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Carbohydrate Polymers 60 (2005) 205–213
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Isolation and structural characterization of protopectin from the skin of *Opuntia ficus-indica* prickly pear fruits

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Received 7 June 2004; revised 28 December 2004; accepted 5 January 2005
Available online 3 February 2005

Abstract

Dilute HCl extraction of *Opuntia ficus-indica* fruits, previously disencrusted by hot water followed by hot chelating treatments, yielded a series of soluble pectic polysaccharides, which were de-esterified and separated into five fractions by anion exchange chromatography. Three of these fractions were thereafter purified by size exclusion chromatography and characterized by sugar analysis combined with methylation and reduction-methylation. Their study was further supported by ^{1}H and ^{13}C NMR spectroscopy. The results showed that one of these fractions (ASP1) was neutral and consisted of a linear β-(1→4)-galactan. One of the acid fractions (ASP2), with a galacturonic content of about 40%, consisted of a disaccharide repeating unit [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] backbone, with short linear β-(1→4)-linked galactan side-chains attached to O-4 of the rhamnosyl residues. Another acid fraction (ASP3), with a high galacturonic content (about 62%), consisted of alternating homogalacturonan blocks (1→4) α-linked and rhamnogalacturonan blocks with approximately the same amount of galactopyranosyluronic acid residues in each block. ^{13}C T_1 relaxation times were measured and noticeable differences in molecular motion of the different sugar rings in the repeating units were found.

Keywords: *Opuntia ficus-indica*; Prickly pear; Pectins; Galactan; Rhamnogalacturonan; ^{1}H and ^{13}C NMR

1. Introduction

Plant cell walls are major contributors to both the form and properties of plant structures. Most current discussions suggest that the primary cell walls that surround all plant cells are fibrillar composites in which cellulose microfibrils are fastened together by cross-linking glycans (Carpita & Gibeaut, 1993). Pectin is the most abundant class of macromolecules within this matrix and, in addition, it is also abundant in the middle lamellae between primary cell walls, where it functions in regulating intercellular adhesion (O'Neill, 1990).

From a structural point of view, pectins are characterized by high content of galacturonic acid. This residue occurs in structural features that form the backbone of three polysaccharide domains that are thought to be found in all pectin species: homogalacturonan, rhamnogalacturonan I and II. Homogalacturonan is a linear chain of α-(1→4)-linked D-galactopyranosyl acid residues generally methyl-esterified. Rhamnogalacturonan type I (RG-I) is composed of a disaccharide repeating unit [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] that can be ramified at O-4 of rhamnosyl residue, with mostly neutral side-chains (1–20 residues) containing mainly L-arabinose and/or D-galactose (arabinan, galactan, arabinogalactan). Despite its name, rhamnogalacturonan II (RG-II) is not structurally related to RG-I but is constituted by a homogalacturonan backbone that can be substituted at O-2 and/or O-3 of galactopyranosyl acid residues, by side-chains containing, in addition to the galactose and arabinose, other residues such as D-xylose, D-fucose, D-glucuronic acid, D-apiose or 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) (O'Neill, 1990; Ralet, Bonnin, & Thibault, 2002; Ridley, O'Neill, & Mohnen, 2001; Stephen, 1983; Thibault & Ralet, 2001; Voragen,

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doi:10.1016/j.carbpol.2005.01.001

Pilnik, Thibault, Axelos, & Renard, 1995; Willats, McCartney, Mackie, & Knox, 2001).

Pectic substances have been isolated and extensively studied from various plant tissues such as grape berries (Saulnier, Brillouet, & Joseleau, 1988; Saulnier & Thibault, 1987), apple (De Vries, Den Uijl, Voragen, Rombouts, & Pilnik, 1983; De Vries, Rombouts, Voragen, & Pilnik, 1982; Renard, Crepeau, & Thibault, 1995; Schols, Vierhuis, Bakx, & Voragen, 1995; Stevens & Selvendran, 1984), sugar beet (Guillon & Thibault, 1989; Guillon, Thibault, Rombouts, Voragen, & Pilnik, 1989; Keenan, Belton, Matthew, & Howson, 1985), citrus (Renard et al., 1995), flax (Davis, Derouet, Herve du Penhoat, & Morvan, 1990), and other materials (Barbier & Thibault, 1982; Bushneva, Ovodova, Shashkov, & Ovodov, 2002; Golovchenko, Ovodova, Shashkov, & Ovodov, 2002; Huisman, Schols, & Voragen, 1999; Odonmazig, Badga, Ebringerova, & Alfoldi, 1992; Polle, Ovodova, Chizhov, Shashkov, & Ovodov, 2002; Polle, Ovodova, Shashkov, & Ovodov, 2002; Redgwell & Selvendran, 1986; Ryden & Selvendran, 1990; Strasser & Amado, 2001; Westerlund, Aaman, Andersson, Andersson, & Rahman, 1991).

