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# Chemical and Physical Properties of an Arabinogalactan-Peptide from Wheat Endosperm

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1. An arabinogalactan-peptide from wheat endosperm was studied by using physicochemical techniques and some aspects of its chemical structure were determined. 2. The arabinogalactan-peptide is a non-associating, polydisperse macromolecule ($\bar{M}_w = 22000$) which exhibits only minor non-ideal effects in aqueous solution. 3. Examination of the products of partial acid hydrolysis of the polysaccharide component showed that arabinose is present in the α-L-arabinofuranosyl configuration, and i.r.-absorption spectroscopy and optical-rotation studies suggest that the D-galactopyranose residues are linked by glycosidic linkages in the β-anomeric configuration. 4. The arabinogalactan is linked to a peptide which represents 8% (w/w) of the arabinogalactan-peptide and which may be present as a molecular core. Partial degradation of the polymer by successive treatment with oxalic acid and NaOH showed that the linkage between polysaccharide and peptide involves galactose and hydroxyproline residues and is glycosidic in nature. A tentative model is proposed for the structure of the wheat endosperm arabinogalactan-peptide. 5. The subcellular location and function of the arabinogalactan-peptide is discussed in relation to previous work with related molecules.

Arabinogalactans have been isolated from plant tissues of diverse origin. They can be readily extracted, usually with water, and have been obtained from coniferous woods (Aspinall, 1959; Timell, 1965), coffee beans (Wolfrom & Patin, 1965; Wolfrom & Anderson, 1967), soya-bean seeds (Morita, 1965*a*,*b*; Aspinall *et al.*, 1967*a*,*b*), aquatic moss (Geddes & Wilkie, 1971), Saguaro cactus cortex (Steelink *et al.*, 1968), apple fruit (Barrett & Northcote, 1965; Knee, 1973), rapeseed (Siddiqui & Wood, 1972), Siratro leaves (Ford, 1972), broad-bean leaves (Pusztai *et al.*, 1971), and from suspension-cultured cells of tomato and sycamore (Lamport, 1970).

Most investigations of arabinogalactans have been concerned with the isolation and structural analysis of the polysaccharide, and any associated protein has been of only passing interest. However, Wolfrom & Patin (1965) did suggest that the arabinogalactan from green coffee beans is associated, in part at least, with the hydroxyproline-containing protein previously described in extracts of ground roasted coffee by Clements & Deatherage (1957). An arabinogalactan that was extracted from broad-bean leaves by Pusztai *et al.* (1971) contained tightly bound protein (4–7%, w/w) of which two-thirds consisted of hydroxyproline, alanine, serine and threonine residues. Lamport (1970) has also reported that arabinogalactans associated with a hydroxyproline-rich protein can be extracted with trichloroacetic acid from the cytoplasmic fraction of several plant tissues.

The present paper reports the results of structural studies of an arabinogalactan-peptide isolated from water extracts of wheat endosperm (Fincher & Stone, 1974*a*). The arabinogalactan-peptide is predominantly polysaccharide (92%, w/w) with galactose and arabinose present in the ratio approx. 1.5:1 (w/w). The arabinogalactan is covalently associated with peptide material (8%, w/w), of which 16–20% (on a molar basis) is hydroxyproline (Fincher & Stone, 1974*a*). The polymer has been characterized by physicochemical methods and the combined physical and chemical data have made possible the formulation of a molecular model.

Other studies on the changes occurring in the water-soluble non-starchy polymers from wheat endosperm during germination suggest that the arabinogalactan-peptide is not significantly altered during germination (Fincher & Stone, 1974*b*).

## Experimental

### *Materials*

*Arabinogalactan-peptide.* The arabinogalactan-peptide from wheat endosperm was prepared as described by Fincher & Stone (1974*a*).

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*Chemicals.* Hydrazine hydrate (98.0–100.0%, w/w) was obtained from Ajax Chemicals Limited, Melbourne, Vic., Australia, and all other chemicals were of analytical grade.

*Enzymes.* Pronase was obtained from E. Merck and Co., Darmstadt, West Germany; thermolysin was from Calbiochem, Los Angeles, Calif., U.S.A.; collagenase was from Schwarz–Mann, New York, N.Y., U.S.A.; trypsin, α-chymotrypsin and β-galactosidase were from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; galactose oxidase (grade x9) was from Hughes and Hughes Enzymes Ltd., London W.1; and Cellulosin AP was from Ueda Kagaku Kogyo Co. Ltd., Osaka-Fu, Japan. The arabinogalactanase preparation from *Bacillus subtilis* was a gift from Professor T. Yamamoto (Osaka City University, Japan) and also contained mannanase, xylanase, amylase and proteinase. The preparation had an arabinogalactanase activity of 0.83unit/mg of freeze-dried material (Emi *et al.*, 1971).

## *Methods*

*Specific refractive increment.* A standard solution of arabinogalactan-peptide (approx. 0.7g/dl) was prepared in water, filtered through a membrane filter (pore size 0.45μm; Sartorius-Membranfilter G.m.b.H., Gottingen, West Germany) and the absolute concentration determined by dry-weight analysis. The stock solution was diluted by weight with water and the refractive-index difference measured at each concentration by using a differential refractometer (Brice–Phoenix, Philadelphia, Pa., U.S.A.).

*Partial specific volume.* The partial specific volume of arabinogalactan-peptide was determined by the classical pycnometric method which involved density measurements of solvent and solution. Solutions of arabinogalactan-peptide (approx. 1g/dl) were prepared gravimetrically in acetate buffer [0.1I (mol/l), pH5.0] and passed through a 0.45μm membrane filter. Exact concentrations were then measured refractometrically and pycnometric determinations were made in duplicate.

*Sedimentation.* The sedimentation-velocity experiments were performed at 24°C in a Spinco model E analytical ultracentrifuge and schlieren optics were used. The speed setting was 59780 rev./min, but the exact speed was determined by timing the revolution counter. The molecular weight of the arabinogalactan-peptide was determined by the sedimentation-equilibrium procedure of Yphantis (1964): solutions were centrifuged at a speed setting of 35600rev./min for 11h at 22°C.

