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Structural features of pectic polysaccharide from *Angelica sinensis* (Oliv.) Diels

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ABSTRACT

The structure of the pectic polysaccharide (ASP3) isolated from roots of *Angelica sinensis* (Oliv.) Diels was investigated using partial acid hydrolysis, enzymic digestion combined with methylation analysis, and further supported by ^{1}H and ^{13}C NMR spectroscopy techniques. The results indicated that ASP3 contained a backbone of linear homogalacturonan fragments as "smooth regions" and rhamnogalacturonan fragments as "hairy regions" with repeating unit of [→4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 →]. A total of 58.8% rhamnopyranose residues in the backbone were substituted at *O*-4 position by the side chains. The side chains contained mainly β-1,6- and β-1,4-galactopyranan bearing 3,6- and 4,6-substituted β-D-galactopyranose residues as branched points and short α-1,5-arabinofuranan possessing 3,5-substituted α-L-arabinofuranose residues as branching points. In addition, β-1,6-galactopyranan side chains were highly branched with α-1,5-arabinofuranan carrying 3-*O*-substituents (1,3,6-Gal) and terminated by the α-arabinofuranose residues which form arabinogalactan.

1. Introduction

Pectins are a family of complex heterogeneous polysaccharides that constitute a large proportion of the cell wall of many higher plants where greatly influence growth, development and senescence (O'Neill, 1990; Ridley, O'Neil, & Mohnen, 2001). Pectins are also traditional gelling and thickening agents for the production of jams and jellies, and the area of the use extends to the production of fruit, dairy, and dessert products and pharmaceuticals (Thakur, Singh, & Handa, 1997; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

In recent years, pectin is increasingly recognized as an important precursor of substrates improving gastrointestinal functions. It plays an important role in the regulation of some physiological processes and therefore in the prevention of hyperlipidemia, as well as bowel cancer (Lim, Yamada, & Nonaka, 1998; Willats, McCartney, Mackie, & Knox, 2001). Earlier, we isolated a pectic polysaccharide named ASP3 from roots of *Angelica sinensis* (Sun, Tang, Gu, & Li, 2005) which is a well-known oriental herb (Zhang & Cheng, 1989). The sugar chain of ASP3 was found to contain residues of galacturonic acid, arabinose, galactose, and rhamnose as the main constituents. Our previous study has shown that ASP3 can protect leucocytes and lymphocytes of mice against radiation-induced damage, which has potential radioprotective effect on acute radiation injured mice.

According to reports, many of the bioactivities of pectins from various sources have been shown to have a relationship with complex branched structures (Wang, Dong, Zuo, & Fang, 2003; Yamada, 1994; Yu, Kiyohara, & Matsumoto, 2001), so elucidation of molecular fine structural features of the pectic substances is necessary for understanding the mechanism of physiological activity and clarifying the structure–activity relationships.

The primary structure of pectin obtained from various sources has been studied extensively by methods of partial chemical or enzymatic degradation (Bushneva, Ovodova, Shashkov, Chizhov, & Ovodov, 2003; Dong & Fang, 2001; Habibi, Mahrouz, & Vignon, 2005; Polle, Ovodova, Chizhov, Shashkov, & Ovodov, 2002a; Polle, Ovodova, Shashkov, & Ovodov, 2002b; Singthong & Cui, 2004). However, the precise chemical structure of pectin remains under debate, although the structural elements of pectin are rather well described (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007). The present work is devoted to further elucidation of the detailed structural features of the pectic polysaccharide ASP3 from *A. sinensis* using mild acid hydrolysis and enzymic digestion followed by NMR spectroscopy and methylation analysis of fragments obtained.

2. Experimental

2.1. Plant materials and preparation of ASP3

The roots of *A. sinensis* (Oliv.) Diels, cultivated in Minxian County, Gansu Province, China, were provided by Shanhe

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Pharmaceutical Co. Ltd. (Wuxi, China). Isolation followed by purification of the pectic polysaccharide ASP3 from roots of *A. sinensis* was performed as described earlier (Sun et al., 2005).

2.2. General methods

Uronic acid content was determined by photometry with *m*-hydroxybiphenyl at 520 nm (Blumenkrantz & Asboe-Hansen, 1973), using D-galacturonic acid as standard. Total neutral sugar content was determined by the reaction with phenol in the presence of sulfuric acid using galactose and arabinose as standards. A correction was made for the response of galacturonic acid in the neutral sugar test. Neutral sugar composition was analyzed by GC after conversion of the hydrolysate into alditol acetates, as described earlier (Sun et al., 2005). The percentage of monosaccharides in the sample was calculated from the peak areas using response factors. The specific optical rotation was determined in H_2O at 25 °C using a WZZ-2A polarimeter.

