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Structural features of a pectic arabinogalactan with immunological activity from the leaves of *Diospyros kaki*

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Abstract

A water-soluble acidic heteroglycan, DL-3Bb, isolated from the leaves of *Diospyros kaki*, had $[\alpha]_D^{20}$ −19.9° (*c* 0.30, water), and contained rhamnose, arabinose, xylose, galactose and galacturonic acid in the molar ratio of 1.0:4.5:0.7:1.5:1.0. About 44% of the galacturonic acid existed as its methyl ester, and *O*-acetyl groups (approx 5.7%) were also identified. Its molecular weight was determined to be 9.0×10^5 Da by high-performance gel-permeation chromatography. Its structural features were elucidated by a combination of methylation analysis, periodate oxidation, two steps of partial acid hydrolysis, and ^{1}H and ^{13}C NMR spectroscopy and ESI mass spectrometry. The data obtained indicated that DL-3Bb possessed a backbone of a disaccharide of [→4)-α-GalA*p*-(1→2)-α-Rha*p*-(1→], with approx 58.7% substitution at O-4 of the rhamnopyranosyl residues by β-(1→4)-linked xylopyranosyl residues, and by β-(1→3) and β-(1→6)-linked galactopyranosyl (galactan) residues. The side chains were further substituted by arabinofuranosyl residues at O-2 by β-(1→4)-linked xylopyranosyl residues and at O-3 by β-(1→6)-linked galactopyranosyl residues. Preliminary tests in vitro revealed that it could stimulate LPS-induced B lymphocyte proliferation, but not for ConA-induced T lymphocyte proliferation. It was proposed that the acid-labile arabinofuranosyl residues in the side chains would not be needed for the expression of the enhancement of the immunological activity, and that the presence of GalAp in the backbone has an important, but not crucial effect on the expression of the activity.

Keywords: *Diospyros kaki*; Polysaccharide; Pectin; Arabinogalactan; Rhamnogalacturonan

1. Introduction

Diospyros kaki, which is native to China, belongs to the family Ebenaceae and is widespread in tropics and subtropics.[1] In Chinese traditional medicine, it has been used mainly in the treatment of hiccough. In recent years, the leaves of *D. kaki* have been favored as a tea with health-conscious persons in Southeast Asia. Up to now, a great diversity of low-molecular-weight constituents including hydrocarbons, steroids, terpenoids, naphthoquinones, etc., from the plant *D. kaki* have been elaborated;[2] however, few reports have been concerned with the polysaccharide constituents from the leaves of this species. In this paper, we reported the structural analysis and immunological activity of a pectic polysaccharide (DL-3Bb) from the leaves of *D. kaki*.

2. Results and discussion

The hot-water extract of the leaves of *D. kaki* was precipitated with ethanol, and the crude polysaccharide, named DL, was obtained. DL was repeatedly subjected to DEAE-cellulose chromatography and Sephacryl S-300 gel-permeation chromatography, which resulted in the isolation of a polysaccharide DL-3Bb (see Section 3 for details).

DL-3Bb was eluted as a symmetrical narrow peak on high-performance gel-permeation chromatography (HPGPC) (Fig. 1a), and gave a single band on PAGE. Its molecular weight was estimated to be 9.0×10^5, in