*Partial acid hydrolysis.* A sample of arabinogalactan-peptide (10mg) was heated at 100°C in a sealed tube with 1ml of freshly prepared 0.0125M-oxalic acid for 5h (Bishop, 1957). The hydrolysate was cooled and a portion chromatographed on a Bio-Gel P-6 column. The remainder was made 80% (v/v) with respect to ethanol and the precipitated material was washed thoroughly with 80% ethanol and dried by solvent exchange (Green, 1963). The supernatant fraction and washings from the ethanol precipitation were evaporated to dryness and redissolved in water for analysis of the monosaccharide composition (Mares & Stone, 1973*a*).

*Chromatography on Bio-Gel P-6.* Samples of arabinogalactan-peptide (0.5–5mg) after the treatments described were dissolved in 0.5ml of 0.3% (w/v) NaCl containing 0.05% $NaN_3$ and loaded on a column (55cm×1.5cm) of Bio-Gel P-6 (200–400 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Columns were run at flow rates of approx. 8ml/h with 0.3% NaCl containing 0.05% $NaN_3$ and fractions (4–5ml) were collected. Carbohydrate in the fractions was measured by the phenol–$H_2SO_4$ method of Dubois *et al.* (1956) as modified by Immers (1964) with galactose as a standard. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) as standard. Hydroxyproline was measured by the colorimetric procedure of Neuman & Logan (1950) as modified by Leach (1960).

*Hydrazinolysis.* The procedure used for hydrazinolysis of the arabinogalactan-peptide was essentially that of Yosizawa *et al.* (1966). A sample of arabinogalactan-peptide (10mg) was sealed in a glass tube with 0.5ml of hydrazine hydrate made 1.5% (w/v) with respect to hydrazine sulphate. The tube was heated at 100°C for 16h, then cooled and the hydrazine removed in a vacuum desiccator over conc. $H_2SO_4$.

*Alkaline degradation.* (*a*) With $Ba(OH)_2$. Samples of arabinogalactan-peptide (10mg) were heated with 0.5ml of saturated $Ba(OH)_2$ (approx. 0.22M) in sealed tubes at 105°C for 6h (Lamport, 1969). Each hydrolysate was neutralized to the Bromothymol Blue end-point with 0.5M-$H_2SO_4$, centrifuged to remove $BaSO_4$ and the supernatant fraction was then evaporated to dryness. (*b*) With NaOH. Samples of arabinogalactan-peptide (10–40mg) were kept at 100°C for 24h in 1–4ml of 5M-NaOH in sealed tubes. Hydrolysates were cooled, neutralized to the Bromothymol Blue end-point with HCl and then centrifuged to remove precipitated sodium silicate (Nottingham, 1955*a*,*b*). The sedimented silicate was washed with water, the supernatant and combined washings were evaporated to dryness and the dry material representing the total neutralized hydrolysate was redissolved in water. Polysaccharide from the neutralized hydrolysate was recovered by precipitation with 4vol. of ethanol and the flocculent precipitate was washed thoroughly with 80% ethanol, dried by solvent exchange, dissolved in water and re-precipitated with ethanol. The com-

bined 80%-ethanol supernatants were evaporated to dryness for amino acid and carbohydrate analysis.

*Treatments with oxalic acid and NaOH.* Arabinogalactan-peptide (47mg) was hydrolysed for 6h at 100°C in 0.0125M-oxalic acid (3.0ml), cooled and the residual polymeric material precipitated with 4vol. of ethanol. After thorough washing and drying by solvent exchange, the precipitated material was treated with 2.0ml of 5M-NaOH and neutralized as described above. The polysaccharide in the neutralized hydrolysate was recovered by precipitation with ethanol as described above and the dried material is termed the oxalic acid–NaOH residue.

*Enzymic hydrolysis of arabinogalactan-peptide.* (*a*) Proteolytic enzymes. Arabinogalactan-peptide (10mg) was dissolved in 0.9ml of the appropriate buffer (see below) to which was added 100$\mu$l of enzyme solution (3mg/ml). After incubation at 40°C for 16h the reaction mixtures, together with substrate and enzyme controls, were heated at 100°C for 10min. All samples were then stored frozen before assay.

Activity of all the enzymes was confirmed against their various protein substrates. The following enzymes and buffers were used: Pronase, 0.2M-sodium phosphate (pH6.8); thermolysin, 1% (w/v) $NH_4HCO_3$ (pH7.9); collagenase, 0.01M-Tris–HCl (pH7.4)–1mM-$CaCl_2$; trypsin–$\alpha$-chymotrypsin (1:1, v/v), 0.01M-Tris–HCl (pH7.8)–10mM-$CaCl_2$.

(*b*) Cellulosin. Cellulosin AP was suspended in 0.1M-sodium acetate buffer (pH5.0), centrifuged to remove insoluble material and then dialysed. After dialysis the non-diffusible material was concentrated in a Diaflo apparatus (Amicon Corp., Lexington, Mass., U.S.A.) fitted with a UM2 ultrafilter. The concentrated solution, which was free of reducing sugars, was then freeze-dried.

Arabinogalactan-peptide (10mg) was dissolved in 0.9ml of 0.1M-sodium acetate buffer (pH5.0) and incubated with 100$\mu$l of Cellulosin solution (3mg/ml) at 40°C for 16h in the presence of toluene. An additional 100$\mu$l of enzyme solution was added after 16h and the incubation continued for a further 24h. The reaction was stopped by heating at 100°C for 10min and any insoluble material was removed by membrane filtration (pore size 0.45$\mu$m).

(*c*) Arabinogalactanase. Arabinogalactan-peptide (10mg) was dissolved in 0.9ml of 0.02M-sodium phosphate buffer (pH6.0), 0.1ml of enzyme solution (3mg/ml in the same buffer) was added and the mixture incubated at 40°C for 16h in the presence of toluene.

