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HYDROXYPROLINE-GALACTOSIDE AS A PROTEIN–POLYSACCHARIDE LINKAGE IN A WATER SOLUBLE ARABINOGALACTAN-PEPTIDE FROM WHEAT ENDOSPERM

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Abstract—A water-soluble arabinogalactan-peptide from wheat endosperm was degraded by treatment with mild acid, alkali and enzymes. 4-Hydroxyproline-galactoside was isolated from the degradation products and was shown to be identical with synthetic *trans*-4-hydroxyproline-β-D-galactopyranoside. This linkage compound connects the arabinogalactan side chains to the peptide core in the arabinogalactan-peptide. A possible structural model for this glycoprotein is discussed.

INTRODUCTION

Arabinogalactans and arabinogalactan-proteins (proteoglycans) have been found in many plant tissues [1]. They have been isolated from aqueous extracts of plant materials by various fractionation methods [1]. The associated peptide or protein moiety cannot usually be removed by physical procedures and often contains high levels of hydroxyproline. Arabinogalactan-peptide preparations from wheat endosperm [2, 3] contain 6–8% polypeptide or protein with a high content (15–20%) of 4-hydroxyproline. Since degradation of the peptide portion by alkali or pronase does not release hydroxyproline, it seems likely that a hydroxyproline-galactoside or -arabinoside forms the linkage compound between the arabinogalactan and the protein moieties. The isolation and synthesis of this linkage compound is described in this paper. While this work was in progress similar results were obtained by McNamara and Stone [4].

RESULTS AND DISCUSSION

The crude arabinogalactan-peptide extracted from wheat flour contained 65–70% of soluble starch [2] which was removed by α-amylase treatment. Mild acid hydrolysis was used to split off the terminal arabinofuranosyl-residues in the arabinogalactan. The resulting galactanpeptide was separated from lower molecular weight material by gel filtration on Sephadex G-25 and had a protein content of 22%. Its amino acid composition is shown in Table 1. Galactose accounted for 96% of the carbohydrate portion, a small amount of glucose, but no arabinose was detected. Incubation of the galactan-peptide with 0.1 M NaOH produced no changes in the serine and threonine content and no β-elimination reaction products were found. This excluded an *O*-glycosyl-serine or -threonine type of linkage [5].

Table 1. Amino acid composition and protein content of the crude arabinogalactan-peptide and its degradation products

	Crude Arabino-galactan-peptide	Galactan-peptide	Galactan-peptide after pronase degradation	Galactan
Hyp	15.5	11.5	27.0	52.8
Asp	6.7	6.6	4.0	0.7
Thr	6.6	6.0	6.7	27.7*
Ser	10.0	8.6	10.1	1.0
Glu	12.9	15.9	14.6	2.0
Pro	1.9	3.9	—	—
Gly	2.8	4.2	1.7	1.5
Ala	26.6	18.0	27.9	1.0
Val	5.9	5.8	6.6	13.2
Met	2.4	1.9	1.6	—
Ile	1.9	2.6	tr†	—
Leu	1.7	3.1	tr	—
Tyr	3.5	2.9	tr	—
Phe	0.5	1.2	tr	—
His	tr	1.0	tr	—
Lys	1.2	3.6	tr	—
Arg	1.0	2.3	tr	—
Protein (%)	5.0	22.1	12.0	1.5

* D-*allo*-hydroxyproline elutes at the same retention time as threonine.
† Trace.

For the isolation of the protein–polysaccharide linkage compound both the peptide and the polysaccharide portions had to be degraded without affecting the

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expected glycosidic linkage. Enzymatic, degradation of the peptide with pronase is either ineffective (Table 1) or incomplete [3, 6]. Alkaline hydrolysis was the only way therefore for the degradation of the peptide, since the postulated hydroxyproline–galactoside linkage could be expected to be alkali stable. Hydrolysis of the galactan-peptide with 5 M NaOH produced a galactan which contained 1.5% residual peptide, the hydroxyproline content of which was 80% (Table 1). The galactan was separated from low-molecular-weight material (amino acids, but no hydroxyproline) by gel filtration on Sephadex G-25. Degradation of the highly branched β-(1→3; 1→6) galactan was achieved by treatment with β-galactosidase (lactase) [6]. Galactose (R_f 0.6), hydroxyproline (R_f 0.43), and a spot (R_f 0.27) which was positive for both amino acid and carbohydrate, were detected by TLC after β-galactosidase degradation. These three degradation products were separated by ion-exchange chromatography on Aminex A-6 (Fig. 1, B). Peaks I and IV were found to be galactose and hydroxyproline respectively. Peak II showed a positive reaction with both orcinol–H_2SO_4- and ninhydrin-reagent, and on TLC it gave one spot (R_f 0.27). Acid hydrolysis of this compound released hydroxyproline and galactose as the only products. The compound was therefore tentatively identified as hydroxyproline-galactoside.

