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Characterisation of secreted polysaccharides and (glyco)proteins from suspension cultures of *Pyrus communis*

Judith M. Webster [1], David Oxley [1], Filomena A. Pettolino, Antony Bacic *

CRC for Bioproducts, School of Botany, University of Melbourne, Victoria 3010, Australia

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Abstract

High molecular weight material recovered from the culture filtrate of cell suspension cultured *Pyrus communis* was composed of 81% carbohydrate, 13% protein and 5% inorganic material. This material was separated into three fractions (one neutral (Fraction A) and two acidic (Fractions B and C)), by anion-exchange chromatography on DEAE-Sepharose CL-6B using a gradient of imidazole–HCl at pH 7.0. The monosaccharide and linkage composition of each fraction was determined after carboxyl reduction of uronic acid residues. From the combined results of the carbohydrate analyses, we conclude that the high molecular weight extracellular material consists of three major and two minor polysaccharides: a (fucogalacto)xyloglucan (36%) in the unbound neutral Fraction A; a type II arabinogalactan (as an arabinogalactan-protein, 29%) and an acidic (glucurono)arabinoxylan (2%) in Fraction B; and a galacturonan (33%) and a trace of heteromannan in Fraction C. The main amino acids in the proteins were Glx, Thr, Ser, Hyp/Pro and Gly. Further separation of Fraction B by solvent partition, SDS–PAGE and analysis by LC-MS/MS identified the major proteins as two chitanases, two thaumatin-like proteins, a β-1,3-glucanase, an extracellular dermal glycoprotein and a pathogenesis-related protein.

Keywords: *Pyrus*; Extracellular polysaccharides; (Glyco)proteins; Cell-suspension cultures; AGP; Mass spectrometry

1. Introduction

Due to the commercial importance of pear (*Pyrus communis*) fruit, the composition of the fruit, the juice and the constituent cell wall polysaccharides have been extensively studied (Pilando and Wrolstad, 1992; Yoshioka et al., 1992; Ahmed and Labavitch, 1980a; Ferreira et al., 2001), especially with regard to the changes that occur during fruit ripening (Martin-Cabrejas et al., 1994; Ahmed and Labavitch, 1980b; Murayama et al., 2002; Hiwasa et al., 2004). Several pear cell wall modifying enzymes and proteins have also been described including galactosidases (Fonseca et al., 2005; Mwaniki et al., 2005; Tateishi and Nagashima, 2005; Sekine et al., 2006) polygalacturonases (Hiwasa et al., 2004; Fonseca et al., 2005; Sekine et al., 2006), pectin methylesterases (Sekine et al., 2006), xylosidase (Fonseca et al., 2005), *endo*-glucanases (Fonseca et al., 2005; Sekine et al., 2006), arabinofuranosidases (Tateishi et al., 2005; Sekine et al., 2006), endotransglycosylase (Hiwasa et al., 2004) and alpha-expansins (Hiwasa et al., 2003; Fonseca et al., 2005). Suspension cultured cells initiated from the flesh of pear fruit have been used as a model system to study a range of physiological processes including cellular senescence, viability and hormone regulation (Tong et al., 1986; Pech and Romani, 1978, 1979; Puschmann and Romani, 1983; Balague et al., 1982; Codron et al., 1979; Puschmann et al., 1985; de Boucaud et al., 1990), pectic oligomer elicitation, ethylene biosynthesis (Campbell and Labavitch, 1991) and cold acclimation (Wallner et al., 1986).

Wallner et al. (1986) showed that during the cell cycle of suspension cultured pear cells, particularly during the logarithmic phase, extracellular polymers (ECP) are released

* Corresponding author. Tel.: +613 8344 5041; fax: +613 9347 1071.
E-mail address: abacic@unimelb.edu.au (A. Bacic).
[1] Present Address: Proteomic Research Group, The Babraham Institute, Babraham, Cambridge CB22 3AT, United Kingdom.

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into the culture medium in a temperature-dependent manner; the composition of the ECPs was largely undefined. ECPs are present in the growth medium of liquid suspension cultures of a diverse range of both dicotyledonous (McNeil et al., 1984; York et al., 1986 and references therein) and monocotyledonous (see Sims et al., 2000 and references therein) plants. Such water soluble polymers consist of neutral and acidic polysaccharides, (glyco)proteins and proteins, which are in general structurally similar to polymers found in plant primary cell walls (McNeil et al., 1984; York et al., 1986). In instances where the ECPs contain polysaccharides that are distinct from those of the primary walls, the cells are not fully de-differentiated. For example, in some suspension cultures of the Poaceae (monocots), where the ECPs are similar to secreted root slime, the cultures appear as root-tissue-like clumps rather than single cells (Sims et al., 2000).

