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Phytochemistry 65 (2004) 609–615
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Structural analysis of a pectic polysaccharide from the leaves of *Diospyros kaki*

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Received in revised form 21 November 2003

Abstract

A pectic polysaccharide DL-2A with a molar mass of 8.5×10^5, was obtained from the boiling water extract of *Diospyros kaki* leaves. It had $[\alpha]_D^{20}$ −21.8° (*c* 0.22, H_2O) and consisted of rhamnose, arabinose, galactose, xylose and galacturonic acid units in the molar ratio of 0.4:3.4:2.4:1.0:0.8, along with traces of glucuronic acid. About 16.7% of galacturonic acid existed as the methyl ester. A combination of linkage analyses, periodate oxidation, partial acid hydrolysis, selective lithium-degraded reaction, ESIMS, ^1H- and ^{13}C- NMR spectral analyses revealed its structural features. It was found that DL-2A possessed an α-(1→4)-galacturonan backbone with some insertions of α-1,2-Rha*p* residues. The side-chains of arabino-3,6-galactan were attached to the backbone via O-4 of Rha*p* residues and O-3 of GalA*p* residues, while 4-linked xylose residues (forming short linear chains) were directly linked to O-4 of rhamnose residues, not as part of the xylogalacturonan. These novel structural features enlarge the knowledge on the fine structure of pectic substances in the plant kingdom.

Keywords: *Diospyros kaki*; Ebenaceae; Pectin; Structure; Polysaccharide; Rhamnogalacturonan

1. Introduction

Pectins are a group of polysaccharides present in all plant primary cell walls, and have been extensively investigated using chemical analysis and enzymatic degradation (Voragen et al., 1995; Schols and Voragen, 1996). Their complicated structure and the retention by plants of large number of genes required to synthesize pectin, indicate that pectins have multiple functions in plant growth and development (Ridley et al., 2001). Pectins from Chinese traditional medicines and herbs have been little investigated except that the group of Yamada has reported several pectic fractions from some species including *Coix lacryma-jobi* L. var. *ma-yuen* (Yamada et al., 1987), *Panax ginseng* C. A. Meyer (Gao et al., 1988) and *Bupleurum falcatum* (Yamada et al., 2000), etc.

Diospyros kaki, belongs to the family Ebenaceae and is widespread in the tropics and subtropics (Mallavadhani et al., 1998). In Chinese herbal medicines, it has been used to treat hiccups. In recent years, the leaves of *Diospyros kaki* have been favored as a tea for healthcare in Southeast Asia. Previously, we have described the chemical properties and immunological activity of a pectic fraction (DL-3Bb) from the leaves of *Diospyros kaki*, which possessed a backbone consisting of the average disaccharide of [→4]α-GalA*p*-(1→2)-α-Rha*p*-(1→) with arabinogalactan and arabinoxylan moieties as side chains linked to O-4 of rhamnose residues (Duan et al., 2003). This paper reports the structural features of another pectic polysaccharide DL-2A obtained from the same species.

2. Results

The boiling water extract of the dried leaves of *Diospyros kaki* was precipitated with 4 vols. EtOH to give the crude polysaccharide DL. After successive separation by DEAEcellulose anionexchange and Sephacryl S-300 gel permeation chromatographic steps, it afforded the carbohydrate fraction DL-2A.

DL-2A showed a symmetrical peak on high performance gel-permeation chromatography (HPGPC), as

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doi:10.1016/j.phytochem.2003.12.002

