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A novel (1 → 4)-α-D-glucan isolated from the fruits of *Opuntia ficus indica* (L.) Miller

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ABSTRACT

A water-soluble polysaccharide (PS-1) was isolated from the fruits of *Opuntia ficus indica* (L.) Miller by hot water extraction, anion-exchange and gel-permeation chromatography (yield 167.5 mg/kg raw fruit; $[\alpha]_D{}^{16}$ ^{1} + 192° (c 1.0, H_2O); total neutral sugar content 96.60% w/w; weight–average molecular weight (M_w) ~360 kDa). Structural characterisation was performed by monosaccharide analysis and linkage analysis (methylation analysis, periodate oxidation and Smith degradation) on full and partial acid hydrolysates, followed by alditol acetylation, and product quantification by GC/GC–MS. Spectroscopic analysis (FT-IR and $^1H/^{13}C$ NMR) was also performed. Polysaccharide PS-1 was found to be an α-D-glucan with a (1 → 4)-linked α-D-Glc*p* backbone, with (1 → 6)-linked (1 → 4)-α-D-Glc*p* side chains, side chains being short in length with no additional branching, and a minimum branching of ~1 in every 9–11 backbone units. The distribution of side chains lengths and corresponding branching density requires further investigation.

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1. Introduction

Opuntia ficus indica (L.) Miller is a tropical or subtropical plant, which belongs to the Cactaceae family and is mainly used for fruit (commonly known as prickly pear and cactus pear, or less commonly as Barbary fig or Indian fig) and stem (commonly known as ‘nopal’) production. It grows wild in arid and semi-arid regions, where production of more succulent food plants is severely restricted, and is therefore routinely consumed in parts of North and Central America, southern Mediterranean countries, and parts of Africa (Gurbachan & Felker, 1998; Meyer & McLaughlin, 1981; Pimienta-Barrios, 1994; Yasseen, Barringer, & Splittstoesser, 1996). Under optimal conditions, annual fruit production can be as high as 50 t/ha, and detailed compositional (proximate) analysis has been performed on the stem, fruit (pulp, skin and seeds) (Dominguez-Lopez, 1995; El Kossori, Villaume, El Boustani, Sauvaire, & Mejean, 1998; Ramadan & Mörsel, 2003; Stintzing, Schieber, & Carle, 2001). Due to its high adaptation to the harsh desert environment and its different applications, the *Opuntia ficus indica* (L.) Miller fruit is an important and abundant potential raw material for industries in many countries (Stintzing & Carle, 2005). Within the last decade, prickly pears have become an important crop in the semi-arid lands of Libya, where they play a strategic role in subsistence agriculture. Efforts are currently being made to develop fruit production and find new food industry applications.

The fruits/stems/juice of *Opuntia ficus indica* (L.) Miller have shown antiulcerogenic (Galati, Monforte, Tripodo, d’Aquino, & Mondello, 2001; Galati et al., 2003), antioxidant (Galati et al., 2003; Gentile, Tesoriere, Allegra, Livrea, & D’Alessio, 2004; Kuti, 2004; Tesoriere, Butera, Pintaudi, Allegra, & Livrea, 2004; Tesoriere, Fazzari, Allegra, & Livrea, 2005; Zourgui, El Golli, Bouaziz, Bacha, & Hassen, 2008), anti-inflammatory/wound healing (Park & Chun, 2001; Park, Kahng, Lee, & Shin, 2001; Panico et al., 2007; Trombetta et al., 2006), anticancer (Zou et al., 2005), neuroprotective (Dok-Go et al., 2003), hepatoprotective (Galati et al., 2005; Hfaiedh et al., 2008; Ncibi, Othman, Akacha, Krifi, & Zourgui, 2008), antiproliferative (Sreekanth et al., 2007), and anti-genotoxicity (Zourgui, Ayed-Boussema, Ayed, Bacha, & Hassen, 2009) activity/effects, and may be used for the treatment of gastritis, hyperglycemia, arteriosclerosis, diabetes, and prostate hypertrophy (Agozzino, Avellone, Caraulo, Ferrugia, & Filizzola, 2005). Different parts of *Opuntia ficus indica* (L.) Miller are used in the traditional medicine of several countries, e.g. in Chinese medicine the fruit is used against inflammation, pain, and snake bite (Zou et al., 2005). The cladodes (branches or portions of the stem that replace leaves as the main photosynthetic plant organs, in this case nopal) have a high water

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content (95% w/w). They are rich in dietary fibre, carbohydrates, minerals, proteins, and vitamins, and are consumed as a vegetable.