In this paper, we describe the separation and characterisation of the ECPs recovered from cell suspension cultures of *P. communis*. The polymers were separated by anion exchange chromatography with a gradient of imidazole–HCl. The polysaccharides were identified after carboxyl reduction of the esterified and non-esterified uronic acids. Methylation analysis enabled both the linkage type and sugar residue to be identified. The proportions and compositions of protein fractions were estimated by amino acid analysis and the major proteins were identified by ESI LC-MS/MS after trypsin digestion.

2. Results and discussion

The ECP isolated from the cell suspension culture medium of *P. communis* was found to consist primarily of carbohydrate (81%), some protein (13%) (Table 1) and inorganic material (5%).

The major monosaccharides present in the ECP are GalA, Glc, Gal, Ara, Xyl, Fuc, with trace amounts of Rha, GlcA and Man (Table 2). This composition was similar when determined by either the conversion of component monosaccharides into alditol acetates, or into partially methylated alditol acetates after the carboxyl reduction of the uronic acids (Tables 2 and 3). Linkage analysis of the ECP after carboxyl reduction (Table 3) revealed a complex set of linkage types consisting primarily of 4-GalA*p*, t-Ara*f*, 3,6-Gal*p*, 4,6-Glc*p*, t-Xyl*p*, 4-Glc*p*, and smaller amounts of 2- and 4-Xyl*p*, t-Fuc*p*, 3- and 5-Ara*f* and t- and 3-Gal*p*. Only 3% of the 4-GalA*p* residues were methyl esterified. The occurrence of the polysaccharides (fucogalacto)xyloglucan, type II arabinogalactan (AG), an acidic heteroxylan and pectic-type galacturonan could be deduced from these types of linkages based on our

Table 1
Recovery of total material, carbohydrate, uronic acid, and protein after anion exchange chromatography (Fig. 1) of *Pyrus communis* ECP

Fraction	Total material[a] (mg)	Neutral carbohydrate[b] (mg)	Uronic acid[c] (mg)	Protein[d] (mg)
ECP	450	206	156	58
A	101	100	2	1
B	79	52	5	21
C	72	10	61	1

[a] Calculated from the weight of fractions collected.
[b] Calculated from the total carbohydrate colorimetric assay adjusted for contribution from uronic acid.
[c] Calculated from the uronic acid colorimetric assay.
[d] Calculated from amino acid analysis.

Table 2
Composition of *Pyrus communis* ECP and fractions obtained by anion-exchange chromatography

Fraction	Monosaccharide composition (wt%)[a]								
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	GlcA
ECP	tr	2	13	12	tr	18	20	35	tr
A	–	4.5	1.5	31.5	1.5	14	47	–	–
B	2	–	34	3	–	55	2	–	4
C	tr	–	5.5	1.5	2	tr	2.5	88.5	–

tr, trace (⩽1%). –, not detected.
[a] From sum of carboxyl reduced, partially methylated alditol acetates.

Table 3
Linkage composition of *Pyrus communis* ECP and fractions obtained by anion-exchange chromatography (Fig. 1)

Monosaccharide	Deduced glycosidic linkage	Linkage composition (mol%)[a] carboxyl reduced				
		ECP	A	B	B'	C
Rha*p*	Terminal	tr	–	2.0	4.0	–
	2-	–	–	–	–	tr
	2,4-	tr	–	–	–	tr
Fuc*p*	Terminal	2.0	4.5	–	–	–
Ara*p*	Terminal	–	–	tr	2.0	–
Ara*f*	Terminal	12.0	1.5	30.0	23.5	4.0
	2-	–	–	tr	tr	–
	3-	2.0	–	3.5	4.0	1.5
	5-	1.5	–	3.0	2.5	1.5
	2,5-	–	–	tr	tr	tr
Xyl*p*	Terminal	9.0	21.0	tr	tr	tr
	2-	3.5	12.0	–	–	–
	4-	1.5	2.5	3.5	3.0	2.0
	2,4-	–	–	tr	tr	–
Man*p*	4-	tr	1.5	–	–	2.0
Gal*p*	Terminal	3.0	6.5	3.0	7.5	tr
	2-	tr	5.0	–	–	–
	3-	2.0	–	3.5	7.5	–
	4-	tr	–	1.5	tr	tr
	6-	tr	–	1.5	3.5	–
	3,4-	tr	–	1.5	tr	tr
	3,6-	12.0	1.5	32.5	30.5	tr
	3,4,6-	tr	–	4.5	1.5	–
Glc*p*	Terminal	tr	tr	tr	tr	tr
	4-	8.0	13.5	2.0	tr	2.5
	4,6-	11.0	29.5	–	–	tr
GalA*p*	Terminal	tr	–	–	–	tr
	4-	31.0	–	–	–	84.0
	2,4-	–	–	–	–	tr
GlcA*p*	Terminal	–	–	1.5	2.5	–
	4-	–	–	2.0	4.5	–

tr, trace (⩽1%); –, not detected.
[a] Average of duplicate determinations.