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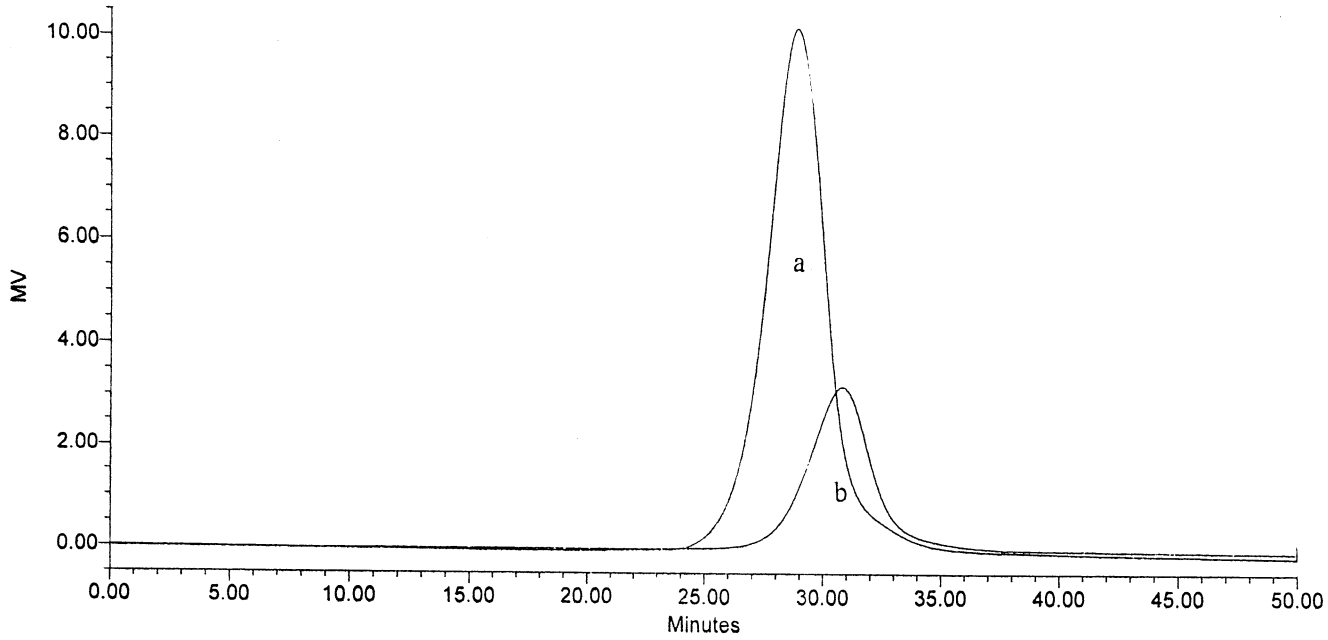

Fig. 1. HPGPC chromatogram on ultrahydrogel columns of DL-3Bb (a) and DL-3BbDe (b).

reference to standard dextrans. It showed a specific rotation of $[\alpha]_D^{20}$ −19.9° (*c* 0.30, water), and was shown to be free of protein by the Lowry method.[3]

Quantitative determination of neutral sugars by gas chromatography (GC) showed that DL-3Bb consisted of rhamnose, arabinose, xylose and galactose in the molar ratio 1.0:4.5:0.7:1.5. It was also shown to contain 11.5% uronic acid by the *m*-hydroxydiphenyl method.[4] The presence of GalA was established by the increase of Gal content in the carboxyl-reduced polysaccharide (data not shown). The content of *O*-acetyl groups was approx 5.7%, according to ^{1}H NMR spectroscopy, and about 44% of the GalA was shown to exist as the methyl ester as determined by the method described.[5]

Methylation of carboxyl-reduced polysaccharide, DL-3BbRe, was performed with the sodium hydroxide–iodomethane (NaOH–MeI) procedure in dimethyl sulfoxide. The permethylated product was hydrolyzed, and the hydrolysate was converted into the partially methylated alditol acetates and analyzed by GC–MS. The results are shown in Table 1.

As shown in Table 1, the total molar recovery of nonreducing terminal residue (Ara*f*, Gal*p*) was approximately equal to that of branched residues (1,2,5-linked Ara*f*, 1,3,6-linked Gal*p*, 1,2,4-Xyl*p* and 1,2,4-linked Rha*p*). Ara was present in the furanose form, with most of the units probably forming a (1→5)-linked arabinan with additional branches at the O-2 of the Ara*f* residues. Although Xyl has been shown to be rare in pectic polysaccharides from parenchymatous tissues, it has been found in pectic fractions from the apple cell wall[6] and the carrot, *Daucus carota*.[7] Xyl was present mainly as (1→4)-linked residues with further substitution at O-2 in DL-3Bb. The total content of Gal*p* residues increased, compared with the result of component analysis, and the molar ratio of (1→4)-linked Gal*p* residues in the methylated products was consistent with the content of GalA, indicating that (1→4)-linked Gal*p* residues were derived from GalA, since (1→2) and (1→2,4)-linked Rha*p* residues and (1→4)-linked GalA residues are commonly found in pectic polysaccharides. An attempt to permethylate the native DL-3Bb ended in

