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Characterization of cell wall polysaccharides from the medicinal plant *Panax notoginseng*

Ying Zhu, Filomena Pettolino, Shaio-Lim Mau, Antony Bacic *

Cooperative Research Centre for Bioproducts, School of Botany, University of Melbourne, Parkville, Vic. 3010, Australia

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Abstract

Panax notoginseng is a commonly used medicinal plant in south-western China. Recent studies indicate that wall polysaccharides are responsible for some of the immunostimulatory activity. Fractionation of the *P. notoginseng* root powder alcohol insoluble residue (AIR) and its compositional analysis enabled us to deduce the polysaccharide and protein composition of the root cell walls. *P. notoginseng* walls are composed primarily of polysaccharide (approximately 97% w/w) and some protein. The polysaccharides include pectic polysaccharides (neutral Type I 4-galactan (21%), arabinan (5%), acidic rhamnogalacturonan I (RG I, 2%) and homogalacturonan (HGA, 24%), non-cellulosic polysaccharides (heteroxylan, 3%), xyloglucan (XG, 3%) and heteromannan (1%)) and cellulose (24%). The root AIR also contains Type II AG/AGPs (5% w/w) typically associated with the plasma membrane and extracellular matrix. Thus, *P. notoginseng* roots contain polysaccharides typical of Type I primary cell walls but are distinguished by their very high levels of Type I 4-galactans and low levels of XGs. The major amino acids in the AIR were Leu (14 mol%), Asx (16 mol%), Glx (10 mol%), Ala (9 mol%), Thr (9 mol%) and Val (9 mol%).

Keywords: Index–*Panax notoginseng*; Cell walls; Composition; Polysaccharides; Protein; Medicinal plant

1. Introduction

Panax notoginseng (Burk) F. H. Chen (Araliaceae) is a well-known medicinal plant found in south-western China (Gao et al., 1996; Lam and Ng, 2001). The AIR root fraction is traditionally used as a tonic and haemostatic agent. Extensive studies have been conducted on the low-molecular-weight biologically active components, especially saponins from this herb (Yang et al., 1983). However, some polysaccharide preparations of *P. notoginseng* roots have also been shown to have immunological activity. A Type II AG with a molecular weight of 1500 kDa, capable of activating the reticuloendothelial system, was isolated from roots of *P. notoginseng* (Ohtani et al., 1987). Gao et al. (1996) fractionated and analyzed the water- and weak alkali-soluble high-molecular-weight (HMW) fraction of *P. notoginseng* roots. The fractions were shown to be composed of the sugars Gal, Glc, Man, Ara and Xyl but these were neither quantified nor analyzed for linkage composition (Gao, 1996; Gao et al., 1996). Sasaki et al. (1990) patented an anti-tumor polysaccharide fraction mainly composed of α-(1,4)-glucan (presumably starch) from roots of *P. notoginseng*.

The lack of detailed structural information on the biologically active polysaccharide fraction from the AIR root fraction was the impetus for the current study. Therefore, we performed a sequential extraction of the polysaccharide-enriched cell wall fraction (AIR) from the roots of *P. notoginseng* in order to elucidate their composition. This fraction will contain both cell wall polysaccharides and proteins as well as cytoplasmic proteins and starch granules.

* Corresponding author. Tel.: +61383445041; fax: +61393471071.
E-mail address: abacic@unimelb.edu.au (A. Bacic).

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2. Results and discussion

2.1. Composition of P. notoginseng root AIR

The HMW components of roots collected as the alcohol insoluble residue (AIR) accounted for 88% w/w of *P. notoginseng* root powder on a dry weight basis. AIR had high starch content (51% w/w), which is common for storage tissues such as roots (Fry, 1988). The monosaccharide analysis of AIR (Table 1) showed it was predominantly composed of Glc (75 mol%), which is mainly derived from starch and cellulose (see below). It also had smaller amounts of Gal (11 mol%), GalA (11 mol%) and Ara (3 mol%). The relative proportions of the monosaccharides determined either as their alditol acetates (Table 1) or deduced from their partially methylated alditol acetates are similar (Table 2).

Linkage composition of AIR (Table 2) showed that it was mainly composed of 4-Glc*p* (72 mol%), indicating high amounts of starch and/or cellulose, together with a smaller content of 4-Gal*p*, representative of Type I 4-galactan, and 4-GalA*p*, typical for HGA. Small amounts of terminal- and 5-Ara*f* (2 and 1 mol%, respectively) were also present in AIR, indicating the presence of some arabinan.

AIR had a protein content of about 3% w/w (Table 3), comprising primarily of the amino acids Ala, Val, Leu, Asx (aspartic acid and/or asparagine), Glx (glutamic acid and/or glutamine) and Thr (Table 3). These amino acids are commonly found in various plant cell wall preparations (Kieliszewski et al., 1992). Although the levels of protein are typical of plant cell walls (Bacic et al., 1988) there is likely to be contamination from cytoplasmic proteins.