The identity of this linkage compound was confirmed by comparison of its properties with those of synthetic *trans*-4-hydroxyproline-β-D-galactopyranoside (Hyp-Gal). (Note that Hyp-Gal was not isolated in pure form.) Thus on ion-exchange chromatography on Aminex A-6 of the synthetic Hyp-Gal (A) and the β-galactosidase degradation products (B, peak II) (Fig. 1), the peaks of both components appeared in corresponding fractions with the same elution time. The two peaks could not be distinguished on TLC (R_f 0.27, R_f of the acetylated

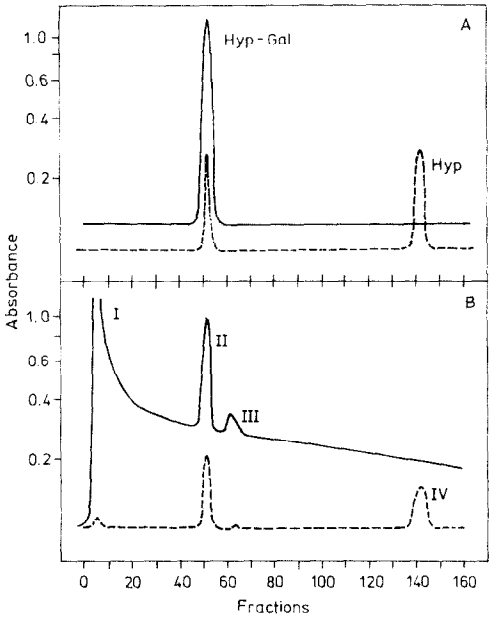

Fig. 1. Ion exchange chromatography on Aminex A-6 of synthetic hydroxyproline-galactoside (Hyp-Gal) (A) and isolated hydroxyproline-galactoside (B). ····, A_{570nm}; ——, A_{420nm}.

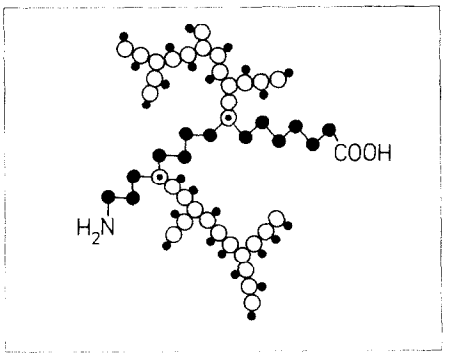

Fig. 2. Possible formula for the arabinogalactanpeptide from wheat endosperm. ●, amino acids; ⊙, hydroxyproline; ○, galactose; •, arabinose.

products 0.75) and both released hydroxyproline and galactose on acid hydrolysis. The identity of the two products was confirmed by MS data ($M^{+\cdot}$ and fragments). The method used for the synthesis of Hyp-Gal is known to give β-glycosides [7]. The β-configuration of the Hyp-Gal isolated is supported also by the release of hydroxyproline on incubation with β-galactosidase. The isolated and synthetic Hyp-Gal are therefore identical and the linkage compound in the arabinogalactan-protein from wheat endosperm is *trans*-4-hydroxyproline-β-D-galactopyranoside.

The arabinogalactan-peptide molecules must be built up of a short peptide backbone chain to which arabinogalactan side chains are bound via the linkage compound. A possible structural model is shown in Fig. 2. The following analytical data are in support of this structure: 1. The average MW of the native arabinogalactan-peptide is 30 000–32 000 [3, 8]. The peptide portion represents about 5–6% of the molecule, the peptide backbone must therefore have a MW of 1500–1800 and consist of 14–18 amino acid residues. The peptide chains are obviously not homogeneous since on hydrolysis they give greatly different mixtures of all the amino acids. 2. Hydroxyproline represents 10–15% of the peptide moiety (Table 1) or an average of two residues per peptide chain. As no hydroxyproline is released from the polysaccharide fraction by proteolytic or alkaline degradation of the peptide core, all hydroxyproline residues must be linked to the polysaccharide [3]. 3. Two arabinogalactan side chains are linked to the peptide core. An average MW of 12 000–14 500 for these side chains can be calculated from the analytical data. This agrees with the MW of the alkali treated arabinogalactan found by gel filtration on Sephadex G-200 (K_{av} = 0.55) [8].

