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Immunological activities and structure of pectin from *Centella asiatica*

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Abstract

Centella asiatica, is mainly found in the south of Asia and the southern hemisphere. The pectin was extracted from *C. asiatica* and isolated by anion-exchange and gel-filtration chromatography with TLC and GLC analyses. It contained arabinose, rhamnose, galactose, xylose and galacturonic acid. The structural features were elucidated by partial acid hydrolysis, enzymatic degradation, methylation, carboxyl-reduction, NMR spectroscopy and ESI-MS experiments. The backbone was presumed to contain 1, 4-linked α-D-GalA and 1, 2/1, 2, 4-linked α-L-Rha with RG-I and homogalacturonan (HG) types. The side chains were neutral chains including arabinosyl, arabinogalactosyl and xylosyl chains. Most of the side chains were attached to Rha, while partial residues were presumed to link to GalA. 14% GalA residues in backbone contained acetyl groups. With deacetylation and carboxyl-reduction, the pectin and its degraded product showed immunostimulating activity to different extent in vitro. These results indicated that the carboxyl and acetyl groups play important roles in the expression of immunological activity.

Keywords: Centella asiatica; Pectin; Immunological activity; Acetyl group; Carboxyl group

1. Introduction

Pectins are complex heterogeneous polysaccharides found in the primary cell wall of most plants, and they are considered to consist of HG (homogalacturonan), RG-I (rhamonogalacturonan I) and RG-II (rhamnogalacturonan II) types (Albersheim et al., 1996). Pectins have been used in the food industry as gelling and thickening agents, and show some pharmaceutical activities (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). The HG segments or 'smooth regions' have linear chains of poly-α-(1→4)-D-galacturonic acid residues. RG-I consists of alternating sequences of Rha and GalA, and the Rha moieties carry side chains ('hairy regions'). These side chains and acetyl groups on galacturonic acid may play an important role in bio-activities (Kravtchenko, Penci, Voragen, & Pilnik, 1993). *Centella asiatica*, as a remedy for sodation and stabilization and against lepra, anabrosis (Chopra, Nayar, & Chopra, 1956; Maquart, Bellon, Gillery, Wegrowski, & Borel, 1990; Yoshinori, Reiko, & Tsumematsu, 1982), has been used in many countries, and in our lab it was isolated and the pectin 1BII was obtained. Using chemical and biochemical methods the structural features and immunological activity were reported. Meanwhile, the effects of acetyl and carboxyl groups in 1BII on immunological activity are investigated. In this study we report and discuss the isolation and structural features and activity of this pectin and its derivatives.

2. Experimental

2.1. Materials and methods

Dried *C. asiatica* was purchased from the Shanghai Medicinal Materials Cooperation Company (No.: 000201). 1-Cyclohexyl-3-(2-morphlinoethy) carbodiimide metho-*p*-toluenesulphonate (CMC), $NaBH_4$ and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium brimoide (MTT) were purchased from Fluka. Concanavalin (ConA) and

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lipopolysaccharide (LPS) were obtained from Sigma. The NMR spectra were recorded on Varian Mercury 400 spectrometers. HPSEC was performed with a Waters system instrument, including GPC software (Millennium^{32}), 515 HPLC pump, 2410 RI detector and 2487 dual λ absorbance detector. GLC was done with a Shimazu-9A apparatus equipped with a 5% OV 225/AW-DMC-Chromosorb W column (2.5 m × 3 mm). ^{3}H-TdR data were counted using a liquid scintillation counter (MicroBeta Trilux, Perkin–Elmer Life Science). Inbred ICR (♀) mice, 6–8 weeks old, weighing 20 ± 2 g, were obtained from Shanghai Experimental Animal Laboratory, Chinese Academy of Sciences.

2.2. Analytical methods

Neutral sugars were determined by GLC after hydrolysis with 2 mol/l TFA at 110 °C for 2 h and conversion of the hydrolysate into alditol acetates, as described previously (Dong, Ding, Yang, & Fang, 1999). Uronic acid content was determined by a modification of the *m*-hydroxylbiphenyl method (Kimberley & Jock, 1992). The D and L configuration of neutral sugars was identified by the Gerwig method (Gerwig, Kamerling, & Vliegenthart, 1978). Methylation analysis was carried out according to the modified NaOH-DMSO method (Needs & Selvendran, 1993), and the methylated product was converted into partially methylated alditol acetates before GLC-MS analysis. Mz of polysaccharide was determined by HPSEC (Ultrahydrogel™ 2000 and 500 columns, 3 mmol/l NaOAc and 30.0 ± 0.1 °C). This was calculated with T-700, 580, 300, 110, 80, 70, 40, 9.3 and 4 (Dextran series, Pharmacia).