There are few studies in the literature concerning *Opuntia ficus-indica* (OFI) prickly pear fruit. It is for these reasons that prickly pear fruits have attracted our attention and we studied the extraction, purification and characterization of pectic and hemicellulosic polysaccharides. From the fruit seeds, we isolated several glucuronoxylans with a molar ratio of xylose to 4-*O*-methyl-glucuronic acid varying from 12:1 to 65:1 (Habibi, Mahrouz, & Vignon, 2002). We also isolated and characterized a 4-*O*-methyl-glucuronoxylan from the fruit skin (Habibi, Mahrouz, & Vignon, 2003). Pectic polysaccharides solubilized with water and calcium chelator agent were previously characterized (Habibi, Heyraud, Mahrouz, & Vignon, 2004). The aim of this paper was the isolation, purification and structural elucidation of several polysaccharides extracted from the fruit skin under acid conditions.

2. Results and discussion

2.1. Extraction of acid soluble pectin (ASP)

The skin tissues of OFI were dried and defatted by refluxing in a Soxhlet apparatus operated with a toluene–ethanol mixture. The resulting material was extracted with de-ionized water at room temperature. The filtrate contained mainly the mucilage exudate, which has been characterized (Habibi, Mahrouz, Marais, & Vignon, 2004). The residue or cell wall material (CWM) contained a high amount of pectin. The majority of these pectic polysaccharides (18.5 wt%) were extracted sequentially by hot water and hot aqueous solution of EDTA, acting as calcium chelator agent. The largest extract was solubilized by EDTA (12.4 wt% of dry matter), the fractions solubilized by

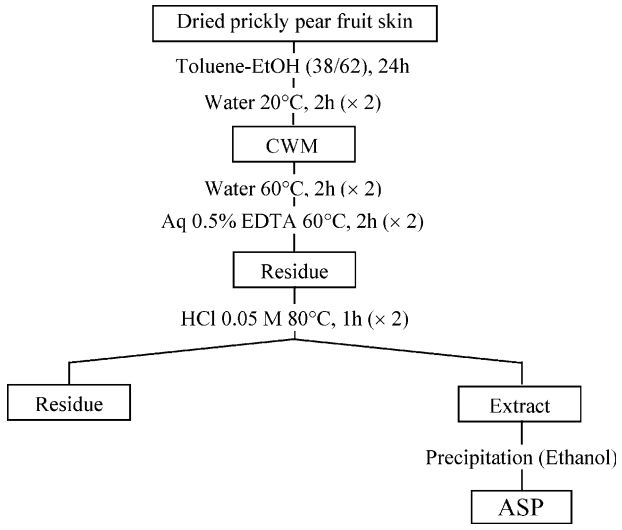

Fig. 1. Scheme of extraction of acid soluble pectin (ASP) from the skin of prickly pear fruits.

distilled water corresponded to 6.1 wt%. Hot water extraction yielded highly esterified pectins with high neutral sugar content. Extraction with chelating agents gave pectins with low degree of methylation. According to Selvendran and O'Neill,(Selvendran & O'Neill, 1987) these pectins originated from the middle lamella, where they are presumed to be present in the form of calcium pectate gel. The pectins remaining in the residue after the foregoing processes are tightly anchored to the cell walls and can be extracted only by degradative extraction. Following established protocols (Renard, Voragen, Thibault, & Pilnik, 1990), the extraction was performed with hot dilute hydrochloric acid solution in order to extract these residuals pectic polysaccharides. During the acidic extraction, some glycosidic linkages, particularly the arabinofuranosyl linkages, can be hydrolyzed even under mild conditions. The polymers solubilized during this treatment were removed by filtration, precipitation and centrifugation. The general fractionation procedure scheme is given in Fig. 1. The resulting polysaccharide, named acid soluble pectin (ASP), accounted for 8.7 wt% (on dry matter), and contained high amount of galacturonic acid (50.7 wt%). Neutral sugars were also present and accounted for 28.2%, with rhamnose (12.5 wt%), galactose (10.8 wt%), glucose (2.9 wt%), arabinose (1.2 wt%) and xylose (0.8 wt%) as shown in Table 1. Furthermore, one can notice the poor yields of total neutral sugars, due to incomplete hydrolysis of the Gal*p*A→Rha*p* linkage, which induced an under-estimation of the rhamnose content, as already reported (Vignon & Garcia-Jaldon, 1996).

2.2. Fractionation of acid soluble pectin (ASP)

After extraction with dilute HCl the ASP fraction was partially esterified and the remaining methyl and acetyl groups were removed by mild alkaline treatment.