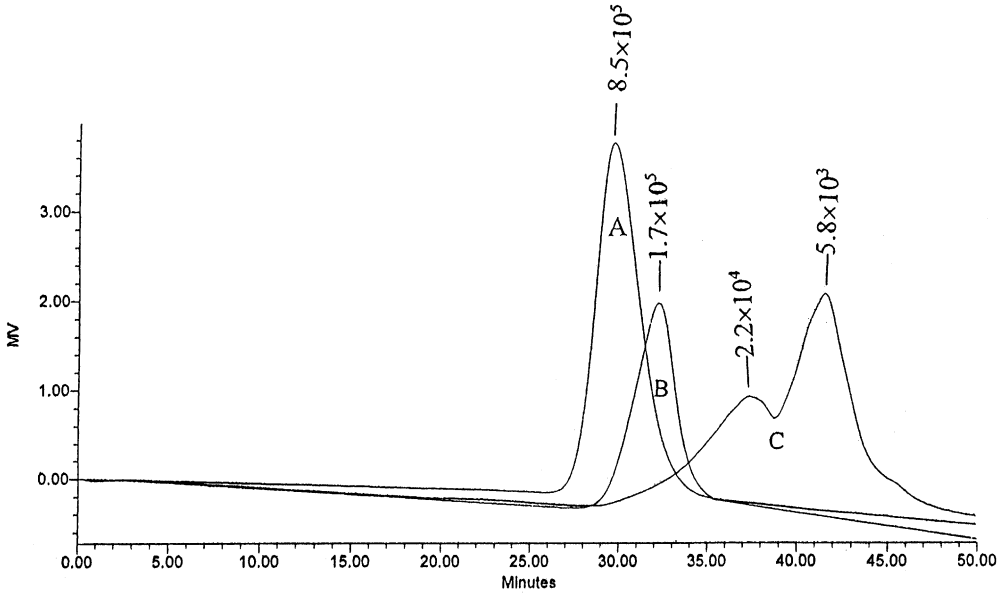

Fig. 1. HPGPC chromatograms on ultrahydrogel columns of DL-2A (A), DL-2Ade2 (B) and DL-2A-Li (C).

shown in Fig. 1, Curve A, and gave a single band at a distance of 50 mm from the origin on PAGE (data not shown). Its molecular weight was determined to be 8.5×10^5, in reference to standard dextrans. It had a specific rotation of $[\alpha]_D^{20}$ -21.8° (c 0.22, H_2O) and was free of protein according to the Lowry method (Bensadoum and Weinstein, 1976).

The presence of 10.6% uronic acid was inferred by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). After reduction with CMC–$NaBH_4$ procedure, the carboxyl-reduced DL-2A was obtained. A combination of sugar composition analysis of native DL-2A and carboxyl-reduced DL-2A revealed that DL-2A contained rhamnose, arabinose, galactose, xylose and galacturonic acid in the molar ratios 0.4:3.4:2.4:1.0:0.8, along with traces of glucuronic acid. About 16.7% of galacturonic acid existed as the methyl ester (Tomoda et al., 1986).

The linkage analysis of the carboxyl-reduced DL-2A was performed by the sodium hydroxide–methyl iodide (NaOH–MeI) procedure. The results (Table 1, Column b) are summarized.

As shown, arabinose residues are mainly present as furanosyls, along with a small amount of pyranosyls. The high amount of terminal Ara*f* residues could not be explained by the low amount of branched Ara*f* residues. Therefore, some terminal Ara*f* residues could be linked to other branched glycosyl residues. The total content of Gal*p* residues was increased compared with the result of the component analysis. The 4-linked and 3,4-linked Gal*p* residues could possibly result from the GalA*p* residues, since 4-linked GalA*p* residues are commonly found in pectic substances from the plant kingdom. Although direct linkage analysis of acidic native polysaccharide does not result in quantitative but only qualitative information under methylation conditions (β-elimination reaction), the disappearance of 4-linked and 3,4-linked Gal*p* residues (Table 1, Column a) confirmed the proposal mentioned above.

DL-2A was completely oxidized with 0.02 M sodium periodate ($NaIO_4$) for 72 h at 4 °C in the dark. A total of 0.72 mol of $NaIO_4$ was consumed and 0.14 mol of formic acid was produced per mole of glycosyl residues, based on the average molar mass (147) of the component residues. The composition analysis of the polyalcohol resulting from periodate oxidation gave a molar

Table 1
Linkage analysis of DL-2A (a), carboxyl-reduced DL-2A (b), carboxyl-reduced DL-2Ade (c), carboxyl-reduced DL-2Ade2 (d) and DL-2A-Li (e)