2.2. Sequential extraction of cell wall polymers

To analyze the composition of the cell wall-derived polysaccharides it was necessary to de-starch the preparation and to perform a sequential extraction of the walls (see Fig. 1) following established procedures (Fry, 1988). Following fractionation, the AIR fractions were analyzed by methylation analysis to determine linkage composition of the polysaccharides, and the protein composition was determined by amino acid analysis. Based on the characteristic linkage patterns established for cell wall polysaccharides (Carpita and Gibeaut, 1993; Shae et al., 1989; Sims and Bacic, 1995), the composition of *P. notoginseng* cell wall fractions was estimated from the mol% of the relevant sugar linkages (see Section 4) and are summarized in Table 4.

2.2.1. Phenol–acetic acid–water (PAW) extracts (Fr_{PAW})

The yield of Fr_{PAW} was 7% w/w. With a protein content of 29% w/w (Table 3), it accounted for the bulk of protein in AIR. The major amino acids were Ala (16 mol%), Asx (13 mol%), Gly (13 mol%) and Leu (12 mol%). Fr_{PAW} had a high proportion of Gal (71 mol%) and Ara (19 mol%), together with a small amount of GalA (6 mol%) and GlcA (4 mol%) (Table 1).

Linkage analysis showed that Fr_{PAW} had a large amount of Ara*f* (terminal) and Gal*p* residues (3-, 6- and 3,6-linked) (Table 2), typical for Type II AG/AGPs (Johnson et al., 2003). The high proportion of 3,6-Gal*p* and terminal Ara*f* residues are indicative of a highly branched core of 3,6-galactan terminating primarily in Ara*f* residues. The high content of Type II AG/AGP (76 mol%, Table 4) in Fr_{PAW} is consistent with the finding of water-extracted Type II AG/AGP fractions in *P. notoginseng* (Ohtani et al., 1987) and closely related species such as *P. ginseng* (Tomoda et al., 1993). Pectic polysaccharides were also found in the Fr_{PAW} fraction. Unbranched Type I 4-galactan (Fry, 1988) was present in Fr_{PAW} as indicated by the presence of 4-Galp residues (18 mol%). There was also a small amount of HGA (4 mol%).

Type II AG/AGPs are the most extensively studied polysaccharides of *P. notoginseng* and other *Panax* species (Gao et al., 1988; Kiyohara et al., 1994; Tomoda et al., 1993). Fr_{PAW} reacted with β-GlcY reagent, a highly sensitive reagent that specifically binds to AGPs (Willats and Knox, 2000; Yariv et al., 1967), indicating the presence of AGPs. Some Type II AGs are also reported to be associated with pectins to form pectic AGs (Yamada, 2000). Two Type II AG-containing fractions from the roots of *P. ginseng* have been found to

Table 1
Monosaccharide composition (mol%)[a] of *P. notoginseng* root AIR and fractions

Monosaccharide	AIR	Fr_{PAW}	Fr_{HW}	Fr_{SC}	Fr_{1MKOH}	Fr_{4MKOH}	Fr_{R}
Rha	tr	–	2	4	tr	2	–
Ara	3	19	5	16	7	11	–
Xyl	tr	–	–	–	38	16	–
Man	tr	–	–	–	–	tr	tr
Gal	11	71	21	51	42	47	5
Glc	75	–	2	–	2	10	88
GalA	11	6	63	26	8	14	2
GlcA	–	4	7	3	3	–	5

[a] Average of duplicate determinations; tr, trace (<0.5 mol%); –, not detected.

Table 2
Linkage composition of *P. notoginseng* root AIR and fractions

Sugar	Deduced glycosidic linkage	Linkage composition (mol%)[a]						
		Fraction						
		AIR	Fr_{PAW}	Fr_{HW}	Fr_{SC}	Fr_{1MKOH}	Fr_{4MKOH}	Fr_{R}
Rha*p*[c]	2-	–	–	1	1	–	–	–
	2,4-	tr	–	1	2	1	2	–
Ara*f*	Terminal	2	25	3	7	4	4	tr
	5-	1	2	2	6	4	3	–
	3,5-	–	tr	tr	3	1	3	–
Xyl*p*	Terminal	–	–	–	–	3	8	–
	2-	–	–	–	–	3	1	–
	4-	tr	–	–	tr	24	11	tr
	2,4-	–	–	–	–	3	1	–
Man*p*	4-	tr	–	–	–	1	2	2
Gal*p*	Terminal	–	2	2	3	3	4	tr
	3-	–	4	–	–	tr	–	–
	4-	14	18	20	47	38	28	3
	6-	–	6	–	–	–	–	–
	3,4-	–	–	tr	tr	–	–	–
	3,6-	tr	35	1	–	tr	–	–
Glc*p*	Terminal	–	–	tr	–	3	tr	3
	4-	72	–	tr	1	1	10	89
	2,4-	–	–	tr	–	–	–	tr
	4,6-	–	–	–	–	1	11	tr
GalA*p*	Terminal	–	–	6	2	tr	tr	–
	4-	11(3)[b]	4	58(22)	26(1)	9	12	1
GlcA*p*	Terminal	–	4	6	2	tr	tr	–
	4-	–	tr	tr	–	1	tr	2