2.3. Partial acid hydrolysis

1BII (600 mg) was dissolved in 150 ml 0.1 mol/l TFA, and kept at 100 °C for 40 min. The resulting solution was cooled, concentrated and dialyzed with distilled water. The retentate was lyophilized to give a degraded polymer (1BIIP, 201 mg), and the dialysate was isolated by gel filtration chromatograph (Sephadex G-10 column) to give a series of oligomer fractions (OA, OB, OC and OD). OD was further acid hydrolyzed with 0.2 mol/l TFA at 100 °C for 1 h to give degraded product OB1.

2.4. Reduction of carboxyl groups

Reduction was carried out with CMC and $NaBH_4$ (Fluka) as described reports (Taylor & Conrad, 1972; Tomoda, Ichikawa, & Shimizu, 1986). The reaction was repeated three times under the same conditions.

2.5. De-acetylation and determination of O-acetyl groups

The degree of acetylation (DA) was determined by ^{1}H NMR and also the method described by Tomoda method (Tomoda, Shimizu, Shimada, & Suga, 1985). For the latter method, 1BII (33 mg) and 1BIIP (31.3 mg) were treated with 0.3 mol/l NaOH (20 ml) at room temperature and stirred for 2 h, respectively. After neutralization with HCl and dialysis, DA of the degraded products was determined.

2.6. Enzymatic hydrolysis

1BII (20 mg) and 1BIIP (21 mg) were treated with pectinase (from Rhizopu E.C. 3.21.15, 467 u/g, Sigma) at 37 °C for 48 h, respectively. The products were boiled at 100 °C for 10 min, centrifuged and dialyzed to give 1BIIE and 1BIIPE, respectively.

2.7. Electrospray ionization mass spectrometry

The ESI-MS was carried out on a VG Quattro MS/MS spectrometer. For positive and negative ESI analyses, oligosaccharide fractions were dissolved in H_2O–MeOH. Other conditions were described as reported previously (Reis, Domingues, Domingues, Ferrer-Correia, & Coimbra, 2003).

2.8. Immunological assays

Different dilutions of the polysaccharide samples (1–100 μg/mL) were incubated directly with mouse splenocytes at 37 °C in humidified 5% CO_2 atmosphere for 48 h. The incubation was expired 5 h later after addition of MTT (5 mg/mL). MTT method was used to measure the cytotoxicity and none specific enhancement of sample (Heeg, Reimann, Kabelitz, Hardt, & Wagner, 1985; Li et al., 1990). Samples were incubated with mouse splenocytes in the presence of mitogen ConA (5.0 μg/mL) or LPS (20 μg/mL) at 37 °C in humidified 5% CO_2 atmosphere. Cells were pulsed with 0.25 μCi/well of ^{3}H-thymidine for the final 12 h (Heeg et al., 1985). The cells were then harvested onto glass filters and the incorporated radioactivity was counted by a liquid scintillation counter. All experiments were performed three times independently.

3. Results and discussion

3.1. Extraction and isolation

C. asiatica alcohol insoluble solids were sequentially extracted with hot water. Protein was removed by

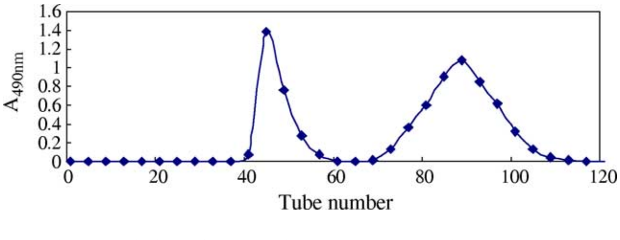

Fig. 1. Isolation of 1BII and 1BI on DEAE-cellulose column.

