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Structure and potential immunological activity of a pectin from *Centella asiatica* (L.) Urban

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Abstract

S3A was a RG-I pectin isolated from *Centella asiatica* that contained Rha, Ara, Gal, Glc and GalA in molar ratio of 1.0:0.6:1.5:0.2:1.1 and had been found to have a backbone composed mainly of the disaccharide repeat unit, →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→. Based on methylation analysis, $NaIO_4$ oxidation, partial acid hydrolysis and lithium-treatment, the structural features were elucidated. Side chains of S3A were predominantly linked to O-4 of 1,2,4-linked α-L-Rha*p*. The side chains are comprised of arabinosyl chains, galactosyl chains, arabinogalactosyl chains and short glucosyl chains. A total of 45% Rha*p* in the backbone was substituted by side chains. The arabinosyl residues were mostly distributed in the arabinosyl side chains. According to the immunological results of S3A and its degraded derivatives, S3A had no immunological activity, but its derivatives had immunostimulating activities to some extent.

Keywords: *Centella asiatica*; Polysaccharide; Pectin; Immunological activity; Structure–activity relationships; Modification

1. Introduction

Centella asiatica, predominantly growing in the Southern hemisphere, has been used as a sedative, as an anti-leprosy drug, and as an anti-ulcer drug in China and India.[1,2] We isolated pectin from this plant and found that it had no immunological activity, while its degraded derivatives showed immunological activities to different extents. This potential bioactive property of pectin has been stated previously.[3] Lemon pectin is a kind of potentially bioactive pectin, which is made up of smooth regions and more complex structure in the hairy regions.[4,5] According to reports, many of the bioactivities of pectins from various sources have been shown to have a relationship with the complex structures within the hairy regions.[3,4,6] Pectic polysaccharides include three different repeating units: HG, RG-I and II, and consist of both hairy and smooth regions.[7] By now, at least 30 side chains have been identified, and these side chains are predominantly attached to O-4 of the rhamnosyl residues.[6] In the present study, we elucidate the structural features of pectin from *C. asiatica* and discuss the relationship between immunological activity and structure.

2. Results and discussion

2.1. Structural analysis

S3A is an acidic polysaccharide and has a carboxyl absorption at ν 1726.0 in the IR (KBr). No absorption at 280 nm and a negative response to the Lowry method showed that S3A did not contain protein. Thin-layer chromatography (TLC) and compositional analysis showed S3A contained Rha, Ara, Gal, Glc and GalA in a molar ratio of 1.0:0.6:1.5:0.2:1.1. HPGPC showed that S3A was homogeneous (Fig. 1).

In the ^{13}C NMR spectrum of S3A (referenced to CH_3OH at δ 49.5 ppm), the signal at δ 21.0 and 57.5 indicated the presence of *O*-acetyl and *O*-methyl groups

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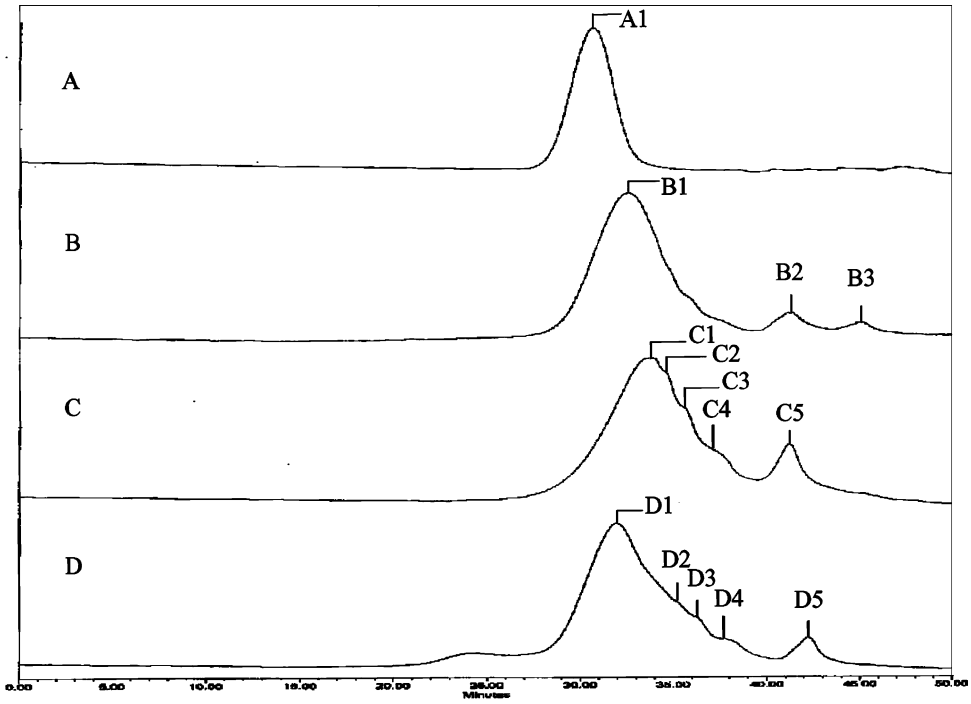


Fig. 1. HPGPC profiles of S3A (A) and its degraded products: S3A-I (B), S3A-S (C) and S3A-L (D) recorded on RI detector. The molecular sizes were recorded as following: A1 = 4.3×10^5; B1 = 1.6×10^5; B2 = 6.4×10^3; B3 < 4×10^3; C1 = 8.4×10^5; C2 = 7.1×10^4; C3 = 4.6×10^4; C4 = 2.8×10^4; C5 = 6.5×10^3; D1 = 2.1×10^5; D2 = 7.6×10^4; D3 = 3.7×10^4; D4 = 2.3×10^4; and D5 = 4.6×10^3.

as carboxylic acid methyl esters in S3A.[8,9] The content of acetyl and methyl groups was estimated to be 3.9 and 0.4%, respectively. In the anomeric region, the signals at δ 109.8–107.9 ppm were assigned to α-Ara*f*,[10] and the signals at δ 103.9 were assigned to β-Gal*p*.[11] The signals at δ 101.2, 99.2 and 98.1 arose from α-Glc*p*, α-Rha*p* and α-Gal*p*A,[12–14] respectively (Fig. 2). Other characteristic signals were assigned in shown in Fig. 2, with reference to literature values[8–14] (Table 1).