Table 1
Methylation analysis of carboxyl-reduced DL-3Bb (DL-3BbRe) and carboxyl-reduced DL-3BbDe (DL-3BbDeRe)

Peak no.	Methylated sugars	Molar ratio		Linkages
		DL-3BbRe	DL-3BbDeRe	
1	2,3,5-Me_3-Ara*f*	5.3	trace	terminal
2	2,3-Me_2-Ara*f*	4.9	trace	1,5-
3	3-Me-Ara*f*	3.1		1,2,5-
4	2,3,4-Me_3-Xyl*p*	trace	0.2	terminal
5	2,3-Me_2Xyl*p*	1.33	2.0	1,4-
6	3-Me-Xyl*p*	0.76		1,2,4-
7	2,3,4,6-Me_4-Gal*p*	1.11	1.16	terminal
8	2,3,4-Me_3-Gal*p*	1.64	2.5	1,6-
9	2,4,6-Me_3-Gal*p*	0.73	0.67	1,3-
10	2,4-Me_2-Gal*p*	1.0	trace	1,3,6-
11	2,3,6-Me_3-Gal*p*	2.31	2.6	1,4-
12	3,4-Me_2-Rha*p*	1.0	1.0	1,2-
13	3-Me-Rha*p*	1.42	1.5	1,2,4-

failure due to the poor solubility of the polysaccharide in dimethyl sulfoxide.

DL-3Bb was partially hydrolyzed under mild acidic conditions and then dialyzed, giving DL-3BbDe (non-dialysate, yield 38.2%) and DL-3Bb-Oli (dialysate). DL-3Bb-Oli was repeatedly fractionated on columns of Bio-Gel P-2 and Sephadex G-10. Four carbohydrate fractions were eluted sequentially in the void vol (I), penta- and tetrasaccharides (II), disaccharide (III) and monosaccharide regions (IV). TLC analysis showed that arabinose was the only component sugar in fractions I–IV. These fractions were identified by methylation analysis and ESIMS as a (1→5)-linked arabinan with branches at O-2 (I), a mixture of a (1→5)-linked arabinose tetrasaccharide, and a (1→5)-linked arabinose pentasaccharide with branches at O-2 (II), and *O*-arabinofuranosyl-(1→5)-arabinose (III).

DL-3BbDe, with a molecular weight of 4.0×10^5, was eluted as a single symmetrical peak on HPGPC (Fig. 1b) and gave a single band on PAGE. It was composed of rhamnose, xylose, galactose and galacturonic acid in a similar molar ratio with DL-3Bb, except that almost all arabinofuranosyl residues were removed. Its specific rotation was $[\alpha]_D^{20}$ +78.5° (*c* 0.229, water). The change of specific rotation from $[\alpha]_D^{20}$ −19.9° to $[\alpha]_D^{20}$ +78.5° after mild acid treatment suggested the presence of α-Ara*f* residues in native DL-3Bb.

In comparison with that of DL-3BbRe, the results of the methylation analysis on carboxyl-reduced DL-3BbDeRe showed that the molar ratios of (1→3,6)-linked Gal*p* residues and (1→2,4)-linked Xyl*p* had decreased, and that the molar ratios of (1→4)-linked Xyl*p* and (1→6)-linked Gal*p* residues had increased. These results suggested that Ara*f* residues should be attached at O-2 of a (1→4)-linked xylan and at O-3 of a (1→6)-linked galactan.