Table 1
Sugar composition[a] of ASP fractions

Fraction	Uronic acid	Neutral sugars					
		Gal 6,6′-d_2	Rha	Ara	Xyl	Gal	Glc
ASP native	50.7	–	12.5	1.2	0.8	10.8	2.9
ASP1 native	–	–	–	–	–	98.3	–
ASP2 native	38.5	–	3.0	1.2	1.3	18.0	3.0
ASP2 reduced	–	40.5	30.6	1.5	1.9	15.0	2.7
ASP3 native	55.0	–	2.0	–	–	0.2	–
ASP3 reduced	–	61.6	32.0	–	–	6.0	–

[a] Expressed in relative weight percentages.

The solution was acidified and ASP, in its acid form ASP^+, was fractionated by anion exchange chromatography according to the scheme presented in Fig. 2. Five fractions (ASP1*, 16 wt%; ASP2*, 15 wt%; ASP3*, 32 wt%; ASP4*, 8 wt% and ASP5*, 7 wt%) were obtained. The three major fractions (ASP1*, ASP2* and ASP3*) were thereafter purified by size exclusion chromatography to give purified fractions ASP1, ASP2 and ASP3.

2.3. Characterization of three of the purified fractions

The sugar composition and methylation data of the three purified fractions were reported in Tables 1 and 2. In the case of acidic fractions (ASP2 and ASP3), the sugar composition and methylation data of their carboxyl reduced forms are also given. The carboxyl groups of ASP2 and ASP3 were reduced with $NaBD_4$ into the corresponding 6,6′-dideutero-D-galactosyl residues before methylation in order to differentiate the galactose arising from the reduction of the galacturonic acid residues and the galactose residues already in the side-chains.

These results were also confirmed by ^1H and ^{13}C NMR spectroscopy, using recent spectral sequences. Among these methods, homonuclear Correlated Spectroscopy (COSY), and shift-correlation using either Heteronuclear Multiple Quantum Coherence (HMQC) or Heteronuclear Multiple Bond Correlation (HMBC) were used in order to assign unambiguously most of ^1H and ^{13}C resonances. The NMR spectra of the two fractions studied are given in Figs. 3 and 4, the corresponding NMR data are reported in Tables 3 and 4.

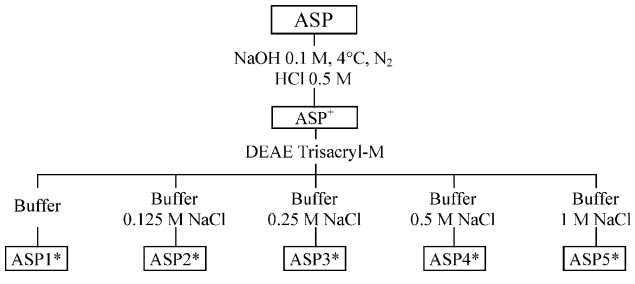

Fig. 2. Scheme of fractionation of acid soluble pectin (ASP).

2.3.1. Characterization of ASP1

The sugar analysis of the fastest eluted fraction revealed that this fraction was composed exclusively of galactose (Table 1). The methylation data (Table 2) showed only the presence of 2,3,6-tri-*O*-methyl galactitol, indicating that this fraction corresponded in fact to a (1→4)-linked galactan. The ^1H and ^{13}C NMR spectra of ASP1 are reported in Figs. 3 and 4. The ^{13}C spectrum showed only six signals at δ 105.17 (C-1), 72.74 (C-2), 74.19 (C-3), 78.44 (C-4), 75.34 (C-5) and 61.63 ppm (C-6). In the ^1H spectrum the relative simplicity of the structure is exhibited by signals at δ 4.58 (H-1), 3.66 (H-2), 3.77 (H-3), 4.12 (H-4), 3.71 (H-5) 3.80 ppm (H-6,6′). All these data collected in Tables 3 and 4, are compatible with a linear structure and are in good agreement with NMR data already reported for β-(1→4)-D-galactan (Arifkhodzhaev, 2000; Herve du Penhoat, Michon, & Goldberg, 1987; Pressey & Himmelsbach, 1984).

2.3.2. Characterization of ASP2

The sugar composition of ASP2 (Table 1) showed that it was composed essentially of galacturonic acid, rhamnose and galactose, in the ratio 40:30:15 suggesting the presence of a rhamnogalacturonan substituted with galactan side-chains. The arabinose was detected in very small amount, indicating the removal of arabinan, known to be present in side-chains of hairy regions of pectin, but hydrolyzed during the acidic conditions of extraction. Very low yields of total neutral sugars were observed for non-reduced ASP2 sample, which can be explained by an incomplete hydrolysis of the Gal*p*A→Rha*p* linkage.