Methylated sugars	Molar ratio					Linkages
	a	b	c	d	e	
2,3,5-Me_3-Ara*f*	10.2	11.2	3.6	n.d	10.4	Terminal
2,3-Me_2-Ara	3.8	4.3	Trace	n.d	4.0	1,5-or 1,4-
3-Me-Ara*f*	3.0	3.2	n.d	n.d	3.0	1,2,5-
2,4-Me_2-Ara*p*	1.4	1.2	0.6	n.d	1.0	1,3
2,3,4-Me-Ara*p*	n.d	n.d	0.4	n.d	0.2	Terminal
2,3,4-Me_3-Xyl*p*	n.d	1.0	1.0	Trace	n.d	Terminal
2,3-Me_2-Xyl*p*	n.d	1.5	1.3	0.2	n.d	1,4-
2,4-Me_2-Xyl*p*	Trace	3.1	2.9	0.4	n.d	1,3-
2,3,4,6- Me_4-Gal*p*	0.5	0.4	1.0	0.3	0.6	Terminal
2,3,4- Me_3-Gal*p*	4.0	4.4	5.4	0.7	4.0	1,6-
2,4,6- Me_3-Gal*p*	3.6	3.3	4.8	0.4	3.1	1,3-
2,4- Me_2-Gal*p*	5.5	5.0	2.3	0.2	5.3	1,3,6-
2,3,6- Me_3-Gal*p*	n.d	2.9	3.1	14.7	n.d	1,4-
2,6- Me_2-Gal*p*	n.d	1.5	1.4	0.4	n.d	1,3,4-
3,4- Me_2-Rha*p*	n.d	n.d	n.d	0.1	n.d	1,2-
2,3- Me_2-Rha*p*	0.4	n.d	n.d	n.d	0.7	1,4-
3- Me-Rha*p*	trace	2.4	2.2	0.2	n.d	1,2,4-

n.d: not detected.

ratio of rhamnose, arabinose, xylose and galactose of 1.0:2.1:1.6:4.0, in addition to a large amount of glycerol. These results were in approximate agreement with the expected results based on linkage analysis of carboxyl-reduced DL-2A ($NaIO_4$ consumption: 0.67; formic acid production: 0.126).

Mild acid hydrolysis of Dl-2A gave a degraded polysaccharide. DL-2Ade was composed of rhamnose, arabinose, galactose, xylose and galacturonic acid in the molar ratio of 0.4:0.88:2.2:1.0:0.7, based on the composition analysis of DL-2Ade and the carboxyl-reduced DL-2Ade.

The results of linkage analysis of the carboxyl-reduced DL-2Ade (Table 1, Column c) indicated that the content of 1,3-linked Ara*p* residues and 1,3,6-linked Gal*p* residues had decreased, and that the molar ratios of terminal Ara*p* residues and terminal, 1,3-linked and 1,6-linked Gal*p* residues had increased, followed by release of most of the Ara*f* residues. These results suggested that Ara*f* residues should be attached to O-3 of Ara*p* residues, and O-3 and O-6 of 3,6-branched Gal*p* residues in arabinogalactan.

A further hydrolysis of DL-2Ade with 0.25M TFA afforded five degraded products (DL-2Ade2, DL-2A-0la, DL-2A-0lb, DL-2A-0lc, DL-2A-0ld) with decreasing molar mass (see details in Experimental).

DL-2Ade2, with a molecular mass of 1.7×10^5, was eluted as a single peak on HPGPC (Fig. 1 Curve B). It contained rhamnose, xylose, galactose and galacturonic acid in a molar ratio of 0.3:0.5:1.0:9.0. Complete carboxyl-reduction of DL-2Ade2 could not be obtained since 13% of uronic acid still remained, even though the procedure of carboxyl reduction was repeated 5 times. However, the results of the linkage analysis of the partial carboxyl-reduced DL-2Ade2 (Table 1, Column d) suggested that the (1→4)-galacturonan moiety could be found in the backbone of DL-2Ade2.

DL-2A-0la contained rhamnose, xylose, galactose and galacturonic acid in the molar ratio of 0.4:0.6:1.0:4.1. The predominate linkages of 4-linked Gal*p*, observed in the linkage analysis of the carboxyl-reduced DL-2A-0la, suggested that DL-2A-0la had a similar structure to DL-2Ade2 except for a difference in the molar ratio of neutral and acidic components.