A significant amount of scientific investigations have been performed on extraction and analysis of polysaccharides from cladodes (Amin, Awad, & El-Sayed, 1970; Majdoub et al., 2001; McGarvie & Parolis, 1981a, 1981b; Paulsen & Lund, 1979; Trachtenberg & Mayer, 1981), fruit pulp (Matsuhiro, Lillo, Sáenz, Urzúa, & Zárate, 2006), and fruit peel/skin (Forni, Penci, & Polessello, 1994; Habibi, Heyraud, Mahrouz, & Vignon, 2004a, 2004b) of *Opuntia ficus indica*, particularly from mucilage, and their biophysical properties (Trachtenberg & Mayer, 1982). Such mucilages (extracted by a variety of methods, e.g. cold water, hot water, ethanol, and acid) contain a complex mixture of glycoproteins and heteropolysaccharides, which have been fractionated and characterised (at least in part). Identified polysaccharides include pectin-type material (galacturonan, some with low methoxyl/high acetyl content), arabinogalactans, rhamnogalactans, arabinoxylans, and rhamnogalacturonans (with arabinan, galactan and/or arabinogalactan side-chains).

The only evidence for the presence of a neutral glucan was observed by Paulsen and Lund (1979), who identified one neutral and two acidic polysaccharide-containing fractions from the cladodes of *Opuntia ficus indica* cv "Burbank's Spineless". From the neutral fraction a very small quantity of glucan with a molecular weight of $\sim 1 \times 10^6$ Da was isolated, but in insufficient quantity to perform more detailed structural characterisation. This article presents the isolation and structural characterisation of a novel $(1 \rightarrow 4)$-α-D-glucan from the fruits of *Opuntia ficus indica* (L.) Miller.

2. Materials and methods

2.1. Materials

Natural fruits from *Opuntia ficus indica* (L.) Miller were utilised in this study (~5 kg). The peeled dried (hot air, 40–60 °C) fruits were uniform in shape, size, and colour and were obtained from the Ejfarah region of Libya at weekly intervals during the period of mid July to mid August. The peeled dried fruits were cut into small ellipsoidal pieces (~7 cm length). The *Opuntia ficus indica* (L.) Miller fruits selected for this study were at the full stage of ripeness/maturity, since these have been previously shown to contain greater amounts of polysaccharides (Ishurd, Sun, Xiao, Ashour, & Pan, 2002).

2.2. General methods

Specific rotation measurements were performed at 20 ± 1 °C using an automatic polarimeter (Model WZZ-2B, China). UV–vis absorption spectra were recorded using a Shimadzu MPS-2000 spectrophotometer. FT-IR spectra (KBr discs) were recorded using a Nicolet 360 FT-IR spectrometer. Elemental analysis (C, H and N) was conducted on an Elementar Vario EL III instrument. Total neutral carbohydrate content was determined by the phenol–sulfuric acid method as D-glucose equivalents (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined according to the *m*-hydroxydiphenyl colorimetric method, in which neutral sugars do not interfere (Filisetti-Cozzi & Carpita, 1991). Protein content was determined by the method of Bradford (1976).