Table 2
Partially methylated alditol acetates[a] of native ASP1 and carboxyl reduced ASP2 and ASP3 fractions

Alditol	ASP1 native	ASP2 reduced	ASP3 reduced
3,4-Me_2-Rha[b]	–	21.8	26
3-Me-Rha	–	6.2	6
Total	–	28.0	32
2,3,4,6-Me_4-Gal	0	6.2	6
2,3,6-Me_3-Gal	99	25.2	–
Total	99	31.4	6
2,3,6-Me_3-Gal 6,6′-d_2	–	38.5	62

[a] Relative mole ratio.
[b] 3,4-Me_2-Rha = 1,2-di-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol, etc.

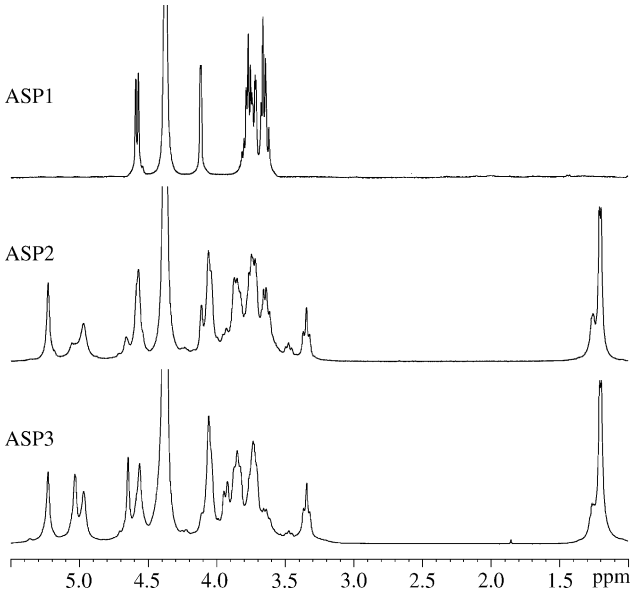

Fig. 3. ^{1}H NMR spectra of ASP1, ASP2 and ASP3 (333 K, 400 MHz).

The results of methylation analysis of $NaBD_4$ carboxyl reduced ASP2 fraction (Table 2), showed that the amount of 2,3,6-tri-*O*-methyl galactitol 6,6′-d_2 arising from the reduced 4-linked D-galacturonic acid, was larger than the sum of 3-*O*-methyl rhamnitol and 3,4-di-*O*-methyl rhamnitol (38.5:28), indicating that ASP2 consisted of alternating 'smooth' homogalacturonan regions (galaturonic acid residues linked (1→4)) and branched 'hairy' or ramified regions, with one-third of the galacturonic acid units involved in smooth regions and two-third in rhamnogalacturonan. The presence in the same proportion of 3-*O*-methyl rhamnitol and 2,3,4,6-tetra-*O*-methyl galactitol indicated that the galactan side-chains were attached to the backbone at the O-4 position of rhamnose residues. The detection of

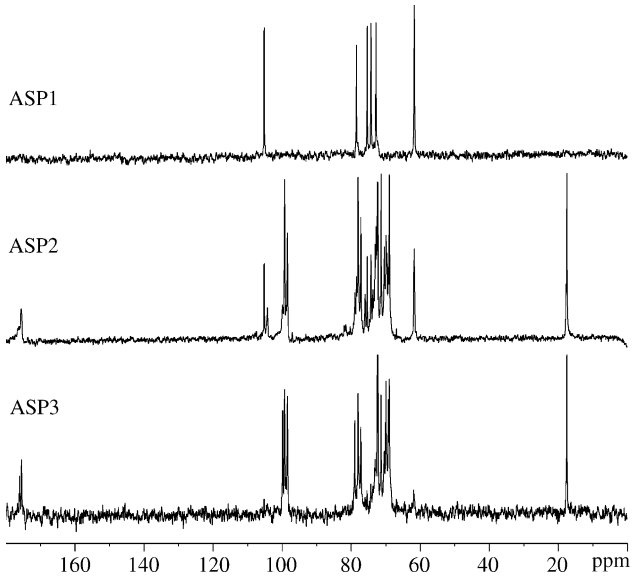

Fig. 4. ^{13}C NMR spectra of ASP1, ASP2 and ASP3 (333 K, 400 MHz).

only 2,3,6-tri-*O*-methyl galactitol and 2,3,4,6-tetra-*O*-methyl galactitol in proportion 25.2:6.2, indicated that the galactan side-chains consisted of an average of five galactose units.