knowledge of ECPs and primary walls. Wallner et al. (1986) found that the ECP from *P. communis* consisted of a small neutral polysaccharide containing Glc, Gal, Ara, Man and Xyl as well as a large molecular weight pectic polysaccharide but their structures were not reported.

The protein content (13%, Table 1) is within the range of 5% and 15%, typically found in ECPs (e.g. *Mentha* (Uchiyama et al., 1993) and *Zea mays* (Bacic et al., 1987)), although there is a report that in the ECP of *Nicotiana tabacum*, protein levels vary between 1.5% in healthy cultures rising to 23% when the cultures are osmotically stressed (Iraki et al., 1989). The major amino acids present in the pear ECP are Glx, Thr, Ser, Hyp/Pro and Gly (Table 4).

In order to gain a greater understanding of the composition of the pear ECP, it was fractionated by anion exchange chromatography (Fig. 1). The unbound material, Fraction A, accounted for 22% of the material loaded onto the column (Table 1). The weakly bound Fraction B was eluted from the column at 0.6 M imidazole–HCl and a more tightly bound Fraction C eluted from the column at 1.8 M imidazole–HCl (Fig. 1). Fractions B and C accounted for 18% and 16%, respectively, of the loaded material by weight (Table 1).

The yield from the column, in terms of measured neutral carbohydrate, was 79% while the yield of uronic acid residues was only 44% (Table 2). Although it is not unusual to recover only 70% of material applied to an anion exchange column, the low recovery of uronic acid residues is probably due to precipitation of pectic polysaccharide during processing, and/or irreversible, binding to the anion exchange resin (see also Sims and Bacic, 1995; Sims et al., 2000). The recovery of arabinogalactan-protein (AGP) (as determined by the specific interaction with β-glucosyl Yariv reagent) in Fraction B was complete (yield 92%, data not shown). Fraction B also contained almost all of the protein recovered from the column although the total protein yield from the column was very low (yield 39%, Table 1). This low yield was probably due to adsorptive losses, particularly during dialysis. Another potential source of protein loss would be through irreversible adsorption to the anion-exchange column. This would be expected to lead to the selective depletion of acidic proteins, but a comparison of the amino acid compositions of ECP and Fraction B (Table 4) shows the opposite effect. The most plausible explanation for this would be adsorption of basic proteins to precipitated pectic polysaccharide.

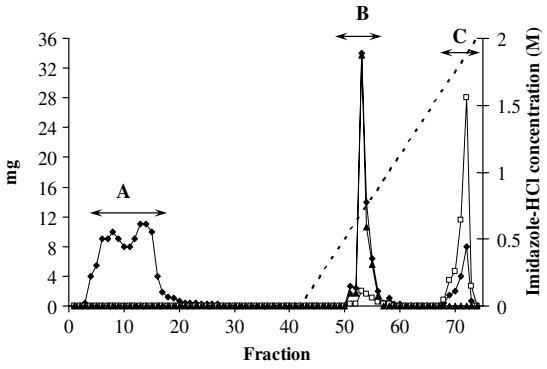

Fig. 1. Anion-exchange chromatography of *Pyrus communis* ECPs on a column of DEAE-Sepharose CL-6B with a gradient of imidazole–HCl (pH 7.0). Fractions (10 ml) were assayed for hexose (♦), uronic acid residues (□) and for AGP (▲) and were pooled as indicated to give Fractions A, B, and C.

Table 4
Amino acid composition of *Pyrus communis* ECPs and fractions obtained by anion-exchange chromatography (Fig. 1)

Amino acid	Composition (mol%)[a]			
	ECP	B	B'	B''
Asx	6.6	9.6	3.8	9.8
Glx	16.3	17.1	10.8	15.4
Hyp	4.1	4.0	17.9	0.3
Ser	8.7	9.4	13.2	9.5
Gly	8.0	6.1	7.5	10.1
His	2.2	0.6	0.6	0.3
Arg	3.0	1.9	1.8	2.3
Thr	8.8	11.2	9.0	8.7
Ala	5.7	6.7	11.6	9.7
Pro	8.3	8.8	5.8	8.5
Tyr	4.9	4.5	3.1	3.4
Val	5.6	5.3	5.0	6.0
Met	1.2	0.7	0.4	0.4
Ile	3.0	2.3	1.6	2.7
Leu	6.8	5.0	3.3	4.3
Phe	3.9	4.3	2.0	4.7
Lys	2.8	2.1	2.4	3.7
Trp	0	0	0	0
Cys	0	0	0	0

[a] Analysis was performed at least in duplicate.