2.3. Polysaccharide extraction and fractionation

Cut dried fruits (4.0 kg) were firstly refluxed with methanol to remove lipophilic compounds, and then successively boiled in distilled water for 6 h at 100 °C. After filtration to remove debris fragments, the filtrate was concentrated by rotary evaporation. Protein was removed with the Sevag method (Alam & Gupta, 1986). The crude polysaccharide fraction (26.0 g) was obtained through precipitation with 3 vol. of ethanol and desiccation in vacuo. The crude polysaccharide precipitate was redissolved in distilled water and applied to a DEAE-Sephadex A-25 column (90 × 5 cm). The column was first eluted with distilled water followed by 0.1, 0.3 and 0.5 M sodium chloride, respectively, and fractions collected accordingly, and their neutral carbohydrate and uronic acid contents confirmed by phenol–sulfuric (DuBois et al., 1956) and *m*-hydroxydiphenyl (Filisetti-Cozzi & Carpita, 1991) methods, respectively. Based on the results of this assay, the neutral carbohydrate-positive fraction eluted with water was further purified/fractionated on a Sephadex G-200 column (100 × 5 cm) eluted with 0.1 M sodium chloride, yielding three further fractions. The neutral carbohydrate and uronic acid contents of these fractions were confirmed by phenol–sulfuric (DuBois et al., 1956) and *m*-hydroxydiphenyl (Filisetti-Cozzi & Carpita, 1991) methods, respectively, and the uronic acid-free neutral carbohydrate-positive fraction was collected, dialysed and lyophilised to obtain a purified polysaccharide (PS-1, 670 mg, 2.58% w/w of the crude polysaccharide).

2.4. Purified neutral polysaccharide characterisation

2.4.1. Homogeneity and molecular weight profile

PS-1 homogeneity and molecular weight profile was determined using a Waters AMLC system (717 plus autosampler and 600 delta AMLC pump) equipped with a TSKgel 4000 PW_{XL} column (7.8 × 300 mm) and a Waters 2414 Refractive Index Detector (RID). PS-1 solution (0.5% w/v, 20 μL sample injections) was analysed using a 0.05 M sodium chloride mobile phase with a flow rate of 0.8 mL/min. The AMLC system was calibrated using T-series Dextran standards (T-10, T-40, T-70 and T-500 kDa standards).

2.4.2. Monosaccharide analysis

PS-1 was hydrolysed to its constituent monosaccharides using trifluoroacetic acid (TFA), and the released monosaccharides derivatised (alditol acetates), and analysed by gas chromatography (GC) (Li et al., 2003). Xylose, glucose, rhamnose, mannose, and galactose were also separately derivatised as standards, and acetyl inositol was utilised as an internal standard. Derivatised monosaccharides (from hydrolysed polysaccharide and monosaccharide standards) were analysed by GC using a GC-9A (Shimadzu, Japan), a capillary column (OV-225, China) and flame ionisation detection (FID). Nitrogen was used as the carrier gas (40 mL/min). The injector temperature was kept at 250 °C (split injection 70:1), and the detector temperature was maintained at 235 °C. GC station software was Zhida N2000 (Zhida, China).

2.4.3. Methylation analysis

Methylation analysis of PS-1 (9.0 mg) was performed using the method of Hakomori (1964). Methylated polysaccharide was treated with 90% formic acid (4 mL) for 14 h at 100 °C in a sealed tube. After removal of formic acid by rotary evaporation, residues were heated with 2 M trifluoroacetic acid (TFA, 2 mL) under the same conditions and the resulting hydrolysate rotary evaporated to dryness. The methylated sugars were reduced with sodium borohydride, acetylated with acetic anhydride, and analysed as alditol acetates by GC (as detailed previously). Identification of methylated sugars was performed by gas chromatography–mass spectrometry (GC–MS) and by relative GC retention time. Molar ratios were estimated from peak area and response factor analysis (Björndal, Lindberg, & Svensson, 1967).

2.4.4. Periodate oxidation and Smith degradation

PS-1 (9.0 mg) was dissolved in 0.014 M sodium metaperiodate (30 mL) and kept in the dark at 4 °C, and the absorption at 223 nm monitored at daily intervals. The reaction was completed after

Table 1
GC/GC–MS data for alditol acetate derivatives from methylated/hydrolysed neutral polysaccharide (PS-1) isolated from *Opuntia ficus indica* (L.) Miller fruit.