For further investigations on the structure of native DL-3Bb, DL-3BbDe was hydrolyzed with 0.25 M TFA at 100 °C for 3 h. After evaporation to remove TFA, the hydrolysate was fractionated on a column of Bio-Gel P-2, and three fractions, i.e., the void vol. (Ol-1), tetrasaccharide (Ol-2) and disaccharide (Ol-3) regions were collected. Ol-1 was further separated on a column of Sephadex G-25, from which two fractions (Ol-4, Ol-5) were obtained.

Ol-4 consisted of Rha and GalA, along with trace of Gal. Methylation analysis of the carboxyl-reduced Ol-4 revealed that it consisted of (1→2)-linked Rha*p* residues and (1→4)-linked Gal*p* residues in the approximate molar ratio of 1.2:1. These results indicate that Ol-4 possibly originated from the backbone of a rhamnogalacturonan, and then neutral side chains would be attached to the acidic backbone via O-4 of Rha*p* residues in native DL-3Bb.

Ol-5 showed a broad peak on a column of Sephadex G-25, indicating that it was a mixture composed of several oligosaccharides. After complete hydrolysis, TLC analysis of the hydrolysate indicated the presence of galactose, rhamnose and galacturonic acid. Methylation analysis revealed that (1→2)-linked Rha*p* residues and terminal, (1→6)- and (1→3)-linked Gal*p* residues (molar ratios, 5:1:3:2) were the major linkages in Ol-5. It was proposed that Ol-5 was a mixture of acidic oligasaccharides, composed of (1→2)-linked Rha*p* residues and GalA*p* residues, and several other linear (1→3) and (1→6) oligosaccharides.

Combined with the results of the composition analysis (TLC) and methylation analysis, Ol-2 was identified as galactopyranosyl-(1→6)-galactopyranosyl-(1→6)-galactopyranosyl-(1→6)-galactose. This was consistent with the results of ESIMS which showed the molecular ions at *m*/*z* 707.3 corresponding to $[M+H_2O+Na]^+$.

TLC analysis revealed Ol-3 was composed of Rha and GalA. The ESIMS of Ol-3 contained a molecular ion at *m*/*z* 339.2 corresponding to $[(GalA \rightarrow Rha)\text{-}1]^-$ or $[(Rha \rightarrow GalA)\text{-}1]^-$. The 1H NMR spectrum of Ol-3 showed three signals in the anomeric region, δ 5.18, 5.02 ($J_{1,2}$ 4.0) and 4.90, which were attributed to H-1 of α-Rha*p* (reducing-end group), α-GalA*p* (glycosidically linked) and β-Rha*p* (reducing-end group), respectively, in reference to the chemical shifts of respective monosaccharides and taking into account the effect of glycosidation.[8] Taken together, Ol-3 was tentatively identified as α-GalA-(1→?)-Rha. Further evidence of the structure of Ol-3 could not be obtained due to the limited quantity of Ol-3 available.

DL-3Bb was oxidized with 0.017 M sodium periodate ($NaIO_4$) at 4 °C in the dark, and the reaction was complete in 72 h. A total of 0.79 mol of $NaIO_4$ was consumed and 0.115 mol of formic acid was produced per mole of glycosyl residues, based on the average molar mass (145) of a glycosyl residue. The composition analysis of the polyalcohol resulting from periodate oxidation revealed that the molar ratio of glycerol, rhamnose, xylose and arabinose is 8.31:0.8:0.5:1.9. These values were in approximate agreement with the theoretical values calculated on the basis of the methylation data.

^1H and ^{13}C NMR resonances of the native DL-3Bb and its degraded DL-3BbDe were assigned insofar as possible based on the component analysis, methylation analysis and literature data.[9–12] The ^1H NMR spectrum of DL-3Bb showed signals of a *C*-methyl proton at δ 1.34, an *O*-acetyl at δ 2.11, and anomeric protons at δ 5.09–5.34, which were all due to α-Ara*f* residues. Signals of other glycosyl residues in the ^1H and ^{13}C NMR spectra of DL-3BbDe became well resolved, followed by the release of Ara*f* residues from native DL-3Bb. H-1 signals corresponding to the α-Rha*p* residues (δ 5.16), the α-GalA*p* residues (δ 4.95–4.99), the β-Xyl*p* residues (δ 4.68) and the β-Gal*p* residues (δ 4.38–4.47) were also assigned.