2.1. Fraction A

Fraction A is composed primarily of neutral polysaccharides with only traces of uronic acid-rich polysaccharides (2%) and protein (0.8%) (Table 1). Glc (47%), Xyl (31.5%) and Gal (14%) are the major monosaccharides present in this fraction with smaller amounts of Fuc, Man and Ara (Table 2). Methylation analysis (Table 3) reveals the major linkages in decreasing order of abundance to be 4,6-Glc*p*, t-Xyl*p*, 4-Glc*p*, 2-Xyl*p*, t-Gal*p*, 2-Gal*p* and t-Fuc*p* indicating this fraction is predominantly (92%) a typical (fucogalacto)xyloglucan (York et al., 1990). The low levels of 4-Xyl*p* (2.5%), 3,6-Gal*p* (1.5%) and t-Ara*f* (1.5%), are suggestive of the presence of small amounts of arabinoxylan and type II AG/AGP. Only a trace amount of protein was found in this fraction and so its amino acid composition was not determined.

2.2. Fraction B

Fraction B is composed of 66% neutral carbohydrate, 6% uronic acid and 27% protein (Table 1). The major monosaccharides present in this fraction are Gal (55%) and Ara (34%) with minor levels of GlcA, Xyl, Rha and Glc (Table 2). Linkage analysis (Table 3) shows the

linkages 3,6-Gal*p*, t-Ara*f*, 3,4,6-Gal*p*, 3-Gal*p*, 3-Ara*f*, 5-Ara*f*, 4-Xyl*p*, t-Gal*p*, 4-GlcA*p*, 4-Glc*p*, t-Rha*p*, t-GlcA*p*, 3,4-Gal*p*, 6-Gal*p*, and 4-Gal*p* to be present. The t-GlcA*p* and 4-GlcA*p* residues are not methyl esterified. These linkage results support the data obtained in the Yariv diffusion assay, that the main polysaccharide present in this fraction is a type II AG present as an AGP (95%), as well as a small amount of acidic (glucurono)arabinoxylan (~5%). At least four major groups of AGPs have been reported to occur in ECP isolated from *P. communis* suspension cultures (Chen et al., 1994). A xylan has also been identified from the walls of pear fruit (Ahmed, 1978).

The major amino acids present in Fraction B are Glx, Thr, Asx, Ser, Hyp/Pro and Ala (Table 4). In order to establish the proportion of this protein covalently associated with the AGPs, Fraction B was further fractionated using phenol extraction into two fractions; Fraction B′ containing the AGPs and Fraction B'' containing the non-AGP proteins. Phenol extraction is used to remove hydrophobic molecules, such as proteins from polysaccharide-rich cell walls (Westphal and Jann, 1965). Heavily glycosylated proteins (such as AGPs) and polysaccharides will not be extracted by the phenol. The aqueous, AGP-rich Fraction B' accounted for 83% of Fraction B, while the phenol soluble, protein-rich Fraction B'' only accounted for 10%. The efficiency of the phenol extraction can be seen in Fig. 2 by SDS–PAGE analysis of Fractions B, B' and B'', double stained with both Yariv reagent (AGPs only) and Coomassie Blue (proteins and some glycoproteins; does not stain AGPs). Fraction B contains both high molecular weight AGPs (as seen by the diffuse red band on the top of the gel) and at least 10 protein bands ranging in size from 65 to less than 14 kDa. Fraction B' contains only the high molecular weight AGP, while Fraction B'' contains the proteins and no detectable AGPs. This conclusion is further supported by the amino acid analysis data of the samples (Table 4). The AGP-rich Fraction B' contains only 7.4% protein whereas Fraction B'' contains 84% protein. The most obvious difference in amino acid composition between the two samples is the level of Hyp; 17.9% in Fraction B' and 0.3% in Fraction B''. High levels of Hyp/Pro, Ser, Thr and Ala typify the amino acid composition of "classical" AGPs (Johnson et al., 2003) as found in Fraction B'. Two extracellular AGPs have been characterized in detail from *P. communis* suspension culture media (Chen et al., 1994; Mau et al., 1995; Youl et al., 1998; Oxley and Bacic, 1999). Both AGPs have characteristic monosaccharide and linkage composition but differ in their protein backbones; one with a sequence consistent with a "classical" AGP protein backbone rich in Hyp/Pro, Ser, Ala, Thr, and the other with a non-classical AGP protein backbone that contains both a Hyp/Pro-rich domain as well as a hydrophilic Asn-rich domain.