Compared with the results of S3A and its carboxyl-reduced derivative (S3A-R) from methylation analysis, the absence of 2,3,6-Me_3-galactose and 2,6-Me_2-galactose in the analysis of S3A suggested that the native pectin should contain 1,4-linked Gal*p*A and 1,3,4-linked Gal*p*A, while not containing 1,4-linked or 1,3,4-linked Gal*p*. Because of β-elimination in methylation analysis,[9,15] the content of Ara and Rha were less than those of S3A (data not listed).

To study the linkages between backbone and side chains in S3A, the native pectin was hydrolyzed with TFA. Compared with S3A and its degraded polymers (S3A-P1 and S3A-P2), the content of Ara*f* decreased and then disappeared, and at the same time the amount of 1,3,6-linked Gal*p* and 1,2,4-linked Rha*p* also decreased. However, the amount of 1,2-linked Rha*p* increased considerably (in Table 2). These results suggested that Ara*f* was linked to O-3 of 1,3,6-linked Gal*p* or O-4 of 1,2,4-linked Rha*p*. In the ^{13}C NMR spectrum of S3A-P1 and S3A-P2 (Fig. 2), the signals of Ara*f* and Gal*p* disappeared and became simpler, respectively, while the signal of 1,2-linked Rha*p* at δ 17.0 became stronger compared with the signal of 1,2,4-linked Rha*p* at δ 17.3. These results confirmed that Ara*f* and Gal*p* were linked to O-4 of 1,2,4-linked Rha*p*. S3A-P2 was further hydrolyzed to give S3A-P3. Gas chromatography (GC) and TLC analyses showed S3A-P3 contained Rha, GalA, Gal and Glc (molar ratio 1.0:1.1:0.1:0.14). Because the terminal glucosyl residues were still retained in S3A-P3, it is suggested that the Glc residues are probably located at the backbone of S3A-P3 or form short chains with other residues. In the ^{13}C NMR spectrum of S3A-P3, the signals of Gal*p*, 1,2,4-linked Rha*p* mostly disappeared, confirming that the galactosyl side chains are linked to O-4 of a 1,2,4-linked Rha*p*.

The oligomers and monomers in hydrolysis were investigated to study the linkage relationship between residues. S3A-O2 was fractionated by Sephadex G-10 to give a series of oligosaccharide fractions. Among them, the disaccharide was identified as β-D-Gal*p* (C-1 δ 104.8)-(1→3)-D-Gal*p* (C-1 δ 98.8) (*m*/*z* 365.3, $[Gal_2 + Na]^+$) with ESIMS and ^1H and ^{13}C NMR (BB-DEPT) experiments. The larger molecular weight oligosaccharide fraction (S3A-O2m, eluted before standard trisaccharide) was a mixture; thus it (40 mg) was further hydrolyzed with 0.2 M TFA (100 °C, 2 h), and gave a

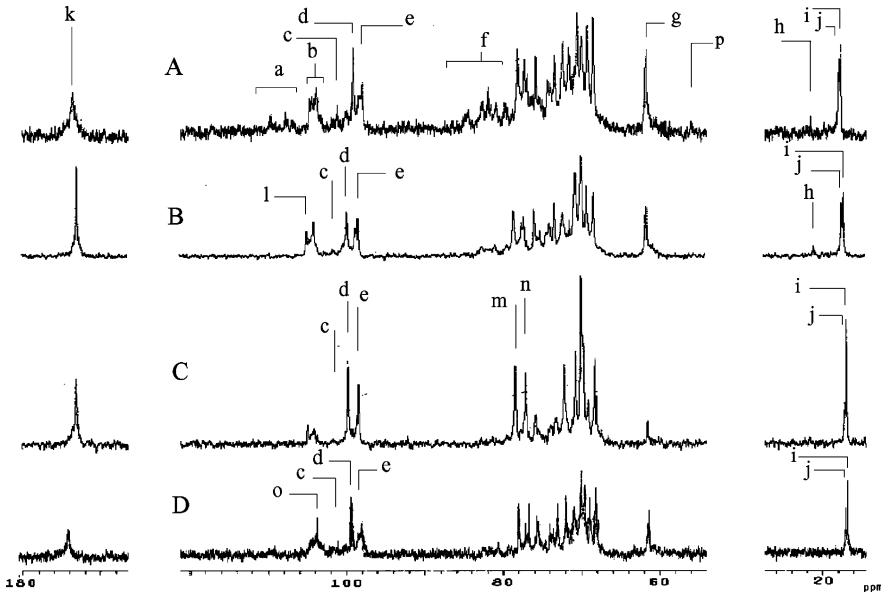


Fig. 2. ^{13}C NMR spectra of S3A (A) and its degraded products: S3A-P1 (B), S3A-P2 (C) and S3A-N (D) (400 Hz, room temperature). The signals were assigned as following: C-1 of α-Ara*f* (a), C-1 of β-Gal*p* (b), C-1 of α-Glc*p* (c), C-1 of α-Rha*p* (d), C-1 of α-Gal*p*A (e), C-2–C-4 of α-Ara*f* (f), C-6 of β-Gal*p* or C-5 of α-Ara*f* (g), *O*-acetyl groups (h), C-6 of 1,2-linked α-Rha*p* (i), C-6 of 1,2,4-linked α-Rha*p* (j), C-6 of α-Gal*p*A (k), C-1 of 1,3-linked Gal*p* (l), C-4 of α-Gal*p*A (m), C-2 of α-Rha*p* (n) and C-1 of 1,6- or 1,3,6-linked β-Gal*p* (o), C of methyl group (p).