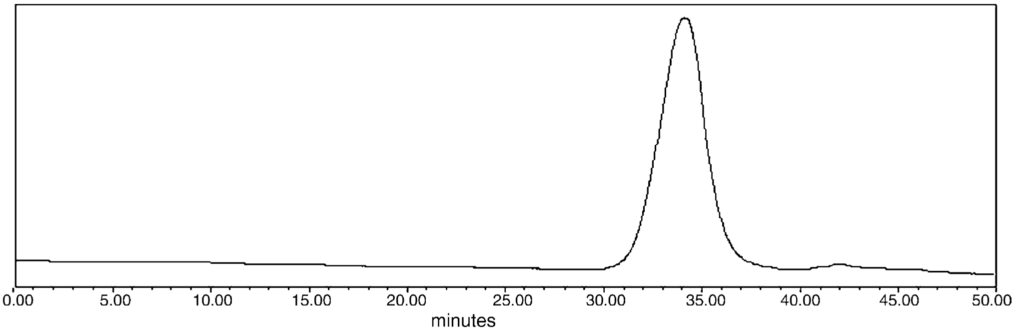

Fig. 2. HPSEC profile of 1BII.

precipitation with trichloroacetic acid and the solution was then dialyzed and precipitated with alcohol as described previously (Wang, Dong, Zuo, & Fang, 2003). Black powder (crude polysaccharide) was obtained. Using DEAE-cellulose (Cl^- form) chromatography with water, 0.1, 0.3 and 0.5 mol/l NaCl eluents, the crude polysaccharide was fractionated to five sub-fractions. The fraction eluted from 0.1 mol/l NaCl solution was further chromatographed on the DEAE-cellulose column (Cl^- form) using a 0–0.3 mol/l gradient NaCl solution. At last two fractions were obtained according to the eluent time, as shown in Fig. 1. The later fraction was further purified on Sephacryl S-300 column (100×2.6 cm) and Sephadex G-150 column (100×2.6 cm) to give a polysaccharide, designated 1BII. 1BII was shown to have symmetrical peak in HPSEC (Fig. 2), and had specific rotation $[\alpha]_D^{20} + 14.7°$ (c 0.50, H_2O). The average Mz was estimated to be 7.74×10^4, according to the calibrated standard curve.

3.2. Structural characterization

1BII had a negative response to the Lowry test, this and the absence of absorption at 280 nm indicated that it did not contain any protein. Weak absorption at ca. 1720 cm^{-1} in the IR spectra suggested a small quantity of uronic acid or acetyl groups presented in this polysaccharide. TLC analysis confirmed that it contained galacturonic acid. TLC and GLC analyses showed the presence of Ara, Gal, Rha, Xyl and GalA residues in 1BII. And the ratio of them was 0.57: 1.44: 1.0: 0.44: 1.07, which were listed in Table 1.

The signal at δ 20.7 ppm in ^{13}C NMR spectrum suggested the native polysaccharide contained *O*-acetyl groups (Shimizu, Tomoda, Kanari, & Gonda, 1991). Meanwhile, the signal at δ 2.14 ppm in ^1H NMR spectra supported the above deduction. The presence of these groups was confirmed by GLC analyses of the hydrolyzates, and the acetyl groups content was determined to be 2.9%. The anomeric configuration of each residue in 1BII was elucidated from the ^{13}C NMR spectrum. The anomeric signal at 99.2 ppm was assigned to the anomeric carbons of α-GalpA (Shimizu and Tomoda, 1985). The signals at 110.2, 108.7 ppm in 1BII were assigned to the anomeric carbons of α-Araf. The anomeric carbons signals of β-Xylp were at 102.0 ppm in 1BII (Nandini & Salimath, 2001). Other signals assignments had been marked in Fig. 3, according to the 2D NMR and previously reported assignment (Cui, Eskin, Biliaderis, & Marat, 1996; Wang et al., 2003).

Both 1BII and its carboxyl-reduced derivatives (1BIIR) were methylated. The methylation analysis of 1BIIR showed 1BII contained 1, 4-linked GalA and Rha residues together with other neutral residues. Because of β-elimination in methylation analyses, the molar ratio of Rha and Ara residues in native polysaccharides (data not be listed) were less than those of the carboxyl-reduced derivatives (Table 2).