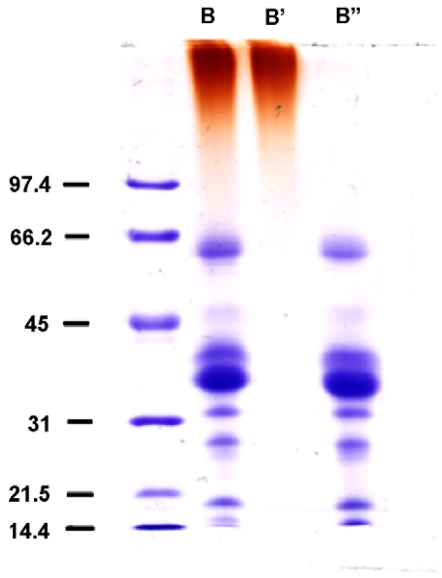

Fig. 2. SDS–PAGE of Fraction B from the anion exchange chromatography of *Pyrus communis ECP* and its sub-fractions B' and B'' (aqueous and phenolic extracts, respectively) stained with Coomassie Blue and β-glucosyl Yariv reagent (red). Molecular weight markers are in kilodaltons (kDa).

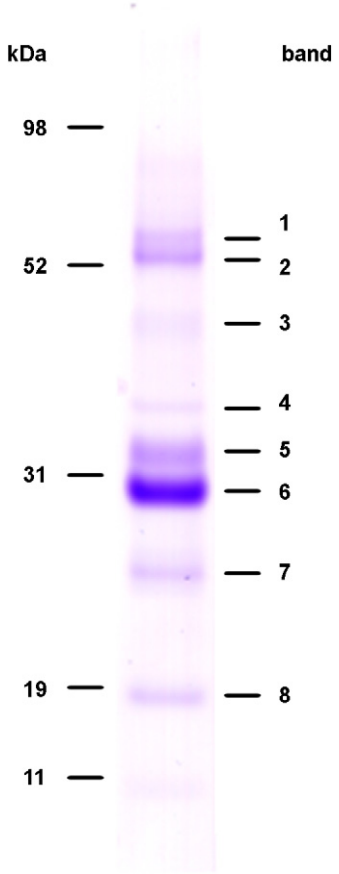

Fig. 3. SDS–PAGE of Fraction B from anion exchange chromatography of *Pyrus communis* ECP stained with the Coomassie-based Imperial protein stain (to visualize only the "non-AGP" protein bands). Each of the bands indicated from 1 to 8 were excised destained, reduced, alkylated prior to trypsin digestion and peptide analysis by LC–MS/MS. Positions of molecular weight markers are indicated.

Table 5
Identification of protein bands by LC–MS/MS

Band	Peptides matched in Mascot search	Peptides matched by manual sequencing	Database match
1, 2	ISTVNPYTVLEASIFK	**DASTLQYTTR**	Dermal glycoprotein (Fragment)
		VVTEMoxFISEAK	(Q6V8M3_MALDO)
		NVDVSQSLIYTR	EDGP (Fragment) (Q05929_DAUCA)
		ALVVPVSK	
3		**SFIDSSISLAR**	Chitinase (Q43576_TOBAC, Q43591_TOBAC)
4	**IYEPNQATLEALR**	*__QSVGVCYGR__	Beta-1,3-glucanase (Q56AP0_FRAAN,
	NVQPYSADVK	**YIAVGNEVR**	Q56AP1_FRAAN, Q84RT6_FRAAN,
	YQLTFG		Q84RT7_FRAAN, Q84Y07_FRAAN,
			Q84JM2_FRAAN)
5, 6	ITFTNNCPNTVWoxPGTLTGDQKPQLSLTGFELASK	**ITFTNNCPN**	Thaumatin-like protein (TP1A_MALDO,
	PQLSLTGFELASK	**SSSCPANVNAACPAQLQVKAACPAQLQVK**	Q3BCT8_MALDO, Q3BCT4_MALDO,
	ASQSVDAPSPWoxSGR		Q3BCT6_MALDO)
	SACLAFGDSK		
	YCCTPPNNTPETCPPTEYSEIFEK		
	QCPQAYSYAYDDK		
	*QCPQAYSYAYDDK		
	VLLSIGGASGSYSLTSADDAR		Endo-chitinase class III (Fragment)
	SQVLPTIK		(Q9LUX6_PYRPY)
	WoxYDINSGYSASIK		
7	**APGGCNNPCTVFK**		Thaumatin-like protein (Q5ND92_ACTDE)
	CPDAYSYPK		Thaumatin (Fragment) (Q70CE5_FAGSY)
8	*__QDTPQDYLNSHNTAR__		Pathogenesis-related protein (Fragment)
	ADYNYESNSCADGK		(Q9LLJ4_PYRPY)

Peptide sequences were identified from mass spectral data by either searching against the Uniprot database using Mascot, or by manual interpretation with the resulting peptide sequences used for BLAST searching of the Uniprot database. Peptides in bold type were the major peptides detected by LC–MS/MS in each band.