series of fractions by Sephadex G-10, from which the disaccharide fraction was identified as the mixture of three disaccharides by ESIMS and ^1H and ^{13}C NMR (BB-DEPT) experiments. The major disaccharide was α-D-Gal*p*A (C-1 δ 100.3, C-6 δ 175.7) -(1→2)-L-Rha*p* (C-1 δ 94.0, C-6 δ 19.1) (*m*/*z* 339.3, $[GalA\text{-}Rha\text{-}H]^-$), the other two were β-D-Gal*p* (C-1 δ 104.9, C-6 δ 62.5) -(1→6)-D-Gal*p* (C-1 δ 98.8, C-6 δ 71.5) (*m*/*z* 365.3, $[Gal_2+Na]^+$) and α-L-Rha*p* (C-1 δ 103.4, C-6 δ 19.1) -(1→4)-D-Gal*p*A (C-1 δ 96.3, C-6 δ 175.7) (*m*/*z* 339.3, $[Rha\text{-}GalA-H]^-$). S3A-O2m (90 mg) was treated with 0.2 M TFA (100 °C, 4 h), and then isolated on a Sephadex G-10 column to give a series of degraded fractions. Among them, a disaccharide fraction was identified as α-D-Gal*p*A-(1→2)-L-Rha*p* (*m*/*z* 339.3, $[GalA\text{-}Rha-H]^-$) (Table 3) with ESIMS and 2D NMR experiments.

S1 and S2, the products of S3A from oxidation, were shown to be the mixtures of monosaccharides and non-sugar components by TLC and GC analyses (Table 4). S3 was identified as α-L-Rha*p*-(1→3)-ThrA (threonic acid) by 2D NMR spectroscopy (Table 3, Fig. 3).

S3A-P2 was also oxidized with $NaIO_4$, and the resulting oligomers were analyzed by ESIMS. Fraction P2S1 was a monosaccharide mixture. P2S4 was analyzed with ESIMS (LC–MS) to present three anions: *m*/*z* 587.5, 497.0, 423.1 that corresponded to $[Gal_2\text{-}Rha\text{-}ThrA-H_2O-H]^-$, $[GalA_2\text{-}Rha-H_2O-H]^-$ and $[Gal\text{-}Rha\text{-}ThrA-H_2O-H]^-$. These fragments should come from 1,3- or 1,3,6-linked Gal*p*, 1,2,4-linked Rha*p*, 1,2,4-linked Gal*p*A and 1,4-linked Gal*p*A, which were proposed to have the following structures: β-Gal*p*-(1→3 or 6)-β-Gal*p*-(1→4)-α-Rha*p*-(1→3)-ThrA, α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-Gal*p*A and β-Gal*p*-(1→4)-α-Rha*p*-(1→3)-ThrA. P2S2 was identified to contain GalA-glycerol (*m*/*z* 268.9, $[GalA\text{-}glycerol+1-H]^-$). The results above indicated the backbone of S3A consists of the repeating unit: →2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→.

S3A-I, the intermediate product in $NaIO_4$ oxidation, was heterogeneous in HPGPC (Fig. 1). The uronic acid assay[16] showed that S3A-I did not contain GalA, which did not agree with the fact that 1,3,4-linked GalA should be kept in $NaIO_4$ oxidation. The reason perhaps was that 1,3,4-linked GalA residues were lost in dialysis after the cleavage of backbone. Thus 1,3,4-linked GalA residues should form a small sequence group and are perhaps located in short side chains as previously described.[17] Composition analysis of S3A-I showed that the ratio of Gly:Ara:Rha:Gal was 2.5:1.4:0.3:1.0, which is not in accord with that from the methylation analysis (Table 2). This could be explained by the fact that some components in S3A-I were relatively lower molecular weight compounds (Fig. 2) and were removed in the dialysis of the methylated products (MW cutoff 3500–5000 Da). Methylation analysis of S3A-S showed that S3A-S contained 1,3-linked, 1,3,6-linked and terminal Gal*p* (Table 2), indicating that the sequence of →3)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(3←1)-β-D-Gal*p* exists in S3A. The distribution of the galactosyl chains is shown in Fig. 1.

Table 1
Glycosidic linkage composition of the pectin (S3A)

Residues	Glycosidic linkage composition			Methylated sugars [a]
		mol%	%	
α-L-Rha*p*	1,2-	13.1	54.6	3,4-Me_2-Rha*p*
	1,2,4-	10.9	45.4	3-Me-Rha*p*
	Total	24.0	100.0	
α-L-Ara*f*	T-	7.1	46.1	2,3,5-Me_3-Ara*f*
	1,5-	3.5	22.7	2,3-Me_2-Ara*f*
	1,3,5-	4.8	31.2	2-Me-Ara*f*
	Total	15.4	100.0	
β-D-Gal*p*	T-	9.8	29.3	2,3,4,6-Me_4-Gal*p*
	1,3-	13.1	39.2	2,4,6-Me_3-Gal*p*
	1,6-	4.8	14.4	2,3,4-Me_3-Gal*p*
	1,3,6-	5.7	17.1	2,4-Me_2-Gal*p*
	Total	33.4	100.0	
α-D-Gal*p*A	1,4-	19.0	84.1	2,3,6-Me_3-Gal*p*
	1,3,4-	3.6	15.9	2,6-Me_2-Gal*p*
	Total	22.6	100.0	
α-D-Glc*p*	T-	4.8	100.0	2,3,4,6-Me_4-Glc*p*
	Total	4.8	100.0	
Neutral sugars			77.4	
Uronic acid			22.6	
Acetyl groups			3.9	
Methyl groups			0.4	