2.3. Partial acid hydrolysis

Fragment ASP3 (150 mg) was hydrolyzed with 0.2 M trifluoroacetic acid (TFA) for 1 h at 121 °C. After cooling, TFA was evaporated under a stream of N_2. The hydrolysate was dissolved in distilled water and dialyzed against distilled water (M_w cut-off 3500 Da). The retentate and dialysate were concentrated and purified respectively on size exclusion chromatography (SEC) of Sepharose CL-6B column (*D*1.0 × 120 cm, Amersham Bioscience) at room temperature and eluted with degassed distilled water (12 mL/h). A purified high-molecular weight fraction (ASP3-PH) and a low-molecular weight fraction (ASP3-PL) were collected, concentrated and then lyophilized.

2.4. Enzymic hydrolysis

Fragment ASP3 (100 mg) was dissolved in 24 mL of water, and 1 M NaOH (6 mL) was added. The solution was kept for 2 h at 25 °C. Excess alkali was neutralized with acetic acid to pH 5.5. Mould endo-α-(1,4)-polygalacturonase (EndoPG, EC 3.2.1.15, Fluka – 467 U/g) was added and the mixture was incubated at 30 °C for 72 h. The enzyme was inactivated by heating at 100 °C for 10 min and the denatured protein was removed by centrifugation. The digestion product obtained was concentrated and subjected to Sepharose CL-6B column (*D*1.0 × 120 cm, Amersham Bioscience) to give an enzyme-resistant fraction (ASP3-EH) and an enzyme-sensitive fraction (ASP3-EL). Fractions were collected, concentrated and then lyophilized.

2.5. Determination of the glycosidic linkage composition

The glycosidic linkage analysis was determined by methylation and gas chromatography–mass spectroscopy (GC–MS). Prior to methylation, the sample containing uronic acid was reduced to the corresponding neutral sugar by using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl-*p*-toluenesulfonate (CMC, Fluka) and sodium borodeuteride ($NaBD_4$, Acpos), following a procedure described by Taylor and Conrad (1972) and York, Darvill, McNeil, Stevenson, and Albersheim (1986) with slight modification (Cui, 2005). After this reduction, methylation analysis was carried out according to the method of Ciucanu and Kerek (1984) with slight modification to give a fully methylated product (Cui, 2005). The methylated product was then converted into partially methylated alditol acetates (PMAA) by hydrolysis, reduction with $NaBH_4$, and acetylation followed by linkage analysis using GC–MS (OV1701 capillary column, 0.25 mm × 30 m, 0.25 mm film thickness coupled to a Trace Mass Spectrometer, Finnigan). The carrier gas was helium; 3 °C/min gradient from 150 to 250 °C. The temperature of the interface was 250 °C; Energy of ionizing electrons was 70 eV. Peak identification was based on retention times using partially methylated alditol acetates as standards. The percentage of the methylated sugars was estimated as ratios of the peak areas (total ion current).

2.6. ^1H and ^{13}C NMR spectroscopy

The polysaccharide samples were exchanged 3 times in D_2O (at concentrations of approximately 40 mg/mL), with intermediate freeze-drying. Finally, samples were dissolved in D_2O. ^1H and ^{13}C NMR spectroscopy were performed on a Brucker AMX500 NMR spectrometer (Germany) using standard pulse sequences with 5 and 10 mm tubes at 65 °C. Internal 1,4-dioxane was used as an internal chemical-shift reference for spectra. Two-dimensional spectra (COSY-45, TOCSY, and HMQC) were recorded using the standard Bruker procedures (Cui, Eskin, Biliaderis, & Marat, 1996).

3. Results and discussion

3.1. Preparation of ASP3

The pectic polysaccharide named ASP3 has been isolated and purified from roots of *A. sinensis* as described earlier (Sun et al., 2005). The sugar composition of ASP3 was listed in Table 1.

3.2. Partial acid hydrolysis of ASP3

Partial degradation of polysaccharides by acid hydrolysis is based on the fact that some glycosidic linkages are more labile to acid than others. For example, linkages between neutral sugars are the most susceptible to acid hydrolysis; hence controlled acid hydrolysis is frequently used to remove neutral sugars (Coenen et al., 2007). To study the linkages between backbone and side chains of polysaccharide, the native polysaccharide ASP3 was partially hydrolyzed with 0.2 M TFA. Partial acid hydrolysis resulted in two subfractions of ASP3: high-molecular (ASP3-PH) and low-molecular (ASP3-PL) ones. The sugar composition of fractions was given in Table 1. It showed that for the polymer fraction ASP3-PH, the amount of arabinose, galactose, mannose and glucose decreased considerably compared with ASP3, whereas the amount of rhamnose and galacturonic acid increased, suggesting that linkages between two GalA sugars are more stable then aldobiuronic linkages (GalA-Rha) or pseudo-aldobiuronic (Rha-GalA) sugars and linkages between neutral sugars are most susceptible to acid hydrolysis. ASP3-PH, yield 70% of the parent ASP3, composed of galacturonic acid (76.5%), rhamnose (2.7%), galactose (19.2%) and small amount of arabinose (0.2%), suggesting the presence of a typical homogalacturonan and rhamnogalacturonan substituted by the side chains of mainly galactosyl and arabinosyl residues.

For the oligomer fraction ASP3-PL, the sugar composition presented in Table 1 showed that the neutral sugars (arabinose, galactose, mannose and glucose) were the main constituent. These results confirmed that galacturonic acid and rhamnose present in the backbone which were not capable of mild hydrolysis, whereas the neutral sugars were attached to the side chains and easy to be hydrolyzed.