Component	T_R[a]	Molar ratio	M_s (m/z)	Linkage
2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl-D-glucitol	1.00	1.0	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	Glc*p*-(1 →
2,3,6-Tri-*O*-methyl-1,4,5,-tri-*O*-acetyl-D-glucitol	2.76	8.9	43, 45, 87, 99, 101, 113, 117, 233	→ 4)-Glc*p*-(1 →
2,3-Di-*O*-methyl-1,4,5,6-tetra-*O*-Acetyl-D-glucitol	4.91	0.9	43, 101, 117, 127, 261	→ 4,6)-Glc*p*-(1 →

[a]Retention time of alditol acetate relative to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-glucitol

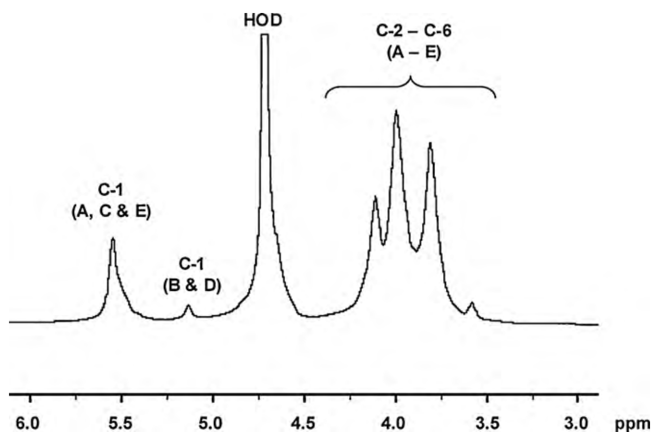

Fig. 1. ^{1}H NMR (500 MHz) spectrum of neutral polysaccharide (PS-1) isolated from *Opuntia ficus indica* (L.) Miller fruit.

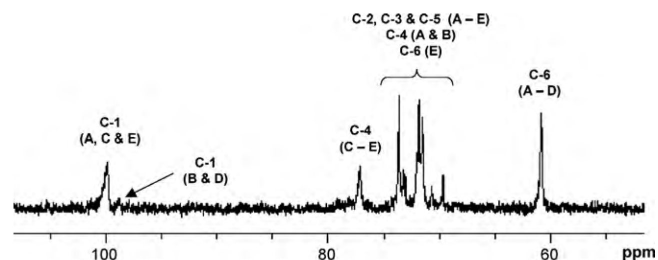

Fig. 2. ^{13}C NMR (500 MHz) spectrum of neutral polysaccharide (PS-1) isolated from *Opuntia ficus indica* (L.) Miller fruit.

120 h and ethylene glycol (0.2 mL) was added to the solution with stirring for 30 min to decompose excess reagent. Consumption of sodium metaperiodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Pazur, 1994) and formic acid production was determined by titration with 0.01 M sodium hydroxide. The reaction mixture was dialysed against distilled water for 2 days to remove small molecules, and the retained material (higher molecular weight material) was reduced with sodium borohydride (25 mg, 12 h). The pH was adjusted to 5.0, the solution was dialysed, and the retained material was lyophilised, and then hydrolysed with 2 M trifluoroacetic acid (TFA, 2 h at 110 °C). The resulting hydrolysate was analysed by GC (as detailed previously).

2.4.5. Partial hydrolysis

PS-1 was partially hydrolysed with aqueous trifluoroacetic acid solution (adjusted to pH 2.0, 20 mL, 18 h at 100 °C). After neutralisation with sodium hydroxide, a polymeric product was obtained by precipitation with excess ethanol from a small volume of water, and then retained on dialysis using a 2 kDa molecular weight cut-off membrane. The isolated (by lyophilisation) residue was subjected to periodate oxidation and Smith degradation as detailed above.

2.4.6. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded using a Bruker 500 MHz instrument. For ^{1}H NMR spectroscopy at 70 °C, PS-1 (8 mg) was repeatedly dissolved in deuterium oxide (D_2O, 99.99%, 5× 5 mL), and the solution repeatedly lyophilised. For ^{13}C NMR spectroscopy at 50°C, PS-1 was dissolved in deuterium oxide (D_2O, 99.99%, 1 mL, 64 mg/mL).