Among the degraded products of S3A with lithium treatment, LO1, LO2 and LO3 corresponded to mono-, di- and tri-saccharide fractions from standard elution times, respectively. S3A-L was the main component from degradation, and it was obtained as the void volume. LO4 was the fraction between S3A-L and LO3, which contained no Glc (Table 4). With the consideration that Glc was linked to the backbone tightly in hydrolysis, terminal glucosyl residues in S3A should be linked to the backbone directly or located in the end of short side chains. These side chains were seldom reported, and the size and composition of these glucose short chains still need further study. LO3 was analyzed by ESIMS, and shown to have main-ion fragments: *m/z* 641.5, 625.4, 349.1, 301.2 and a small amount of anion fragments: *m/z* 325.3, 339.5. These fragments corresponded to $[\text{Ara-Hex}_4 - H_2O + Na]^+$, $[\text{Ara-Hex}_2\text{-Rha} - H_2O + Na]^+$, $[\text{Hex-Rha} + Na]^+$, $[\text{Ara-Rha} - H_2O + Na]^+$, $[\text{Ara-GalA} - H]^-$ and $[\text{GalA-Rha} - H]^-$. Methylation analysis of LO3 showed the presence of 1,2,3,5-Me_4-Rha and 1,3,4,5-Me_4-Rha; thus these rhamnosyl residues should be located at the reducing end. Compositional analysis of S3A-L and other products in from the lithium treatment (Tables 2 and 4) showed that arabinosyl residues mostly appeared in LO3 and LO4, but only a small amount of Ara*f* and no acetyl groups appeared in S3A-L. The removal of acetyl groups was caused by the basic solution when the lithium treatment was quenched by ice-water.[8,18] The loss of Ara*f* probably arose from the cleaved backbone that led to some short chains being isolated by chromatography. These results suggested that a large amount of arabinosyl residues formed arabinosyl chains that were directly linked to the backbone. S3A was resistant to degradation by lithium treatment for 30 min, which was similar to the behaviour of pectin P.[19]

2.2. Immunological activity and structure

S3A and its derivatives were tested in an immunological assay, and the results are listed in Table 5. All the samples had no cell toxicity under the test conditions. The pharmacological results showed that the native polysaccharide (S3A) and S3A-P1 and S3A-P2 had either no or only a slight effect in the immuno-stimulating tests, while other derivatives showed remarkable immuno-enhancing activity. Among the samples, S3A-N enhanced the proliferation of B-lymphocytes much more remarkably than that shown by T-lymphocytes, and S3A-L, S3A-I, S3A-S and S3A-P3 had similar proliferative effects on both T- and B-lymphocytes in vitro.

S3A was treated with H_3PO_4–$NaNO_2$ (Scheme 1), which is a procedure similar to that found in the literature.[20,21] With this treatment 1,3-linked galactosyl and arabinosyl residues were removed more easily than 1,6-linked galactosyl residues, as described in Table 2 and Fig. 2. S3A-N had similar molecular size in the HPGPC, as well as in specific rotation and residues with S3A-P1 (Tables 2 and 6). The difference was that S3A-P1 contained a high ratio of 1,3-linked Gal*p*, while S3A-N contained a high ratio of 1,6-linked Gal*p*. In the immunological test, S3A-N showed more remarkable activities than S3A-P1. Thus, the major contributor of immunological activity in S3A-N should be 1,6-linked galactosyl moieties, which was different from the previous reports where arabino-(1→3,6)-galactosyl chains were proposed as the major contributors.[22–24]

In comparing the structural analyses of S3A-L and S3A (Tables 1 and 2), S3A-L contains no acetyl groups and a small amount of Ara*f*. In pharmacological tests, it had immuno-stimulating activities, indicating that the Ara*f* moieties or acetyl groups in S3A affected the expression of immunological activity.[4,6] Treatment with $NaIO_4$ to remove 1,6-linked Gal*p* gave S3A-S, which also showed immuno-stimulating activities. These results showed that polysaccharides with a large proportion of 1,3- and 1,3,6-linked Gal*p* moieties had immuno-stimulating activity in high concentrations

Table 2
Glycosyl linkage composition of the S3A's derivatives[a]

Residues	S3A-P1		S33-P2		S3A-N		S3A-L		S3A-I[b]		S3A-S	
	mol%	%	mol%	%	mol%	%	mol%	%	mol%	%	mol%	%
Rha												
1,2-	16.9	58.7	29.9	71.5	15.7	59.9	9.0	49.7	n.d.	n.d.	n.d.	n.d.
1,2,4-	11.9	41.3	11.9	28.5	10.5	40.1	9.1	50.3	18.0	100.0	n.d.	n.d.
	28.8	100.0	41.8	100.0	26.2	100.0	18.1	100.0	18.0	100.0	n.d.	n.d.
Ara												
T-	<0.1		n.d.	n.d.	<0.1		1.9		n.d.	n.d.	0.7	100.0
1,5-	<0.1		n.d.	n.d.	n.d.		<0.1		n.d.	n.d.	n.d.	n.d.
1,3,5-	n.d.		n.d.	n.d.	n.d.		<0.1		13.8	100.0	n.d.	n.d.
			n.d.	n.d.					13.8	100.0	0.7	100.0
Gal												
T-	12.7	36.5	9.3	58.1	14.5	35.2	15.5	26.3	n.d.	n.d.	23.8	24.0
1,3-	10.2	29.3	6.0	37.5	7.4	17.9	25.3	42.9	35.9	52.6	57.1	57.5
1,6-	8.5	24.4	0.4	2.5	11.6	28.2	8.2	13.9	n.d.	n.d.	n.d.	n.d.
1,3,6-	3.4	9.8	0.3	1.9	7.7	18.7	10.0	16.9	32.3	47.4	18.4	18.5
	34.8	100.0	16.0	100.0	41.2	100.0	59.0	100.0	68.2	100.0	99.3	100.0
GalA												
1,4-	28.8	89.4	40.8	99.5	26.9		18.2		n.d.	n.d.	n.d.	n.d.
1,3,4-	3.4	10.6	0.2	0.5	<0.1		<0.1		n.d.	n.d.	n.d.	n.d.
	32.2	100.0	41.0	100.0					n.d.	n.d.	n.d.	n.d.
Glc												
T-	4.1	100.0	1.2	100.0	5.7	100.0	2.7	100.0	n.d.	n.d.	n.d.	n.d.
	4.1	100.0	1.2	100.0	5.7	100.0	2.7	100.0	n.d.	n.d.	n.d.	n.d.
Acetyl		4.4		2.4		2.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] n.d., not detected.
[b] The content of hexose and pentaose and the data for Rha, Ara and Gal came from methylation analysis; data for GalA came from a galacturonic acid assay.