(*d*) Galactose oxidase. The method of Roth *et al.* (1965) was used to measure the action of galactose oxidase on the arabinogalactan-peptide (300$\mu$g/ml). Recrystallized D-galactose was used as a standard.

*I.r.-absorption spectroscopy.* Thoroughly dried samples of the arabinogalactan-peptide and the galactan-peptide residue after oxalic acid treatment were pressed into KBr discs. Spectra were determined by using a Perkin–Elmer model 457 spectrophotometer.

*Optical rotatory dispersion.* Optical rotations were measured in a Perkin-Elmer 141 polarimeter at 20°C in a 1ml jacketed cell (10cm light-path).

*Amino acid analysis.* Samples were hydrolysed as described by Fincher & Stone (1974*a*) and amino acids were analysed by the accelerated method of Spackman *et al.* (1958) with a Beckman model 120B amino acid analyser. Hydroxyproline in hydrolysates was resolved from aspartic acid by the method of Mashburn & Hoffman (1970).

*Monosaccharide composition.* Monosaccharides in the hydrolysates of polysaccharides were determined by g.l.c. of the alditol acetate derivatives (Albersheim *et al.*, 1967; Mares & Stone, 1973*a*).

*Gel filtration chromatography on Sephadex G-200.* Samples of polysaccharides (1–4mg) were dissolved in 0.5ml of 0.3% NaCl containing 0.05% $NaN_3$, applied to a column (50cm×1.5cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted at flow rates of approx. 6ml/h with 0.3% NaCl containing 0.05% $NaN_3$. The column was calibrated with Blue Dextran 2000 (Pharmacia) and galactose.

*Precipitin tests.* Arabinogalactan-peptide [0.05–0.4ml, 1mg/ml in 0.85% (w/v) NaCl containing 1mM-$CaCl_2$] was added to a solution of purified castor-bean (*Ricinus communis*) lectin (200$\mu$g in 0.1ml of the same salt solution). All volumes were made to 0.6ml, and the solutions were mixed and incubated at 37°C for 1h and left overnight in a refrigerator. Precipitates were centrifuged and washed twice in the cold with 0.5ml of 0.85% NaCl solution containing 1mM-$CaCl_2$. The tubes were drained and the precipitates were dissolved in 0.6ml of 0.1M-NaOH and their protein content was determined by the method of Lowry *et al.* (1951). Inhibitors of the precipitin reaction were tested by mixing with the lectin and incubating at 37°C for 1h before addition of arabinogalactan-peptide.

## Results

### *Physicochemical properties*

Table 1 summarizes the physicochemical parameters determined for the arabinogalactan-peptide.

The behaviour of the arabinogalactan-peptide at sedimentation equilibrium was analysed as described by Yphantis (1964). The results indicate that the arabinogalactan-peptide is polydisperse with respect to molecular weight and exhibits minimal self-interaction in aqueous solution. During velocity sedimentation the material exhibited a single symmetrical boundary which was not hypersharp. The dependence of $s_{20,w}$ on concentration was linear over the range 0.2–1.2g/dl. The frictional ratio suggests that the

Table 1. *Physicochemical parameters of the arabinogalactan-peptide from wheat*

For details see the text.

| Parameter | Value |
|---|---|
| Specific refractive increment | 0.142ml/g |
| Partial specific volume ($\bar{v}$) | 0.62ml/g |
| Intrinsic viscosity ([$\eta$])* | 0.108dl/g |
| Sedimentation coefficient at zero concentration ($s^0_{20,w}$) | 3.10S |
| Weight-average molecular weight ($\bar{M}_w$) | 22000 ± 1000 |
| Scheraga-Mandelkern function ($\beta$) | $2.70\times10^6$ |
| Frictional ratio ($f/f_0$) | 1.46 |
| Stokes radius ($a$) | 2.45nm (24.5Å) |
| Diffusion coefficient at zero concentration ($D^0_{20,w}$) | $9.5\times10^{-7}$cm$^2\cdot$s$^{-1}$ |

* See Fincher & Stone (1974*a*).

Table 2. *Determination of the apparent molecular weights, by chromatography on Sepharose G-200, of the arabinogalactan-peptide and the galactan-peptide residue recovered after oxalic acid hydrolysis*

$K_{av.} = \frac{V_e - V_0}{V_t - V_0}$ where $V_e$ = elution volume, $V_0$ = void volume and $V_t$ = total volume. $M_{app.}$ values were obtained from data published by Pharmacia Fine Chemicals relating $K_{av.}$ values to the molecular weights of dextrans. See the text for details of chromatography.

| Polymer | $K_{av.}$ | $M_{app.}$ |
|---|---|---|
| Arabinogalactan-peptide | 0.34 | 32000* |
| Galactan-peptide | 0.53 | 17000 |
| Dextran 20 | 0.45 | 21000† |

* Arabinogalactan-peptide has $\bar{M}_w = 22000$ (cf. Table 1).
† Dextran 20 (Pharmacia) has $\bar{M}_w = 22700$ (manufacturer's data).

macromolecule is essentially spherical in shape (Tanford, 1961) and the value for intrinsic viscosity, which is higher than those for globular proteins of similar molecular weight (Yang, 1961), may mean that the arabinogalactan-peptide has a somewhat expanded form.

*Partial acid hydrolysis*

The elution profile of an oxalic acid hydrolysate of the arabinogalactan-peptide on Bio-Gel P-6 is shown in Fig. 1(*a*). A carbohydrate component with a molecular weight greater than 6000 was eluted in the fractions near the void volume of the column together with most of the protein. A second carbohydrate component was eluted at the total bed-volume of the column, and intermediate fractions contained smaller amounts of both carbohydrate and protein.