1BIIP, the product of 1BII's partial acid hydrolysis, was shown to have a symmetrical peak in HPSEC (Mz, 7.0×10^4). The composition and methylation analyses showed that most of Ara residues and partial Gal residues were removed, but 1, 3, 6-linked Gal remained in a small amount. Compared with these changes, Xyl residues were largely retained. All the results above indicated that most of the Ara in side chains are located at the end of the side chains. ^{13}C NMR spectrum of 1BIIP showed peak associated with Ara, which was in agreement with the results of methylation analysis.

1BII was treated with pectinase to give the degraded product 1BIIE. GLC analysis showed 1BII contained much less Xyl residues than 1BII. This result suggested Xyl residues should be located in the short side chains and lost on enzymatic cleavage and dialysis (MWCO 3500–5000 Da). 1BIIP had been treated with pectinase, and most of Xyl residues had also been removed (Table 1). The result confirmed the above assumption.

Table 1
Glycosyl residues compositions of 1BII, 1BIIP and their carboxyl-reduced derivatives

Residues	Molar ratio						
	1BII	1BIIR	1BIIE	1BII-O	1BIIP	1BIIPR	1BIIPE
Rha	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ara	0.57	0.58	0.47	0.77	0.11	0.1	0.19
Xyl	0.44	0.43	0.15	0.22	0.43	0.4	0.26
Gal	1.44	2.51	1.46	1.87	1.12	2.2	1.53

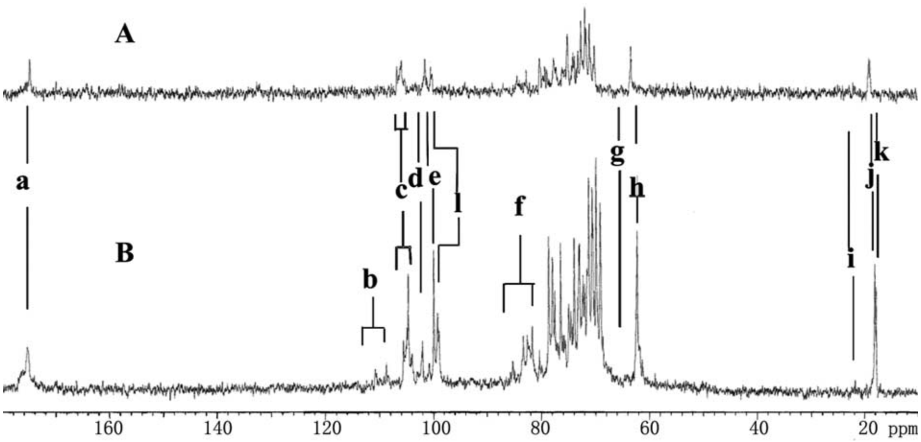

Fig. 3. ^{13}C NMR spectra of 1BII (B) and 1BIIP (A): (a) C6 of GalA, (b) C1 of Ara, (c) C1 of Gal, (d) C1 of Xyl, (e) C1 of GalA, (f) C2-C4 of Ara, (g) C5 of Xyl, (h) C6 of Gal or C5 of Ara, (i) O-acetyl group, (j) C6 of 1, 2, 4-Rha, (k) C6 of 1, 2-Rha, (l) C1 of Rha.

3.3. ESI-MS analysis

1BII was hydrolyzed with HCl to give a series of oligo- or mono-saccharide fractions: OD, OC, OB and OA by Sephadex G-10 column, according to the eluent time. OA was a mixture of monosaccharides, and therefore it was not analyzed further. OB was a mixture of disaccharides and consisted of Gal, Rha and GalA residues mostly, according to the TLC and GLC analyses. ^{1}H NMR of OC indicated the anomeric area was very complex, and showed OC contained much more components. Thus OC was not investigated further. In ^{1}H NMR of OD, the anomeric signals mainly distributed from δ 5.2–5.0 ppm. TLC and GLC analyses of OD showed it mainly contained GalA, Rha, Gal and Ara residues. Positive and negative ESI-MS were carried out, and the negative electrospray ion spectrum was shown in Fig. 4. The main fragment in negative ESI-MS spectrum was *m*/*z* 661which was identified as [Rha$_2$-GalA$_2$-1]$^-$, and its positive fragment was *m*/*z* 685. Other fragments were attributed in Table 3. From the ESI-MS spectra Rha$_2$-GalA$_3$, Rha-GalA$_2$ and Rha-GalA fragments were observed, and these results suggested that the backbone of 1BII mainly consisted of repeated Rha-GalA units. The presence of the weak fragment GalA$_2$ showed the backbone of 1BII contained small ‘smooth region’. While the presence of fragments GalA-Gal$_2$, GalA-Gal and Gal-Pent suggested partial side chains in 1BII were linked to GalA. In positive ESI-MS spectrum of OD, *m*/*z* 683.2 (30%), 461.5 (100%) and 577.4 (19%) were also observed and identified as [Ara$_5$-H$_2$O+Na]$^+$, [Gal$_2$-Ara-H$_2$O+Na]$^+$ and [GalA$_2$-Gal-1+2Na]$^+$, respectively.