3. Results and discussion

3.1. Extraction, separation, isolation and basic analysis of purified neutral polysaccharide

The yield of the crude water-soluble polysaccharide (26.0 g) from the dried cut fruits (4.0 kg) of the *Opuntia ficus indica* (L.) Miller was 0.65% w/w. The crude polysaccharide was separated and sequentially purified through DEAE–Sephadex A-25 and Sephadex G-200, and a purified neutral polysaccharide (PS-1, 670 mg, ∼2.6% w/w of crude polysaccharide) was chromatographically separated and isolated (based upon spectrophotometric assessment of fractions for their total neutral carbohydrate and uronic acid contents).

PS-1 appeared as an off-white powder, $[\alpha]_D^{16\ 1}$ +192° (*c* 1.0, H_2O). The relatively high positive value of optical rotation suggested the dominating presence of α-D-form glycosidic linkages (Zhao, Kan, Li, & Chen, 2005). PS-1 had a negative response to the Bradford test and no absorption at 280 or 260 nm in its UV spectrum, indicating the absence of protein and nucleic acid material. Elemental analysis found it to be free of nitrogen, confirming it to be a neutral polysaccharide (i.e. confirming the absence of aminosugar residues, uronic acids having already been eliminated). The GPC profile showed a single and symmetrically sharp peak, indicating

Table 2
Potential monosaccharide residues present in the neutral polysaccharide (PS-1) isolated from *Opuntia ficus indica* (L.) Miller fruit.

Residue code	Monosaccharide residue	Description of monosaccharide residue
[A]	α-D-Glc*p*-(1 → 4)-	Main chain or side chain non-reducing end unit
[B]	α-D-Glc*p*-(1 → 6)-	Non-reducing end unit attached directly to branching point unit
[C]	→ 4)-α-D-Glc*p*-(1 → 4)-	Main chain or side chain unbranched unit
[D]	→ 4)-α-D-Glc*p*-(1 → 6)-	Side chain unbranched unit attached directly to branching point unit
[E]	→ 4,6)-α-D-Glc*p*-(1 →	Main chain branching unit
[F]	→ 4)-α-D-Glc[a]	Reducing end unit

[a]An equilibrium of various forms will be present (α, β, open chain, furanose, and pyranose).

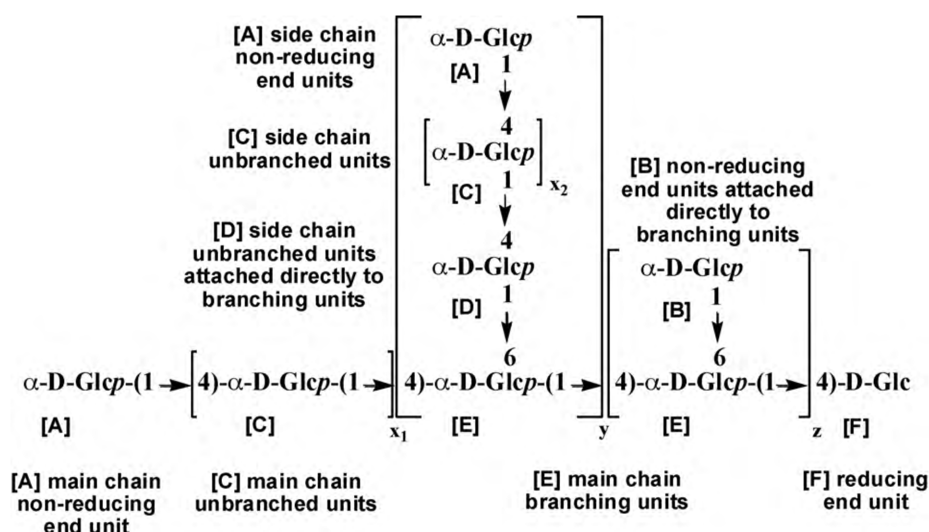

Fig. 3. Proposed structural features of the neutral (1 → 6)-branched, (1 → 4)-linked α-D-glucan polysaccharide (PS-1) isolated from *Opuntia ficus indica* (L.) Miller fruit.

that PS-1 was a homogeneous polysaccharide, with a weight average molecular weight (M_w) of ~360 kDa (from dextran T-series calibration).

