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A GALACTOGLUCOMANNAN FROM THE LEAF AND STEM TISSUES OF RED CLOVER (*Trifolium pratense*)

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ABSTRACT

A galactoglucomannan has been isolated by fractionation of the alkali-soluble hemicelluloses of the leaf and stem tissues of red clover (*Trifolium pratense* L.) The hemicellulose contains galactose, glucose, and mannose residues in the molar ratios 0.25·1.0:1.1 and accounts for *ca.* 25% of the mannose residues present in the clover tissues. Structural studies showed that the hemicellulose has a main chain of β-(1 → 4)-linked D-glucopyranosyl and D-mannopyranosyl residues, to which are attached α-(1 → 6)-linked D-galactopyranosyl residues.

INTRODUCTION

Galactoglucomannans, glucomannans, or galactomannans have often been assumed to be present in the vegetative tissues of leguminous plants. Recently[1], a pure galactoglucomannan was isolated from the stems of the tropical legume *Stylosanthes humilis*. This hemicellulose, which has *ca.* 6% of galactose residues, is unusual in that that the ratio of glucose to mannose is 1:1, but is otherwise similar to the galactoglucomannans found in softwoods[2]. Gaillard and Bailey[3] studied the distribution of galactose and mannose residues in the cell-wall polysaccharides of red clover, and partially purified a hemicellulose that was concluded to contain a galactomannan or a galactoglucomannan. We now report on the structure of a galactoglucomannan present in the leaf and stem tissues of red clover.

RESULTS AND DISCUSSION

Gaillard and Bailey[3] found that there was no marked difference in the monosaccharide composition between the corresponding polysaccharides from clover leaves and stems. In the present study, the plant material consisted of a mixture of leaves, petioles, and stems. The ethanol-extracted plant material was treated with ethylenediaminetetra-acetic acid (EDTA) to remove pectic substances, and the residue was delignified by the method of Wise *et al.*[4]. The resultant holocellulose was treated successively with aqueous 10% potassium hydroxide and 24% sodium hydroxide containing 4% of boric acid. On acid hydrolysis, the hemicellulosic material isolated from the 10% extract released arabinose, galactose, glucose, mannose, rhamnose,

and xylose, in the molar ratios 1 0 1 0 2 9 0 3 0 2 5 1, and also uronic acids This material was not further studied

The 24% sodium hydroxide–4% boric acid extract, on acid hydrolysis, released arabinose, galactose, glucose, mannose, rhamnose, and xylose, in the molar ratios 1 0 1 1 1 4 1 0 0 3 1 4, and also uronic acids Partial fractionation could be achieved by the method of Gaillard[5], but precipitation with Cetavlon[6] was more effective. Acid hydrolysis of the precipitate obtained on the addition of Cetavlon to a solution of the hemicellulosic material in 10% aqueous potassium hydroxide gave only small quantities of mannose The hemicellulose remaining in solution gave galactose, glucose, and mannose, in the molar ratios 0 25 1.0 1 1, and traces of xylose Attempted fractionation of this material with barium hydroxide[7] or Fehling's solution[8] did not change the hexose ratios but resulted in the elimination of the pentose-containing material

The purified hemicellulose had $[\alpha]_D^{23}$ −8 9° (*c* 1 1, sodium hydroxide) and was only slightly soluble in cold water Electrophoresis (cellulose acetate[9]) of the derivative dyed with Procion indicated that the hemicellulose was probably a pure triheteropolysaccharide

The galactoglucomannan was methylated successively by the methods of Haworth[10] and Hakomori[11], and the product showed no i r absorption for hydroxyl groups G l c examination of a methanolysate showed the presence of the methyl glycosides of the following sugars 2,3,4,6-tetra-*O*-methylhexoses (glucose and mannose), 2,3,6-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylmannose, 2,3-di-*O*-methylglucose, 2,3-di-*O*-methylmannose, and 2,3,4,6-tetra-*O*-methylgalactose A sample of the methylated polysaccharide was hydrolysed[12], and the hydrolysate was examined by paper chromatography and t l c The identities of the above sugars were further confirmed, and it was evident that only traces of tetra-*O*-methylglucose were present A sample of the hydrolysate was reduced with sodium borohydride and acetylated according to the method of Bjorndal *et al*[13] The products were examined by g l c, and the ratios of the peak areas for the above methyl sugars were found to be 0 06 1 00 1 17 0 09 0 08 0 16, respectively

The galactoglucomannan reduced 1 05 moles of periodate per mole of "anhydrohexose" residue, and acid hydrolysis of the reduced oxopolysaccharide released erythritol and glycerol in the molar ratio 5 8 1 0 Controlled, mild hydrolysis of the polysaccharide with acid gave galactose as the main product Further hydrolysis of the polysaccharide gave the following oligosaccharides cellobiose, cellotriose, 4-*O*-β-D-glucopyranosyl-D-mannose, 4-*O*-β-D-mannopyranosyl-D-glucose, and the β-(1 → 4)-linked-D-mannose di-, tri-, and tetra-saccharides The isolation of these oligosaccharides shows that there are both contiguous glucosyl and mannosyl