DL-2A-0lb consisted of rhamnose, galactose and galacturonic acid in the approximate molar ratio of 2.0:1.0:1.0. The ESI–MS of DL-2A-0lb showed a pseudomolecular ion at *m*/*z* 671.2, corresponding to [(Hex) 1 (HexA) 1 (6deoxy) $2+Na]^+$. In addition, the fragments ions at *m*/*z* 525.3 and 349.3 corresponded to [(Hex) 1 (HexA) 1 (6 deoxy) $1+Na]^+$ and [(Hex) 1 (6 deoxy) $1+Na]^+$, respectively. After reduction with $NaBD_4$, GCMS analysis of the methylated derivative revealed that terminal Gal*p*, 4-linked Rha*p* and 2-linked Rha*p*-1-d were detected. Therefore, DL-2A-0lb could be identified as Gal*p*-(1→4)-Rha*p*-(1→?)-GalA*p*-(1→2)-Rha*p*.

Component analysis showed that DL-2A-0lc contained GalA, Xyl and Rha in the molar ratios 2:0.4:0.3. The ESI–MS of DL-2A-0lc gave two pseudomolecular ions at *m*/*z* 545.3 and 471.4 with the sequence decreasing abundance, which could be attributed to [(HexA) 3-H]$^-$ and [(Pen) 1 (HexA) 1 (6 deoxy) 1-H]$^-$, respectively. The results of linkage analysis of the oligosaccharide-alditol-1-d indicated the presence of terminal Xyl*p* and 4-linked Rha*p*. Therefore, the peak *m*/*z* 471.4 in ESI-MS should be originated from Xyl*p*-(1→4)-Rha*p*-(1→?)-GalA*p*.

DL-2A-0ld consisted of galactose and galacturonic acid in 1.2:1.0 molar ratios. The pseudomolecular ion *m*/*z* 355.3 attributed to [(Hex) 1 (HexA) 1-H]$^-$ in ESI–MS. After reduction with $NaBD_4$, only terminal Gal*p* was found in the methylated derivative of the oligosaccharide-alditol-1-d. Thus, DL-2A-0ld perhaps possessed the structural sequence Gal*p*-(1→?)-GalA*p*.

In order to determine the substituted position of the GalA*p* in the oligosaccharides mentioned above, DL-2A-0lb, DL-2A-0lc and DL-2A-0ld were oxidized respectively with 0.025M sodium periodate ($NaIO_4$) at 4 C in dark for 4 days, prior to addition of ethylene. Uronic acid was detected only in the end product of DL-2A-0ld. Since only 4- and 3,4-linked GalA*p* could be found in native DL-2A, the linkages of GalA*p* in the linear oligosaccharides were either terminal, 3-substituted or 4-substituted. DL-2A-0lb could be identified as Gal*p*-(1→4)-Rha*p*-(1→4)-GalA*p*-(1→2)-Rha*p*, and DL-2A-0lc as the mixture of (1) Xyl*p*-(1→4)-Rha*p*-(1→4)-GalA*p* and (2) GalA*p*-(1→4)-GalA*p*-(1→4)-GalA*p*, and DL-2A-0ld as Gal*p*-(1→3)-GalA*p*.

Treatment of DL-2A with metal lithium dissolved in ethylenediamine resulted in selective cleavage of galacturonic acid residues (Lau et al., 1987). The two degraded products, DL-2A-Li and DL-2A-LiOl were collected.

DL-2A-Li showed two major peaks on HPGPC (Fig. 1. Curve C) with molecular masses of 2.2×10^4 and 5.8×10^3. Composition analysis of DL-2A-Li revealed it was composed of rhamnose, arabinose and galactose in the molar ratio of 0.3:3.2:2.2, and traces of galacturonic acid (<1%) were detected according to the m-hydroxydiphenyl method. The results of the linkage analysis of DL-2A-Li indicated that little changes occured in the side chains of arabino-3,6-galactan, and that almost all Xyl*p* residues had disappeared (Table 1, Column e). Furthermore, a small amount of 4-linked Rha*p* residues were identified, where possibly resulted from 1,2,4-linked Rha*p* residues being converted into 4-linked rhamnitol after treatment with lithium. The significant decrease of the molecular masses (from 8.5×10^5 of native DL-2A to 2.2×10^4 and 5.8×10^3 of DL-2A-Li) indicated that GalA*p* residues were in the backbone of native DL-2A. In addition, some GalA*p* residues were attached to O-2 of Rha*p* residues.