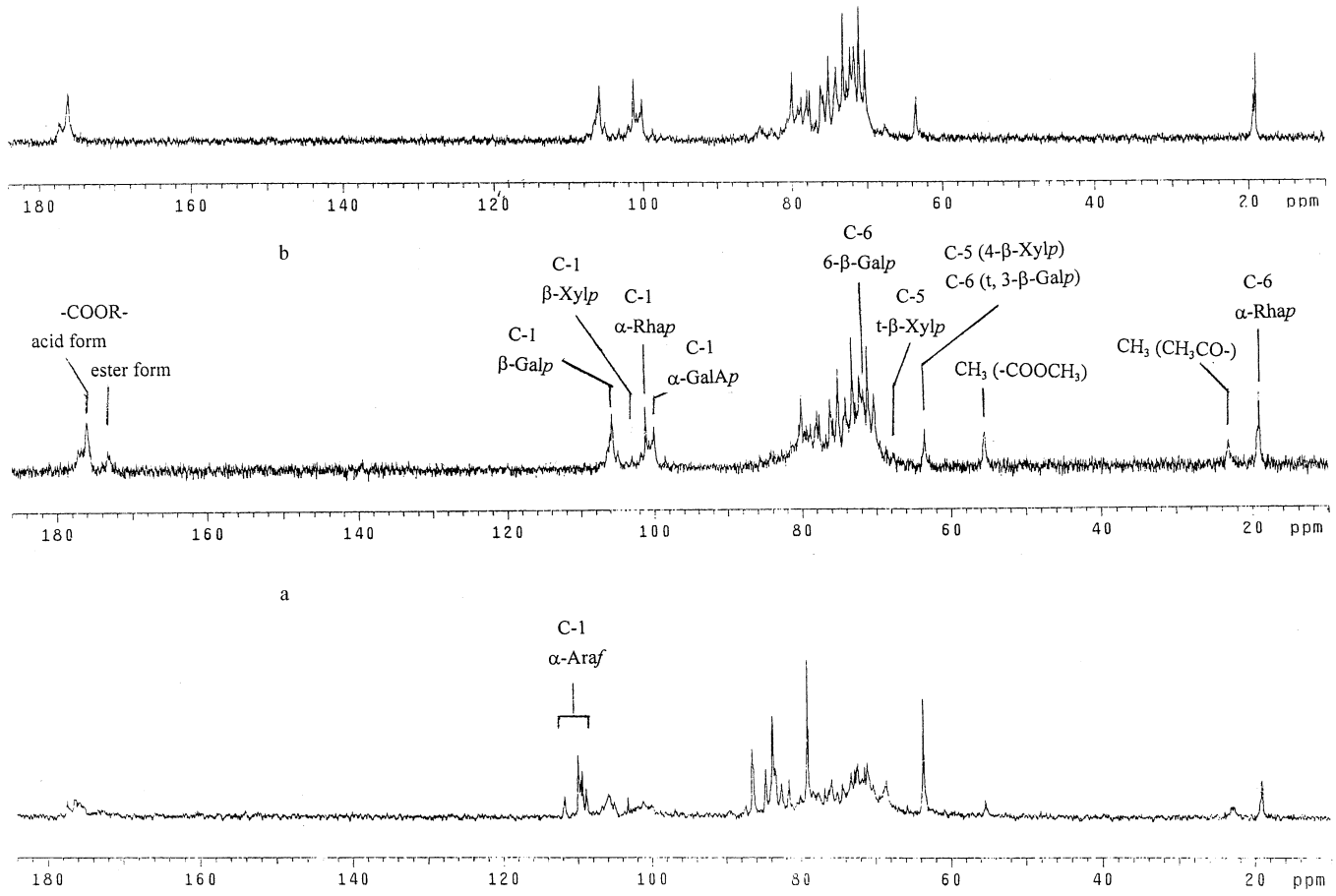

Fig. 2. ^{13}C NMR spectra of DL-3Bb (a), Dl-3BbDe (b) and alki-treated Dl-3BbDe (c).