The NMR data for ASP2 are reported in Tables 3 and 4. The ^{1}H and ^{13}C spectra of ASP2 (Figs. 3 and 4) presented a relative simplicity. The ^{13}C NMR spectrum contained in the anomeric region two major peaks at 99.23 and 98.43 ppm characteristics, respectively, of C-1 of rhamnopyranosyl α-(1→2) linked and galactopyranosyl acid α-(1→4)-linked residues. Medium signals at 105.16, 75.82, 74.92 and 61.67 ppm were assigned to C-1, C-5, C-2 and C-6, respectively, of β-(1→4)-linked galactosyl residues of the galactan side-chain. A less intense signal, present in the anomeric region at 98.63 ppm, is characteristic of C-1 of substituted rhamnosyl residues. The one-bond correlation of the ^{13}C and the ^{1}H NMR (^{1}H/^{13}C-HMQC experiment) allowed the attribution of all the protons of (1→4)-linked galactose residues at δ 4.58 (H-1), 3.63 (H-2), 3.92 (H-3), 4.11 (H-4), 3.65 (H-5) and 3.78 ppm (H-6,6′).

The NMR results corroborated the methylation data and demonstrated that ASP2 consisted of smooth α-(1→4) galacturonic acid blocks alternating with [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] blocks, 33% of the galacturonic acid units being involved in the galacturonic acid regions. Among the rhamnose residues, 20–25% bear in O-4 short β-(1→4)-galactan side-chain with an average of five galactose units. We can propose for ASP2 the following repeating units (Fig. 5), but other isomer structures are possibles.

2.3.3. Characterization of ASP3

The sugar composition of ASP3 presented in Table 1 showed that it is composed of galacturonic acid, rhamnose and small amount of galactose, in the ratios (62:32:6) suggesting the presence of a rhamnogalacturonan substituted by galactan side-chains. The lack of arabinose is noticed and can be explained by the hydrolysis of the arabinofuranosyl linkages during the acidic conditions of extraction. The very poor yield in total neutral sugars is more likely due to an incomplete hydrolysis of the Gal*p*A→Rha linkage. The results of methylation analysis of $NaBD_4$ reduced ASP3 fraction, showed that the amount of 2,3,6-tri-*O*-methyl galactitol 6,6′-d_2 (62%) arising from the reduced 4-linked D-galacturonic acid, was larger than the sum of 3-*O*-methyl rhamnitol (6%) and 3,4-di-*O*-methyl rhamnitol (26%), indicating that ASP3 consisted of alternating 'smooth' homogalacturonan (galaturonic acid residues linked (1→4)) regions and rhamnogalacturonan regions, with 50% of the galacturonic acid units involved in homogalacturonan and 50% in the rhamnogalacturonan regions.

Also, 2,3,6-tri-*O*-methyl galactitol residues corresponded exclusively to the (1→4)-linked galacturonic acid residues in the main chain. The proportion of 2,3,4,6-tetra-*O*-methyl galactitol was found to be equal to the proportion of 2,4-linked L-rhamnosyl residue (3-*O*-methyl

Table 3
^{1}H NMR data[a] for related glycosyl residues of ASP1, ASP2 and ASP3 fractions

Fraction	Glycosyl residues	^{1}H assignment					
		1	2	3	4	5	6
ASP1	β-Galactosyl residues						
	→4)-β-D-Gal*p*-(1→	4.58	3.66	3.77	4.12	3.71	3.80
ASP2	α-Galacturonosyl residues						
	→4)-α-D-Gal*p*A-(1→	5.05	na[b]	3.97	4.37	4.67	–
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	4.97	3.95	4.00	4.39	4.58	–
	α-Rhamnosyl residues						
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	5.22	4.06	3.88	3.34	3.78	1.20
	→4)-α-D-Gal*p*A-(1→2,4)-α-L-Rha*p*-(1→	5.22	4.12	na	na	na	1.25
	β-Galactosyl residues						
	→4)-β-D-Gal*p*-(1→	4.58	3.63	3.92	4.11	3.65	3.78
	T-β-D-Gal*p*-(1→	na	3.47	na	na	na	Na
ASP3	α-Galacturonosyl residues						
	→4)-α-D-Gal*p*A-(1→	5.03	na	3.97	4.37	4.65	–
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	4.96	3.95	4.00	4.35	4.56	–
	α-Rhamnosyl residues						
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	5.22	4.06	3.88	3.33	3.78	1.20
	→4)-α-D-Gal*p*A-(1→2,4)-α-L-Rha*p*-(1→	5.22	4.12	na	na	na	1.25
	β-Galactosyl residues						
	→4)-β-D-Gal*p*-(1→	4.56	3.63	3.92	4.06	na	3.78

[a] In ppm relative to the signal of internal acetone (CH_3) in deuterium oxide at 2.1 ppm.
[b] na, Not assigned.

rhamnitol), which showed that there is only one galactose unit as side-chain.