(100 μg/mL). S3A-P3 mainly contained 1,4-linked Gal*p*A and 1,2-linked Rha*p*, as well as a small amount of Glc*p* and Gal*p*, however, it showed a stronger immuno-stimulating activitiy than S3A, S3A-P1 or S3A-P2. These results suggested that the RG backbone with a small number of side chains showed activity to some extent. S3A showed no effect on immunological activities in vitro. The reason probably arose from its three-dimensional structure and conformation in solution.

Taken together, S3A is a typical RG-I polysaccharide. The backbone of S3A is composed mainly of the disaccharide repeat unit, →2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→. A total of 45% of the rhamnosyl residues are linked to side chains via O-4. The side chains contain galactosyl chains, arabinogalactosyl chains, arabinosyl chains, glucosyl short chains and 1,3,4-linked GalA chains, which form complex hairy regions. Arabinosyl residues are mostly distributed in the arabinosyl side chains and are seldom linked to galactosyl residues. This potentially bioactive pectin was first obtained from *C. asiatica*. Structural and immunological studies on this pectin will be helpful to future investigations in food technology and biochemistry.

Table 3
Chemical shifts and spin–spin coupling constants for oligosaccharides O2D and S3[a]

	Residues	$J_{1,2}$ (Hz)	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
O2D	Gal*p*A-(1→	3.9	100.4/5.1	74.5/3.4	71.9/3.9	70.7/3.85	73.3/4.3	177.8
	→2)-Rha*p*	1.3	94.3/5.2	80.0/4.0	71.9/3.9	74.7/3.45	71.3/3.85	19.6/1.25
S3	Rha*p*-(1→	1.2	99.7/5.0	70.9/3.6	70.5/3.9	72.4/3.4	69.6/3.7	17.1/1.2
	→2)-ThrA		176.9	70.7/4.4	78.0/4.1	60.7/3.8	-	-

[a] δ-units, room temperature, downfield from Me_4Si.

Table 4
Content and compositional analysis of oligomers and monomers in lithium treatment and $NaIO_4$ oxidation

Fractions	Weight ratio	Neutral sugars composition in GC analyses (molar ratio)			
		Ara	Rha	Gal	Glc
LO1	32				
LO2	24	n.d.	3.9	5.9	0.9
LO3	15	3.6	2.1	3.3	1.1
LO4	27	6.7	0.7	2.6	n.d.
S1/S2	6/30	1.0	2.2	n.d.	n.d.
S3	20				
S4/S5	30/14	0.16	1.0	0.47	n.d.

3. Experimental

3.1. Materials

The dried plant, *C. asiatica*, was purchased from Shanghai Medicinal Materials Cooperation Company (code: 000201). 1-Cyclohexyl-3-(2-morphlinoethy)carbodiimide metho-*p*-toluene-sulfonate (CMC), TFA, $NaBH_4$ and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Fluka. Concanavalin (ConA) and lipopolysaccharide (LPS, from *Escherichia coli* Serotype 055: B5) were obtained from Sigma. Me_2SO was from E. Merck. ^{3}H-TdR was obtained from Shanghai Institute of Nuclear Research. All other reagents were of analytical grade as commercially available.

3.2. General methods

The ^{1}H, ^{13}C and 2D NMR spectra were recorded on Varian Mercury 400 and Inova 600 NMR spectrometers. A polarization transfer pulse of 135° was used in the DEPT experiments. IR spectra and specific rotations were obtained with a Perkin–Elmer 591B spectrophotometer and a Perkin–Elmer 241 M digital polarimeter, respectively. HPGPC was performed with a Waters instrument, including GPC software (Millennium[32]), 515 high-performance ion chromatography (HPLC) pump, 2410 RI detector and 2487 dual-wavelength

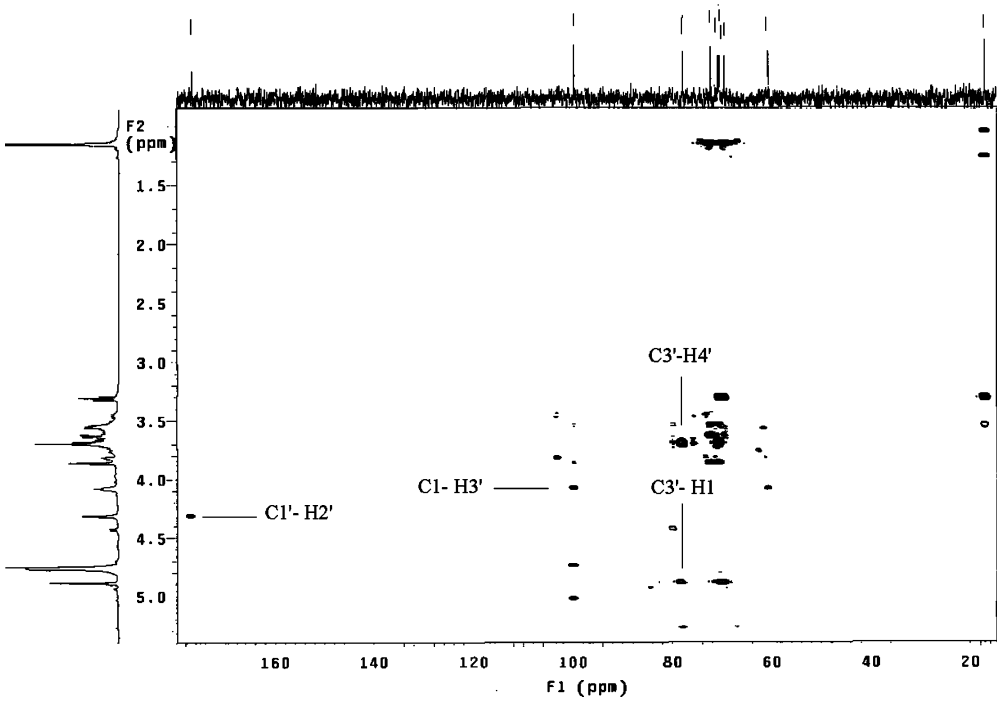


Fig. 3. HMBC spectrum (600 Hz, room temperature) of the oligosaccharide S3; ‘’ refers to threonic acid.