The subfraction ASP3-PH possessed higher positive rotation $[\alpha]_D^{25}$ + 194° than that of the raw fraction ASP3 (+131°) measured at the same conditions (*c* 0.1; H_2O; 25 °C) due to the prevalence of D-Gal*p*A, the main constituent of ASP3-PH.

Table 1
Sugar composition of fractions from ASP3 by partial acid hydrolysis and degradation of EndoPG.

Fragments	Uronic acid (wt.%)	Content of the sugar residues (wt.%)				
		Rha	Ara	Man	Glc	Gal
ASP3	58.3	1.9	10.5	0.4	0.9	24.9
ASP3-PH	76.5	2.7	0.2	Trace	0.4	19.2
ASP3-PL	6.3	Trace	31.9	1.9	3.4	62.8
ASP3-EH	35.6	4.6	9.4	2.0	1.3	46.0
ASP3-EL	83.0	Trace	1.0	Trace	3.7	1.8

3.3. Enzymic digestion of ASP3

Fraction ASP3, which previously shown a symmetrical SEC peak on Sepharose CL-6B (Fig. 1A), was de-esterified with 0.5 M NaOH, digested with EndoPG, and then subsequently fractionated and purified on the same chromatography.

The SEC elution profile of modified ASP3 is shown in Fig. 1B. A small proportion of enzyme-resistant fraction with high-molecular weight (ASP3-EH) and a large proportion of enzyme-sensitive fraction with low-molecular weight (ASP3-EL) were obtained. ASP3-EH, yield 29.71% of the parent ASP3, $[\alpha]_D^{25}$ + 16° (*c* 0.1; H_2O), was polymeric and eluted in the void volume. It contained residues of galactose (46.0%), arabinose (9.4%), galacturonic acid (35.6%), and rhamnose (4.6%). ASP3-EL, yield 62.3% of ASP3, contained large amount of free galacturonic acids or oligomers (83.0%) which were determined by HPAEC-PAD (results not shown). The results confirmed that the linear homogalacturonan was present in the parent ASP3 (Yu et al., 2001).

Sugar composition of the fractions was given in Table 1. It showed that the Gal*p*A content of ASP3-EH decreased (35.6%) compared with the parent ASP3 (58.3%), indicating a significant degradation of the polygalacturonan backbone. However, the presence of Gal*p*A residue also indicated the occurrence of methyl esters, acetyl groups or neutral side chains attached to the backbone of Gal*p*A units, thus limited the action of EndoPG by steric hindrance (Bonnin, Dolo, & Goff, 2002). Fragment ASP3-EH was differed in content of neutral sugar residues, especially rhamnose (4.6%), in comparison with fragment ASP3 (1.9%). With an increase of rhamnose residues and other neutral sugars in ASP3-EH, we presumed that some of Gal*p*A residues were connected with rhamnose residues in repeating units to give a typical rhamnogalacturonan backbone, and the neutral sugars (arabinose, galactose, mannose and glucose) were attached to the backbone as side chains, thus resistant to the digestion of EndoPG.

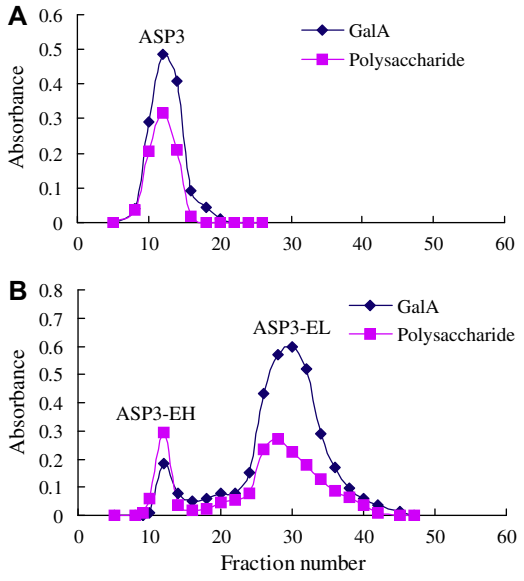

Fig. 1. Elution profiles of the polysaccharide fraction ASP3 before (A) and after enzymatic digestion with EndoPG (B).

3.4. Determination of the glycosidic linkage composition of ASP3 and ASP3-PH

To differentiate galactose arising from the reduction of galacturonic acids residues and galactose existed in the side chains, the carboxyl groups of ASP3 were reduced with $NaBD_4$ into the corresponding 6,6′-d_2-D-galactosyl residues before methylation.