The fraction of the oxalic acid hydrolysate that was soluble in 80% ethanol contained arabinose as the only monosaccharide. The amount of arabinose was not increased on hydrolysis with 0.5M-$HNO_3$, indicating that arabinose had been released as single residues during oxalic acid treatment of the arabinogalactan-peptide. This suggests that all arabinose residues are present in the furanose form in the arabinogalactan-peptide. The 80%-ethanol-soluble fraction, which presumably corresponds to the low-molecular-weight carbohydrate eluted from Bio-Gel P-6 (Fig. 1*a*), was free from protein and hydroxyproline.

Galactose was the only sugar present in the material precipitated by 80% ethanol after oxalic acid hydrolysis, and all the hydroxyproline of the native arabinogalactan-peptide was found in this fraction.

The removal of arabinose from the arabinogalactan-peptide markedly decreased its solubility in ethanol. The native arabinogalactan-peptide is soluble in 60% (v/v) ethanol (Fincher & Stone, 1974*a*), whereas the galactan-peptide residue was completely insoluble in 50% (v/v) ethanol even though its molecular weight was lower. Perlin (1951) observed that the removal of arabinose from arabinoxylans leads to their precipitation from aqueous solutions.

Table 2 shows the apparent molecular weights of native arabinogalactan-peptide and the galactan-peptide residue recovered after oxalic acid hydrolysis. If all the arabinose were removed from the arabinogalactan-peptide ($\bar{M}_{app.} = 32000$) by the oxalic acid treatment the apparent molecular weight of the galactan-peptide residue would be approx. 21000. The finding of a lower value ($\bar{M}_{app.} = 17000$) suggests either that some pyranosidic linkages are cleaved during mild acid hydrolysis (cf. intermediate fractions shown in Fig. 1*a*) or that the residual molecule has undergone a conformational change after the removal of arabinose residues.

The removal of arabinose units by oxalic acid hydrolysis leaving a galactan associated with peptide suggests that galactose residues constitute a molecular backbone in the polysaccharide portion of the arabinogalactan-peptide and implicate galactose as the sugar involved in the polysaccharide–peptide linkage.

*Hydrazinolysis*

An aqueous solution of the hydrazine-treated arabinogalactan-peptide was chromatographed on Bio-Gel P-6 (Fig. 1*b*). The polysaccharide component was still associated with the protein material and was completely excluded. A major Folin–Lowry-positive component was detected at the total bed-volume of the column, but further investigation revealed that hydrazine sulphate reacts strongly in the Folin–Lowry test (Lowry *et al.*, 1951) and it is also likely that any amino acid hydrazides present would also give

Table 3. *Amino acid composition of the arabinogalactan-peptide and fractions obtained after hydrazinolysis, NaOH and oxalic acid–NaOH treatments*

Results are expressed as molar % of recovered amino acids.

| Amino acid | Arabinogalactan-peptide | Hydrazinolysis residue | Residue after NaOH hydrolysis (80%-ethanol insoluble) | Low-molecular-weight material released by NaOH (80%-ethanol soluble) | Residue after oxalic acid–NaOH hydrolysis (80%-ethanol-insoluble residue) |
|---|---|---|---|---|---|
| Lys | 2.1 | 0 | 1.4 | 2.1 | 0 |
| His | 0.6 | 0 | 0 | 0 | 0 |
| Arg | 0.9 | 0 | 0 | 0 | 0 |
| Hyp | 16.7 | 35.0* | 76.1† | 0 | 83.3‡ |
| Asx | 6.3 | 3.2 | 2.2 | 9.0 | 2.2 |
| Thr | 6.1 | 3.8 | 2.6 | 0.6 | 0.4 |
| Ser | 9.6 | 11.2 | 3.1 | 4.6 | 1.4 |
| Glx | 12.2 | 11.2 | 2.9 | 15.6 | 1.9 |
| Pro | 1.3 | 2.8 | 0 | 3.3 | 0 |
| Gly | 4.8 | 9.9 | 3.0 | 11.5 | 1.6 |
| Ala | 24.3 | 8.1 | 1.9 | 35.1 | 1.6 |
| ½Cys | 0 | 0 | 0 | 0 | 0 |
| Val | 6.0 | 9.1 | 4.6 | 7.7 | 6.6 |
| Met | 1.0 | 0.2 | 0 | 1.6 | 0 |
| Ile | 1.4 | 1.6 | 0.8 | 1.2 | 0.4 |
| Leu | 1.4 | 2.1 | 1.3 | 2.1 | 0.7 |
| Tyr | 3.4 | 1.0 | 0 | 4.5 | 0 |
| Phe | 0.5 | 0.9 | 0 | 1.0 | 0 |
| Hexosamine | 1.3 | Not determined | 0 | 0 | 0 |

* *cis* form not included in calculation.
† 45.6% *trans*-4-hydroxyproline, 30.5% *allo*-4-hydroxyproline.
‡ 46.5% *trans*-4-hydroxyproline, 36.8% *allo*-4-hydroxyproline.

a positive test. Although these results do not conclusively show whether the peptide portion of the arabinogalactan-peptide was degraded during the hydrazinolysis treatment, they indicate that there was no extensive degradation of polysaccharide (cf. Heath & Northcote, 1971).

Another sample of hydrazine-treated arabinogalactan-peptide was dialysed exhaustively against water to remove hydrazine sulphate and any amino acid hydrazides before amino acid analysis and Bio-Gel P-6 chromatography. Table 3 compares its amino acid composition with that of the parent arabinogalactan-peptide and shows that whereas hydrazinolysis only partially degraded the peptide, the residual peptide which remained associated with the polysaccharide was considerably enriched in hydroxyproline. Amino acid analysis of the low-molecular-weight component eluted from the Bio-Gel P-6 column showed that no hydroxyproline was released during hydrazinolysis.

The Bio-Gel P-6 profile after hydrazinolysis of the galactan-peptide residue from oxalic acid treatment of the arabinogalactan-peptide was similar to that shown in Fig. 1(*b*), but in this case the residual polysaccharide was retarded by Bio-Gel P-6 and its apparent molecular weight was approximately one-quarter that of the original galactan-peptide.

