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FINE STRUCTURE OF THE ARABINOGALACTAN–PROTEIN FROM *Lolium multiflorum*

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ABSTRACT

The extracellular arabinogalactan–protein from suspension-cultured cells of *Lolium multiflorum* (ryegrass) was purified in a single step by affinity chromatography of the culture medium on myeloma protein J539–Sepharose. The product obtained after two Smith-degradations had an apparent molecular size corresponding to that of galactoheptaose and was mainly (1→3)-linked. The Smith-degradation products were essentially monodisperse with respect to molecular size, indicating that periodate-oxidisable residues were distributed regularly along the polysaccharide chains. The polysaccharide chains in the arabinogalactan–protein were probably attached to hydroxyprolyl residues of the protein core *via* *O*-glycosylic linkages. No carbohydrate appears to be linked to serine or threonine residues. No oligo-arabinosides linked to hydroxyproline could be detected.

INTRODUCTION

Arabinogalactan–proteins (AGPs), which are widely distributed in plant tissues and their secretions[1], are proteoglycans containing high proportions of polysaccharides rich in L-arabinofuranosyl and D-galactopyranosyl residues, but may also contain smaller proportions of L-rhamnopyranosyl, D-mannopyranosyl, D-xylopyranosyl, D-glucopyranosyl, D-glucosyluronic acid, and D-galactosyluronic acid residues[1]. The polysaccharides comprise a backbone of (1→3)-linked β-D-galactopyranosyl residues branched through positions 6 with (1→6)-linked β-D-galactopyranosyl side-chains which are substituted with arabinofuranosyl residues, arabinopyranosyl residues, and the other less-abundant monosaccharides, often in terminal positions[1]. Using Smith-degradation procedures, it has been shown, for many AGPs, that the β-D-galactan framework contains blocks of galactopyranosyl

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residues which are interrupted at regular intervals by periodate-sensitive residues[2–4]. The blocks which remain resistant to Smith degradation consist largely of (1→3)-linked galactopyranosyl residues[1].

The protein content of AGPs is usually <10%, but is characteristically rich in hydroxyproline, alanine, and serine. The nature of the carbohydrate–protein linkage has been demonstrated unequivocally only for the AG-peptide from wheat endosperm and involves galactopyranosyl residues linked *O*-glycosylically to hydroxyprolyl residues[5,6]. However, the hydroxyproline of rice-bran proteoglycan is substituted with oligo-arabinosides[7], and alkali-stable arabinosyl-hydroxyproline linkages have been detected in an AGP from Timothy grass pollen[8]. In *Cannabis sativa* leaf, both galactosyl-serine and alkali-resistant linkages have been detected[9], and carbohydrate is attached to serine, threonine, and hydroxyproline in the AGP from *Phaseolus vulgaris*[10]. A large proportion of the polysaccharide in radish-leaf AGP may be linked to the protein through 3-*O*-D-galactosylserine[11].

The AGP secreted by suspension-cultured ryegrass endosperm cells is typical of other plant AGPs in that the carbohydrate component consists mainly of arabinosyl (36%) and galactosyl (64%) residues, arranged as a (1→3),(1→6)-β-D-galactan framework terminating primarily in arabinofuranosyl residues[12]. The protein, which accounts for 7% of the AGP, contains 22.5% of alanine, 14.8% of hydroxyproline, and 9.8% of serine, on a molar basis[12].

We now report on the nature of the carbohydrate–protein linkage and the carbohydrate chains in ryegrass AGP.

EXPERIMENTAL

Cell cultures. — Liquid suspension cultures of ryegrass *(Lolium multiflorum)* endosperm cells were maintained at 28°, in the dark, on a modified White's medium which contained 4% of sucrose as the carbon source[13].

Affinity chromatography. — A Balb/c mouse bearing a myeloma protein J539 ascites tumour was kindly provided by Dr. Michael Potter (National Institutes of Health, U.S.A.) The myeloma protein J539 was purified from ascites fluid by chromatography[14] on propionic acid-treated Sepharose 4B, and covalently bound to CNBr-activated Sepharose 4B as specified by the manufacturers (Pharmacia Fine Chemicals, Ltd.); the swollen gel contained ~3 mg of bound protein/mL. The myeloma protein J539–Sepharose was equilibrated in phosphate-buffered saline (PBS; pH 7.4, 0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.01% $CaCl_2 \cdot 2\ H_2O$, 0.01% $MgCl_2 \cdot 6\ H_2O$).