The results of methylation analysis of carboxyl reduced ASP3 and ASP3-PH were given in Table 2. It showed that the purified ASP3 was composed mainly of 1,4-D-linked galacturonic acid, 1,2- and 1,2,4-linked rhamnose, terminal, 1,5-, 1,3,5-linked arabinose, terminal, 1,6-, 1,3,6-, 1,4- and 1,4,6-linked galactose, as was commonly reported in pectic polysaccharide (Habibi, Heyraud, Mahrouz, & Vignon, 2004; Polle et al., 2002b). Methylation analysis revealed that 2,3,6-tri-*O*-methyl galactitol (6,6′-d_2) arising from the reduced 1,4-D-galacturonic acid was the main sugar component. The low amount (1.8%) of 3-*O*-methyl rhamnitol and 3,4-di-*O*-methyl rhamnitol suggested that ASP3 contained the main part of homogalacturonan fragments as "smooth regions" (a linear chain of 1,4-D-Gal*p*A units) and certain amount of rhamnogalacturonan segments as "hairy regions". Regarding the rhamnosyl residues, The ratio 41.2:58.8 of 1,2- to 1,2,4-linked rhamnose indicated a total of 58.8% rhamnose residues in the backbone were substituted at *O*-4 position by side chains.

The proportion of terminal, 1,6-, 1,3,6-, 1,4-, and 1,4,6-linked galactose was, respectively, in the ratio of 14.9:30.7:34.9:12.3:7.2. These results suggested that the galactan side chains contained a central core of 1,6-linked galactose residues, as 65.6% of the units were 1,6-linked, more than half of which (53.2%) were substituted at *O*-3 position (1,3,6-linked galactose). In addition, there was a core of 1,4-linked galactose residues (19.5%), in which

Table 2
Glycosidic linkage composition of methylated ASP3 and ASP3-PH.

Residues	Linkage	PMAA	Mol.%		Mol.%	
			ASP3	ASP3-PH	ASP3	ASP3-PH
D-Gal*p*A	1,4-	2,3,6-Me_3-Gal*p*	100.0	100.0	52.1	76.9
L-Rha*p*	1,2-	3,4-Me_2-Rha*p*	41.2	73.8	1.8	2.4
	1,2,4-	3-Me-Rha*p*	58.8	26.2		
L-Ara*f*	T-	2,3,5-Me_3-Ara*f*	62.4	53.2	13.6	0.3
	1,5-	2,3-Me_2-Ara*f*	19.3	46.8		
	1,3,5-	2-Me-Ara*f*	18.3	—		
D-Gal*p*	T-	2,3,4,6-Me_4-Gal*p*	14.9	42.4	27.9	18.9
	1,6-	2,3,4-Me_3-Gal*p*	30.7	23.2		
	1,3,6-	2,4-Me_2-Gal*p*	34.9	12.2		
	1,4-	2,3,6-Me_3-Gal*p*	12.3	12.0		
	1,4,6-	2,6-Me_2-Gal*p*	7.2	10.2		
D-Glc*p*A	T-	2,3,4,6-Me_4-Gal*p*	100.0	100.0	3.9	1.3
D-Glc*p*	1,4-	2,3,6-Me_3-Glc*p*	100.0	100.0	0.7	0.2

36.9% of the units (1,3,6-linked galactose) were substituted at *O*-6 position. The low proportion of terminal galactosyl residues (14.9%) indicated that a part of the galactopyranan side chains were terminated by the α-arabinofuranose residues which form arabinogalactan.

The proportion of terminal, 1,5-, 1,3,5-linked arabinose was, respectively, in the ratio of 62.4:19.3:18.3. These results suggested that the arabinan side chains contained a central core of 1,5-linked arabinosyl residues, as 37.6% of the units were 1,5-linked. The degree of branching (around 33.3%) was relatively high, for almost half of which (48.7%) were branched at *O*-3 position. 62.4% of arabinosyl residues were at the nonreducing ends. The high proportion of terminal arabinose residues suggested that not all of the terminal arabinose units present in the arabinan side chains, but also attached to the highly branched galactan side chains or connected to the backbone directly (Ros, Schols, & Voragen, 1996).

Compared with the parent ASP3, carboxyl reduced ASP3-PH was relatively enriched in galacturonic acid and rhamnose (76.9% and 2.4%, respectively) whereas the amount of arabinose, galactose, mannose and glucose decreased considerably. The results confirmed that galacturonic acid and rhamnose were present in the backbone, whereas other neutral sugars were attached to the side chains which were easy to be hydrolyzed under weak acid. From the GalA:Rha ratio 32:1 it can be concluded that homogalacturonan (HG) and rhamnogalacturonan I (RG I) fragments remain present in the sample after TFA hydrolysis (Coenen et al., 2007).

Methylation analysis (Table 2) also showed that the content of 1,2,4-linked rhamnose (26.2%) of the total rhamnose in the backbone of ASP3-PH decreased compared with that of ASP3 (58.8%), whereas the amount of 1,2-linked rhamnose increased, indicating the branching points of the backbone with neutral side chains appeared to be at *O*-4 position of 1,2,4-linked rhamnose residues. Further information was then deduced that 1,2- and 1,2,4-linked rhamnose residues were substituted in *O*-2 position by 1,4-D-linked galacturonic acid.