In the anomeric carbon region, signals at δ 108.71–111.54 (Fig. 2a), 105.09–105.846, 103.07, 101.237 and 100.117 were assigned to the C-1s of α-Ara*f*, β-Gal*p*, β-Xyl*p*, α-Rha*p* and α-GalA*p*, respectively. The ^{13}C NMR spectra (Fig. 2) of DL-3Bb and DL-3BbDe showed signals of *C*-methyl at δ 18.97, *O*-acetyl methyl at δ 23.76, *O*-methyl of carboxylic acid methyl esters at δ 55.42, the carboxyl in the acid form at δ 177.39 and carboxyl in the ester form at δ 175.04. After DL-3BbDe was deesterified with 0.2 M sodium hydroxide for 2 h at room temperature, the disappearance (Fig. 2c) of signals of carboxyl groups in the ester form at δ 175.04, *O*-methyl in methyl esters at δ 55.42 and methyl of *O*-acetyl at δ 23.76 confirmed the presence of the *O*-acetyl group and carboxylic acid methyl esters.

The methylene signals of C-5 of the Ara*f* residues and C-6 of the Gal*p* residues were identified in the DEPT-mode of the ^{13}C NMR spectrum as negative peaks. Therefore, the resonances (Fig. 2a) at δ 63.52 were assigned to C-5 of terminal Ara*f* and C-6 of terminal Gal*p* and (1→3)-linked Gal*p*. Signals at δ 68.46 (C-5 of 5- and 2,5-linked Ara*f*) and δ 71.05–71.39 [(1→6)-linked and (1→3,6)-linked Gal*p*] were also assigned as shown in the ^{13}C-NMR spectrum of native DL-3Bb. In Fig. 2b, the resonances at δ 71.11 were assigned to C-6 of (1→6)-linked Gal*p*, the weaker signals at δ 67.66 were attributed to C-5 of terminal Xyl*p*, and the signals at δ 63.42 were assigned to C-5 of (1→4)-linked Xyl*p* and C-6 of terminal and (1→3)-linked Gal*p*.

Based on the results described above, it could be concluded that DL-3Bb possesses a backbone of the average disaccharide of [→4]-α-GalA*p*-(1→2)-α-Rha*p*-(1→)], having a degree of methyl esterification of 44%. The side chains are attached at the O-4 of Rha*p* residues, including β-(1→4)-linked Xyl*p* residues, and β-(1→3) and β-(1→6)-linked Gal*p* (galactan) residues. The highly branched α-(1→5) arabinan was attached to O-2 of β-(1→4)-linked Xyl*p* and O-3 of β-(1→6)-linked Gal*p* residues, forming arabinoxylan and arabinogalactan moieties, respectively. These partial structural features resemble those of the pectic polysaccharides isolated from the carrot, *Daucus carota*,[7] the seed of *Coix lacryma-jobi* L.var. ma-yuen,[13] the root of *Angelica acutiloba* Kitagawa [14] and the leaves of *Plantago major* L.[15] These acidic arabinogalactans contain a higher content of (1→3,6)-linked Gal*p* residues and/or contain some (1→4)-linked Gal*p* residues in the degraded polysaccharide after treatment with mild acid hydrolysis and/or arabinofuranosidase. Differently, it was found that few (1→3,6) and (1→4)-linked Gal*p*

residues existed in the degraded polysaccharide DL-3BbDe.

Some pectic polysaccharides with immunomodulating activity have been isolated from plants, and side chains such as arabino-3,6-galactan were suggested to be important for the activity of complement activation.[13,14,16,17] The effects of DL-3Bb and its degraded products, DL-3BbDe and DL-3BbDeR, on the proliferation of ConA- or LPS-induced lymphocytes were tested in vitro, respectively. These results are shown in Table 2.

