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POLYSACCHARIDES LEACHED FROM THE SURFACE OF PEA ENDOCARP

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Key Word Index—*Pisum sativum*; Leguminosae; pea endocarp; surface polysaccharides; *Monilinia fructicola*; arabinogalactan.

Abstract—Polymers (apparent M_r 1.1–1.23 × 10^6) containing *ca* 80% carbohydrate and 16% protein are leached by water from the surface of pea endocarps. These polymers were examined by hydrolysis, methylation analysis and Trisacryl gel column chromatography and were found to be primarily acidic arabinogalactans (Type II) with a 3-linked galactan backbone branched through C-6 to side chains of 6-linked galactopyranose terminating in 4-*O*-methyl glucuronic acid and arabinofuranose residues. The main polymeric component obtained by gel filtration of the polymers gave a positive reaction to the presence of the β-glucosyl Yariv antigen. The presence of small amounts of xyloglucans and xylans was suggested by the detection of 4,6-linked glucopyranose and 4-linked xylopyranose residues. Pectic polysaccharides were suggested by the presence of 5-linked arabinofuranose and 4-linked galacturonic acid residues. Inoculation of pea endocarps with a conidial suspension of *Monilinia fructicola* resulted in reduced amounts of 4-linked galacturonic acid residues.

INTRODUCTION

In previous studies on metabolic fluxes in infection-droplets (diffusate) associated with the inoculation of pea endocarp with *Monilinia fructicola* [1] major differences in filtration characteristics were observed between infection-droplets containing conidia and the water droplet (leachate) controls. After 18 hr/20° incubation chemical analysis indicated that the carbohydrate-containing polymers present in the water droplets had almost completely disappeared from the infection-droplets and that arabinose > galactose > xylose appeared as the major free monosaccharides [1]. These free monosaccharides were not present in uninoculated leachates from the endocarp surface. The above plant–fungus model system has been used in studies of the *in vivo* elicitation of pisatin, the primary phytoalexin of *Pisum sativum* [2]. In this paper we have shown that these water-soluble polymers are primarily acidic arabinogalactans (Type II) [3] and that some infection-related modifications occur within the first 6 hr of incubation with fungal conidia. These changes do not appear on present evidence [2] to be associated directly or indirectly with pisatin elicitation *in vivo*.

RESULTS AND DISCUSSION

Small quantities (3–8 mg/100 ml diffusate) of polymeric material were precipitated by addition of 4 vols EtOH to the diffusate preparations. The yield of polymers leached by water from the surface by 18 hr was estimated to represent *ca* 25 μg/cm² of wetted surface. The isolated material was primarily composed of carbohydrate (*ca* 80% w/w) with a smaller proportion of protein (16% w/w). The monosaccharide compositions are indicated in Table 1. The polymers leached by water from the endocarp surface were largely composed of galactose, arabinose and uronic acid residues (1:0.9:0.4, molar ratios) with a low proportion of xylose residues (0.1). The relative monosaccharide composition did not change appreciably in the presence of *M. fructicola* conida except for galacturonic acid which decreased by *ca* 40%.

The linkage composition of the polymeric material leached from the pea endocarp surface, as determined by methylation analysis, is shown in Table 2. A complex array of glycosidic linkages were detected with only minor differences in the molar proportions of linkages between the material isolated after different treatments. The major linkages present in all samples were 3-, 6-, 3,6- and terminally linked galactopyranose, and arabinofuranose as 5- and terminally linked residues. The 4-*O*-methyl glucuronic acid was exclusively terminal and galacturonic acid was present primarily as 4-linked with a low proportion of the 3,4-linked residue. There was a small but progressive drop in the proportional level of 4-linked galacturonic acid residues upon treatment with the conidia of *M. fructicola*. Xylose was present primarily as 4-linked with some 2- and terminally linked residues. A small proportion of glucose, not detected during monosaccharide analysis, was present as 4- and 4,6-linked residues.

The polymers isolated from pea × H_2O 18 hr incubations were chromatographed by gel filtration on a

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Table 1. Component sugar residues in hydrolysates of polysaccharides leached from the pea endocarp in the presence or absence of *M. fructicola* conidia

Interaction polymers isolated from	(mol %) MeGlcA	GalA	Gal	Xyl	Ara
Pea × H_2O 18 hr	6.6	10.2	41.2	5.5	36.5
Pea × *M. fructicola* 2hr	7.7	9.1	44.9	3.5	34.8
Pea × *M. fructicola* 4 hr	7.8	5.9	46.3	3.0	37.0
Pea × *M. fructicola* 6 hr	8.1	6.0	47.4	3.5	35.1

Table 2. Methylation analyses of polysaccharides leached from the pea endocarp in the absence or presence of conidia of *M. fructicola*