* Q = pyroGlu, Mox = oxidized methionine (Met-sulphoxide), Wox = oxidized tryptophan (*N*'-formylkynurenine), Leu and Ile are indistinguishable in low-energy MS/MS spectra. Ile is only used here where indicated by the database sequence.

The identities of the major proteins in Fraction B were further investigated after SDS–PAGE analysis (staining only with a Coomassie-based stain for proteins) (Fig. 3). Excised bands 1–8 were destained, reduced, alkylated, digested with trypsin (in-gel) and the resultant peptide mixtures analysed by ESI-LC-MS/MS. Mass spectral data were submitted to Mascot for searching against the Uniprot database. Due to the very low database coverage of the *P. communis* genome, proteins were either not matched to any database entry, or were matched with relatively low scores to database entries from other plant species. Therefore, in order to identify the unmatched proteins, and to ensure that low scoring protein matches were from the major protein in each band rather than minor components, MS/MS spectra of all major peptides from each band were manually interpreted and the resulting peptide sequences used for BLAST searching of the Uniprot database (Table 5).

The most abundant protein detected ran as a doublet at around 30 kDa (bands 5 and 6), and was matched to an apple thaumatin-like protein (TLP) (Gao et al., 2005). A second major protein, which ran as a ~55 kDa doublet (bands 1 and 2), was matched to sequences from extracellular dermal glycoprotein (EDGP) from apple and carrot species. Other proteins detected were relatively minor and were matched to two different classes of chitinase, a β-1, 3-glucanase, a pathogenesis-related protein and a second class of thaumatin-like protein.

The identified proteins are commonly observed proteins secreted into the medium of plant cell suspension cultures and are considered defense related (Robertson et al., 1997), although there is evidence to suggest that they may be involved in the initiation of embryogenesis, (Matthys-Rochon, 2005; de Jong et al., 1993). More recently, the advent of proteomic approaches has resulted in the identification of hundreds of secreted proteins from cell suspension cultures of *Arabidopsis thaliana* (Oh et al., 2005; Ndimba et al., 2003; Borderies et al., 2003), *Lupinus albus* (Schwend et al., 2003) and *Nicotiana tabacum*, (Okushima et al., 2000). These studies reveal an array of protein classes are secreted and include a range of hydrolytic enzymes, proteins involved in signalling and regulation, protein degradation and protection, structural proteins and proteins of unknown function.

2.3. Fraction C

Fraction C is predominantly uronic acid-rich carbohydrate (85%) with a trace of protein (1%) (Table 1). The main monosaccharide present in this fraction is GalA (88.5%) with minor amounts of Ara, Glc, Man, Xyl and trace amounts of Rha and Gal (Table 2). Methylation analysis confirms that Fraction C is predominantly a homopolymer of GalA, containing the linkages 4-GalA*p* (84%) and t-Ara*f*, 4-Glc*p*, 4-Xyl*p*, 4-Man*p*, 3-Ara*f* and 5-Ara*f* (Table 3). As observed for the total ECP, only 3% of the 4-GalA*p* residues were methyl esterified. The major polymer present in this fraction is therefore a linear unbranched homogalacturonan. Trace amounts of other polysaccharides are also present in this fraction including a rhamnogalacturonan (2-Rha*p*), an AGP (3,6-Gal*p*), a xyloglucan (4-Glc*p*) and a heteromannan (4-Man*p*).

The occurrence of a homogalacturonan is consistent with the results presented by Dick and Labavitch (1989), who describe the most abundant soluble polysaccharide from ripening *P. communis* fruit to be a homogalacturonan, although the degree of esterification of this molecule was significantly higher (60%) than that secreted from suspension cultured cells. They also described a less abundant soluble polysaccharide from ripening pear fruit as a rhamnogalacturonan I type polysaccharide with large highly branched arabinan side chains, most of which are lost during the ripening process. Studies on the composition of ECPs from cultures of other members of the Rosaceae have been conducted. AGP and galactoglucomannan were isolated from the ECP of *Rubus fruticosis* (Cartier et al., 1987, 1988) and the ECP of apple (*Malus domestica*) is predominantly composed of AGP and xyloglucan, but unlike pear, is low in homogalacturonan (Reid et al., 1999).