After partial acid hydrolysis, most arabinose residues (97.5%. mainly T- and 1,3,5-Ara*f*) were removed and partial *O*-6 substituted galactosyl residues (32.4%. mainly 1,6-Gal*p* and 1,3,6-Gal*p*) were hydrolyzed, while the proportion of terminal galactose residues (42.4%) increased considerably, compared with ASP3 (14.9%). All the results above indicated that most of arabinose residues in side chains were located at the end of the side chains. The high molar ratio of terminal arabinose (62.4%) in the original polysaccharide ASP3 suggested that in addition to connecting directly with *O*-4 position of 1,2-linked rhamnosyl in the backbone or attached to *O*-5 position of 1,5-L-Ara*f* residues into short arabinan chains, most of T-L-Ara*f* should be attached to *O*-3 of 1,6-linked D-galactosyl residues which forms arabinogalactan. During the course of partial acid hydrolysis, these furanosyl rings are usually considered as weak glycosidic linkages that are more labile to acid than pyranosyl rings (Cui, 2005; Habibi et al., 2005), which cause an increase of terminal galactose. There was a minor change in the amount of 1,4- and 1,4,6-linked galactose residues in ASP3-PH after partial acid hydrolysis, which suggested that the side chains consisted by these two kinds of galactose residues were stable in weak acid condition. The detection of 2,3,4,6-Me_3-Gal*p* (6,6′-d_2) indicated that Gal*p*A were 1,4-linked.

Based on the results of partial acid hydrolysis and enzymic digestion, we supposed that the backbone of the pectic polysaccharide ASP3 consisted of galacturonic acid and rhamnose residues. The neutral side chains attached to *O*-4 position of rhamnose residues. In addition to the α-1,5-linked arabinofuranan side chains substituted at *O*-3 position (1,3,5-Ara), there were dominantly two other types of neutral side chains existed in ASP3: one was arabinogalactan in which 1,6-D-linked galactopyranan was highly branched with a main core of 1,6-D-linked galactose carrying 3-*O*-substituents (1,3,6-Gal) and terminated by α-arabinofuranose residues. The other was 1,4-D-linked galactopyranan branched with a main core of 1,4-D-linked galactose carrying 6-*O*-substituents (1,4,6-Gal).

3.5. ^{1}H and ^{13}C NMR spectroscopy analysis

The 1D and 2D NMR spectra of the three fragments were analyzed, respectively. According to the characteristic signals, the ^{1}H and ^{13}C spectra of the polysaccharide fraction ASP3 were completely assigned by two-dimensional COSY-45, TOCSY and HMQC NMR experiments, and the corresponding chemical shifts of signals were summarized in Table 3.

The ^{1}H and ^{13}C NMR spectra of ASP3 were given in Fig. 2. In the proton spectrum (Fig. 2A), signals at 5.0–5.4 and 4.4–5.0 ppm were corresponded to the anomeric protons of α-arabinofuranose and β-galactopyranose residues. H-1 of α-rhamnopyranose and α-galacturonic acid had signals at 5.25 and 5.08/4.95 ppm, respectively. Two weak resonance signals at 1.28 and 1.38 ppm were identified to H-6 from methyl group of the rhamnopyranose units. These methyl rhamnose signals appeared generally as two well-resolved doublets, due to the presence of two different rhamnose residues, which were, respectively, the rhamnosyl residues linked only at *O*-2 and the rhamnosyl residues linked both at *O*-2 and *O*-4 (Cui et al., 1996; Habibi et al., 2005). This assignment was in agreement with the results of methylation analysis.

Table 3
Chemical shifts for the resonances of glycosyl residues of ASP3 in ^{1}H and ^{13}C NMR spectra.

Glycosyl residues	Chemical shifts, δ (ppm)						
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	CH_3O
→ 4)-α-Gal*p*A-(1 → 4	5.08/102.3	3.70/70.7	4.10/71.3	4.41/81.2	n.d.[a]/73.2	173.5	3.78/55.6
→ 4)-α-Gal*p*A-(1 → 2	4.95/103.0	3.70/70.9	4.00/71.3	n.d./81.0	n.d./n.d.	176.9	–
→ 2)-α-Rha*p*-(1 →	5.25/n.d.[a]	4.10/78.5	3.86/72.2	3.41/76.0	3.70/70.2	1.25/19.5	–
→ 2,4)-α-Rha*p*-(1 →	5.25/n.d.	4.15/79.0	3.90/72.2	3.82/84.7	3.47/72.5	1.38/19.7	–
β-Gal*p*-(1 →	4.42/105.5	3.53/73.8	3.64/73.5	3.93/71.4	3.69/77.4	3.78/63.3	–
2-*O*-Me-β-Gal*p*-(1 →	4.49/105.0	3.29/84.5	3.36/75.7	3.96/71.4	3.73/78.6	3.70/63.3	3.48/62.1
→ 6)-β-Gal*p*-(1 →	4.61/106.5	3.47/73.0	3.66/73.5	3.91/70.9	3.88/75.3	3.98/3.72/71.3	–
→ 3,6)-β-Gal*p*-(1 →	4.68/105.5	3.79/72.9	3.83/84.6	4.21/69.9	3.76/74.2	3.72/71.3	–
→ 4,6)-β-Gal*p*-(1 →	4.46/105.5	3.54/73.5	3.66/73.5	3.95/78.5	3.66/75.3	3.72/71.3	–
→ 4)-β-Gal*p*-(1 →	4.69/105.5	3.69/73.0	3.94/73.0	4.14/78.5	3.71/n.d.	3.88/3.82/63.3	–
α-Ara*f*-(1 → 3	5.40/111.0	4.19/84.3	3.91/76.9	4.05/87.0	3.91/3.79/64.0	–	–
α-Ara*f*-(1 → 3	5.23/112.0	4.22/84.3	3.93/76.9	4.12/86.8	3.82/3.69/64.0	–	–
α-Ara*f*-(1 → 5	5.13/110.0	4.12/83.8	3.95/76.9	4.02/86.5	3.82/3.72/64.0	–	–
→ 5)-α-Ara*f*-(1 →	5.07/108.0	4.12/83.8	4.02/77.0	4.21/86.8	3.80/3.89/71.6	–	–
→ 3,5)-α-Ara*f*-(1 →	5.09/108.0	4.28/82.8	4.09/84.0	4.44/82.0	3.94/3.82/72.0	–	–