Ryegrass AGP was isolated by affinity chromatography on a 50-mL column of myeloma protein J539–Sepharose 4B. The extracellular medium of ryegrass cell cultures was filtered and portions (200–250 mL) were applied to the affinity column (prior dialysis did not improve the binding efficiency). Unbound material was eluted with PBS, and AGP was subsequently eluted with 0.1M citric acid (pH 3.0) containing M NaCl[15]. Fractions containing carbohydrate were combined, dialysed

for 24 h against distilled water at 4°, and freeze-dried.

Analytical procedures. — P.c. was conducted with *A*, 1-butanol–ethanol–water (4:1:5, upper phase); and *B*, ethyl acetate–pyridine–water (8:2:1); with detection by ammoniacal silver nitrate (polyols) or *p*-anisidine hydrochloride (sugars).

Carbohydrate was determined by the phenol–sulphuric acid method[16], with standards prepared from a 1:2 mixture of arabinose and galactose (total concentration, 100 μg/mL) in water saturated with benzoic acid (as a preservative).

Protein was determined by the method of Lowry *et al.*[17], using crystalline bovine serum albumin as standard.

The monosaccharide compositions of the AGP and its degradation products were determined by g.l.c. of their alditol acetate derivatives[18] on a support-coated open-tubular (SCOT) glass-capillary column (28.5 m × 0.5 mm i.d.) coated with Silar 10C and the temperature programme 180° for 4 min, 180°→210° at 1°/min. Other details of the g.l.c. procedure are given elsewhere[18].

Methylation analysis. — Methylation was performed using the method of Hakomori[19] as described by Björndal *et al.*[20]. The methylated polysaccharide was formolysed and hydrolysed[21], and the partially methylated sugars were converted[22] into their alditol acetates and subjected to g.l.c.–m.s. using a vitreous silica WCOT column (25 m × 0.22 mm i.d.) with bonded phase BP-75 (ref. 23) directly coupled to a Finnigan MAT mass spectrometer[24]. Identifications were based on retention times and comparison of mass spectra with published spectra[25].

Smith degradation. — Freeze-dried AGP (66 mg) was treated with 0.12M sodium metaperiodate for 72 h; the product was then reduced with 0.79M sodium borohydride for 96 h and treated with M trifluoroacetic acid at ~20° for 72 h as described previously[2,26]. Fractionation of the products with methanol–acetone (1:2) yielded the Smith-degraded polysaccharide SD1 (19 mg) and a syrup which consisted [p.c. (solvent *A*) and chromotropic acid assay[27]] mainly of glycerol with traces of sugars and a strongly fluorescent component [p.c. (solvent *B*)] which was probably proteinaceous[4]. SD1 was subjected to steric-exclusion chromatography (s.e.c.) on Bio-Gel P-10 (elution with M NaCl, dextran standards).

A portion (10.6 mg) of SD1 was submitted to a second Smith-degradation. Because of the small quantity of material available, oxidation (in 15mM $NaIO_4$ for 96 h) was monitored spectrophotometrically[28]. The periodate consumption was 4.3 mmol/g. Reduction with $NaBH_4$ and hydrolysis with M trifluoroacetic acid at room temperature were carried out as before. After treatment with acid for 72 h, an aliquot (100 μL) of the solution (1 mL) was assayed for polyol[27], and another aliquot (0.4 mL) was neutralised with $NaHCO_3$ and subjected to s.e.c. (Bio-Gel P-2). The remaining solution was freeze-dried and the residue was fractionated (1:3 methanol–acetone), yielding the product SD2 (1 mg) and a syrup consisting almost entirely of glycerol.

Treatment of the AGP with alkaline-borohydride. — Ryegrass AGP (5 mg) was added to saturated aqueous barium hydroxide (1 mL) and the mixture was

heated at 105° for 6 h. A solution of sodium borotritide (8.7 μCi, 15.9 Ci/mmol) in 0.52M NaOH (4 μL) was added and the solution was kept for 16 h at room temperature. Sodium borohydride (5 mg) was added and the incubation was continued[10] for 90 min at 35°. The mixture was neutralised with 0.5M H_2SO_4 and centrifuged, and the supernatant solution was dried *in vacuo* at 37°. A solution of the residue in 200 μL of deionised water was stored at −15°. Products were fractionated on a column of Bio-Gel P-2 calibrated with D-glucose and cello-oligosaccharide standards[29]; fractions were assayed for carbohydrate and radioactivity.