*Alkaline degradation*

*With $Ba(OH)_2$.* Gel-filtration chromatography on Bio-Gel P-6 showed that no low-molecular-weight carbohydrate or protein material was released by treatment of the arabinogalactan-peptide with saturated $Ba(OH)_2$.

*With NaOH.* After treatment of arabinogalactan-peptide with 5M-NaOH, chromatography of the neutralized hydrolysate on Bio-Gel P-6 (Fig. 1*c*) indicated that all the carbohydrate was excluded from the gel, but that some low-molecular-weight peptide material had been released from the arabinogalactan-peptide.

Table 3 compares the amino acid composition of the 80%-ethanol-soluble and -insoluble fractions of neutralized, NaOH-treated arabinogalactan-peptide with that of the parent arabinogalactan-peptide, and it is apparent that some amino acids have undergone partial or total degradation during the treatment with alkali (Nottingham, 1955*a*,*b*). However, without making any correction for amino acid losses, calculation shows that 81% (on a molar basis) of the recovered amino acids were present in the fraction soluble in 80% ethanol, and 19% in the 80%-ethanol precipitate. No hydroxyproline or carbohydrate was released to the 80%-ethanol-soluble fraction by NaOH treatment, and of the peptide material that

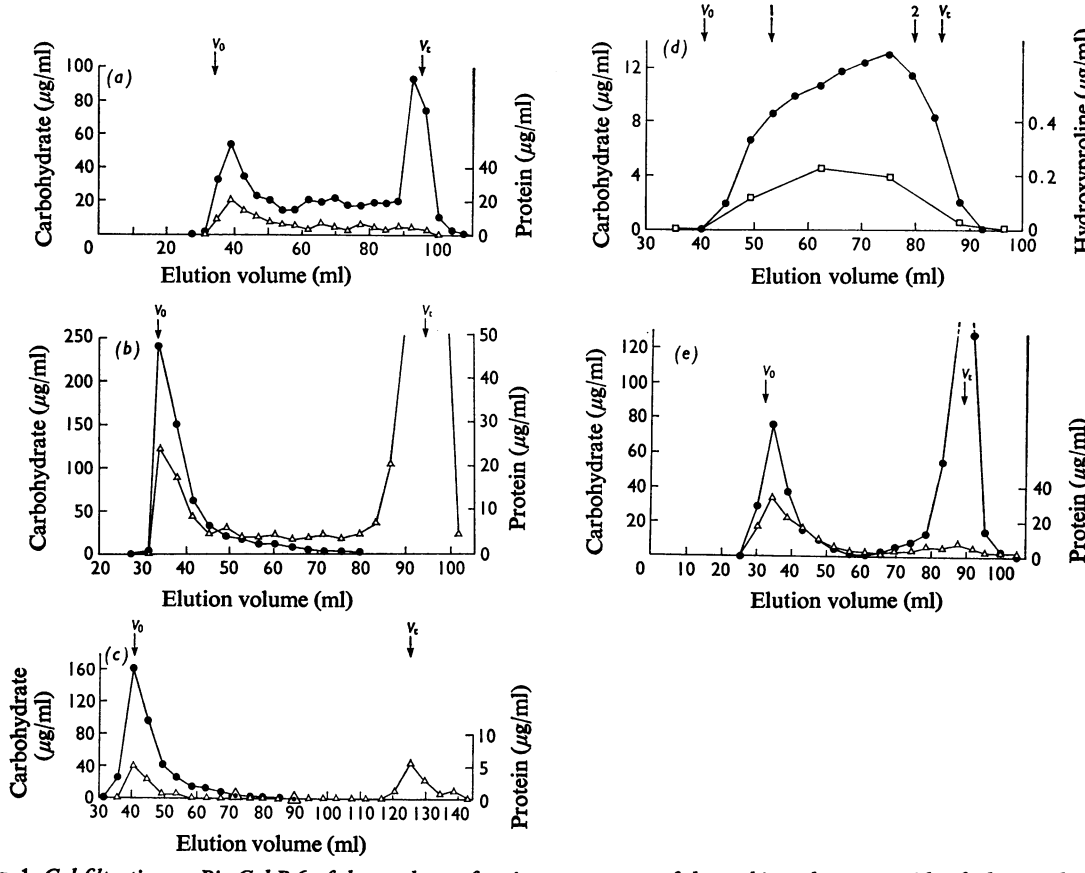


Fig. 1. *Gel filtration on Bio-Gel P-6 of the products of various treatments of the arabinogalactan-peptide of wheat endosperm*

Treated material (0.5–5mg) was dissolved in 0.5ml of 0.3% (w/v) NaCl containing 0.05% $NaN_3$ and loaded on a column (55cm×1.5cm), which was run at a flow rate of approx. 8ml/h. Fractions (4–5ml) were collected. Analyses for carbohydrate (●) and protein (△) were made on individual fractions and for hydroxyproline (□) on pools of three adjacent fractions. (*a*) Arabinogalactan-peptide after hydrolysis in 0.0125M-oxalic acid for 5h at 100°C in a sealed tube. (*b*) After hydrazinolysis for 16h at 100°C in a solution of hydrazine hydrate [1.5% (w/v) with respect to hydrazine sulphate]. (*c*) After treatment with 5M-NaOH for 24h at 100°C in a sealed tube. (*d*) After successive treatment with oxalic acid and NaOH. (*e*) After hydrolysis with a Cellulosin preparation for 16h at 40°C. For further details see the text. Arrows show: $V_0$ (void volume); $V_t$ (total volume); 1 ($M_{app.}$=3000) and 2 ($M_{app.}$=1000, based on manufacturer's data for globular proteins).

remained associated with the polysaccharide 76% (on a molar basis) was hydroxyproline (Table 3). These results provide strong evidence that hydroxyproline is the amino acid residue involved in the linkage of polysaccharide to protein.