The NMR data of ASP3 are reported in Tables 3 and 4, and the ^{1}H and ^{13}C spectra in Figs. 3 and 4. The ^{13}C spectrum of ASP3 presented a relative simplicity. It showed three major peaks in the anomeric region. The signal at 99.79 ppm corresponded to C-1 of α-(1→4)-GalA (homogalacturonan regions). The signals at 99.24 and 98.40 ppm, are characteristics, respectively, of C-1 of rhamnopyranosyl α-(1→2) linked and galactopyranosyl acid α-(1→4) linked

Table 4
^{13}C NMR data[a,b] for related glycosyl residues of ASP1, ASP2 and ASP3 fractions

Fraction	Glycosyl residues	^{13}C assignment					
		1	2	3	4	5	6
ASP1	β-Galactosyl residues						
	→4)-β-D-Gal*p*-(1→	105.17	72.74	74.19	78.44	75.34	61.63
ASP2	α-Galacturonosyl residues						
	→4)-α-D-Gal*p*A-(1→	99.80 (203)[b]	69.85 (nd[c])	69.90 (nd)	78.92 (nd)	72.34 (nd)	176.01
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	98.43 (307)	69.11 (313)	69.70 (315)	77.99 (313)	72.46 (318)	175.57
	α-Rhamnosyl residues						
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	99.23 (272)	77.93 (282)	71.27 (276)	72.73 (263)	68.86 (287)	17.45 (685)
	→4)-α-D-Gal*p*A-(1→2,4)-α-L-Rha*p*-(1→	98.63 (nd)	78.44 (nd)	na[d]	81.02 (nd)	na	17.55 (nd)
	β-Galactosyl residues						
	→4)-β-D-Gal*p*-(1→	105.16 (325)	72.12 (328)	74.92 (325)	78.84 (nd)	75.82 (337)	61.67 (524)
	T-β-D-Gal*p*-(1→	104.20 (509)	72.29 (nd)	73.62 (nd)	69.18 (nd)	75.90 (449)	61.64 (nd)
ASP3	α-Galacturonosyl residues						
	→4)-α-D-Gal*p*A-(1→	99.79 (304)	69.85 (304)	69.90 (308)	78.92 (311)	72.29 (304)	176.05
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	98.40 (376)	69.11 (366)	69.70 (366)	77.13 (376)	71.28 (376)	175.48
	α-Rhamnosyl residues						
	→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→	99.24 (336)	77.90 (326)	70.30 (326)	72.26 (336)	68.79 (325)	17.47 (747)
	→4)-α-D-Gal*p*A-(1→2,4)-α-L-Rha*p*-(1→	99.01 (nd)	78.35 (nd)	na	81.02 (nd)	na	17.65 (nd)
	β-Galactosyl residues						
	→4)-β-D-Gal*p*-(1→	105.16 (nd)	72.73 (nd)	74.21 (nd)	68.84 (nd)	75.38 (nd)	61.74 (nd)

[a] In ppm relative to the signal of internal acetone (CH_3) in deuterium oxide at 31.5 ppm (^{13}C).
[b] Carbon-13 T_1 in millisecond appear in brackets.
[c] nd, Not determined.
[d] na, Not assigned.

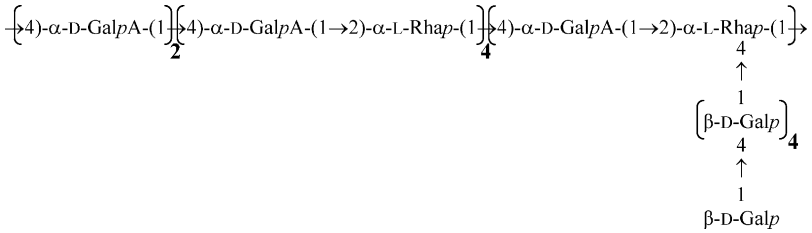

Fig. 5. Schematic structure of ASP2.

residues of rhamnogalacturonan regions. These results confirmed that ASP3 is constituted of alternating homogalacturonan regions and rhamnogalacturonan regions.

The presence of one galactose unit as side-chain was indicated by minor signals at δ 105.16 (C-1), 74.21 (C-2), 75.38 (C-5) and 61.74 ppm (C-6). The one-bond correlation of the ^{13}C and the ^{1}H NMR ($^{1}H/^{13}C$-HMQC) allowed the assignment of the protons of galactosyl unit at δ 4.56 (H-1), 3.63 (H-2), 3.92 (H-3), 4.06 (H-4) and 3.78 ppm (H-6,6′). These assignments are in good agreements with published data (Colquhoun, de Ruiter, Schols, & Voragen, 1990; Davis et al., 1990; Huisman et al., 2001; Keenan et al., 1985; Ryden, Colquhoun, & Selvendran, 1989; Vignon & Garcia-Jaldon, 1996).