RESULTS AND DISCUSSION

Purification of ryegrass AGP. — Affinity chromatography of cell culture medium on myeloma protein J539–Sepharose 4B gave a bound fraction that could be eluted by decreasing the pH to 3 after all of the non-bound material had been removed (Fig. 1). The bound material contained 7% of protein, and methylation analysis showed that the polysaccharide moiety consisted mainly of →3.6)-Gal*p*

TABLE I

METHYLATION ANALYSIS OF PURIFIED NATIVE AGP FROM *Lolium multiflorum*

Sugar linkage	*Proportion (mol %)*	*Approximate molar ratio*	*Required by model*[a]
Ara*f*→	32	11	11
→5)-Ara*f*	7	2	2
Gal*p*→	6	2	2
→3)-Gal*p*	7	2	2
→6)-Gal*p*	8	3	4
→3,6)-Gal*p*	37	12	11
→3,4.6)-Gal*p*	3	1	1

[a]See Fig. 3.

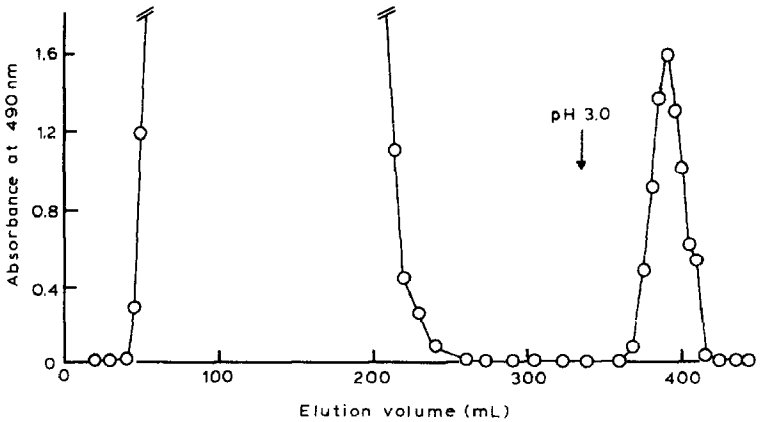

Fig. 1. Elution profile of ryegrass endosperm culture-filtrate on myeloma protein J539–Sepharose 4B.

and terminal Ara*f* residues (Table I). These data are similar to those reported[12] for the extracellular ryegrass AGP prepared by precipitation with the Yariv artificial antigen, and confirm that the affinity chromatography procedure provides a simple, one-step purification of the ryegrass AGP. G.l.c.–m.s. permitted the positive identification of →3,4,6)-Gal*p* residues in this AGP preparation (Table I; *cf.* ref. 12).

Smith degradation. — The product (SD1) of the first Smith-degradation had $[\alpha]_D$ +29° and consisted almost entirely of Gal*p* residues with only a trace of protein and ~4 mol% of Ara. The survival of some Ara may have been due to the presence in the AGP of a few internal periodate-resistant residues or to incomplete oxidation. S.e.c. showed a single peak with an apparent mol. wt. of ~1700. A second Smith-degradation yielded SD2, which had an apparent mol. wt. of ~1200 (single monodisperse peak in s.e.c.). This decrease in molecular weight indicated the removal of three Gal*p* residues from SD1 which was consistent with the amounts of glycerol released (3 mol/mol) and periodate consumed by SD1 (~7 mol/mol). The periodate consumption corresponded to the value expected for oxidation of three Gal*p* residues [end group or →6)-linked] and a terminal glycerol group arising from cleavage of →6)-Gal*p* or →5)-Ara*f* residues in the main chain of the parent polysaccharide.

Methylation analysis of SD2 showed terminal Gal*p*, →3)-Gal*p*, and →3,4,6)-Gal*p* in the molar ratios ~1:5:2.

Treatment of the AGP with alkaline-borohydride. — The elution profile of ryegrass AGP on Bio-Gel P-2 after treatment with saturated $Ba(OH)_2$ in the presence of sodium borotritide is shown in Fig. 2. Several poorly resolved carbohydrate peaks are present in the region of the void volume and presumably correspond to polysaccharides of various chain lengths. The apparent absence of included carbohydrate suggests that no short oligosaccharides were released. The profile of radioactivity contains three peaks (Fig. 2); the first was eluted near the void volume (Peak I, K_{av} 0.06), just after the major carbohydrate peaks, and the others (II and III) were eluted at K_{av} 0.78 and 1.08, respectively. This might be taken as evidence for the attachment of polysaccharide, oligosaccharide, and possibly monosaccharide units to serine or threonine of the protein core. However, commercial prepara-