*Residue of arabinogalactan-peptide after successive oxalic acid and NaOH treatments*

The results of partial acid hydrolysis and of treatment with strong alkali point to galactose and hydroxyproline as the residues involved in the linkage of polysaccharide to peptide in the arabinogalactan-peptide. This was further investigated by subjecting arabinogalactan-peptide to successive treatment with oxalic acid and NaOH.

The Bio-Gel P-6 elution profile of the oxalic acid–NaOH residue (Fig. 1*d*) shows that hydroxyproline and carbohydrate are present in the same fractions. Further, monosaccharide analysis of the oxalic acid–NaOH residue showed that galactose was the only sugar present, whereas hydroxyproline (83.3%) was the predominant amino acid. The only other amino acid present in significant amounts was valine (6.6%) (Table 3).

These results provide further evidence for a polysaccharide–peptide linkage in arabinogalactan-peptide involving galactose and hydroxyproline residues.

*Action of enzymes on the arabinogalactan-peptide*

After treatment with each of four proteolytic enzymes (see under 'Methods'), both the peptide and polysaccharide components were completely excluded from Bio-Gel P-6, indicating that very little, if any, degradation of the peptide portion of the arabinogalactan-peptide had occurred.

*Cellulosin.* Prolonged digestion with Cellulosin (72h) caused considerable degradation of the arabinogalactan-peptide as shown by Bio-Gel P-6 chromatography of the hydrolysate (Fig. 1*e*). The low-molecular-weight material that was released contained both arabinose and galactose and represented 80% of the polysaccharide in the arabinogalactan-peptide. Only small amounts of peptide material were found in these low-molecular-weight fractions. The higher-molecular-weight fraction eluted near the void volume of the column represented 15–20% (w/w) of the parent arabinogalactan-peptide. This fraction contained all the hydroxyproline and all the peptide material of the original molecule, together with residual polysaccharide consisting of 11% arabinose and 89% galactose.

These results do not show conclusively whether or not the peptide remained covalently associated with the residual higher-molecular-weight carbohydrate component, but if Cellulosin had cleaved the polysaccharide–peptide linkage it would be expected that the peptide portion would be retarded by Bio-Gel P-6.

The galactan-peptide residue from oxalic acid treatment of the arabinogalactan-peptide was also treated with Cellulosin and the hydrolysate chromatographed on Bio-Gel P-6. Again the residual polysaccharide-peptide was eluted near the exclusion volume. Pronase treatment of the excluded material under the conditions described (see the Experimental section) caused no release of peptide material.

*Arabinogalactanase.* The arabinogalactanase from *B. subtilis* released no low-molecular-weight oligosaccharides from the arabinogalactan-peptide, as determined by gel-filtration chromatography on Bio-Gel P-6.

*Galactose oxidase.* Galactose oxidase oxidized approx. 4% of the galactose residues in the arabinogalactan-peptide. The result is expressed as the percentage of anhydrogalactose residues oxidized, the number of galactose residues being estimated from molecular-weight data (Table 1) and the chemical composition of the arabinogalactan-peptide (Fincher & Stone, 1974*a*).

*I.r. absorption*

A broad band in the region 895–875cm$^{-1}$ was observed in the spectrum of the arabinogalactan-peptide and this band was particularly strong in the galactan-peptide spectrum. Derivatives of β-D-galactopyranose are known to absorb in this region (Barker *et al.*, 1956).

*Optical rotatory dispersion and specific optical rotation*

The optical rotatory dispersion curves are shown in Fig. 2 and the specific rotations for the polysaccharide-peptides were:

Arabinogalactan-peptide:

$[\alpha]_D^{20} = -60°$ ($c = 0.99$ in $H_2O$)

Galactan-peptide:

$[\alpha]_D^{20} = -7°$ ($c = 0.74$ in $H_2O$)

The interpretation of the optical rotatory dispersion data is complicated by the presence in both preparations of peptide material, accounting for approx. 8% of the arabinogalactan-peptide and for as much as 15–20% of the galactan-peptide. Nevertheless, the value of $[\alpha]_D^{20}$ for the galactan-peptide provides strong evidence that galactose is present as β-D-galactosyl residues (Hirst & Jones, 1946; Timell, 1965; Bailey, 1965).

Fig. 2 indicates that the specific rotation of the native arabinogalactan-peptide is more laevorotatory than the specific rotation of the galactan-peptide residue. The ease with which arabinose could be removed by hydrolysis with dilute oxalic acid indicates that arabinose is present in the furanose form, and the increased laevorotation of the arabinogalactan-peptide compared with the galactan-peptide further suggests that the arabinofuranose is present in the α-anomeric configuration (Timell, 1965). This conclusion is based on the assumption that the arabinofuranose residues are present as the L-stereoisomer, as has been previously reported in a wheat arabinogalactan (Ford & Peat, 1941), larchwood arabinogalactans (Timell, 1965) and arabinogalactans from other plant tissues (Unrau, 1964; Wolfrom & Patin, 1965; Wolfrom & Anderson, 1967).

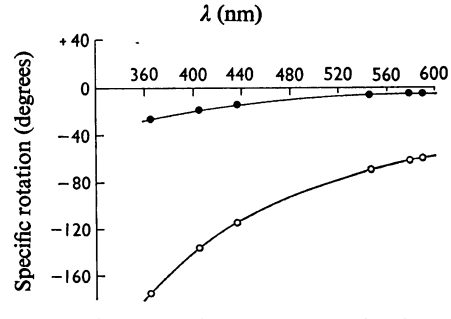


Fig. 2. *Optical rotatory dispersion curves for the arabinogalactan-peptide* (○) *and galactan-peptide* (●)

For details see the text.