OD was further acid hydrolyzed to give oligosaccharides mixture OB1. ESI-MS spectrum of OB1 showed it mainly contained *m*/*z* 339.4 which was identified as [GalA-Rha-1]$^-$, as shown in Fig. 5. The presence of fragments *m*/*z* 355.3 (15%), 325.7 (9%), which were identified as [GalA-Gal-1]- and [GalA-Pent-1]- respectively, conformed that a small amount of side chains were linked to GalA.

3.4. Immunological activity and structure

Structure–activity studies showed the pectin with unbranched regions had a low activity while the highly branched regions had a high activity (Samuelsen et al., 1996). Further studies discovered the arabino β-3, 6-galactan moiety in RG-I contributed in the expression of immune system modulating activity (Yu et al., 1998, 2001). 1BII and its degraded product 1BIIP had similar ratios of 1, 2-Rha, 1, 2, 4-Rha and GalA, and the difference between them was that 1BIIP contained trace amount of Ara residues compared with 1BII. In the immunological activity assay (Table 4), 1BII had no activity even at the 1 μg/ml level, while 1BIIP had some proliferation enhancement in 100 μg/ml dose on T and B lymphocytes. These results suggested that the galactosyl or arabinosyl side chains of pectin had effect on the expression of immunological activity.

When 1BII and 1BIIP were deacetylated with NaOH, their immunological activities on B lymphocytes increased to some extent. These results indicated that the acetyl

Table 2
Glycosyl linkages composition of the carboxyl-reduced derivatives of 1BII, 1BIIP

Linkages	1BIIR	1BIIPR
→2)-Rha$_p$-(1→	10.5	13.0
→2,4)-Rha$_p$-(1→	11.6	14.0
Ara$_f$-(1→	7.8	2.7
→5)-Ara$_f$-(1→	2.8	trace
→3,5)-Ara$_f$-(1→	2.2	trace
Gal$_p$-(1→	5.2	3.1
→4)-Gal$_p$-(1→	1.7	8.8
→3)-Gal$_p$-(1→	7.8	11.4
→6)-Gal$_p$-(1→	10.2	5.7
→3,6)-Gal$_p$-(1→	2.1	0.6
→3,4)-Gal$_p$-(1→	4.9	0.6
Xyl$_p$-(1→	3.8	8.5
→2)-Xyl$_p$-(1→	5.7	2.3
→4)-Gal$_p$A-(1→	20.0	27.2
→3,4)-Gal$_p$A-(1→	3.7	2.0