DL-2A-LiOl gave a broad peak beyond the trisaccharide region on a column of Bio-Gel P-2, and contained xylose and rhamnose in an 5.3:1.0 molar ratio. After reduction with $NaBD_4$, DL-2A-LiOl was subjected to linkage analysis. Terminal Xyl*p*, 4-linked Xyl*p* and 4-linked Rha*p*-1-d were identified in the molar ratio 1.0:4.3:0.8. This result suggested that DL-2A-LiOl was a mixture of a series of oligosaccharides having the following structure: Xyl*p*-(1→4)-[Xyl*p*]$_n$-(1→4)-Rha*p* ($n>1$).

Signals in the ^{1}H NMR and ^{13}C NMR spectra of the native DL-2A were assigned as completely as possible, based on component analysis, linkage analysis and on literature values (Dong et al., 1999; Dong et al., 2001; De Pinto et al., 2001; Polle et al., 2002; Duan et al., 2003). The ^{1}H NMR of DL-2A showed resonances corresponding to a C-methyl proton at δ 1.26 and anomeric protons δ 5.12-5.25 due to the α-Ara*f* residues. H-1 signals were assigned to the α-Rha*p* residues (5.10), the α-GalA*p* residues (5.02), the β-Xyl*p* residues (4.74) and the β-Gal*p* residues (4.52-4.55), respectively.

The ^{13}C NMR spectra (Fig. 2, spectra a) of DL-2A identified signals of the C-methyl of Rha*p* at δ 18.82, the O-methyl as carboxylic acid methyl esters at δ 55.42, the carboxyl, in acidic form at δ 177.4 and the carboxyl, in ester-form at δ 176.0. In the anomeric carbon region, signals at δ 109.2–111.53 could be attributed to C-1 of α-Ara*f*; δ 104.9–105.6 to C-1 of β-Gal*p*; δ 103.06 to C-1 of β-Xyl*p*; δ 101.68 to C-1 of α-GalA*p*, respectively. The methylene signals of C-5 of Ara*f*, Xyl*p* and C-6 of Gal*p* residues were identified by the application of DEPT ^{13}C-NMR analysis (Bao et al., 2001), in which they appeared as negative peaks. Therefore, the resonance at δ 62.8 was assigned to C-5 of terminal Ara*f* and C-6 of terminal and 3-linked Gal*p*. Signals at δ 68.3 (C-5 of 5- and 2,5-linked Ara*f*) and signals at δ 71.6 (6-linked and 3.6-linked Gal*p*) were also assigned. The weaker signals at δ 63.6 were attributed to C-5 of terminal Xyl*p*.

The signals of the ^{13}C-NMR spectrum of DL-2Ade2 (Fig. 2, Curve b) were also assigned, according to the literature (Sun et al., 1987). δ 175.8 was attributed to C-6 of the carboxyl in the units →4)-α-GalA*p*-(1→2)-α-Rha*p*-(1→, δ 175.2 attributed to that in the units →4)-α-GalA*p*-(1→4)-δ-GalA*p*-(1→ and δ 173.5 attributed to carboxyl groups in ester-form . The resonances at δ 102.7, 81.2, 73.1, 70.8 and 70.6 were assigned to C-1, C-4, C-5, C-3 and C-2 of the repeating unit →4)-α-GalA*p*→, respectively. In addition, after release of neutral sugar residues, the O-methyl signal δ 55.7 from carboxylic acid methyl esters became stronger.

3. Discussion

Up to now, only the group of Amadò claimed that galactose might possibly be attached to the O-3-position of galacturonic acid in pectic substances (Strasser et al., 2001). The characterization of the degraded olisaccharide Gal-(1→3)-GalA suggested that this linkage does indeed exist in pectins. This new finding enlarges the knowledge on pectic substance fine structure.