Table 5
Effect of S3A and its derivatives on immunological activity of lymphocyte in mouse splenocytes in vitro

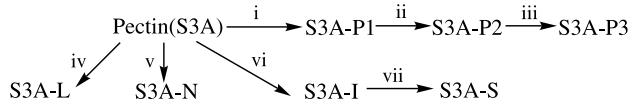
Fraction	Conc. (μg/mL)	MTT test [b]	Control	^{3}H-TdR test			
		Mean ± SD (OD_{570nm})		T cell [c]		B cell [d]	
				Mean ± SD (cpm)	Prolif. (%) [a]	Mean ± SD (cpm)	Prolif. (%) [a]
Control		0.401 ± 0.028	Negative	6586 ± 551		9691 ± 395	
			Positive	55,813 ± 2302		67,791 ± 973	
S3A	1	0.411 ± 0.012		54,854 ± 1337	− 2	70,090 ± 860	3
	10	0.412 ± 0.024		55,372 ± 1670	− 1	68,787 ± 1673	1
	100	0.497 ± 0.007		57,502 ± 2087	3	73,520 ± 2320	8
Control		0.150 ± 0.014	Negative	5900 ± 423		5632 ± 401	
			Positive	32,639 ± 3512		56,742 ± 718	
S3A-P1	1	0.149 ± 0.005		27,588 ± 1880	− 15	54,116 ± 1508	− 5
	10	0.129 ± 0.009		32,669 ± 445	0	57,866 ± 2006	2
	100	0.169 ± 0.010		34,025 ± 94	4	62,602 ± 2117	10
S3A-P2	1	0.158 ± 0.012		33,813 ± 2181	4	53,434 ± 1480	− 6
	10	0.158 ± 0.006		31,769 ± 1249	− 3	57,146 ± 2657	1
	100	0.258 ± 0.019 **		45,578 ± 2847	40	73,485 ± 4388	30
Control		0.318 ± 0.004	Negative	3781 ± 251		2743 ± 379	
			Positive	89,428 ± 579		30,132 ± 1262	
S3A-N	1	0.336 ± 0.017		87,777 ± 2554	− 2	50,452 ± 3193	67
	10	0.341 ± 0.008 *		87,248 ± 4650	− 2	58,254 ± 3945	93
	100	0.401 ± 0.022 *		115,011 ± 3235	29	95,417 ± 3104	217
Control		0.235 ± 0.006	Negative	8484 ± 458		8812 ± 177	
			Positive	31,457 ± 1618		42,312 ± 852	
S3A-L	1	0.273 ± 0.002 **		20,629 ± 1391	− 34	40,714 ± 2321	− 4
	10	0.418 ± 0.016 ***		30,917 ± 2346	− 2	45,041 ± 4074	17
	100	0.605 ± 0.047 **		66,881 ± 4132	113	70,133 ± 3888	140
S3A-I	1	0.281 ± 0.006 ***		34,601 ± 1071	10	40,714 ± 2321	− 4
	10	0.294 ± 0.018 *		31,987 ± 1021	2	45,041 ± 4074	6
	100	0.426 ± 0.002 ***		51,406 ± 1340	63	70,133 ± 3888	66
Control		0.184 ± 0.006	Negative	2074 ± 177		2361 ± 291	
			Positive	90,126 ± 1494		39,310 ± 1952	
S3A-P3	1	0.187 ± 0.003		91,791 ± 5471	2	37,009 ± 1995	− 6
	10	0.208 ± 0.006 **		111,151 ± 849	23	36,906 ± 1668	− 6
	100	0.272 ± 0.011 **		135,909 ± 4984	51	58,986 ± 2714	50
S3A-S	1	0.233 ± 0.009 **		104,018 ± 4949	15	36,538 ± 3023	− 7
	10	0.297 ± 0.009 ***		107,783 ± 1941	20	49,741 ± 1032	27
	100	0.397 ± 0.013 ***		161,085 ± 5935	79	67,871 ± 121	73

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
[a] The ' − ' indicates samples had inhibition activity, ≥ 15% showed the sample was effective.
[b] Effect on activity of lymphocyte without any induction.
[c] Effect on ConA-induced mitogenic activity of T-lymphocyte.
[d] Effect on LPS-induced mitogenic activity of B-lymphocyte.

absorbance detector. GC was done with a Shimazu-9A apparatus equipped with a 5% OV 225/AW-DMC-Chromosorb W column (2.5 m × 3 mm). GC–MS was performed with a Shimadzu QP-5050A apparatus. ESIMS's were obtained with a Finnigan LCQ-DECA spectrometer. ^{3}H-TdR data were counted using a liquid scintillation counter (MicroBeta Trilux, Perkin–Elmer Life Science).

3.3. Isolation and purification of the native polysaccharide

The dried *C. asiatica* (6 kg), previously defatted with 95% EtOH, was extracted with hot water, deproteinated with TCA, dialyzed against running water for 3 days and distilled water for 1 day, and then precipitated with 4 vols of EtOH. The precipitate was washed with EtOH



Scheme 1. Modification of S3A: (i) 0.1 M TFA, 100 °C, 20 min; (ii) 0.2 M TFA, 100 °C, 1 h; (iii) 0.2 M TFA, 100 °C, 2 h; (iv) Li, 1,2-diaminoethane, room temperature 10 h; (v) H_3PO_4, $NaNO_2$; (vi) (a) $NaIO_4$, (b) $NaBH_4$; (vii) 0.2 M TFA, 40 °C, 24 h.

and acetone, and then vacuum-dried at 40 °C for 2 days to give a black powder (crude polysaccharide, yield: 2.1%). The crude polysaccharide was fractionated on a DEAE-cellulose column (Cl^- form, 50 × 10 cm), and eluted stepwise with distilled water, 0.1, 0.3, and 0.5 M NaCl. After dialysis and lyophilization, the fraction of elution with 0.3 M NaCl was further purified on DEAE-cellulose (Cl^- form), eluting stepwise with distilled water and 0.2 M NaCl. The portion of elution with 0.2 M NaCl was collected, dialyzed, and lyophilized. The purification was carried out on Sephacryl S-300 columns (100 × 2.6 cm) with 0.2 M NaCl as eluent. After rechromatography on S-300, a fraction (S3A, yield: 15.7% from crude polysaccharide) was obtained. S3A was homogeneous by HPGPC.