Table 4. *Precipitation of castor-bean lectin by the arabinogalactan-peptide*

Each test contained 200 $\mu$g of lectin (see text for details).

| Arabinogalactan-peptide ($\mu$g) | Lectin precipitated ($\mu$g) |
|---|---|
| 50 | 70 |
| 100 | 89 |
| 200 | 108 |
| 300 | 120 |
| 400 | 170 |

Table 5. *Inhibition of lectin-arabinogalactan-peptide precipitation by galactose and methyl β-D-galactoside*

The inhibitors were mixed with the lectin and incubated at 37°C for 1 h before adding the arabinogalactan-peptide.

| | Inhibition (%) | |
|---|---|---|
| Inhibitor concn. ($\mu$mol) | Galactose | Methyl β-D-galactoside |
| 0.25 | 31 | 35 |
| 0.5 | 40 | 46 |
| 1.0 | 46 | 54 |

*Cross-reaction of arabinogalactan-peptide with castor-bean lectin*

Table 4 shows the amount of castor-bean lectin precipitated by increasing amounts of arabinogalactan-peptide. The precipitation was strongly inhibited by both galactose and methyl β-D-galactoside (Table 5).

## Discussion

The following conclusions can be drawn about the structure of the arabinogalactan-peptide.

The material is polydisperse and has a weight-average molecular weight of 22000±1000. The absence of a hypersharp boundary during velocity sedimentation and the linear dependence of $s_{20,w}$ on concentration indicate the absence of significant non-ideal effects in solution. Little non-ideality could be detected on applying the tests devised by Yphantis (1964). The molecule is fairly symmetrical ($f/f_0 = 1.46$), but the intrinsic viscosity (0.108 dl/g) suggests that it may be slightly expanded in form compared with a globular protein. From its molecular weight (22000) and its peptide content (8%, w/w) it may be calculated that a maximum of approx. 20 amino acid residues are present. Of these residues three or four are hydroxyproline. It is not known whether the peptide is homogeneous with respect to amino acid composition, but the detection of some amino acids at proportions of less than 5% (Table 3) indicates either that contaminant protein is present or that there is heterogeneity in the amino acid composition of the peptide.

Since both strong-alkali treatment and hydrazinolysis failed to release hydroxyproline, it may be concluded that all the hydroxyproline residues are substituted by polysaccharide chains. On this basis it is proposed that the arabinogalactan-peptide consists of a peptide core to which polysaccharide chains are appended through each of the hydroxyproline residues.

Further evidence for such a structure is found in the decrease which takes place in the molecular weight of both the arabinogalactan-peptide and the galactan-peptide residue on treatment with hydrazine. Amino acids comprise approx. 8% (w/w) of the total arabinogalactan-peptide molecule, and yet after removal of 50% of these by hydrazinolysis the apparent molecular weight is decreased to approximately one-quarter of that of the untreated arabinogalactan-peptide. Similar relative size changes occur with the galactan-peptide.

The resistance of the peptide core to proteolytic enzymes suggests either that the peptide has an unusual structure or that polysaccharide chains shield it from enzymic attack. It was found that after Cellulosin digestion the residue was also resistant to proteolysis even though the polysaccharide chains were considerably shorter than in the parent molecule.

Since all the arabinose can be removed from the arabinogalactan-peptide by oxalic acid treatment without disrupting the polysaccharide–peptide linkages, the peptide must be linked to galactose. The further finding that these linkages are not cleaved during prolonged exposure to hot 5M-NaOH indicates that the polysaccharide and peptide are linked glycosidically, presumably through the hydroxyl group of hydroxyproline. Such a linkage would not be cleaved by the base-catalysed β-elimination reaction (cf. Gottschalk, 1972) and indeed it has been shown that, in contrast with the lability of the corresponding serine and threonine glycosides, the 4-hydroxy-L-proline glycoside of 2-amino-2-deoxy-D-glucose is stable in alkaline conditions (Vercellotti & Just, 1967). It is possible, however, that in addition to galactosylhydroxyproline linkages there could be other types of polysaccharide–protein linkages present in the arabinogalactan-peptide. Lamport *et al.* (1973) presented evidence that galactosylserine and arabinosylhydroxyproline are both involved in the linkage of carbohydrate to protein in glycopeptides from tomato cell walls. Although all the hydroxyproline residues in the arabinogalactan-peptide are linked to galactose, the present evidence does not entirely eliminate the possibility that a few serine (or threonine) residues could be glycosidically linked to single sugars as reported by Lamport *et al.* (1973). If any carbohydrate was

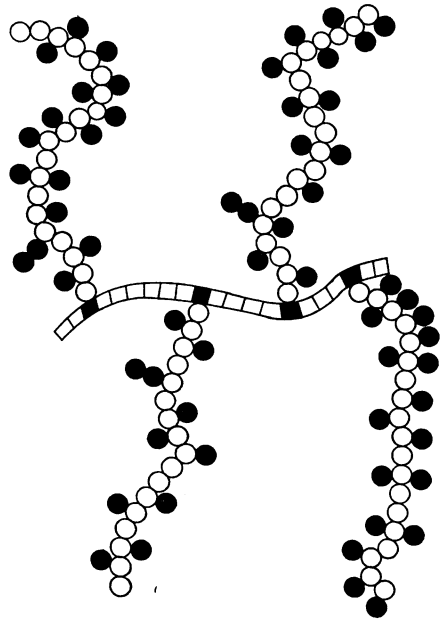

Fig. 3. *Proposed model of the arabinogalactan-peptide*

Galactose (○); arabinose (●); hydroxyproline (■); other amino acids (□).

present in this form it would be expected to be released and subsequently destroyed in the hot alkaline conditions.

The results of i.r.-absorption spectroscopy and optical rotation studies show that the glycosidic linkages between the D-galactopyranosyl units in the polysaccharide chains are in the β-anomeric configuration. The resistance of arabinogalactan-peptide to the *B. subtilis* arabinogalactanase further suggests that the galactose residues are not joined by β-1,4-linkages (Emi *et al.*, 1971), this being consistent with the work of Ford & Peat (1941) in which hydrolysis of methylated polysaccharides prepared from water extracts of wheat flour resulted in the isolation of 2,4-dimethylgalactose as the only galactose derivative. However, the specificity of the arabinogalactanase has not been completely defined (Emi *et al.*, 1971) and it is likely that both the chain length and the positional linkage of arabinofuranosyl residues in the polysaccharides may also influence susceptibility to attack by this enzyme. Further structural analysis is needed to define the linkage position between galactose residues.