The other unusual structural feature of DL-2A was that DL-2A contained a small but significant proportion of 1,4-linked Xyl*p* residues, which were attached to O-4 of rhamnose residues, not as part of xylogalacturonan (Schols et al., 1995). This observation was also

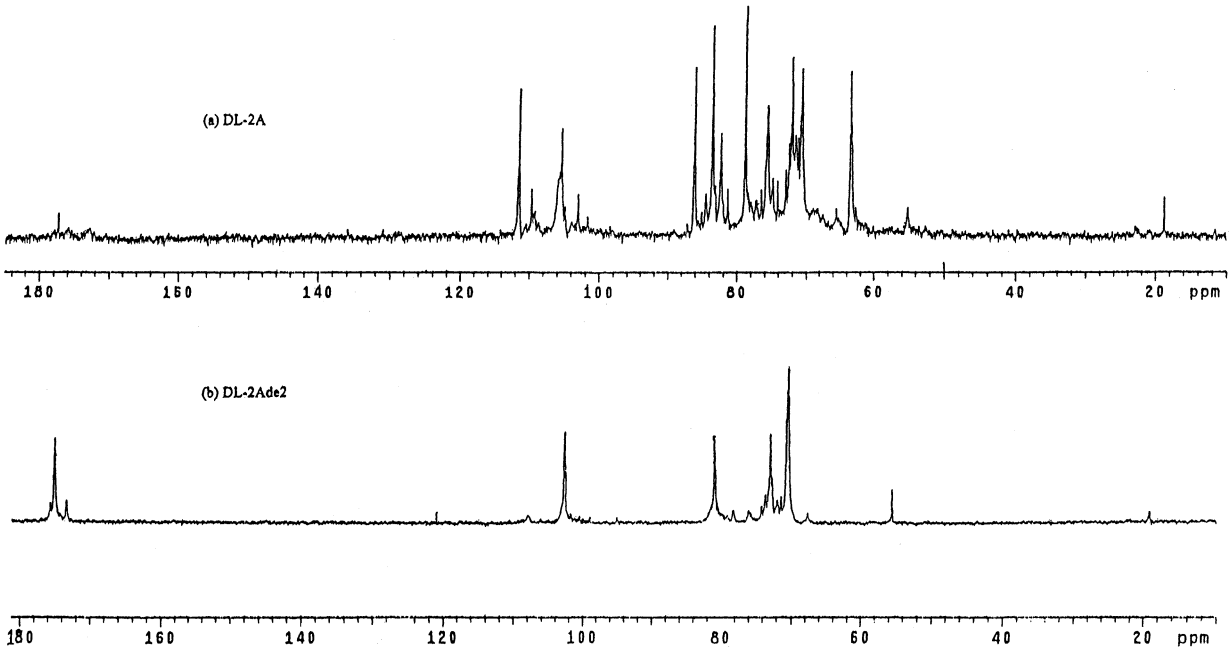

Fig. 2. ^{13}C NMR spectrums of DL-2A (a) and DL-2Ade2 (b).

found in the pectic substance CA-2 from the seed of *Coix lacryma-jobi* var. *ma-yuen* (Yamada et al., 1987) and the pectic fraction DL-3Bb previously reported (Duan et al., 2003).

4. Conclusion

A pectic polysaccharide, DL-2A isolated from the boiling water extract of *Diospyros kaki* contained rhamnose, arabinose, galactose, xylose and galacturonic acid in the molar ratios 0.4:3.4:2.4:1.0:0.8. Based on linkage analysis, periodate oxidation, partial acid hydrolysis, selective lithium-degraded reaction, ESI–MS, ^{1}H- and ^{13}C- NMR spectral analysis, it could be deduced that the native DL-2A possessed a backbone of α-1,4-galactopyranosyluronan blocks with some insertions of α-1,2- Rha*p* residues. In the neutral side chains, Gal*p* residues were attached to O-3 of GalA*p* residues and O-4 of Rha*p* residues at the same time, and were further substituted by Ara*f* residues to form arabino-3,6-galactan. While 4-linked Xyl*p* residues were found to be present as short linear chains probably attached to O-4 of rhamnose residues.