[a] Average of duplicate determinations; tr, trace (<0.5 mol%); –, not detected.
[b] Numbers in brackets represent the mol% of methyl esterified 4-GalA*p*.
[c] 2-Rha*p* is deduced from 1,3,4,5-tetra-O acetyl, 2-O-Me deoxyhexitol, etc.

Table 3
Protein content and amino acid composition of *P. notoginseng* root AIR and fractions

Protein content (w/w%):	AIR	Fr_{PAW}	Fr_{HW}	Fr_{SC}	Fr_{1MKOH}	Fr_{4MKOH}	Fr_{R}
	3	29	1	2	15	8	tr
Amino acid (mol%)[a]							
Ala	9	16	14	21	14	17	40
Arg	tr	tr	–	–	tr	tr	–
Asx	16	13	13	5	11	8	–
Cys	2	1	14	3	–	–	–
Glx	10	8	9	3	8	7	–
Gly	6	13	16	20	11	10	–
His	tr	–	–	–	–	2	–
Hyp	1	tr	–	–	1	–	–
Ileu	6	6	4	4	7	8	12
Leu	14	12	7	12	14	17	31
Lys	1	–	–	7	–	–	–
Met	1	1	–	–	5	1	–
Phe	6	5	–	2	4	4	–
Pro	3	5	–	2	4	6	–
Ser	5	4	6	4	5	4	–
Thr	9	6	9	5	3	3	–
Tyr	2	2	–	1	2	2	–
Val	9	8	8	11	11	11	17

[a] Average of duplicate determinations; tr, trace (<0.5 mol%); –, not detected.

have relatively high contents of β-(1,4)-linked galactans (Tomoda et al., 1993), but whether these are covalently associated remains to be determined.

2.2.2. Water extracts (Fr_{HW})

Fr_{HW} accounted for 14% w/w of AIR and was essentially polysaccharide (99% w/w) that contained

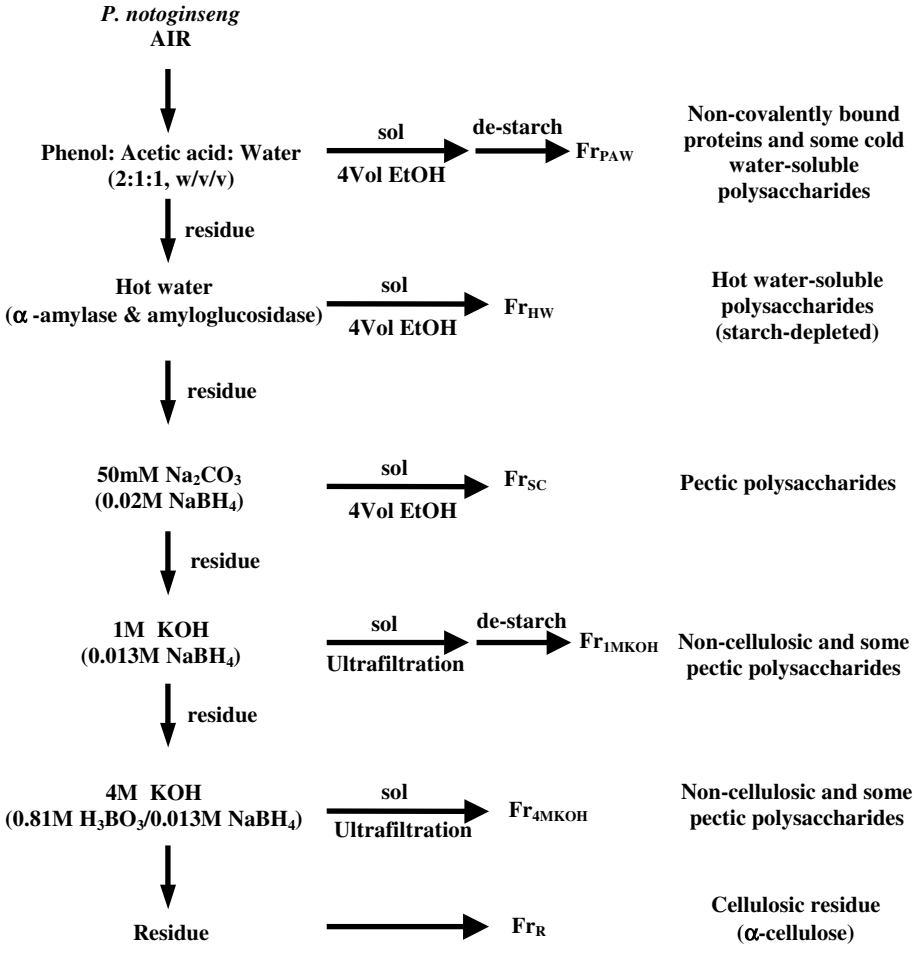

Fig. 1. Scheme for the sequential extraction of high-molecular-weight material from *P. notoginseng* alcohol insoluble residue (AIR).