As observed in Table 2, when ConA was used as mitogen for T lymphocytes, and the polysaccharides, including DL-3Bb, DL-3BbDe and DL-3BbDeR at different concentrations, were added, no significant change was found (line 5). On the contrary, the proliferation of B lymphocytes increased significantly when LPS was used as mitogen (last line). These results suggested that Dl-3Bb, DL-3BbDe and DL-3BbDeR could enhance the LPS-induced B lymphocyte proliferation at concentrations of 1, 10 and 100 μg/mL, while they had little effect on the ConA-induced T lymphocyte proliferation. In addition, after the release of Ara*f* residues from the native DL-3Bb, the enhancing ratio of B lymphocyte proliferation increased by 68% at a concentration of 100 mg/mL. However, when GalA*p* residues were reduced to Gal*p* residues, the enhancing ratio of B lymphocyte proliferation (100 μg/mL) was reduced by 28–95% . These results indicate that: (a) the Ara*f* residues had no positive effect on the immunological activity; and (b) the presence of GalA*p* residues in the backbone could contribute to the activity, but they were not the key factor in the expression of the activity of B lymphocyte proliferation. This observation was different from that of the anti-complementary pectic arabinogalactans from *Coix lacryma-jobi* L. var. mayuen,[13] and *A. acutiloba* Kitagawa,[14] in which the anti-complementary activity decreased, followed by treatment with mild acid hydrolysis. Similar to the pectic polysaccharide with the anti-complementary activity from *Plantago major* L,[15] it was proposed that the linear galactan with higher β-6-linked Gal*p* residues in the side chain was responsible for the expression of the immunological activity.

3. Experimental

3.1. Material

Dried leaves of *D. kaki* were collected in He-nan province in China. T-series Dextran, 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. Concanavalin A (ConA) and lipopolysaccharide (LPS) was from Sigma Chemical Co., and Medium RPMI-1640 was purchased from Gibco Laboratories. All other reagents were of analytical grade as available and were used without further purification.

3.2. General methods

Specific rotations were determined on a Perkin–Elmer 241M digital polarimeter in water at $20\pm1\,^{\circ}C$. IR spectra (KBr or Nujol pellets) were recorded on a Perkin–Elmer 599B FTIR spectrometer. GC was carried out with a Shimadzu GC-14B apparatus, equipped

Table 2
Effect of DL-3Bb, DL-3BbDe and DL-3BbDeRe on the proliferation of ConA-induced T lymphocytes and LPS-induced B lymphocytes in vitro

Sample name	Concentration (μG/mL)	ConA			LPS		
		Average CPM	SD	Percent (%) [a]	Average CPM	SD	Percent (%) [a]
Control	blank	9451	1884		9207	290	
	with mitogen	108,113	1395		40,451	1110	
DL-3Bb	1	96,268	401	−11	56,927	3731	41
	10	105,740	351	−2	55,971	2550	38
	100	92,293	1960	−15	62,513	2298	55
DL-3BbDe	1	95,663	2262	−12	54,606	3624	35
	10	102,044	2291	−6	63,899	1980	58
	100	98,383	4029	−9	90,311	8843	123
DL-3BbDeRe	1	93,239	9372	−14	55,460	4411	37
	10	93,895	88	−13	59,710	4201	48
	100	109,478	91	1	79,041	3697	95

[a] Values $>15\%$ indicate the sample could stimulate mitogen-induced lymphocyte proliferation; values $<-15\%$ indicate the sample could inhibit mitogen-induced lymphocyte proliferation.

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. 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 (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained with a Bruker AM 400 spectrometer with a dual probe in the FT mode at room temperature. All the chemical shifts are reported relative to Me_4Si. The DEPT experiments were done using a polarization-transfer pulse of 135°. Protein content was measured by the Lowry method.[3] Reduction of carboxyl groups was carried out with CMC–$NaBH_4$ for three times as described by Taylor and Conrad.[18]

3.3. Separation and purification of DL-3Bb

The dried leaves of *D. kaki* were percolated with cold EtOH for 2 weeks and then extracted with hot water. After addition of EtOH (4 vols), the crude polysaccharides DL (yield: 7.26%) were precipitated from the solution. DL was applied to a column of DEAE-cellulose (Cl^- form), and eluted stepwise as four fractions (DL-1, DL-2, DL-3 and DL-4) with water, followed by 0.1, 0.2 and 0.4 N NaCl. DL-3 was further purified on DEAE-cellulose (OAC^- form) chromatography and Sephacryl S-300 gel-permeation chromatography to give the purified polysaccharide, DL-3Bb (recovery of 1.22% from DL).