5. Experimental

5.1. Material

Dried leaves of *Diospyros kaki* were collected in Henan province in China. T-series Dextran, Cellulose phosphate P?, DEAE-cellulose, Sephacryl S-300 and Sephadex G-10 were purchased from Pharmacia Co., Bio-Gel P-2 was from Bio-Rad Laboratories. 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMC) and trifluoroacetic acid (TFA) were from E. Merck. Sodium borodeuteride was from Fluka Co., All other reagents were of analytical grade as available and were used without further purification.

5.2. General methods

Specific rotations were determined on a PerkinElmer 241M digital polarimeter in water at 20±1 C. IR spectra (Nujol pellets) were recorded on a PerkinElmer 599B FTIR spectrometer. GC was carried out with a Shimadzu GC-14B apparatus, equipped with a 5% OV225/AW-DMCS-Chromosorb W (80–100 mesh) column (2.5 m × 3 mm), as well as a hydrogen-flame ionization detector. The ESIMSs were recorded with VG Quattro MS/MS spectrometer. Solutions of the sample (1 μg/μL) in aq 30% MeOH containing 1% HCl were introduced into the ES source at 1 μL/min using a Harvard 22 syringe infusion pump. GC–MS was conducted with a Finnigan Model MD-800 combined with GC–MS spectrometry equipped with an HP-1 capillary column. The ^{1}H and ^{13}C NMR spectra were obtained at 400 MHz in D_2O, with a Bruker AM 400 spectrometer with a dual frequency probe in the FT mode at room temperature. All chemical shifts are reported relative to Me_4Si. The NMR DEPT experiments were carried out using a polarization-transfer pulse of 135°. HPGPC was performed with a Waters 515 instrument fitted with the GPC software (Millennium^{32}), using a Waters 2410 RI as the detector. Protein content was measured by the Lowry method (Bensadoum and Weinstein, 1976). Reduction of carboxyl groups was carried out with CMC–$NaBH_4$ for three times as described (Taylor and Conrad, 1972).

5.3. Separation and purification of native DL-2A

The dried leaves (4.75 kg) of *Diospyros kaki*, after percolation with cold EtOH for 2 weeks, were dried and the residues (3 kg) were extracted 6 times with boiling water (30 L H_2O, 6–7 h/per time). After concentration, EtOH (4 vols) was added. The precipitate was dried *in vacuo* at 40 °C and the crude polysaccharide DL (345 g) was obtained. An aliquot (15 g) was then applied to a column (80 × 6 cm) of DEAE-cellulose (Cl^- form), and eluted stepwise as four fractions [DL-1 (30 mg), DL-2 (525mg), DL-3 (1.4 g) and DL-4 (607 mg)] with water, 0.1, 0.2 and 0.4 N NaCl, respectively. DL-2 was further purified using Sephacryl S-300 gel permeation chromatography to give the purified polysaccharide, DL-2A (recovery of 82.9% from DL-2).

5.4. Gel permeation chromatography and molecular weight

Measurements were performed by HPGPC on two columns of Ultrahydrogel™ 1000 in series. The columns were calibrated with T-series Dextran T-2000, T-500, T-110, T-70, T-40, T-10. NaAc (3mM) was used as eluant and the flow rate was kept at 0.5 mL/min. A 20 μL aliquot was injected for each run.

5.5. Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed on an apparatus with a gel (7.5%) tube (124 × 4mm) and 5mM Tris–glycine buffer (pH 8.3) at 5 mA for 40 min. The resulting gel was stained by the periodate-Schiff's reagent (PAS) procedure and with Coomassie blue reagent. DL-2A gave a distinct band at a distance of 50mm from the origin.

5.6. Composition analysis and determination of O-methyl groups in methyl ester

(a) Sugar composition: The acidic polysaccharides (oligosaccharides) were hydrolyzed with 2 M TFA at

121 °C for 2 h and the neutral for polysaccharides 1.5 h. Neutral sugars were analyzed by GC after conversion of the hydrolysate into alditol acetates as described (Dong et al., 1999). TLC analysis was performed on a PEI-cellulose plate (E. Merck), developed with 5:5:1:3 EtOAc–pyridine–HOAc–water. The plate was visualized by spraying with *o*-phthalic acid reagent and heating at 100 °C for 5 min. Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973).