[a] Not determined.

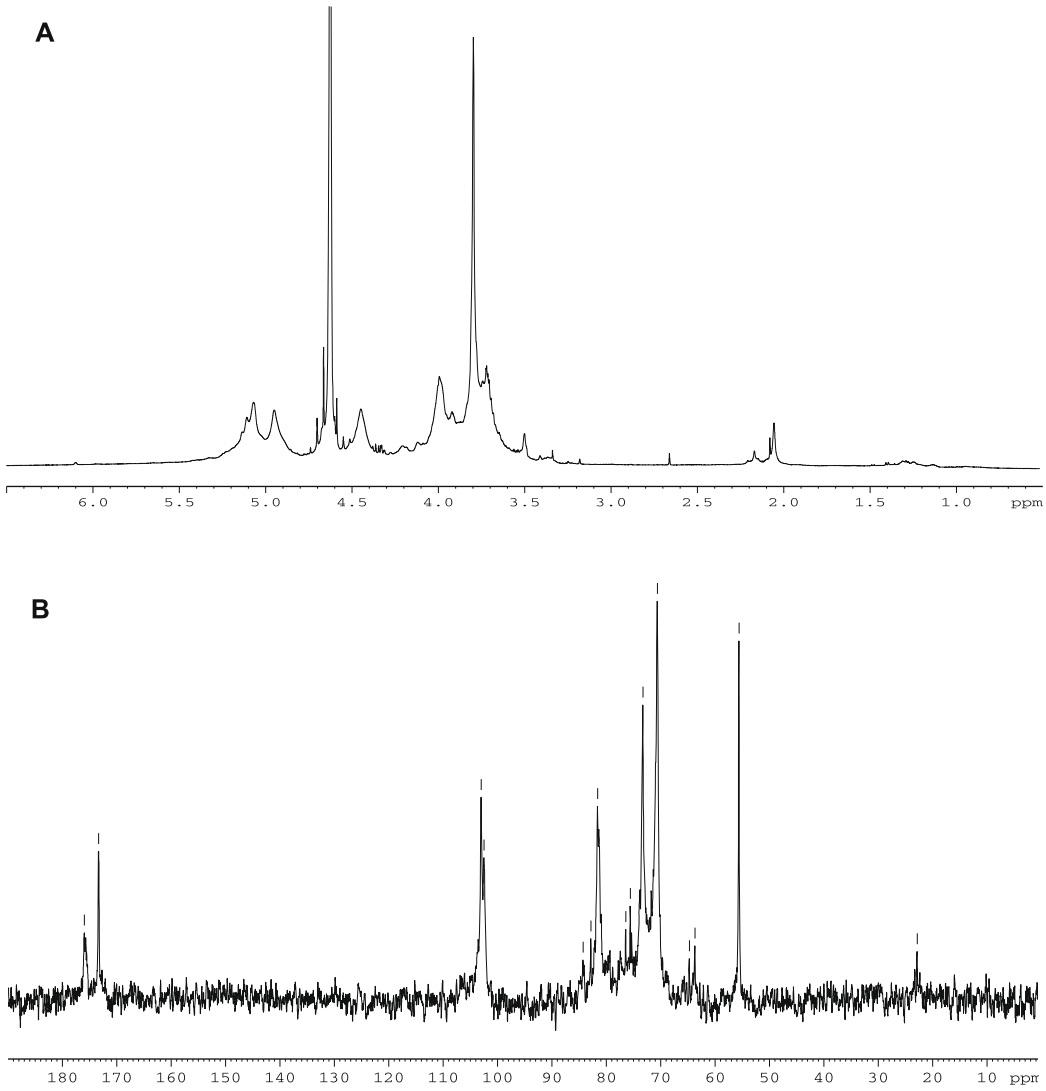

Fig. 2. ^{1}H (A) and ^{13}C (B) NMR spectra of the polysaccharide fraction ASP3.