3.4. Gel-permeation chromatography and molecular weight

Measurements were performed by HPGPC with a Waters 515 instrument fitted with GPC software (Millennium[32]), using a Waters 2410 RI detector. The column was calibrated with T-series Dextran T-2000, T-500, T-110, T-70, and T-40 (Pharmacia Co.). NaAc (3 mM) was used as eluant, and the flow rate was kept at 0.5 mL/min. A 20-mL aliquot was injected for each run.

3.5. Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed on an apparatus with gel (7.5%) tubes (124 × 4 mm each) and 5 mM Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the periodate–Schiff (PAS) procedure and with Coomassie blue reagent. DL-3Bb gave a distinct band at a distance of 49 mm from the origin, and DL-3BbDe moved 52 mm.

3.6. Composition analysis and determination of *O*-methyl groups in methyl esters

3.6.1. Sugar composition. The acidic polysaccharide (oligosaccharide) was hydrolyzed at 121 °C for 2 h and the neutral polysaccharide for 1.5 h. Neutral sugars were analyzed by GC after conversion of the hydrolysate into alditol acetates as described.[19] 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.[4]

3.6.2. Determination of *O*-methyl groups in methyl esters. The sample (2.5 mg) was dissolved in 0.5 mol/L NaOH (0.1 mL) containing EtOH as an internal standard, and the solution was left at room temperature for 30 min. The mixture was analyzed by the procedures described.[5]

3.7. Methylation analysis

The samples were methylated three times by the method of Needs and Selvendran,[20] and the resulting permethylated product was hydrolyzed, reduced, acetylated and analyzed by GC–MS as described previously.[21] The partially methylated alditol acetates were identified by their fragment ions in EIMS. Data are provided in Table 1.

3.8. Partial acid hydrolysis

DL-3Bb (150 mg) was treated with 0.020 M TFA at 100 °C for 2 h. The mixture was evaporated to dryness, and the residue was dialyzed against distilled water (3 × 500 mL). The degraded polysaccharide DL-3BbDe was further hydrolyzed with 0.25 M TFA at 100 °C for 3 h, and the product was applied to gel-permeation chromatography (Bio-Gel P-2, Sephadex G-25).

3.9. Periodate oxidation

The polysaccharide (8.0 mg) was oxidized with 0.017 M $NaIO_4$ at 4 °C in the 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 measured by a spectrophotometric method,[22] and HCOOH production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ (25 mg, 12 h). The pH was adjusted to 5.0, the solution was dialyzed, and the nondialysate was lyophilized, then hydrolyzed with 1 M TFA at 100 °C for 6 h. The hydrolysate was analyzed by GC (170 °C for determina-

tion of glycerol and 210 °C for determination of sugar composition, respectively).

3.10. De-esterification of DL-3BbDe[23]

DL-3BbDe (50 mg) was kept in 0.2 M NaOH for 2 h at room temperature, and the solution was neutralized with AcOH and dialyzed.

3.11. Lymphocyte proliferation test in vitro[24,25]

Spleen cells (1×10^5/well) were seeded into a 96-well plate in the presence of mitogen ConA (5.0 mg/mL) or LPS (10.0 mg/mL). After incubation for 44 h at 37 °C in a humidified 5% CO_2 incubator, 46 kBq of ^{3}HdR was added to each well, and the plate was further incubated for another 4 h, and then the resolver (100 mL/well) was added. The polysaccharide samples in various concentrations (1, 10, 100 mg/mL) were incubated with mouse splenocytes in the presence of ConA or LPS, respectively. The proliferation of cells were determined by the 3H-TdR incorporation method. Results are shown in Table 2.

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