(b) Determination of *O*-methyl groups in methyl ester: The sample (2.5mg) was dissolved in 0.5 M NaOH (0.1mL) containing ethanol as internal standard and the solution was left at room temperature for 30 min. The mixture was analyzed by the procedure described (Tomoda et al., 1986).

5.7. Linkage analysis

The samples of polysaccharides were methylated three times by the method of Needs and Selvendran (1993), and the resulting permethylated products were hydrolyzed, reduced, acetylated and analyzed by GC-MS as described previously (Sweet and Shapiro, 1975). The partially methylated alditol acetates were identified by their relative retention times on GC and fragment ions in EI-MS, and the molar ratios were estimated from the peak areas and response factors. The GC temperature program was isothermal at 140 °C for 3 min, followed by a 3 °C/min gradient up to 250 °C. The oligosaccharides, after reduction with $NaBD_4$, were methylated once by the method described above. The data are presented in Table 1.

5.8. Periodate oxidation

The polysaccharide (16.3 mg) was oxidized with 0.02 M sodium periodate ($NaIO_4$, 25 mL) at 4 °C in dark and the absorption at 224 nm was measured. The reaction was complete in 72 h, and ethylene glycol (0.1 mL) was added to the solution with stirring for 0.5 h. Consumption of $NaIO_4$ was calculated from the absorption at 224 nm, and HCOOH production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the retentate was reduced with $NaBH_4$ (50 mg, 12 h). The pH was adjusted to 5.0, and the solution was dialyzed and the retentate lyophilized, then hydrolyzed with 1 M TFA at 100 C for 6 h. The hydrolysate was analyzed by GC (170 C for determination of glycerol and 210 C for determination of sugar composition, respectively).

5.9. Two steps partial acid hydrolysis

DL-2A (333mg) was treated with 0.02 M TFA at 100 C for 3 h. The mixture was evaporated to dryness using methanol, and the residue was dissolved with a small amount of water and dialyzed (cut off 3000 Da) against distilled water (3×500 ml). The degraded polysaccharide DL-2Ade (yield, 63.1% from DL-2A) was further hydrolyzed with 0.25 M TFA at 100 °C for 3 h and the mixture was treated as described above. The retentate was concentrated, lyophilized and then DL-2Ade2 was obtained (yield 11.7% from DL-2Ade). The dialysate was concentrated, and then separated on two columns (100 × 1.6 cm /each) of Sephadex G-10 and Bio-Gel P-2 successively, using Shodex RI SE-51 (Shodex Co., Japan) as the detector. Four fractions were collected in the void volume (DL-2A-0la, 9.5 mg), and in the elution volumes respectively corresponding to tetrasaccharide (DL-2A-0lb, 3.1 mg), trisaccharide (DL-2A-0lc, 2.5 mg) and disaccharide (DL-2A-0ld, 2.1 mg).

5.10. Selective degradation of galacturonic acid residues with lithium dissolved in ethylenediamine

The procedure was performed as previously reported (Lau et al., 1987). Briefly, the native polysacharide, DL-2A (50 mg), was dried *in vacuo* at 40 °C, and dissolved in ethylenediamine (25 ml, dried over 4 Å molecular sieves before used). An additional amount of lithium (130 mg) was added to the solution and the whole was allowed to stand for 3 h. The reaction was quenched by water and evaporated to dryness by addition of toluene repeatedly. The resulting powder was cooled in ice and dissolved in water. The solution was titrated to pH 4.5 with glacial acetic acid and passed through a column of Cellulose phosphate PIIion-exchange resin to remove lithium ions. The eluate from the column was lyophilized to yield "lithium-treated" DL-2A (DL-2A-Li). The end product was passed through a column of Sephadex G-10 and fractionated on a column of Bio-Gel P-2 using Shodex RI SE-51 as the detector. Two fractions, in the void volume (DL-2A-Li, 10.4 mg), and the included region (DL-2A-LiOl, beyond the trisaccharide region, 1.8 mg) were collected.

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