The NMR results are in good agreement with the methylation data and indicate that ASP3 contained both α-(1→4)-α-D-galacturonic blocks and [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] blocks, with half of the galacturonic acid units involved in galacturonan blocks and the other half in the rhamnogalacturonan blocks. The ratio of rhamnosyl residues either linked at O-2 or both at O-2 and O-4 are very similar and close to 4:1 in ASP2 and ASP3, but the side chains of ASP3 are very short as they contained only one galactose unit. Finally, we can propose the following repeating unit for ASP3 (Fig. 6), but other isomer structures are possibles.

2.4. Carbon-13 NMR spin lattice relaxation times

We measured the ^{13}C spin–lattice relaxation times of ASP2 and ASP3 (Table 4). The ^{13}C T_1-values of ASP2 showed an important discrepancy between the backbone and side-chain sugar residues. Carbons of galacturonic acid and rhamnosyl units in the backbone presented shorter T_1 than carbons of galactose units located in the side chains.

The longest T_1 values corresponded to the terminal galactose unit of the side-chain, which is in agreement with additional degrees of freedom provided by its terminal side-chain character. Carbons of the four galactose units in the side-chain have an average T_1 value of 325 ms. Carbons of galacturonic blocks exhibited the shortest T_1 values (203 ms), which can be easily explained by a greater rigidity, due to inter-molecular interactions between galacturonic acid blocks, whereas glycosyl units involved in rhamnogalacturonan blocks presented longer T_1 values, 272 (Rha) and 307 ms (GalA) as they are in more flexible zones. The rhamnosyl residues presented shorter T_1 (272 ms) than the galacturonic units (307 ms), which is most likely caused by less segmental motion because of its location at branch point which makes it relax faster.

The ^{13}C T_1-values of ASP3 presented longer T_1 values than corresponding carbons of ASP2 sugar residues. This is in agreement with the fact that removal of lateral side-chains in ASP3 induced a lower molecular weight and thus a greater mobility of the sample. Furthermore one can notice a lower discrepancy in the ^{13}C T_1-values of ASP3, 304–376 ms compare to the T_1 values of backbone glycosyl units of ASP2 (203–307 ms). This phenomenon can also be explained by the presence of lateral side-chain in ASP2 which induced restricted mobility for the sugar residue to which the side-chain is attached, and thus less segmental motion for the backbone glycosyl residues.

3. Experimental

3.1. Materials

Small pieces of skin of fresh mature *Opuntia ficus-indica* prickly pear fruits, collected from an experimental station plantation (Marrakech, Morocco), were dried in a ventilated oven monitored at 50 °C. After drying, the samples were ground for a few minutes in a domestic coffee grinder and sieved to produce a homogeneous powder.

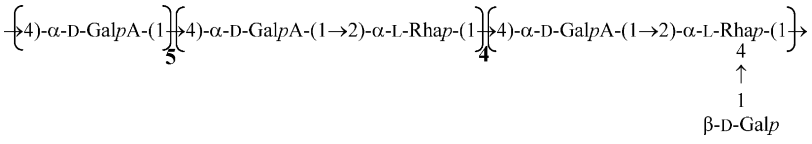

Fig. 6. Schematic structure of ASP3.

3.2. Analytical methods

Neutral sugars were analyzed, after H_2SO_4 hydrolysis, by GLC as their corresponding alditol acetates, using a Packard and Becker 417 instrument coupled to a Hewlett-Packard 3380 A integrator. Glass columns (3 mm×2 m) packed with 3% SP 2340 on Chromosorb W-AW DMCS (100–120 mesh), or 3% OV 17 on the same support were used. Uronic acid content was determined according to Blumenkrantz and Asboe-Hansen method (Blumenkrantz & Asboe-Hansen, 1973). The carboxyl groups of the D-galactosyluronic acid were reduced according to the method of Taylor & Conrad (1972). The carboxyl-reduced and the neutrals samples were methylated twice by the Hakomori procedure, as described by Jansson, Kenne, Liedgren, Lindberg, & Lonngren (1976). The partially methylated carbohydrates were then converted into their alditol acetates by successive treatments with $NaBH_4$ and pyridine-Ac_2O and analyzed on a fused-silica wide-bore column (30, 0.53 m) half bonded with SP-2380. Peak identification was based on retention times using partially methylated alditol acetate standards and confirmed by GLC by using a SP 2380 capillary column (0.32 mm) coupled to a Nermag R1010C mass spectrometer. Peak areas were corrected by using the molar response factors according to Sweet, Shapiro, & Albersheim (1975).