Monosaccharide	Deduced glycosidic linkage	Mole % Pea × H_2O 18 hr	Pea × *M.* fructicola 2 hr	4 hr	6 hr
Ara*f*	Terminal*	9	12	13.5	9
	2-	tr†	tr	tr	tr
	3-	1	1	1	1
	5-	7	7	9	8
Ara*p*	Terminal	1	5	3.5	3
Xyl*p*	Terminal	1	1	tr	tr
	2- and 4-‡	4	2	3	3
	2,4-	tr	0.5	tr	1
Gal*p*	Terminal	5	7	4	6
	2-	1	tr	tr	tr
	3-	10	11	9	9
	4-	0.5	1	1	1
	6-	22	17	21	21
	3,4-	0.5	tr	tr	tr
	4,6	tr	1	1	tr
	3,6	23	19.5	22.5	27
	3,4,6	tr	tr	1	2
	2,3,6-	—	tr	1	tr
Glc*p*	4-	2	4	1.5	1
	4,6-	1.5	1	tr	1
GalA*p*	4-	5	3	2	2
	3,4	1.5	1	1	1
4-OMeGlcA*p*	Terminal	4	3	3	4

*Terminal Ara*f* is deduced from 1,4-di-acetyl-2,3,5-tri-*O*-methylarabinitol etc.

†tr = trace (< 0.5%).

‡2- and 4-Xyl*p* coelute on BP-75; the ratio of 2- as to 4-linked calculated from the GC-MS by the ratio of the unique ion fragments *m/z* 117 and 118, respectively, was 1:3.

Trisacryl column (Fig. 1). Two main peaks were obtained: the major peak, which had a trailing edge, eluted with an apparent M_r of *ca* 1.1–1.23 × 10^6 whilst the second minor peak eluted with an apparent M_r of 5.9 × 10^5. Samples from each, corresponding to pooled fractions A, B and C as indicated in Fig. 1, were quantitated for arabinogalactan-proteins (AGP) by single radial gel diffusion using the β-glucosyl Yariv antigen and a gum arabic standard. Both fractions A and B gave a positive reaction and were estimated to contain *ca* 100 μg/ml AGP. Fraction C did not give any detectable reaction with the Yariv antigen.

The free monosaccharides present in the diffusate during the first 6 hr of the pea × *M. fructicola* interaction were essentially glucose and fructose (Glc + Fru in diffusate; 4 > 2 > 6 hr). Sucrose was detected and some oligosaccharides appeared after 6 hr incubation with the fungus.

The data presented are consistent with the presence of an acidic arabino-3,6-galactan (Type II) as the major component of the polymeric material leached into water from pea endocarp. Whether the protein found in the diffusate is covalently linked to the arabinogalactan was not determined. The arabino-3,6-galactans typically consist of a 3-linked galactan backbone branched through C(*O*)6 to side chains of 6-linked galactopyranose which commonly terminate in uronic acid or arabinofuranose residues [3, 4]. Arabinogalactans (Type II) are common

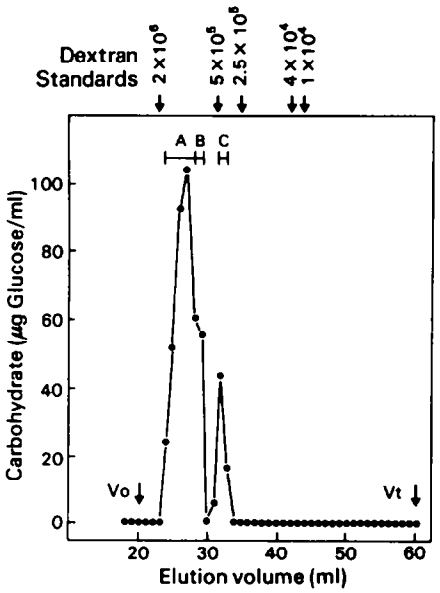

Fig. 1. Gel filtration of pea × H_2O 18 hr water soluble polymers on Trisacryl (40 × 2.5 cm). The column was equilibrated and components were eluted with 6 M urea. Collected fractions were analysed for carbohydrate (glucose standard).

constituents of plant secretions and mucilages and are often synthesized and secreted by some plant tissues in response to wounding [4].

Two types of water-soluble polymers have been shown to be released from cell walls of pea stem (epicotyl) [5], a pectin (Gal/Ara/Rha/GalA) and a xyloglucan. Brillouet and Carré [6] found that the water-soluble nonstarchy polysaccharides of cell walls from pea cotyledon contained arabinose, galactose, galacturonic acid, xylose, glucose and low proportions of rhamnose and mannose. Neither rhamnose nor mannose residues were detected in polymers leached from pea endocarp.

The arabinogalactans leached from pea endocarp were very similar to those extracted from *Dolichos lablab* hulls with cold water [7] but were different from other Type 1 pectic polysaccharides extracted from the same *D. lablab* pods by hot water [8]. The presence of 5-linked arabinofuranose and 4-linked galacturonosylpyranose and of 4,6-linked glucopyranose, and 4-linked xylopyranose may indicate small amounts of water-soluble cell wall derived polysaccharides including pectic polysaccharides and xyloglucans and xylans, respectively [9].

