added. The precipitated polysaccharide, WSTP-I (2.5 g), was collected by centrifugation, washed with 50% EtOH followed by acetone and dried in vacuo. To the combined centrifugate was added 5 vol. of EtOH, and the precipitated polysaccharide, WSTP-II (1.5 g), was recovered as described above.

SDS-polyacrylamide gel electrophoresis.—WSTP-I, WSTP-II, and the crude extract of dry powder of *T. procumbens* leaves (20–200 μg dry weight) were electrophoresed on 10, 7, and 5% (w/v) SDS-polyacrylamide gels in the presence or absence of β-mercaptoethanol according to Laemmli [5]. Electrophoresis was performed with a stacking gel of 4.5% (w/v) SDS-polyacrylamide, and a constant current of 50 mA. Samples were electrophoresed until the tracking dye was within 1 cm of the bottom of the gel. After SDS-PAGE, the gels were fixed and stained with Coomassie Blue and/or periodate–Schiff's reagent [6].

Mild delignification [7] and gel-filtration chromatography.—A solution of WSTP-I (1 g) in water (10 mL) was treated with glacial AcOH (30 μL) and sodium chlorite (500 mg) for 1 h at 60°C. The solution was cooled to 4°C and ultrafiltered with an Amicon series 8000 10-mL stirred cell with a membrane having a mol wt cutoff of 500.

The filtrate was examined by colorimetric analysis [16] for the loss of monosaccharides. The ultrafiltered solution was concentrated (~ 1 mL) and chromatographed on a column (1.2 × 104 cm) of Bio-Gel P-10. The column was eluted with 0.05 M pyridine–AcOH buffer (pH 5.0) and the fractions (2 mL) were analyzed by phenol–H_2SO_4 reagent [16]. The polysaccharide containing fractions were combined, concentrated, and freeze-dried.

A solution of WSTP-IA (100 mg) in 0.05 M pyridine–AcOH buffer, pH 5.0 (1.0 mL) was chromatographed on a column (1.0 × 110 cm) of Sephadex G-200, calibrated with dextran standards [8] T-2000, T-500, T-40, and T-10 (Pharmacia), and the column operations were performed as described above. The polysaccharide eluted as a single symmetric peak corresponding to a mol wt of 72000.

WSTP-IB (100 mg) was solubilized in 0.05 M pyridine–AcOH buffer pH 5.0 (1.0 mL) and chromatographed on a Bio-Gel P-6 column (1.0 × 110 cm). The column was eluted as described above. Two fractions, one eluting at the column void volume (major, 70 mg) and a broad included volume (minor, 30 mg), were obtained. The fraction eluting at the void volume was used for further studies.

Compositional analysis.—These analyses were performed on alditol acetate derivatives. Polysaccharide samples (100–200 μg) in a 1-mL vial were hydrolyzed with CF_3CO_2H (2 M, 0.5 mL) for 6 h at 100°C. The acid was removed by evaporation to dryness in a stream of air and the residue was treated with $NaBD_4$ in water (~ 2 M, 0.2 mL). Excess deuteride was destroyed by the addition of aq 50% AcOH and the solution was repeatedly evaporated to dryness with MeOH containing 5% of AcOH. The alditols with residual NaOAc were acetylated with Ac_2O (0.3 mL) for 1 h at 100°C. EtOH was added to the solution which was concentrated to dryness for analyses by GLC and/or GLC–MS using program A.

Butanolysis.—The absolute configuration of the monosaccharides was established by conversion into the 2-(*S*)- and 2-(*R*)-butyl glycosides [27], and GLC and GLC–MS analysis of their acetate derivatives using program C. Briefly, the polysaccharides (100–150 μg) were solvolysed with butanolic 2 M HCl for 5 h at 100°C, treated with silver carbonate (~ 5 mg) in the presence of a drop of Ac_2O at ambient temperature overnight, centrifuged, and the centrifugate was evaporated to dryness. The residue was acetylated with 1:1 pyridine–Ac_2O (0.3 mL) for 1 h at 100°C, evaporated to dryness, and analyzed by GLC, and GLC–MS. The polysaccharides (50–100 mg) were also hydrolyzed with 2 M CF_3CO_2H for 6 h at 100°C, the acid was removed and the resulting monosaccharides were separated by paper chromatography on a Whatman 3 MM paper using solvent *C*. Specific rotation of the isolated monosaccharides was determined. Rha and Ara had the L configuration, all other sugars had the D configuration.