Table 4
Estimated relative proportions of polysaccharides present in *P. notoginseng* root wall fractions

Polysaccharide	Relative proportion (%)[a]					
	Fraction					
	Fr_{PAW}	Fr_{HW}	Fr_{SC}	Fr_{1MKOH}	Fr_{4MKOH}	Fr_R
Yield:[b]	7	14	7	2	5	13
Pectins						
Arabinan	2	4	16	6	9	–
4-Galactan	18	22	50	37	28	3
RG-I	–	3	5	3	4	–
HGA	4	64	27	9	12	1
Type II AG						
II AG	76	2	–	–	–	–
Non-cellulosic polysaccharides						
Heteroxylan	–	–	–	31	13	–
XG	–	–	–	8	30	–
Mannan	–	–	–	1	2	2
Cellulose						
Cellulose	–	–	–	–	–	90
Undefined						
Undefined	–	6	2	5	2	4

[a] Calculated as sum of mol% of individual sugar linkages as described in Section 4.
[b] The yield of the fraction was based on the recovered weight of each fraction. The recovery only adds up to 48% since starch was removed during the hot water/amylase treatment. –, not detected.

predominantly GalA (63 mol%) and Gal (21 mol%) and small amounts of Rha (2 mol%), Ara (5 mol%), Glc (2 mol%) and GlcA (7 mol%) (Table 1). Linkage analysis showed Fr_{HW} mainly contained pectins (Tables 2 and 4). It had a high content of unsubstituted 4-Gal*p*A, suggesting the presence of HGA (64 mol%) (Table 4). The HGA of Fr_{HW} had a high degree of methyl esterification, with nearly 40% of the carboxyl groups being methyl esterified (22 of 58 mol%) (Table 2). There was a small amount of 2-Rha*p* and 2,4-Rha*p*, suggesting the presence of RGs (Ridley et al., 2001). Several unusual or unusually modified glycosyl residues, such as apiose and aceryl acid, 3-deoxy-D-manno-2-octulosonic acid (Kdo) and 3-deoxy-D-lyxo-2-heptulosaric acid (Dha), which are characteristic of RG-II, have been reported for pectin preparations (Fry, 1988; Ridley et al., 2001). However, the method for linkage analysis used here is known to destroy Kdo and Dha (Strasser and Amado, 2002). Although they can be determined by a modified method (York et al., 1985), this was not attempted due to the low content of RGs in Fr_{HW}. We assume that RG-I is the predominant RG in Fr_{HW}, since in most dicot cell walls, RG-I is more abundant than RG-II (Carpita and McCann, 2002). The linear backbone of RG-I is composed of the repeating disaccharide [–4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p*-(1,–] (Ridley et al., 2001). The degree of branching of the backbone can be deduced from the ratio of 2-Rha*p* to 2,4-Rha*p* since the side chains of RG-I are attached to C-4 of the 2-Rhap residues (McNeil et al., 1984; Zablackis et al., 1995). Approximately half of the Rha*p* residues in Fr_{HW} fraction are branched (Table 2). There was a substantial amount of unbranched 4-linked galactan, as indicated by a relatively high proportion of 4-Gal*p* (20%) and arabinan, as evidenced by the presence of 5- and 3,5-Ara*f*. A proportion of the arabinan (4 mol%) and 4-linked galactan (22 mol%) (Table 4) present in Fr_{HW} might form the side chains of RG-I. Ohtani et al. (1989) reported the isolation of two polysaccharide fractions rich in 4-galactan from *P. japonicus*. 4-Linked galactans were also found in pectic fractions of flax fibers (Goubet et al., 1995), the cotyledons of *Lupinus angustifolius* (Cheetham et al., 1993), as well as carrot root cell walls (Massiot et al., 1988a,b). Fr_{HW} contained only a small amount of protein (1% w/w) that was rich in Ala (14 mol%), Asx (13 mol%), Cys (14 mol%) and Gly (16 mol%) (Table 3).