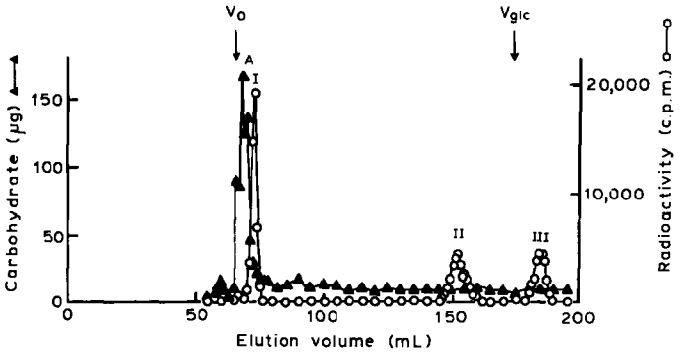

Fig. 2. Elution profile of alkali-treated ryegrass AGP on Bio-Gel P-2.

tions of sodium borotritide may contain tritiated contaminants[30]. Accordingly, the experiment was repeated in the absence of AGP, and peaks showing radioactivity were again detected at K_{av} 0.05 and 1.07, in proportions similar to those in Fig. 2. Radioactive peak II (K_{av} 0.78, Fig. 2) was also detected in the control, in relatively small proportion, but disappeared with increasing time. Thus, the radioactive peaks appear to be derived from contaminants in the sodium borotritide.

Fine structure of the polysaccharide component of ryegrass AGP. — The presence in the AGP molecule of uniform blocks of periodate-resistant Gal*p* residues is demonstrated by the products of Smith degradation. Analysis and s.e.c. suggested that SD1 is a simple galactan, containing ~10 Gal*p* residues only 3 of which are vulnerable to periodate. The production of this apparently monodisperse fragment from the native AGP (mol. wt. $\sim 2.5 \times 10^5$)[12] after only one Smith-degradation, which also removes virtually all of the protein (*cf.* ref. 4), indicates the presence in the AGP of regularly repeating sub-units, as has been demonstrated for other arabinogalactans[2–4,11].

The apparent molecular weight of SD2 is close to the value expected for a molecule containing 7 Gal*p* residues and a terminal glycerol group. The ratio of terminal to internal Gal*p* residues, indicated by methylation analysis data, is consistent with a d.p. of ~7. The →3,4,6)-Gal*p* detected, in a proportion not balanced by that of terminal Gal*p*, appears to be spurious, possibly arising from under-methylation. Spurious derivatives are frequently found in methylation analyses of Smith-degradation products and have been attributed[31–33] to incomplete hydrolysis. However, in the present work, this step was monitored[26] by s.e.c.

The periodate-resistant blocks in the arabinogalactan core are separated by periodate-susceptible residues, which may be →6)-Gal*p* or →5)-Ara*f*. The relatively high degree of freedom of rotation around such glycosidic linkages may result[1] in flexible "kinks" in the polysaccharide chain, which could be important determinants of the overall conformation. Also, each arabinogalactan sub-unit could be linked to amino acid residues in the peptide chain rather than be a component of a large polysaccharide.

The structure for the sub-unit shown in Fig. 3 is one of innumerable possibilities consistent with the available experimental evidence.

Carbohydrate–protein linkage region of the ryegrass AGP. — The products from alkaline-borohydride-treated AGP were eluted from Bio-Gel P-2 close to the exclusion limit; significant levels of carbohydrate of low mol. wt. were not detected (Fig. 2). Treatment of a glycoprotein or proteoglycan with saturated $Ba(OH)_2$ at 105° for 6 h will result not only in the β-elimination of carbohydrate from serine or threonine residues, but also in the cleavage of peptide linkages. Using similar conditions, Miller *et al.*[34] degraded the protein of plant cell-wall glycoproteins and released hydroxyproline–arabinosides. Thus, the alkali cleaved the peptide chain but not the arabinosyl-hydroxyproline linkage. If the ryegrass AGP contained any monosaccharides or short oligosaccharides linked *O*-glycosylically to hydroxyproline, as observed in the secretory AGP from *Phaseolus vulgaris*[10], hydrolysis