Methylation linkage analysis.—Methylation analyses were performed on 0.3–1.0 mg samples using the method of Ciucanu and Kerek [9]. Briefly, the polysaccharide samples were suspended in pure dimethyl sulfoxide (0.3–0.5 mL), stirred at ambient temperature for 15 min, and then ultrasonicated at ~40°C for 10 min. This step was repeated until a clear solution of polysaccharide was obtained. Then the solution was treated with pulverized NaOH (~5 mg) and MeI (0.3–0.5 mL) for 30 min at ambient temperature. The permethylated product was isolated by partition with CH_2Cl_2 and, after conversion into partially methylated alditol acetates by hydrolysis, reduction, and acetylation as described above, subjected to linkage analysis by GLC and GLC–MS using program B.

Partial hydrolysis.—WSTP-I (100 mg) was hydrolyzed with aq 2 M AcOH (5 mL) at 100°C for 4–5 h. The hydrolysate was lyophilized and the residue was dissolved in 0.05 M pyridine–AcOH buffer pH 5.0 (2.0 mL). The solution was chromatographed on a column (1.2 × 104 cm) of Bio-Gel P-4, pre-equilibrated with 0.05 M pyridine–AcOH acid buffer pH 5.0, and then eluted with the same buffer. Fractions (2 mL) were monitored by use of the phenol–H_2SO_4 reagent. The degraded polysaccharide eluted as a single symmetric peak at the column void volume and the released L-Ara eluted at the monosaccharide elution volume. The degraded polysaccharide was analyzed to determine its composition, and subjected to methylation linkage analysis.

Smith degradation.—Smith degradation of WSTP-IA, WSTP-IA$_1$ and WSTP-IB was carried out under the conditions reported by Pritchard et al. [10]. Polysaccharide (20 mg) was treated with 40 mM sodium metaperiodate in 0.1 M NaOAc (5 mL, pH 4) at 4°C for 72 h. The reaction was monitored spectrophotometrically at 222 nm. Excess periodate was destroyed by the addition of 1,2-ethanediol and the solution was desalted by elution with water through a column (1.2 × 104 cm) of Bio-Gel P-4. The oxidized polysaccharide was reduced with $NaBH_4$, excess hydride was destroyed and borate was removed as described previously, and a solution of the modified polysaccharide in water was desalted by passage through a column (1.2 × 104 cm) of Bio-Gel P-4. The oxidized–reduced polysaccharide (polyalcohol) was divided into two portions. One portion was methylated, converted into partially methylated alditol acetates, and analyzed by GLC as described earlier. The other portion was hydrolyzed with 2 M CF_3CO_2H for 6 h at 100°C, and the hydrolysate was divided into two portions. One portion was analyzed by PC using solvent *A*, and the other portion was converted into alditol and acetates and analyzed by GLC. The results showed the presence of only D-Gal.

Sedimentation analysis [28].—A solution (1%) of WSTP-IA in 0.1 M NaCl was analyzed in a Beckman analytical ultracentrifuge Model E at 25°C at 59780 rpm. The movement of the boundary was followed using Schlieren optics.

Chromium trioxide oxidation [11].—Polysaccharide samples WSTP-IA$_1$ and WSTP-IB (~1 mg each) were each acetylated with 2:1 trifluoroacetic anhydride–AcOH (300 mL) at room temperature for 10 min as reported by Khoo and Dell [12]. The peracetylated products were isolated by partition with dichloromethane and oxidized with chromium trioxide in glacial AcOH for 1 h at 50°C. The oxidized products were repeatedly extracted with CH_2Cl_2, evaporated to dryness, and the residues were hydrolyzed with 2 M CF_3CO_2H. After conversion to alditol acetates, the residues were analyzed by GLC as described above.

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