3.4. Homogeneity and molecular size

Determination was done by HPGPC on a linked column of Ultrahydrogel™ 2000 and 500 columns, eluting with 0.003 M NaOAc at a flow rate of 0.5 mL/min. The column was kept at 30.0 ± 0.1 °C, and was pre-calibrated by standard Dextran (T-700, 580, 300, 110, 80, 70, 40, 9.3 and 4, Pharmacia). All samples were prepared as 0.1% (w/v) solutions, and 20 μL of solution was analyzed in each run.

3.5. Sugar composition analyses

The neutral sugars were analyzed by GC after conversion of the hydrolysate into alditol acetates, as described previously.[25] Uronic acid content was determined by a modification of the *m*-hydroxylbiphenyl method.[26] Reduction of uronic acid was carried out with CMC and $NaBH_4$ as reported.[26,27] Methylation analysis was according to the modified NaOH–Me_2SO method.[28] The per-*O*-methylated product was hydrolyzed, reduced and acetylated to give partially methylated alditol acetates, which were analyzed by GC–MS (DB-1 capillary column, 0.25 mm × 30 cm). The degree of acetylation and degree of methylation were determined by the method described previously.[29,30] The configuration was identified by GC of the per-*O*-trimethylsilylated (−)-2-butylglycosides derivatives.[31]

3.6. Partial acid hydrolysis

S3A (590 mg) was hydrolyzed in 0.1 M TFA (150 mL, 100 °C, 20 min) to give a polymer fraction (S3A-P1, 400 mg, homogenous by HPGPC), and an oligomer fraction (S3A-O1). S3A-P1 (300 mg) was further hydrolyzed by 0.2 M TFA (75 mL, 100 °C and 1 h) to give S3A-P2 (119 mg, polymer fraction, homogenous in HPGPC) and S3A-O2 (oligomer). S3A-P2 (60.6 mg) was hydrolyzed by 0.2 M TFA (15 mL, 100 °C and 2 h) to give S3A-P3 (30 mg, polymer fraction, homogenous in HPGPC).

3.7. Treatment with lithium

The lithium-degradation procedure was carried out according to Lau and co-workers[32,33] S3A was dried in vacuum for 24 h over P_2O_5. S3A (105.8 mg) was dissolved and stirred in 1,2-diaminoethane (20 mL) under a nitrogen atmosphere, and pieces of lithium wire (2–3 × 5 mm) were added at room temperature, keeping the solution blue for 10 h. The reaction was quenched by pouring the solution onto ice (100 mL). The desalting was carried out on Dowex 50 (H^+) resin and a Bio-Gel P2 column (100 × 2.6 cm), as described previously[19] to give five portions: LO1, LO2, LO3, LO4 and S3A-L according to the standard oligosaccharides elution times on Bio-Gel P2. S3A-L was analyzed by HPGPC (Fig. 2). Another lithium-treatment was performed with 100 mg of S3A, 20 mL of 1,2-diaminoethane, and lithium wire for 30 min.

3.8. Periodate oxidation and Smith-degradation

S3A (301.5 mg) was oxidized by 0.02 M $NaIO_4$ (300 mL, 4 °C) in the dark with the absorption at 224 nm monitored every day. After 7 days the reaction was quenched with ethylene glycol (2 mL). Consumption of $NaIO_4$ was measured spectrometrically.[34,35] The result-

Table 6
Specific rotation and molecular size of S3A and its derivatives

Fraction	S3A	S3A-P1	S3A-P2	S3A-P3	S3A-N	S3A-L
$[\alpha]_D^{15}$ (*c* H_2O)	+80.0 (0.91)	49.9 (0.77)	51.3 (0.67)		37.1 (0.91)	42.9 (0.11)
M_W (Da) [a]	4.3×10^5	2.6×10^5	1.6×10^5	1.0×10^5	2.0×10^5	2.1×10^5 [b]

[a] Fraction had a similar molecular size to the corresponded standard T-dextran in HPGPC.
[b] The value came from the highest peak in HPGPC.

ing product was reduced, dialysed and lyophilized to give S3A-I (145 mg). S3A-I (100 mg) was mildly acid hydrolyzed with 0.2 M TFA (40 °C, 24 h). After dialysis against distilled water, the retentate was lyophilized to give S3A-S (23 mg), and the dialysate was isolated to give a series of fractions (S1, S2, S3, S4 and S5) on a Sephadex G-10 column.

S3A-P2 (50 mg) was oxidized by 0.02 M $NaIO_4$ (50 mL, 4 °C and 7 days). The resulting product was dealt with as described above. This gave a polymer (S3A-P2I, 30.5 mg), which was mildly acid hydrolyzed with 0.2 M TFA (40 °C, 24 h) to give a series of oligomer fractions (P2S1, P2S2, P2S3 and P2S4), which were isolated by a Sephadex G-10 column.

3.9. Treatment with phosphoric acid and sodium nitrite

Powdered S3A (205.6 mg) and 80% (w/w) phosphoric acid (1.5 mL) were dissolved together at rt. $NaNO_2$ (200 mg) was added with continuous stirring. After 24 h, the resulting product was treated with ethyl ether, alcohol and water (each 5 mL × 3), and then dissolved into 20 mL Na_2CO_3 ice-water. The solution was neutralized and reduced with $NaBH_4$ (407 mg). After neutralization and centrifugation, the supernatant was dialyzed and lyophilized to give S3A-N (74.5 mg, homogenous in HPGPC).

3.10. Lymphocyte proliferation test in vitro

Inbred ICR (♀) mice, 6–8 weeks old, weighing 20±2 g, were obtained from Shanghai Experimental Animal Laboratory, Chinese Academic of Sciences. Different dilutions of the polysaccharide samples (1–100 μg/mL) were incubated directly with mouse splenocytes at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. The incubation was terminated 5 h later after addition of MTT (5 mg/mL). The MTT method was used to measure the cytotoxicity and non-specific enhancement of the sample[36,37] 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 a humidified 5% CO_2 atmosphere. Cells were pulsed with 0.25 μCi/well of [^{3}H]-thymidine for the final 12 h. The cells were then harvested onto glass filters, and the incorporated radioactivity was counted by a liquid scintillation counter[37,38] The data of the controls were different, because S3A and its derivatives were assayed in different groups. In each group assay of S3A's derivatives, S3A was assayed in parallel (data are not listed). All experiments were performed three times independently.