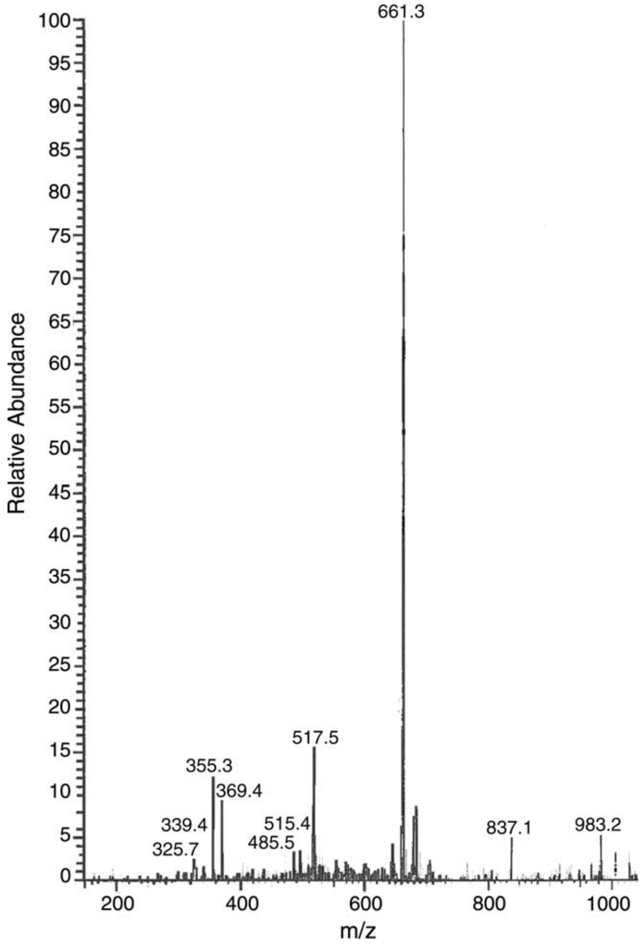

Fig. 4. Negative ESI-MS of the mixture of OD oligosaccharides obtained by partial acid hydrolysis.

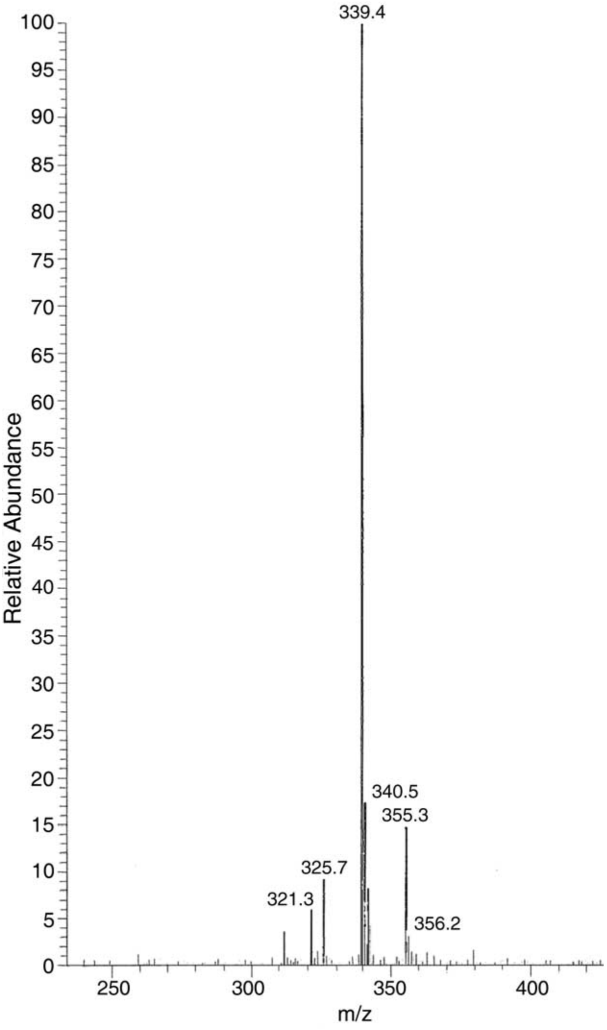

Fig. 5. Negative ESI-MS of the mixture of OB1 oligosaccharides obtained from OD by acid hydrolysis.

groups played an important role in B lymphocytes. The deacetylated 1BII and 1BIIP were further reduced three times to give 1BII-daR1, 1BII-daR2, 1BII-daR3, 1BIIP-daR1, 1BIIP-daR2 and 1BIIP-daR3. All the products were assayed for immunological activity, and the results indicated the carboxyl-reduced products had a more remarkable immuno-activities enhancement than 1BII-da or 1BIIP-da. The results above suggested carboxyl groups played an important role in the properties if pectin.

Table 3
Positive and negative ESI-MS data of OD mixture

M	Fragments (*m/z*, relative abundance)	
	$[M-1]^-$	$[M+Na]^+$
Rha_2-$GalA_2$	661.3 (100)	685.2 (69)
AcGalA-Rha-Gal_2-Pent	983.2 (5)	1007.1 (20)
Rha_2-$GalA_3$	837.1 (5)	861.0 (8)
GalA-Gal_2	517.5 (16)	541.2 (58)
Rha-$GalA_2$	515.4 (5)	n.r.
$GalA_2$	369.4 (9)	393.4 (35)
GalA-Gal	355.3 (12)	379.3 (35)
Rha-GalA	339.4 (4)	n.r.
GalA-Pent	325.7 (2)	349.4 (18)
$GalA_2$-Gal	n.r.	555.2 (37)

'n.r.' Not resolved.