2.2.3. Na_2CO_3 extracts (Fr_{SC})

Fr_{SC} accounted for 7% w/w of root AIR and was mostly polysaccharide (98% w/w) that had high proportions of Gal (51 mol%), GalA (26 mol%) and Ara (16 mol%) (Table 1). It also contained low levels of Rha (4 mol%) and GlcA (3 mol%). Fr_{SC} was composed of 26% of 4-Gal*p*A and 47% of 4-Gal*p* (Table 2). Thus, like Fr_{HW}, Fr_{SC} contained HGA (27 mol%) and RG-I (5 mol%) (Table 4). However, the HGA in Fr_{SC} had a low degree of methyl esterification (1 of 26 mol%). This is consistent with the fact that the pectins with relatively low DE are more likely to become cross-linked via Ca^{2+} bridges and therefore harder to be extracted with water (Fry, 1988). The high proportion of 4-Gal*p* indicates the existence of unbranched 4-galactan (50 mol%, Table 4). Fr_{SC} also contained smaller proportions of terminal, 3-, and 3,5-linked Ara*f* residues arising from 3,5-arabinan. Fr_{SC} contained similar types of pectic polysaccharide as Fr_{HW} except that pectins solubilized by aqueous Na_2CO_3 are more tightly associated with the cell wall (Seymour et al., 1990). Similar to Fr_{HW}, Fr_{SC} contained only a small amount of protein (2% w/w) that is rich in Ala (21 mol%), Gly (20 mol%), Leu (12 mol%) and Val (11 mol%) (Table 3).

2.2.4. Alkali extracts (1 and 4 M KOH) (Fr_{1MKOH} and Fr_{4MKOH}, respectively)

Although starch was absent from Fr_{HW} and Fr_{SC} fractions, it was found in the 1 M KOH extract, suggesting an incomplete removal of starch by the enzymes. Since an excessive amount of enzyme was used during the hot water treatment, it is unlikely that the incomplete removal of starch was a result of insufficient enzyme used. It is known that the effectiveness of enzymatic degradation of starch is dependent on the degree of gelatinization of starch granules (Zobel and Stephen, 1995). During the gelatinization process, the hydrogen bonds are broken, granules take up water and swelling occurs, thus allowing the enzymes to access the starch chains (Zobel and Stephen, 1995). It is possible that in the present study, during the enzyme treatment, the starch granules were only partially gelatinized, resulting in an incomplete enzymatic breakdown. The intact starch granules remained insoluble in hot water and weak alkali. Since the treatment of strong alkali can increase the swelling of starch granules (Chen and Jane, 1994), the remaining starch was then solubilized by 1 M KOH. In order to overcome this starch contamination, the 1 M KOH extract was re-treated with starch-degrading enzymes to give a starch-free fraction, Fr_{1MKOH}.

Fr_{1MKOH} accounted for only 2% w/w of the root AIR and was mainly polysaccharide (85% w/w) that had high proportions of Xyl (38 mol%) and Gal (42 mol%), together with low levels of GalA (8 mol%), Ara (7 mol%), Glc (2 mol%) and GlcA (3 mol%) (Table 1). A high proportion of 4-Gal (38 mol%) (Table 2) was present in Fr_{1MKOH}, indicating a high 4-galactan content. In addition, heteroxylans (31 mol%) (Table 4) were present in Fr_{1MKOH} as indicated by 4-Xyl*p* and 2,4-Xyl*p* (24 and 3 mol%, respectively). The heteroxylan might carry either single terminal-Ara*f* and/or -Glc*p*A residues as substitutions on the 4-Xyl*p* backbone (Bacic et al., 1988). Fr_{1MKOH} also contained a small amount of HGA as indicated by 4-Gal*p*A (9 mol%), and a low level

of 4-Man*p* (1 mol%), indicating the presence of some heteromannan. This fraction contained a high proportion of protein (15% w/w) that was rich in the amino acids Ala (14 mol%), Asx (11 mol%), Gly (11 mol%), Leu (14 mol%) and Val (11 mol%) (Table 3).

The yield of Fr_{4MKOH} was 5% w/w and was predominantly polysaccharide (92% w/w) composed primarily of Gal (47 mol%), with smaller but significant amounts of Xyl (16 mol%), GalA (14 mol%), Ara (11 mol%), Glc (10 mol%) and some Rha (2 mol%) (Table 1). Besides a high content of 4-galactan as indicated by 28 mol% of 4-Gal*p*, Fr_{4MKOH} also contained high proportions of 4,6-Glc*p* (11 mol%) and t-Xyl*p* (8 mol%) (Table 2), indicative of the presence of significant levels of XG (30 mol%) (Table 4). Like Fr_{1MKOH}, Fr_{4MKOH} also had significant amounts of 4-Xyl*p* (11 mol%), typical for heteroxylan; as well as 5- and 3,5-Ara*f* typical for arabinan. In addition, HGA and RGI were present in Fr_{4MKOH} as indicated by the presence of 4-GalA*p* (12 mol%) and 2,4-Rha*p* (2 mol%). The presence of pectic polysaccharides such as HGA and 4-galactan in KOH extracts suggests that these polysaccharides are tightly associated with other cell wall components, possibly through covalent (e.g., ester) linkage.