Both galactose oxidase and the castor-bean lectin interact with the arabinogalactan-peptide, indicating that a few terminal unsubstituted galactosyl residues are present in the molecule (cf. Hajra *et al.*, 1966; Pardoe & Uhlenbruck, 1970; Estrada-Parra & Gomez, 1972; Sharon & Lis, 1972; Suzuki & Suzuki, 1972).

The marked decrease in molecular weight of the arabinogalactan-peptide after removal of arabinose with oxalic acid and the decreased solubility in ethanol of the resultant galactan-peptide suggest that the arabinose is an integral part of the molecule and is almost certainly not present as admixed arabinan of the type detected in pectic substances of higher plants (Aspinall, 1970). The removal of an admixed arabinan would not result in a decrease in molecular weight of the galactan-peptide fraction, and its removal would be unlikely to affect the ethanol solubility of a separate galactan-peptide component.

The optical rotation data and the ease of hydrolysis with dilute oxalic acid indicate that arabinose is present in the α-L-arabinofuranosyl form. Since single arabinose residues are released during oxalic acid hydrolysis of the arabinogalactan-peptide it is likely that the arabinose is present exclusively in the furanose configuration, but it is not known whether the arabinofuranosyl units are present as single residues or as short side chains distributed along the length of the galactan chains.

The model for the arabinogalactan-peptide which is shown in Fig. 3 is based on the considerations discussed above and shows the peptide core to which four arabinogalactan chains are attached through the four hydroxyproline residues. The number of galactose (approx. 75) and arabinose (approx. 60) residues in the model are calculated from the molecular weight of the arabinogalactan-peptide (22000) and the proportion of each sugar present. It should be emphasized that these values represent averages, since the physicochemical studies showed that the polymer was polydisperse with respect to molecular weight. The galactan chains have been assumed to be unbranched, but further work is required to define chain lengths in the polymer.

The intracellular location and function of the arabinogalactan-peptide from wheat endosperm is uncertain, but several possibilities exist. One relates to the storage role of endosperm tissue, in which the arabinogalactan-peptide is found in amounts up to 0.1% (w/w). The finding of similar galactose- and hydroxyproline-rich polymers in barley and *Lolium perenne* seeds (Fincher & Stone, 1974*a*) lends some support to the suggestion that it has a storage function. However, as the arabinogalactan-peptide does not appear to undergo significant depolymerization during endosperm modification (Fincher & Stone, 1974*b*) and since molecules with a similar organization have been detected in other non-storage tissues it is perhaps more likely to have a broader function in plant cells.

Evidence for protein–polysaccharide complexes located both in cell walls and intracellularly have been

put forward by a number of workers. Lamport (1967, 1969) presented evidence that in plant cell walls a hydroxyproline-rich protein is linked to arabinose through an *O*-glycosidic linkage involving hydroxyproline. Hydroxyproline–arabinose glycosides were isolated after enzyme treatment of cell-wall preparations. Lamport & Miller (1971) later reported that these hydroxyproline arabinosides could be isolated from cell-wall preparations from a phylogenetic spectrum of plants ranging from the green alga *Chlorella* to the spermatophytes. Hydroxyproline arabinosides have also been obtained by Talmadge *et al.* (1973) from cell walls of sycamore suspension cultures. In addition to hydroxyproline arabinosides, hydroxyproline *O*-galactoside has been found in alkaline extracts of cell-wall preparations of *Chlamydomonas reinhardtii* (Miller *et al.*, 1972; Roberts *et al.*, 1972) and Lamport *et al.* (1973) reported that serine *O*-galactosides are present in tomato cell walls. Other investigators have noted that hydroxyproline-rich material is present in 'cytoplasmic' fractions (Clarke *et al.*, 1968; Cleland, 1968; Jennings & Watt, 1967; Pusztai & Watt, 1969; Lamport, 1970; Chao & Dashek, 1973) in addition to cell-wall fractions.

Since the wheat endosperm arabinogalactan-peptide has been isolated without recourse to degradative enzyme or chemical extraction procedures (Fincher & Stone, 1974*a*) it is unlikely that it is the same hydroxyproline-rich material reported to be covalently associated with plant cell-wall polysaccharides.

Mares & Stone (1973*a*,*b*) found no galactose or hydroxyproline in wheat endosperm walls isolated in a 70% (v/v) ethanol medium, whereas galactose-rich polysaccharides were present in the water extract of the finely divided endosperm material passing through 75$\mu$m-nylon-mesh sieves during the isolation of the walls in 70% ethanol (Mares & Stone, 1973*c*). It was concluded that if these polysaccharides are associated with the endosperm cell walls they must be very readily dissociated from them in the ethanolic medium. This would be consistent with the partial solubility of the arabinogalactan-peptide in 70% ethanol (Fincher & Stone, 1974*a*).

Pectin has not been detected in water extracts of wheat (Preece & Hobkirk, 1953; MacLeod & McCorquodale, 1958) or in wheat endosperm cell walls (Mares & Stone, 1973*a*), but it is conceivable that the arabinogalactan-peptide could be functionally equivalent to the neutral arabinogalactan which is frequently extracted in the pectic fraction of other plant tissues. Ginzburg (1958) and MacLeod & McCorquodale (1958) have reported the presence of protein in the middle lamella of some plant cells, which suggests the possibility that arabinogalactan-peptide could be a component of the middle lamella in mature endosperm.

At present it remains uncertain whether the arabinogalactan-peptide is associated with endosperm cell walls or is a cytoplasmic component, and detailed information as to its subcellular localization will be necessary before a functional role can be ascribed to this molecule.

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