3.2. Monosaccharide composition and linkage analysis of polysaccharide PS-1

The total neutral sugar content of PS-1 was determined to be 96.60% w/w, and it was composed of only glucose monomers, as detected by GC analysis of the alditol acetate derivatives of the components of the PS-1 hydrolysates. PS-1 did not contain any uronic acid material (as determined by spectrophotometric assay and confirmed by GC analysis of its hydrolysates).

The fully methylated product of PS-1 was hydrolysed with acid, converted into alditol acetates, and analysed by GC and GC–MS, yielding three types of glucitol derivative with a relative molar ratio of 1.0:8.9:0.9 (according to peak area), corresponding to Glc*p*-(1 →, → 4)–Glc*p*–(1 →, and → 4,6)-Glc*p*-(1 → residues, respectively (Table 1). Periodate oxidation of PS-1 resulted in periodate consumption and formic acid production of 1.04 and 0.10 mol, respectively, per residue, which is in relatively close agreement with the theoretical values (1.09 and 0.09 mol, respectively) calculated from the monosaccharide relative molar ratios from the methylation analysis results above.

Smith degradation of the periodate-oxidised PS–1 produced glycerol and erythritol with a molar ratio of 1.1:8.5 (determined by GC after conversion to the corresponding alditol acetates). Therefore terminal non-reducing Glc*p*-(1 → residues from the main backbone (and any side chains) and → 4,6)-Glc*p*-(1 → residues (main backbone and possibly side chain, branch points) amounted to a total of 11.5%, with → 4)–Glc*p*–(1 → residues (main chain and side chain unbranched units) amounting to 88.5%, respectively. This is slightly different from the equivalent values from methylation analysis (17.6 and 82.4%, respectively). PS-1 was partially hydrolysed with TFA, and after periodate oxidation and Smith degradation of the partial hydrolysate only erythritol was found, confirming that PS-1 was a polysaccharide with a (1 → 4)-linked backbone, with relatively acid labile side chains (i.e. of relatively short length and without further branching).

From these results there are six potential monosaccharide residue environments ([A]–[F]) present in the neutral polysaccharide PS-1 isolated from *Opuntia ficus indica* (L.) Miller fruit (Table 2).

3.3. Spectroscopic analysis of polysaccharide PS-1

3.3.1. FT-IR spectroscopic analysis

FT-IR spectroscopic analysis (data not shown) of PS-1 showed a strong band at 3420.19 cm^{-1} attributed to hydroxyl stretching vibrations; a band at 2930.83 cm^{-1} was due to C–H stretching vibrations; the broad band at 1636.52 cm^{-1} was due to bound water (Park, 1971); the band at 850.81 cm^{-1} was ascribed to α-type glycosidic linkages (Barker, Bourne, Stacey, & Whiffen, 1954), and the bands at 850.81 and 915.56 cm^{-1} are characteristic of (1 → 4)-α-D-glucans. Therefore, the FT-IR spectroscopy results, together with the high positive specific rotation, indicate the presence of α-D-glycosidic linkages in PS-1 (Bao, Duan, Fang, & Fang, 2001). The FT-IR absorptions at 1020.85, 1046.68 and 1154.57 cm^{-1} also indicate the α-D-pyranose form of the glucosyl residues.