The proton signals near 2.1 ppm arise from the $-CH_3$ of the *O*-acetyl groups (Fig. 2A). The two doublets suggested that ASP3 contained two kinds *O*-acetyl groups at the different positions of the sugar residues (Bushneva, Ovodova, Shashkov, & Ovodov, 2002). Meanwhile, the carbon signal at 22.8 ppm (Fig. 2B) in ^{13}C NMR spectra supported the above deduction (Wang, Liu, & Fang, 2005).

The $^{1}H/^{13}C$ HMQC and TOCSY spectra of fragments demonstrated the presence of regions consisting of α-1,4-linked homogalacturonan. In the ^{1}H and ^{13}C NMR spectra of fraction ASP3-PH, a group of intensive signals were observed for the residues of α-1,4-linked galacturonic acid (Table 3). According to the literature (Keenan, Belton, Matthew, & Howson, 1985), these resonances were characteristic for a pure α-1,4-D-galactopyranosyluronan which occupied the main part of the fraction ASP3.

In the low-field region of ^{13}C NMR, typical signals were observed for the C-6 carboxyl group of galacturonic acid units at 176.9 and 173.5 ppm. The occurrence of two carboxyl signals confirmed the presence of free and esterified carboxyl groups of α-D-Gal*p*A (Catoire, Goldberg, & Pierron, 1998; Wang et al., 2005). The carbon signal at 55.6 ppm was assigned to methyl ester groups of the native polysaccharide. The presence of methyl esterified Gal*p*A residues was also confirmed by the signal of 55.6/3.78 ppm in HMQC spectrum of fraction ASP3-PH (Bushneva et al., 2002). In addition, the COSY-45 correlation peak at 4.95/4.41 ppm observed for fraction ASP3-PH indicates the H2/H4 interaction of neighbor α-(1,4)-linked Gal*p*A residues of linear homogalacturonan regions (Bushneva et al., 2002).

The anomeric carbon signal of rhamnopyranose was not observed in ^{13}C NMR spectrum for its low content. However, analysis of the homonuclear COSY-45, TOCSY and HMQC spectra of the digested fraction ASP3-EH revealed the presence of α-1,2-linked rhamnopyranose residues. In the HMQC spectrum of fraction ASP3-EH (Fig. 3), the minor response at 19.5 ppm was easily identified to C-6 from methyl group of rhamnopyranose units, and the COSY-45 and TOCSY spectra showed the corresponding proton signals associated with α-1,2-linked rhamnopyranose residues. In particular, some of residues were observed to be substituted at *O*-4 position from the correlation peak of C4/H4-atoms at 84.7/3.82 ppm in the HMQC spectrum (Fig. 3), which agree well with the results of the methylation analysis. Other characteristic signals were assigned and listed (Table 3) according to the 2D NMR spectra and reference (Cui et al., 1996). Finally, a complete assignment of the signals originating from α-1,2- and α-1,2,4-rhamnopyranose residues was obtained by comparison of the observed chemical shifts with corresponding values in literature (Habibi et al., 2005; Keenan et al., 1985).

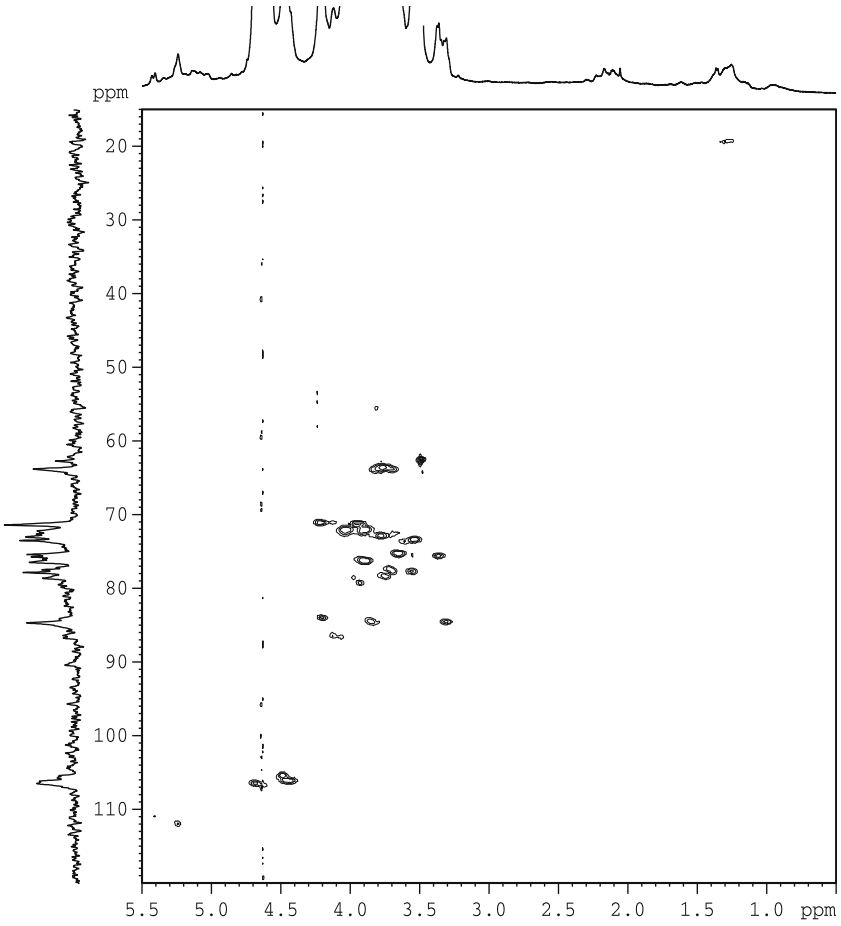

Fig. 3. $^{1}H/^{13}C$ HMQC spectrum of the polysaccharide fraction ASP3-EH.