3.3. Preparation of cell wall material (CWM)

The skin powder was defatted by refluxing in a Soxhlet apparatus during 24 h with 38:62 (v/v) toluene–EtOH. The mucilaginous exudate was removed from defatted residue of skin by treatment with water (2×2 h) at room temperature in order to prepare CWM.

3.4. Isolation of acid soluble pectin (ASP)

Water and EDTA soluble pectic polysaccharides were sequentially extracted from CWM by water (2×2 h at 60 °C) and aqueous solution of EDTA 0.5% (2×2 h at 60 °C). The residue was then extracted twice by dilute acid HCl solution (0.05 M) at 80 °C for 1 h. All extracts, recovered by filtration, were precipitated with EtOH (four volumes) and the resulting precipitate was recovered by centrifugation. The precipitate was extensively washed with ethanol and then re-dissolved in water. The solution was dialyzed against distilled water (2×24 h) and freeze-dried to give acid soluble pectin (ASP).

3.5. Ion exchange chromatography

ASP was saponified with 0.1 M NaOH (overnight, N_2, 4 °C) in order to hydrolyze the acetyl and methyl esters still present. The solution was then acidified to pH 4–5 by addition of 0.5 M HCl solution and extensively dialyzed against distilled water and freeze-dried to give ASP^+ in H^+ form. Five hundred milligrams of ASP^+ was solubilized in 100 ml of 0.05 M phosphate buffer (pH 6.3) and loaded onto a column of DEAE-Trisacryl M (20×200 mm, phosphate form), which was eluted sequentially with phosphate buffer and then with a NaCl gradient (0.125, 0.25, 0.50 and 1 M) in the same buffer. Each fractions were then dialyzed against distilled water and freeze-dried to give five fractions in different amounts: buffer, 80 mg (ASP1*, 16%); 0.125 M, 75 mg (ASP2*, 15%); 0.25 M, 160 mg (ASP3*, 32%); 0.5 M, 40 mg (ASP4*, 8%) and 1 M, 35 mg (ASP5*, 7%). Fractionation procedure is summarized in Fig. 1.

3.6. Size exclusion chromatography

The major fractions (ASP1*, ASP2* and ASP3*) were purified by size-exclusion chromatography using Shodex-OHpak B-804 (7.5×500 mm) and Shodex-OHpak B-803 (7.5×500 mm) columns connected in series and differential refractive index detector. 0.05 M $NaNO_3$ solution was used as the eluent and the flow rate was kept at 1 ml/min. The salts were removed by dialysis and the solution freeze-dried, to give the purified fractions ASP1, ASP2 and ASP3.

3.7. NMR spectroscopy

^1H experiments were recorded on a Bruker Avance 400 spectrometer (operating frequency of 400.13 MHz). Samples were examined as solution in D_2O at 333 °K in 5 mm o.d. tube (internal acetone ^1H (CH_3) at 2.1 ppm relative to Me_4Si). ^{13}C NMR experiments were obtained on the same spectrometer (operating frequency: 100.57 MHz). Samples were recorded as solution in D_2O at 333 °K in 5 mm o.d. tube (internal acetone ^{13}C (CH_3) at 31.5 ppm relative to Me_4Si). Two dimensional spectra COSY, HMBC and HMQC were recorded using the standard Bruker procedures. COSY experiments were performed in the phase-sensitive mode. A 2048 (t_2)×512 (t_1)×2 data matrix was used with spectral widths of 2.5×2.5 kHz. A double quantum filter was used so that all signals could be phased to the pure absorption mode. $^{13}C^1$-H Shift-correlation experiments were performed using both the conventional Bruker sequence (with ^{13}C detection). A 2048 (t_2 ^1H) ×256 (t_1 ^{13}C) data matrix was used, with spectral widths of 2.5 kHz (^1H) ×2.5 kHz (^{13}C). Delay times were 0.7 s between scans and HMBC experiments were performed with a low-pass J-filter value of 145 Hz. A conventional ^{13}C-^1H dual probe was used and the 90° pulse lengths were 8 μs (^{13}C) and 16 μs (^1H). Measurements of the ^{13}C relaxation times T_1 were achieved according to inversion recovery method by varying the spin locking pulse delay $[(T-(180°)-t-(90°)-]_n$ with 12 t values ranging from 50 ms to 3 s.

Acknowledgements

We acknowledge the financial help of the Comité Mixte Franco-Marocain (Action Intégrée 236/SVS/00) and also thank Mrs Marie-France Marais and Dr Claude Bosso for their help in CPG-SM sugar analysis.

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