3. Conclusion

The ECPs secreted by suspension culture cells of *P. communis* are predominantly polysaccharides and proteoglycans and some proteins. Based upon a combination of the yields of different classes from anion exchange chromatography and the deduced linkage analysis of the unfractionated ECP, we have estimated the proportions of the different polysaccharide types (Table 6). Thus, (fucogalacto)xyloglucan (36%), AGP (29%) and galacturonan (33%) comprise 98% of the polysaccharide present in the ECP with minor amounts (~2%) of acidic (glucurono)arabinoxylans and heteromannans. With the exception of the AGPs, these polysaccharides would reflect the relative proportions found in a typical dicot-type cell wall (Bacic et al., 1988). Generally AGPs are in low abundance in cell walls but are commonly found in high levels secreted into the medium of cell suspension cultured cells (eg Sims and Bacic, 1995; Sims et al., 2000). The ECPs also contain minor amounts of a mixture of (glyco)proteins including chitinases, TLPs, β-(1,3)-glucanases, a PR-protein and a dermal glycoprotein (EDGP). The examination of the complex composition of conditioned culture medium, rich in

Table 6
Relative proportions of polysaccharides present in *Pyrus communis* ECPs

Polysaccharides	Relative proportions[a] (% w/w)
(Fucogalacto)xyloglucan	36
Arabinogalactan-protein (AGP)	29
Acidic (glucurono)arabinoxylan	2
Galacturonan	33
Heteromannan	tr

tr, trace.
[a] Calculated as sum of mol% of individual monosaccharide residues.

secreted cell wall polysaccharides, AGPs, PR proteins and cell wall enzymes, as well as other secreted molecules (e.g. peptides mono/oligosaccharides, hormones, other signaling molecules such as fatty acids), provides an insight into the roles these secreted molecules may play in plant growth, development, stress and defense and demonstrates the utility of such *in vitro* model systems.

4. Experimental

4.1. Chemicals

The β-glucosyl Yariv reagent (1,3,5-tris [4-β-D-glucopyranosyloxyphenolazo]−2,4,6-trihydroxybenzene) was from Biosupplies Australia (Parkville, Victoria, Australia). All other chemicals are of analytical grade.

4.2. Plant Material

ECPs from *P. communis* were produced by Dr. David McManus at Tridan-Albright and Wilson (Australia) Ltd., Yarraville, Victoria, Australia (Mau et al., 1995). Suspension cultures were initiated from fruit (Pech et al., 1979) and grown for 7 days in an airlift fermentor (1000 L) at 27 °C. The cells were removed from the medium by rotary drum vacuum filtration, and the ECPs recovered by ultrafiltration. The ECPs were diafiltered (molecular weight cut-off 3000 Da) and then spray-dried. ECPs were dissolved in Na_2–EDTA (0.5 mg/ml, pH 7.5) containing NaN_3 (0.02% w/v) and left overnight at 4 °C. The solution was clarified by vacuum filtration using a series of filter membranes (Whatman 541, 542, GF/A, GF/F, 0.45, 0.22 μm), dialysed against deionised water (molecular weight cut-off 6–8000 Da), concentrated under reduced pressure (35 °C) and then freeze dried.

4.3. Fractionation of ECPs

Solubilized ECPs (450 mg) in 20 mM imidazole–HCl buffer (225 ml, pH 7.0) were loaded onto a DEAE-Sepharose CL-6B column (42 × 2.0 cm), equilibrated in the same buffer, and eluted at 1 ml/min until no more carbohydrate washed from the column. Polymers which bound to the column were eluted with a linear gradient (0.02–2 M) of imidazole–HCl (pH 7.0) over 750 ml and fractions (10 ml) assayed for hexose, uronic acid and AGP. Fractions were pooled to yield one unbound fraction (A), and two bound fractions (B and C) (see Fig. 1). The pooled fractions were concentrated and dialysed against deionized water and freeze dried.

Fraction B (10 mg in 1 ml deionised water) was extracted with 90% aqueous phenol (1 ml) at 68 °C for 30 min with shaking (Oxley, 1988). The solution was centrifuged at 11,000 rpm for 10 min to separate the layers. The upper aqueous layer (B') was further extracted with fresh 90% phenol twice more. The lower phenol layers (B'') were pooled. Both aqueous (B') and phenol layers (B'') were dialysed (molecular weight cut-off 10,000 Da) against distilled water for three days until the smell of phenol was no longer present. Both solutions were freeze dried. The yield of Fraction B' was 8.3 mg and B'' was 1.0 mg.