3.3.2. NMR spectroscopic analysis

The potential monosaccharide residue environments ([A]–[F]) present in polysaccharide PS-1 (Table 2) have been used to assist with interpretation of ^{1}H and ^{13}C NMR spectra (Figs. 1 and 2, respectively), as detailed below. The ^{1}H NMR spectrum of PS-1 (Fig. 1) contains numerous peaks, which were assigned based upon the results discussed above (identification of residues, linkages, etc.) and comparison with ^{1}H NMR spectroscopic assignments for α-D-glucans by Papp-Szabó, Kanipes, Guerry, and Monteiro (2005) and van Leeuwen et al. (2008). The relatively broad unresolved signal at ~δ 5.5 ppm corresponds to the anomeric (C-1) protons of [A], [C] and [E] residues (Table 2), i.e. linked to the C-4 of the next residue along the chain (main backbone or side chain) forming a (1 → 4)-linkage between the α-D-Glc*p* residues. The smaller signal at ~δ 5.1 ppm corresponds to the anomeric (C-1) protons of [B] and [D] residues (Table 2), i.e. linked to the C-6 of the next residue, forming the (1 → 6)-linkage between the α-D-Glc*p* residues (the branch point linkage). The ratio of these peak areas (1:9) gives an indication of the relative branching ratio/density. The collection of broad unresolved peaks in the ~δ 3.5–4.5 ppm region are assigned to the remaining (C2–C-6) protons in residues [A]–[E] (Table 2). The large peak at ~δ 4.7 ppm corresponds to partially deuterated water (HOD).

The ^{13}C NMR spectrum of PS-1 (Fig. 2) contains numerous peaks, which were assigned based upon the results discussed above (identification of residues, linkages, etc.) and comparison with ^{13}C NMR spectroscopic assignments for α-D-glucans (specifically) by Seymour, Knapp, and Bishop (1976), Seymour, Knapp, Chen, Jeans,

and Bishop (1979) and Uzochukwu, Balogh, Loefler, and Ngoddy (2002). The relatively broad signal at ∼δ 100 ppm corresponds to the anomeric (C-1) carbons of [A], [C] and [E] residues (Table 2), i.e. linked to the C-4 of the next residue along the chain (main backbone or side chain) forming a (1 → 4)-linkage between the α-D-Glc*p* residues. The smaller signal at ∼δ 98 ppm corresponds to the anomeric (C-1) carbons of [B] and [D] residues (Table 2), i.e. linked to the C-6 of the next residue, forming the (1→6)-linkage between the α-D-Glc*p* residues (the branch point linkage). The signal at ∼δ 77 ppm corresponds to the C-4 carbons of residues [C]–[E] (Table 2), i.e. linked to the C-1 of the preceding residue along the chain (an internal link in the main backbone or side chain) forming a (1 → 4)-linkage between the α-D-Glc*p* residues. The relatively large signal at ∼δ 61 ppm corresponds to unlinked C-6 carbons of residues [A]–[D] (Table 2), i.e. not involved in branching points. The collection of poorly resolved peaks in the ∼δ 70–75 ppm region are assigned to the remaining carbon atoms, namely the C-2, C-3 and C-5 carbons in residues [A]–[E], the C-4 carbons in residues [A] and [B], and the C-6 carbons in residue [E] (Table 2).

It should be noted that no assignment of signals in both ^{1}H and ^{13}C NMR spectra have been discussed for the non-reducing ends (residue [F] in Table 2), since these are rarely observed due to their very low concentration with respect to other residue signals.

4. Conclusions

The neutral polysaccharide (PS-1), isolated from the fruits of *Opuntia ficus indica* (L.) Miller is a lightly branched α-D-glucan, with a weight average molecular weight (M_w) in the region of ∼360 kDa. The potential structural features of PS-1 polysaccharide are presented in Fig. 3. PS-1 is composed of a (1 → 4)-linked α-D-Glc*p* backbone, with (1 → 6)-linked α-D-Glc*p* side chains. Linkage analysis suggests a minimum branching of ∼1 in every 9–11 backbone units (∼1 in 9 from periodate oxidation/Smith degradation (ratio ∼1:8), and ∼1 in 11 from methylation analysis (ratio ∼1:10) results, and ∼1 in 10 from ^{1}H NMR analysis). This is if $y = 0$ (Fig. 3), i.e. side chains are composed of only terminal non-redusing α-D-Glc*p* units linked to the backbone via (1 → 6)-linkages. Periodate oxidation/Smith degradation of partial hydrolysates of PS-1 suggests that any side chains must be relatively acid labile (i.e. short in length, with no additional branching). The distribution of side chain lengths (and corresponding branching density) requires confirmation (by analysis of hydrolysates prepared using a debranching enzyme, such as pullulanase or isoamylase).

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