4. Conclusion

1BII was a complex pectin. The backbone composed of 1, 4-linked GalA and 1, 2-linked Rha, which contained RG-I (hairy regions) and HG regions (smooth regions). The side chains included arabinosyl, arabinogalactosyl and xylosyl chains. Most of the side chains in 1BII were linked to O4 of Rha, and a small amount of side chains were linked to GalA directly. 1BII contained 2.9% *O*-acetyl groups. These acetyl groups played important roles in immunological activity in vitro. With carboxyl-reduction and immunological activity assays, it was confirmed that carboxyl groups had remarkable effect on the immunological activities. These conclusions are useful to the study on activity of pectins and the mechanism of immunological activity, as well as the use of *Centella asiatica*.

Table 4
Effect of CA-1, 1BII and CA-3 on ConA- or LPS-induced mitogenic activity of lymphocyte in mouse splenocytes in vitro

	Conc. (μg/ml)	MTT test	3H-TDR test			
		Mean ± s.d. ($OD_{570\ nm}$)	T cell		B cell	
			Mean ± s.d. (CPW)	Prolif. (%)[a]	Mean ± s.d. (CPW)	Prolif. (%)[a]
Control	Negative		6586±551	–	9691±395	–
	Positive		55813±2302	–	67791±973	–
1BII	1		46044±1242	−18	66772±1536	−2
	10		49787±3671	−11	65097±3380	−4
	100		56554±207	1	77497±1174	14
Control	Negative		3360±135	–	3670±288	–
	Positive	0.222±0.007	17224±703	–	12838±1050	–
1BII-da	1	0.231±0.010	16167±1749	−6	14849±1138	16
	10	0.249±0.008*	17361±1763	1	17184±635	34
	100	0.293±0.023*	18594±24	8	21776±1284	70
1BII-daR1	1	0.260±0.038	23632±1605	37	16460±394	28
	10	0.301±0.012**	50322±4045	192	30575±2017	138
	100	0.467±0.019***	37604±3634	118	29176±1390	127
1BII-daR2	1	0.264±0.015*	25821±1035	50	24799±1212	93
	10	0.409±0.025**	47936±2836	178	35765±2170	179
	100	0.561±0.039**	25789±1580	50	27623±1024	115
1BII-daR3	1	0.279±0.012**	20229±1608	17	13981±258	9
	10	0.355±0.018**	26177±2367	52	23649±76	84
	100	0.508±0.037**	26284±1602	53	39418±575	207
IBIIP	1	0.228±0.009	21532±454	25	11192±1027	−13
	10	0.254±0.018	22824±1176	33	12200±1255	−5
	100	0.284±0.003**	41295±2135	140	24560±1116	91
IBIIP-da	1	0.253±0.026	14303±556	−17	15461±1518	20
	10	0.262±0.008**	22090±1627	28	17020±1036	33
	100	0.425±0.025**	37385±3604	117	29035±1745	126
IBIIP-daR1	1	0.223±0.038	20796±1046	21	15563±386	21
	10	0.292±0.026*	27179±1455	58	26650±331	108
	100	0.462±0.058*	25638±1625	49	33488±1137	161
IBIIP-daR2	1	0.247±0.026	19489±910	13	16868±1411	31
	10	0.284±0.012**	28157±1211	63	26268±1065	105
	100	0.483±0.026**	40104±1141	133	39576±3897	208
1BIIP-daR3	1	0.242±0.010*	22241±1616	29	19058±300	48
	10	0.340±0.003***	35818±1189	108	33302±1724	159
	100	0.443±0.020**	19527±1436	13	30219±1640	135
1BII-O	1	0.344±0.008***	19981±1019	16	17610±495	37
	10	0.445±0.034**	27404±2285	59	24416±1679	90
	100	0.648±0.035**	52549±3847	205	50857±209	296

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. T lymphocyte proliferation was induced on ConA, B lymphocyte proliferation was induced on LPS.

[a] '–' showed proliferation inhibition, ≥15% showed that the sample was effective.

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