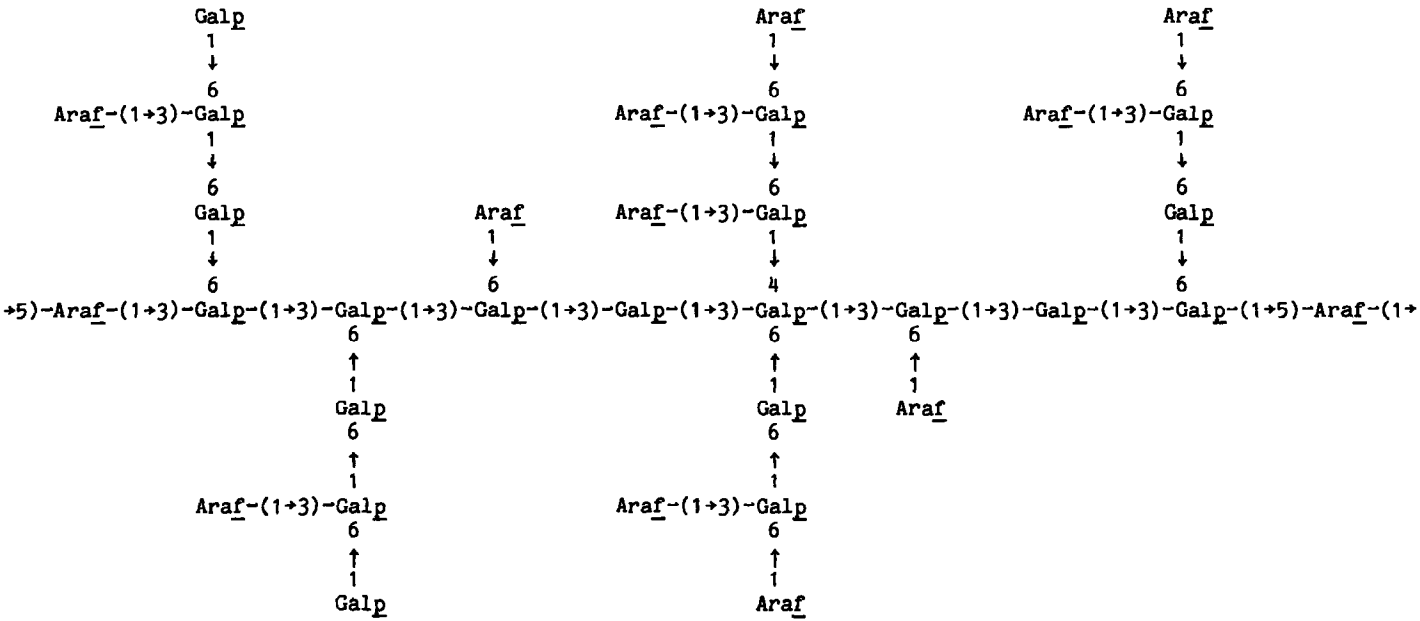

Fig. 3. One of many possible structures for the repeating sub-unit of the carbohydrate moiety in ryegrass AGP.

with $Ba(OH)_2$ would have released similar oligomers, included in the Bio-Gel P-2 pores. However, since no reducing ends would be produced, the oligomers would not be labelled by reduction with sodium borotritide. Polysaccharide chains linked to hydroxyproline would be released from the peptide core of the AGP by a similar mechanism, but would not be radioactively labelled and would not be included in Bio-Gel P-2.

On the other hand, if carbohydrate chains were covalently associated with serine or threonine residues, treatment with $Ba(OH)_2$ would catalyse their release by β-elimination, and sodium borotritide would reduce and label the reducing ends of the released saccharide chains. Single Gal*p* residues of the type found linked to serine in the cell-wall glycoproteins[35] and in the AGPs from *Cannabis sativa*[9], opium poppy[36], and radish leaf[11] would be released, and labelled galactitol would be recovered. Polysaccharide chains linked to serine or threonine would also be released from the polypeptide and reduced with sodium borotritide at the reducing end, but would not be included in Bio-Gel P-2.

With ryegrass AGP (Fig. 2) carbohydrate is eluted near the void volume, suggesting that it exists mainly as polysaccharide chains. The radioactivity detected in the fractions eluted from Bio-Gel P-2 (Fig. 2) is attributable to contaminants in the sodium borotritide[30]. It may be concluded that few, if any, oligosaccharide or monosaccharide substituents are attached to the protein core of the ryegrass AGP and that serine or threonine residues are not involved in the linkage of carbohydrate to protein. In view of the high content of hydroxyproline of the ryegrass AGP[12], and by analogy with the well-characterised AG-peptide from wheat endosperm[5,6,37], hydroxyproline residues remain the prime candidates for the attachment of arabinogalactan chains to the protein core of the AGP.

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