References

1. Jiangsu New Medical College. *Dictionary of Chinese Materia Medica*, Shanghai Science and Technology Publishers: Shangai, 1995, p. 1873.
2. Chopra, R. N.; Nayar, S. L.; Chopra, I. C. *Glossary of Indian Medicinal Plants*; CSIR: New Delhi, 1956; p 58.
3. Yamada, H. In *Pectins and Pectinases*; Visser, J.; Voragen, A. G. J., Eds.; Elsevier Science: Amsterdam, 1996; p 173.
4. Ros, J. M.; Schols, H. A.; Laenceina, J.; Voragen, A. G. J. In *Bioactive Carbohydrate Polymers*; Paulsen, B. S., Ed.; Kluwer Academic Publishers: Dordrecht, 2000; pp 121–128.
5. Schols, H. A.; Voragen, A. G. J. *Carbohydr. Res.* **1994**, *256*, 83–95.
6. Voragen, A. G. J.; Daas, P. J. H.; Schols, H. A. In *Bioactive Carbohydrate Polymers*; Paulsen, B. S., Ed.; Kluwer Academic Publishers: Dordrecht, 2000; pp 129–145.
7. Ridley, B. L.; O'Neill, M. A.; Mohnen, D. *Phytochemistry* **2001**, *57*, 929–967.
8. Shimazu, N.; Tomoda, M.; Kanari, M.; Gonda, R. *Chem. Pharm. Bull.* **1991**, *39*, 2969–2972.
9. Bao, X. F.; Wang, Z.; Fang, J. N.; Li, X. Y. *Planta Med.* **2002**, *68*, 237–243.
10. Nandini, C. D.; Salimath, P. V. *Food Chem.* **2001**, *74*, 417–422.
11. Sims, I. M.; Craik, D. J.; Bacic, A. *Carbohydr. Res.* **1997**, *303*, 79–92.
12. Bao, X. F.; Duan, J. Y.; Fang, X. Y.; Fang, J. N. *Carbohydr. Res.* **2001**, *336*, 127–140.
13. Shimazu, N.; Tomada, M. *Chem. Pharm. Bull.* **1985**, *33*, 5539–5542.
14. Cui, W. W.; Eskin, M. N. A.; Biladeris, C. G.; Marat, K. *Carbohydr. Res.* **1996**, *292*, 173–183.
15. Dong, Q.; Fang, J. N. *Carbohydr. Res.* **2001**, *332*, 109–114.
16. Kimberley, A. T.; Jock, G. B. S. *Anal. Biochem.* **1992**, *201*, 190–196.
17. Oechslin, R.; Lutz, M. V.; Amado, R. *Carbohydr. Polym.* **2003**, *51*, 301–310.
18. Bedouet, L.; Courtois, B.; Courtois, J. *Carbohydr. Res.* **2003**, *338*, 379–383.
19. Fransen, C. T. M.; Haseley, S. R.; Huisman, M. M. H.; Schols, H. A.; Voragen, A. G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **2000**, *328*, 539–547.
20. Painter, T. J. *Carbohydr. Res.* **1977**, *55*, 95–103.
21. Bitter, T.; Muir, H. M. *Anal. Biochem.* **1962**, *4*, 330–334.
22. Yamada, H. In *Bioactive Carbohydrate Polymers*; Paulsen, B. S., Ed.; Kluwer Academic Publishers: Dordrecht, 2000; pp 15–24.
23. Yu, K. W.; Kiyohara, H.; Matsumato, T.; Yang, H. C.; Yamada, H. *Planta Med.* **1998**, *64*, 714–719.
24. Yu, K. W.; Kiyohara, H.; Matsumato, T.; Yang, H. C.; Yamada, H. *Carbohydr. Polym.* **2001**, *46*, 147–156.
25. Dong, Q.; Ding, S. W.; Yang, X.; Fang, J. N. *Phytochemistry* **1999**, *50*, 81–84.
26. Taylor, A. B.; Conrad, H. E. *Biochemistry* **1972**, *11*, 1385–1388.

27. Tomoda, M.; Ichikawa, M.; Shimizu, N. *Chem. Pharm. Bull.* **1986**, *34*, 4992–4996.
28. Needs, P. W.; Selvendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.
29. Tomada, M.; Shimizu, N.; Shimada, K.; Suga, M. *Chem. Pharm. Bull.* **1985**, *33*, 16–21.
30. Tomada, M.; Ichikawa, M.; Shimizu, N. *Chem. Pharm. Bull.* **1986**, *34*, 4992–4996.
31. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
32. Lau, J. M.; McNeil, M.; Albersheim, D. P. *Carbohydr. Res.* **1987**, *168*, 245–274.
33. Mort, A. J.; Bauer, W. D. *J. Biol. Chem.* **1982**, *257*, 1870–1875.
34. Vaishnav, V. V.; Bacon, B. E.; O'Neill, M.; Cherniak, R. *Carbohydr. Res.* **1997**, *306*, 315–330.
35. Tomada, M.; Gonda, R.; Kasahara, Y.; Hikino, H. *Phytochemistry* **1986**, *25*, 2817–2820.
36. Heeg, K.; Reimann, J.; Kabelitz, D.; Hardt, C.; Wagner, H. *J. Immunol. Methods* **1985**, *77*, 237–246.
37. Li, X. Y.; Wang, J. F.; Zhu, P. P.; Liu, L.; Ge, J. B.; Yang, S. X. *Acta Pharmacol. Sin.* **1990**, *11*, 542–545.
38. Kang, S. J.; Li, J. H.; Meng, Y. R.; Wang, X. N. *Chin. J. Clin. Oncol.* **1996**, *23*, 309–311.