Whilst these data can be best expressed by the structure shown in Fig. 2, various similar models can be visualized with different degrees and locations of branching [8]. This model is also in support of the original, less detailed, proposal by Fincher *et al.* [3] for the structure of the arabinogalactan-peptide. The proposed structures look very similar to the proteoglycans of animal connective tissues which have however a much higher MW and a higher degree of branching [9].

EXPERIMENTAL

Plant material. A white wheat flour obtained from a local mill was used as a source for wheat endosperm.

General methods. Sugars were quantitatively analysed by the H_2SO_4–orcinol method [10] in a Technicon Auto Analyzer. The qualitative sugar analyses were carried out by GLC of the alditol acetates [11] on a column of 1.5% ECNSS-M and 1.5% OV 225 on Chromosorb Q. Peptides and amino acids were assayed by the ninhydrin method in a Technicon Auto Analyzer, measuring $A_{570\,nm}$. Hydroxyproline was detected at 420 nm. The amino acid analyses were carried out on a Biocal Amino Acid Autoanalyzer BC 201 by a single column/four buffer system after hydrolysis of the samples with 6M HCl [12]. TLC was carried out on Si gel developed with *iso*-PrOH–HOAc–H_2O (3:1:1). After development the plates were sprayed with ninhydrin or H_2SO_4. Gel filtration on Sephadex G-25: 15 ml samples containing 30–40 mg material/ml were centrifuged (4000 rpm, 30 min) and the clear supernatants were loaded on a column (7 × 85 cm). The columns were eluted (against gravitation) with H_2O at a flow rate of 86 ml/hr and fractions of 18–19 ml were collected.

Isolation of the galactan peptide. The crude arabinogalactan-peptide was extracted and treated with α-amylase as described by Neukom and Markwalder [2]. The crude extract was adjusted to pH 1.1 with 1 M H_2SO_4 and refluxed at 100° for 1 hr to hydrolyse off the arabinofuranosyl residues. The solution was neutralized, lyophilized and then purified by gel filtration.

Alkaline degradation of the peptide. 300–400 mg of galactan-peptide were dissolved in 500 ml of 5 M NaOH, refluxed at 100° for 24 hr, neutralized with 6 M HCl and the precipitated silicic acid removed by centrifugation. The sediment was washed (× 3) with 10 ml H_2O, the combined supernatants desalted by ultrafiltration (Diaflo UM 10 membrane), concn to 10–15 ml and loaded on a Sephadex G-25 column for purification.

Degradation of the galactan by β-galactosidase. Galactan (100 mg) was dissolved in 45 ml H_2O to which was added, 5 ml 0.1 M $MgSO_4$ and 2.5 g immobilized β-galactosidase [13]. The mixture was adjusted to pH 4 and incubated at 40–45°. The reaction (monitored by TLC) was stopped after 2.5 hr by filtration, and the filtrate lyophilized.

Isolation of the hydroxyproline-galactoside. The dried reaction products of either the β-galactosidase degradation or the synthesis were dissolved in 5 ml H_2O. Samples of 0.5 ml were used for ion exchange chromatography on Aminex A-6. The column (0.9 × 53–55 cm) was eluted with 0.2 M HCl at 50 ± 2° at a working pres. of 16.7 ± 1.3 kg/cm^2 (flow rate 100 ml/hr). Fractions of 50 drops (3.12 ml) were collected and immediately analysed for carbohydrate and hydroxyproline content respectively.

Synthesis of trans-4-*hydroxyproline-β-*D-*galactoside.* Acetobromogalactose (1-bromo-(2,3,4,6-tetra-*O*-acetyl)-α-D-galactopyranose) was prepared by the method used for the synthesis of acetobromoglucose [14]. The product was purified by recrystallization from dry Et_2O: mp 78.5 – 78.8°. *N*-CBZ-hydroxyproline methyl ester (Sigma Chemical Corp., St. Louis, Miss., USA) was reacted with acetobromogalactose under conditions described for the synthesis of disaccharides [15]. The reaction mixture was evapd to dryness and deacetylated in 2 M KOH at 100° for 1 hr. The alkaline soln was neutralized on a Dowex 50 (H^+) column (1.5 × 18 cm). The column was washed with H_2O to remove free galactose, free hydroxyproline and the hydroxyproline-galactoside were eluted with 1 M NH_3 soln. This mixture was evapd to dryness, redissolved in H_2O and separated on Aminex A-6.

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