Strong alkali extracts of typical Type I walls usually contain predominantly XGs and some heteroxylans, together with smaller amounts of pectins (Zablackis et al., 1995). Although the two KOH-solubilized fractions of *P. notoginseng* root cell walls had substantial XG and heteroxylan content, 4-galactan is the most abundant type of polysaccharide. The presence of such high contents of 4-galactan in strong alkali extracts is not commonly found in sequential extracts of plant cell walls. 4-Galactans were found in flax cell walls however they were essentially absent from the 4 M KOH extract of these walls (Gorshkova et al., 1996, 1998). High levels of unbranched 4-galactan are also a major component of the cotyledon cell walls of white lupin (*Lupinus albus*) (Carre et al., 1985) where they are extracted with the pectic fraction. Relatively high levels of Gal*p* also appear in the "hemicellulose A" and "hemicellulose B" fractions of these walls (Carre et al., 1985). It is likely that some of the 4-galactans of *P. notoginseng* are either physically entrapped or chemically (covalently or through hydrogen bonding) bonded to other cell wall components so that they could only be solubilized with strong alkali. The Fr_{4MKOH} had some protein (8% w/w) that was rich in the amino acids Ala (17 mol%), Gly (10 mol%), Leu (17 mol%) and Val (11 mol%) (Table 3).

2.2.5. Cellulose residue (Fr_R)

Thirteen percent of the starting root material (AIR) of *P. notoginseng* remained after the sequential extractions. Fr_R was essentially composed of polysaccharide. The predominant sugar was Glc (88 mol%), together with small amounts of Gal (5 mol%), GlcA (5 mol%) and GalA (2 mol%) (Table 1). Linkage analysis showed that Fr_R was mainly composed of the load bearing cell wall polysaccharide cellulose, as indicated by 89 mol% of 4-Glc*p* (Table 2). This cell wall residue contained only minor amounts of 4-Gal*p*, 4-Man*p*, 4-Gal*p*A and 4-Glc*p*A (Table 2), indicating that small amounts of the non- cellulosic and pectic polysaccharides were not completely solubilized during the sequential extraction. Incomplete extraction of non-cellulosic and pectic cell wall polysaccharides is commonly observed, for example, in the extraction of Arabidopsis (Zablackis et al., 1995) and sycamore cell walls (Koller et al., 1991). This might reflect either the heterogeneity in the bonding of the polysaccharides to other components of the cell walls (Zablackis et al., 1995) or physical entrapment during assembly of walls. Fr_R only had a trace of protein, indicating that most of the proteins of *P. notoginseng* cell wall have been removed during the sequential extractions.

3. Conclusions

The polysaccharide composition of the cell walls of *P. notoginseng* roots was deduced by recalculating the data to reflect the recovery of each type of polysaccharide in each fraction (excluding starch content, which accounted for 51 mol% of AIR) (see Table 5). Thus, the cell walls of *P. notoginseng* roots are composed of cellulose (24%), pectic polysaccharides (52%), including HGAs and RGs together with Type I 4-galactans and arabinans, and non-cellulosic polysaccharides (7%), including heteroxylans, XGs and heteromannans, and Type II AG/AGPs (10%). A small proportion of the residues (7%) could not be assigned but would be expected to arise from pectic and non-cellulosic matrix polysaccharides. Compared with a typical Type I cell wall from *Arabidopsis* leaves (Zablackis et al., 1995) which is composed of pectins (42%), non-cellulosic polysaccharide (24%) and cellulose (14%), the most significant difference of *P. notoginseng* cell walls is their high content of 4-galactan and the low content of XG and RG I. The cellulose content also differs, but highly variable levels of cellulose are common between different types of plant cell walls (Bacic et al., 1988).

As a root storage tissue, carrot root cell walls show a higher similarity to *P. notoginseng* root than *Arabidopsis* leaf cell walls (see Table 5). Cell walls prepared from carrot roots are also rich in 4-Galp residues (Massiot et al., 1988a,b). They contain cellulose (~25%), non-cellulosic polysaccharides (10–15%) such as XGs, heteroxylans and heteromannans, and pectic polysaccharides with associated neutral arabinose- and galactose-containing polysaccharides (45–50%). The levels of pectin in both carrot and *P. notoginseng* cell walls

Table 5
Comparison of *P. notoginseng* roots (deduced from cell wall polysaccharide composition) with *Arabidopsis* leaf cell walls and carrot root cell walls