Incubation of pea endocarp with conidial suspensions of *M. fructicola* for 18 hr caused the disappearance of water-soluble polysaccharides [1] consistent with an arabinogalactan polymer being hydrolysed by enzymes from the germinating conidia; however, these polymers were still present in the diffusates examined in this paper and presumably substantial hydrolysis of the pea arabinogalactans occurs after longer incubation periods with the fungus. Here we have shown that minor changes in the polysaccharide structure occurred as early as 2 hr after inoculation with *M. fructicola* conidia and before the appearance of the first germ tubes (3 hr) or the accumulation of protein in diffusates. These modifications were presumably due to endohydrolase attack on pectins as evidenced by a decrease in intra-chain (4-linked) galacturonic acid residues. The filtration characteristics of the diffusate were altered, but there was no concomitant detection of free arabinose, galactose and xylose. These free sugars were predominant in 18 hr diffusates of the pea × *M. fructicola* interaction [1]. Polygalacturonases of fungal origin are present in extracts of plum and peach tissue infected with the closely related *M. fructigena* and *M. laxa* [10].

Neither the acidic arabinogalactan polymers leached from pea endocarp nor products of their breakdown by the fungus could be demonstrated to elicit *in vivo* the biosynthesis of pisatin in the pea × *M. fructicola* interaction [2]. Therefore, the elicitation of pisatin did not appear to be due to the action of a polygalacturonase from *M. fructicola* releasing fragments from the pea epidermal cell walls by the type of mechanism proposed by Nothnagel *et al.* [11].

EXPERIMENTAL

Plants. Endocarp surface of detached pea pods was used [12].

Fungus. The culture of *M. fructicola* (wint.) Honey and the preparation of wet-harvested conidial suspensions used for inoculum were as previously described [12].

Incubation. A washed conidial suspension (4 × 10^5 conidia/ml; *ca* 1 ml/pod cavity) was topically applied to the endocarp surface of detached half pea pods from which seeds had been removed, and incubated in shallow humidity chambers at 20° in the dark for 2, 4 or 6 hr. The H_2O control consisted of pods treated with sterile dist. H_2O and incubated for 18 hr; 200 ml of conidial suspension were used for each incubation and *ca* 150 ml diffusate was recovered at each time of harvest.

Isolation of polysaccharide from diffusate. The diffusate harvested at each time was centrifuged for 1 hr at 6780 *g* to remove plant debris and fungal spores. To the supernatant was added 4 vol. EtOH and the aq. EtOH soln allowed to stand for 7 days at −25°. The precipitated polysaccharide was collected by centrifugation for 30 min at 1300 *g*, washed with 80% EtOH, freeze dried and stored *in vacuo* over P_2O_5. The EtOH supernatants were concd *in vacuo* by rotary film evaporation (35°) and examined by PC.

Determination of monosaccharide residue composition of polymer fractions. Duplicate samples (1 mg) from each preparation were hydrolysed by methanolysis (1 N HCl in MeOH) for 12 hr at 82° and the TMSi derivatives of the Me glycosides prepared as in ref. [13]. TMSi derivatives were dissolved in hexane and analysed by GC on 3% OV 225 (1 m × 4 mm; 150°, 5 min initial then temp. programmed at 2°/min to 220°) and 3% OV 101 (1 m × 4 mm; 150° initial then temp. programmed at 2°/min to 220°). The detector (FID) was at 250° and carrier gas N_2 at 50 ml/min.

Methylation and GC-MS. Linkage analysis was by methylation using the procedure of ref. [14]. Partially methylated alditol acetates were separated and quantified by GC-MS as described in ref. [15]. GC-MS was carried out using a fully automated instrument and EI as described in ref. [16].

Chromatography. The pea × H_2O 18 hr polymers (0.6 mg) were dissolved in 6 M urea then applied to a Trisacryl (LKB, Bromma) column (40 × 2.5 cm) and eluted with 6 M urea. The flow rate was 12 ml/hr and 1 ml fractions were collected. The V_0 and V_t of the column were 20 and 60 ml, respectively. The total carbohydrate content of each fraction was determined by the method of ref. [17]. M_r calibration of the column was achieved with Dextran M_r Standards (Pharmacia). T2000 (M_r 2 × 10^6), T500 (M_r 5 × 10^5), T250 (M_r 2.5 × 10^5), T40 (M_r 4 × 10^4) and T10 (M_r 1 × 10^4).

Radial gel diffusion. Each sample was injected into a well (*ca* 3.5 mm diam.) in an agarose gel containing β-glucosyl Yariv reagent (10 μg/ml), 1% w/v agarose, 0.15 M NaCl 0.02%, w/v NaN_3 and incubated as described in ref. [18].

Examining of low M_r fraction by PC. Diffusate concentrates were applied to Whatman No. 1 paper and chromatographed in EtOAc–PrOH–H_2O (4:1:2, upper layer) or to Whatman 3MM paper and chromatographed in PrOH–EtOAc–H_2O (6:1:3). Sugars were detected by the alkaline $AgNO_3$ method [19, 20].

Total protein was estimated by the Coomassie Brilliant Blue G-250 binding technique [21] with BSA as a std.

Total carbohydrate was determined by the pHoH–H_2SO_4 method of ref. [17] using galactose as a std.

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