In the HMQC spectrum of ASP3-PH (Figure not shown), the signals of α-arabinofuranose and partial β-galactopyranose almost disappeared and became simple. Compared with these changes, α-galacturonic acid and α-rhamnopyranose residues were largely retained, and the signal of 1,2-linked rhamnopyranose at 1.25 ppm became stronger compared with the signal of 1,2,4-linked rhamnopyranose at 1.29 ppm. These results confirmed that α-arabinofuranose and β-galactopyranose residues were side chains linked to *O*-4 of 1,2,4-linked rhamnopyranose.

Galacturonic acid, rhamnose, arabinose and galactose were detected as the main sugars in fragment ASP3-EH, suggesting the presence of a rhamnogalacturonan. The signals in the heteronuclear $^{1}H/^{13}C$ HMQC spectrum of fragment ASP3-EH (Fig. 3) also indicated the occurrence of linear α-1,2-L-rhamno-α-1,4-D-galacturonan region.

In the anomeric region, the signals at 105–107 ppm corresponded to the anomeric carbons of terminal or branched galactopyranose residues, and signals at 108–110 ppm were assigned to the C-1 of terminal or branched arabinofuranosyl units (Cui, 2005). The signals at 102.3 and 103.0 ppm arose from C1 of α-Rha*p* and α-Gal*p*A, respectively (Cui et al., 1996) (Fig. 2B). Resonance signals in the region of 75–85.1 ppm were corresponded to secondary hydroxyl groups (C-2, C-3) of arabinofuranosyl units, while the signals of C-2–C-4 of β-D-Gal*p* appeared in the region of 65–85 ppm (Cui, 2005). Resonance signals in the region of 60–64 ppm arose from C-5 of terminal arabinofuranosyl units (64.0 ppm) and C-6 of terminal galactopyranose residues (63.3 ppm).

According to the literature data published for pectic substances, ^{1}H and ^{13}C NMR spectroscopy allowed detection of the terminal, 6-, 3,6-, 4- and 4,6-substituted β-galactopyranose residues in the polysaccharide fragment ASP3. In addition, the residues of α-arabinofuranose substituted in 5-, and 3,5-positions as well as the terminal α-arabinofuranose residues were identified (Table 3). The connectivity from H-1 to H-5 was clearly established from COSY-45 spectra. These data were confirmed by two-dimensional homonuclear COSY-45, TOCSY, and the heteronuclear HMQC spectrum.

In addition, the residues of 2-*O*-methyl-β-D-galactopyranose were detected. The resonance of C-2 at 84.5 ppm in the $^{1}H/^{13}C$ HMQC spectrum of the fragment ASP3-EH (Fig. 3) indicated that the *O*-methyl group (62.1/3.48 ppm) was linked at this position, which otherwise would be in the range of 72–74 ppm (Polle et al., 2002a, 2002b). A correlation peak of the *O*-methyl group with H-2 of the terminal β-galactopyranose residue (OMe/H2 3.48/3.29 ppm) in the COSY-45 spectrum of ASP3-EH confirmed this interpretation. Other signals for these substituted residues were fully assigned and listed (Table 3) from homonuclear COSY-45, TOCSY spectra and heteronuclear HMQC, compared with literature data (Bushneva et al., 2002; Catoire et al., 1998; Habibi et al., 2005; Polle et al., 2002a, 2002b).

4. Conclusion

The structural features of ASP3 were studied using partial acidic hydrolysis and enzymic digestion, combined with methylation analysis and NMR spectral data. The results indicated that α-1,4-D-galactopyranosyluronan fragments occupied the main part of the pectic polysaccharide ASP3 as "smooth regions". Some residues of galacturonic acid in the linear region were methoxylated and contained *O*-acetyl groups on C-2 and/or C-3. The ramified region appear to be rhamnogalacturonan blocks with repeating unit of

[→ 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 →]. A total of 58.8% rhamnosyl residues in the backbone were substituted at *O*-4 position by the side chains. The side chains, which contained residues of terminal, β-1,6-, β-1,3,6-, β-1,4- and β-1,4,6-linked galactopyranose and terminal, α-1,5-, α-1,3,5-linked arabinofuranose, were linked as blocks of galactan, arabinan, and arabinogalactan, which were the mainly constitution of the branch, attached to *O*-4 of the backbone rhamnose residues. A part of terminal Gal*p* were substituted at *O*-2 position by methyl into 2-*O*-Me-β-D-Gal*p*.

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