Polysaccharide	Relative proportion (%)		
	Plant and tissue		
	P. notoginseng roots	*Arabidopsis* leaf[a]	*Daucus carota. roots*[b]
Pectins			
Arabinan	5	–	~5
4-Galactan	21	–	~7[c]
RG I	2	11	~2
RG II	–	8	–
HGA	24	23	~35
Type II AG			
Type II AG	10	–	–
Non-cellulosic polysaccharides			
Heteroxylans	3	4	~3
Xyloglucans	3	20	~6
Mannans	1	–	~6
Cellulose			
Cellulose	24	14	~25
Undefined			
Undefined	7	20	~11

[a] Data are taken from Zablackis et al. (1995).
[b] Data are calculated from reported linkage composition (Massiot et al., 1988a,b).
[c] Includes Type I AG; –, not detected.

are similar (52% vs. 49%), while the latter has a higher proportion of 4-galactan and a lower content of HGA.

P. notoginseng AIR had a protein content of about 3% w/w, comprised primarily of the amino acids Leu (14 mol%), Asx (16 mol%), Glx (10 mol%), Ala (9 mol%), Thr (9 mol%) and Val (9 mol%). The amino acid composition of *P. notoginseng* root AIR was similar to that of a typical Type I cell wall, Arabidopsis leaf cell wall (Zablackis et al., 1995). *P. notoginseng* AIR Hyp content (1 mol%), derived from cell wall proteins (Johnson et al., 2003), is similar to that of *Arabidopsis* cell wall (1 mol%), although compared to other plant tissues, for example, tobacco suspension cultured cells (21 mol%; Johnson et al., 2003), *P. notoginseng* roots are relatively low in Hyp.

4. Experimental

4.1. Material

P. notoginseng dried root powder was a gift from the Yunnan Chinese Medicine Corporation. The root powder was ground with a mortar and pestle to pass through a 100 μm screen.

4.2. Preparation of the alcohol insoluble residue (AIR)

P. notoginseng root powder (10 g) was sonicated with 2×100 mL of 80% (v/v) MeOH for 30 min at RT and the extracts centrifuged (3373*g*, 5 min). The supernatants were pooled, concentrated at 50 °C by rotary evaporation under reduced pressure, and dried under vacuum to give the total LMW fraction enriched in saponins (Yoshikawa et al., 1997), which was not analyzed in the present study. The HMW pellet was dried under vacuum to give the alcohol insoluble residue, AIR.

4.3. Sequential extraction of the AIR

P. notoginseng root AIR was sequentially extracted according to the scheme in Fig. 1. AIR (10 g) was successively extracted with 2×70 mL aliquots of PAW (20 mL of glacial acetic acid and 50 mL of 80% (w/v) aqueous phenol), for 6 h at RT for the first extraction, and overnight at 4 °C for the second extraction. The soluble extracts were neutralized with 4 M NaOH while the residue was washed with ice-cold water until neutral. The washes were neutralized and combined with the extract, all of which was precipitated with 4 vols. of EtOH at 4 °C overnight. Precipitated polymers were resuspended in water, de-starched with heat stable α-amylase (from *Bacillus licheniformis*, Sigma #A3306) followed by amyloglucosidase (Megazyme), and freeze–dried to give the PAW-soluble fraction (Fr_{PAW}).

The residue from PAW extraction was suspended in 700 mL of Mops buffer (50 mM, pH 7.0) and placed in a boiling water bath (100 °C) for 30 min. α-Amylase (30,000 U) was added to the suspension and incubated with occasional stirring in a boiling water bath for 15 min. The suspension was then transferred to a 50 °C water bath, temperature equilibrated, and amyloglucosidase (2000 U) added and the mixture incubated for 30 min. The extract was then concentrated to 100 mL by rotary evaporation and precipitated with 4 vols. of EtOH. The precipitated HMW pellet was resuspended in water and freeze–dried to give the hot water-soluble fraction (Fr_{HW}).

The residue from hot water extraction was successively extracted with 2×400 mL of 50 mM Na_2CO_3 (supplemented with 20 mM of $NaBH_4$ and 0.2% (w/v) of NaN_3), overnight at 4 °C for the first extraction and for 6 h at RT for the second extraction. The extracts were adjusted to pH 5.0 with glacial acetic acid, combined, concentrated to 100 mL by rotary evaporation and then precipitated with 4 vols. of EtOH. The precipitated HMW polymers were resuspended in water and freeze–dried to give the weak alkali-soluble fraction (Fr_{SC}).

The residue from 50 mM Na_2CO_3 extraction was extracted with 300 mL of 1 M KOH followed by 300 mL of 4 M KOH overnight at RT. The alkali was

supplemented with 0.013 M $NaBH_4$ and 0.2% NaN_3. Boric acid (0.81 M) was also included in the 4 M KOH extraction to aid the extraction of heteromannans (Fry, 1988). The extracts were neutralized with glacial acetic acid, de-salted by ultrafiltration and freeze–dried to give the 1 M and 4 M KOH soluble fractions Fr_{1MKOH} and Fr_{4MKOH}, respectively. The 1 M KOH extract was further de-starched with α-amylase followed by amyloglucosidase and the HMW material recovered by precipitation with 4 vols. of EtOH as described previously.