4.4. Analytical methods

AGP was determined by radial diffusion Mancini assay against β-glucosyl Yariv reagent (Van Holst and Clarke, 1985) using gum arabic (Sigma) as a standard. Total carbohydrate was determined according to the method of Dubios et al. (1956), using Gal as a standard. Hexoses were determined by the anthrone method (Dische, 1962) using Gal as a standard and uronic acids determined by the 3-phenyl phenol method (Blumenkrantz and Asboe-Hansen, 1973) using GalA as a standard. Inorganic material was determined as ash content and was performed by National Analytical Laboratories (Melbourne, Australia). Monosaccharide compositions were determined by GC–MS following carboxyl reduction (Kim and Carpita, 1992) and the conversion of the neutral monosaccharides to either their alditol acetates or their partially methylated alditol acetates (Sims and Bacic, 1995).

4.5. Linkage analysis

Methylation was performed using NaOH and CH_3I as described by Sims and Bacic (1995). The alditol acetates and partially methylated alditol acetates were separated on a fused-silica capillary column (25 m × 0.22 mm i.d.) with bonded phase BPX70 (SGE, Australia) on an Agilent 6890 Series GC System with a 5973 mass selective detector (Lau and Bacic, 1993). Identifications were based on peak R_t's and by comparison of EI-MS spectra with published spectra. The types of polysaccharides present in the ECPs and fractions were deduced by comparison of the linkage analyses to the linkage analysis of individual polysaccharides purified from ECPs and cell walls (Shea et al., 1989; Sims and Bacic, 1995). Levels of uronic acid methyl esterification were determined as described previously (Kim and Carpita, 1992; Sims and Bacic, 1995).

4.6. Amino acid analysis

The amino acid composition of the ECP and fractions was determined as their phenylthiocarbamyl (PTC) derivatives, following acid hydrolysis (6 M HCl, 110 °C, 18 h) using reversed-phase HPLC as described by Oxley and Bacic (1995).

SDS–PAGE was performed as described by Laemmli (1970) using a PhastGel (Pharmacia) for the AGP-rich Fraction B and an Invitrogen XCell SureLock Mini-Cell for the proteins in Fraction B''. For the PhastGel system a 12% polyacrylamide separating gel with a 5% polyacrylamide stacking gel was used. The gel was stained first with Coomassie Blue, destained and then stained with 0.2 mg/ml

β-glucosyl Yariv reagent in 1% w/v NaCl overnight followed by washing in 1% w/v NaCl (4 × 10 min). For the Invitrogen system, a pre-cast NuPAGE Novex 10% Bis–Tris, 1.0 mm thick gel was used and stained with the Coomassie-based Imperial Protein stain (Pierce Biotechnology, IL, USA).

4.7. Identification of (glyco)protein bands by LC–MS/MS

Bands were excised from Coomassie-stained SDS–polyacrylamide gels and washed with 25 mM ammonium bicarbonate (2 × 10 min) and 25 mM ammonium bicarbonate in 50% acetonitrile (2 × 10 min). Proteins were then reduced (10 mM DTT/25 mM ammonium bicarbonate, 1 h 50 °C) and alkylated (50 mM iodoacetamide/50 mM ammonium bicarbonate, 1 h room temperature). After further washing as above, the gel slices were dried under vacuum, then rehydrated in 25 mM ammonium bicarbonate containing trypsin (10 ng/μl, Promega sequencing grade) and incubated overnight at 30 °C. The digests were acidified with 1% trifluoroacetic acid and aliquots used directly for LC–MS.

Protein digests were separated by RP-HPLC on a Vydac C18 column (0.075 × 100 mm) with a gradient of 5–35% aqueous acetonitrile (containing 0.1% formic acid) at a flow rate of 250 nL/min over 15 min. The column outlet was connected to a nanoelectrospray interface (Protana Engineering) attached to a quadrupole time-of-flight MS (Applied Biosystems MDS Sciex QSTAR pulsar i). The MS was operated in "Information Dependent Acquisition" mode such that fragment-ion spectra were acquired on the three most intense doubly, triply or quadruply charged ions detected in the previous TOF scan. TOF scans were acquired for 1 s each over the m/z range 350–1500 and MS/MS scans for 2 s each over the m/z range 100–1700. Peptides were identified by searching the "other green plant" entries of the Uniprot 8.9 database (http://www.expasy.org/sprot/) with the mass spectral data using Mascot software (Matrix Science, London, UK, http://www.matrixscience.com) and by manual interpretation of fragment-ion spectra.

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