The residue of the sequential alkali extractions was washed with water until the pH reached 7.0. It was then freeze–dried to give the "α-cellulose" residue fraction (Fr_R).

4.4. Analytical methods

Total carbohydrate content was determined by the $PhOH–H_2SO_4$ method (Dubois et al., 1956), using galactose as the standard. Protein was assayed by the Bio-Rad micro-assay method (Bradford, 1976) with bovine serum albumin as the standard. The amino acids obtained by acid hydrolysis of proteins were derivatized to the corresponding *N*,*O*-(*S*)-*tert*-butyldimethylsilyl (TBDMS) derivatives (Chaves das Neves and Vasconcelos, 1987). The AGP content was determined by radial gel diffusion in agarose-containing β-GlcY reagent [1,3,5-tri-(*p*-β-D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene] (van Holst and Clarke, 1985). β-GlcY reagent was prepared from *p*-aminophenyl-β-D-glucoside using the methods described by Ganjian and Basile (1997). Gum arabic was used as a standard. Starch content was determined enzymatically using a commercial starch assay kit (Megazyme).

Carboxyl-reduction of uronic acids was achieved by activating the uronic acids with carbodiimide (Taylor and Conrad, 1972) and reducing with $NaBD_4$, essentially using the method described by Kim and Carpita (1992) as modified by Sims and Bacic (1995). Constituent monosaccharides of carboxyl-reduced polysaccharides were converted to their corresponding alditol acetates (Blakeney et al., 1983; Harris et al., 1984; Kim and Carpita, 1992; Sims and Bacic, 1995) following Saeman acid-hydrolysis (Saeman et al., 1954). To determine their linkage and substitution patterns, carboxyl-reduced polysaccharide preparations were methylated with CD_3I according to the protocol described by Ciucanu and Kerek (1984). Permethylated alditol acetates were generated by trifluoroacetic acid hydrolysis, reduction with $NaBD_4$, and acetylation essentially following the protocol described by Harris et al. (1984). The alditol acetates and the permethylated alditol acetates were recovered in CH_2Cl_2 and separated on a Hewlett-Packard HP 6890 gas chromatograph equipped with an autosampler on a CPSil5 (25 m × 0.3 mm, Chrompack, Middelburg, The Netherlands) column. They were detected by electron impact ionization (70 eV) mass spectrometry on a Hewlett-Packard 5973 mass selective detector. Data were acquired in full scan mode (total ion chromatogram, TIC) to detect ions from *m*/*z* 100 to 700. Uronic acids were analyzed as their di-deuterated parent hexoses following a primary reduction with sodium borodeuteride then carbodiimide activation and reduction with either sodium borohydride to give total uronic acids, or sodium borodeuteride to give the proportion of esterified uronic acids (Kim and Carpita, 1992). The alditol acetates of the deuterium-reduced uronic acids co-eluted with those of their parent hexose during GC but were identified by mass spectrometry and quantified by the corresponding di-deutero bearing fragment ions of C-6.

4.5. Estimation of polysaccharide composition of *P. notoginseng* root cell wall fractions

To estimate the proportion of a certain polysaccharide present in an extracted fraction, the mol% of sugar linkage specifically attributed to that polysaccharide was summed (Carpita and Gibeaut, 1993; Shae et al., 1989; Sims and Bacic, 1995). For RG-I, the 2-Rha*p* and 2,4-Rha*p* together with an equal amount of 4-Gal*p*A were attributed to the RG-I backbone. The 4-Gal*p*A which is not accounted for in RG-I, together with all methyl esterified Gal*p*A and terminal Gal*p*A, was attributed to HGA. All 5-Ara*f* and 3,5-Ara*f* together with the equivalent branch terminal Ara*f* were assigned to arabinan; 4-Gal*p* was attributed to 4-galactan; while 4-Xyl*p*, 2,4-Xyl*p* and 2,3,4-Xyl*p* with the equivalent branch terminal Ara*f*/Glc*p*A were assigned to heteroxylans (AXs/GAXs). Type II AG was estimated by summing 3-, 6-, and 3,6-Gal*p* together with the equivalent branch terminal Ara*f* (Aspinall and Cottrell, 1971). For XG, 2-Xyl*p*, 4,6-Glc*p* with an equal amount of 4-Glc*p* together with equivalent branch terminal Xyl*p*, Fuc*p* and Gal*p* were added. The 4-Glc*p* not assigned to XG was assigned to cellulose. Those linkages that were not included in the above categories were attributed to unidentified polysaccharides.

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