The methylation data can be interpreted to give an average molecule of d p *ca* 41, which consists of a backbone of (1→4)-linked D-glucopyranosyl and D-mannopyranosyl residues The correspondence of di-*O*-methylhexose residues with the tetra-*O*-methylgalactose residues, and the absence of 2,3,4-tri-*O*-methylhexoses, shows that there is no main-chain branching It is also evident that the main chain is almost exclusively terminated by mannose residues The presence of nearly equal quantities of 2,3-di-*O*-methylglucose and 2,3-di-*O*-methylmannose shows that the galactosyl residues are attached at position 6 of the glucose and mannose residues to an equal extent The periodate-oxidation results suggest that the d p for the parent galactoglucomannan is slightly lower than the value obtained from the methylation analysis

The galactoglucomannan from red clover has a structure similar to those found in the softwoods[2], but the ratio of glucose to mannose is *ca* 1 1 In this respect, the structure is similar to the hemicellulosic galactoglucomannan isolated from *Cordyline indivisa*[14] and to the endospermic polysaccharides isolated from various *Liliaceae* and *Iridaceae*[15–17] The galactoglucomannan from the stems of *Stylosanthes humilis* is also structurally similar, but it was shown that the non-reducing, terminal galactose residues are attached exclusively to mannose residues[1]

EXPERIMENTAL

Paper chromatography was carried out on Schleicher and Schuell No 2043b paper and t l c on Kieselgel G (Merck), using the following irrigants (*1*) ethyl acetate–pyridine–water (8 2 1), (*2*) ethyl acetate–pyridine–water (2 1 2), (*3*) 1-butanol–pyridine–water–benzene (5 3 3 1), (*4*) 1-propanol–ethyl acetate–water (7 2 1), (*5*) 1-butanol–ethanol–water–ammonia (4 1 5 trace), (*6*) butanone–water–ammonia (10 1 trace), (*7*) benzene–ethanol–water–acetic acid (200 47 15 1) Chromatographic detection reagents were alkaline silver nitrate, 3% *p*-anisidine hydrochloride, and 5% naphth-1-ol/conc sulphuric acid G l c was performed on a Perkin–Elmer F 30 chromatograph, using columns (2 m × 2 mm i d) containing (*A*) 10% *m*-bis(*m*-phenoxyphenoxy)benzene on AW DMCS Chromosorb W (100–120 mesh) and (*B*) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) Electrophoretic examination of the polysaccharide, as its derivative dyed with Procion MGS red, was carried out on cellulose acetate[9] Polysaccharides were hydrolysed in sealed tubes with 0 5M sulphuric acid for 12–16 h at 100°; hydrolysates were neutralized with barium carbonate Neutral sugars in hydrolysates were determined by g l c of their derived glycitol acetates (column *B*)

Isolation of the hemicellulosic material — Ethanol-extracted, milled leaf and stem tissues (150 g) of red clover (*Trifolium pratense* L) were treated three times with 2% EDTA di-sodium salt (2 l, pH 6 8) for 2 h at 70°. The yield of pectic material was *ca* 15 g The residual material was delignified with acid chlorite[4], and the resultant holocellulose (75 g) was treated with 10% potassium hydroxide (2 × 2 l) to give hemicellulosic material (10 9 g) Further treatment with 24% aqueous sodium

hydroxide (2 l) containing 4% of boric acid led to the isolation of hemicellulosic material (2 4 g), which was the source of the galactoglucomannan. The α-cellulose (27 5 g) still contained *ca.* 3% of mannose residues

Isolation of the galactoglucomannan — Preliminary fractionation of the above hemicellulosic material, by the method of Gaillard[5], resulted in some losses Recombined sub-fractions (2 g) were dissolved in 10% aqueous potassium hydroxide (50 ml), and 2% aqueous Cetavlon was added until precipitation was complete The soluble and insoluble fractions were separated by centrifugation and treated with a large excess of 5% acetic acid, and the solutions were dialysed. The acid hydrolysate of the soluble fraction (300 mg; 0 2% of the plant material) contained galactose, glucose, mannose, and xylose in the molar ratios 0 25 1 0 1.1 trace No alternation in the proportion of the hexose residues was obtained on attempted fractionation with barium hydroxide or Fehling's solution

Periodate oxidation of the galactoglucomannan — A sample (25 mg) of the hemicellulose was oxidised in the dark at 5° with 0 05M sodium metaperiodate The periodate consumed after 28 days was 1 05 moles per mole of hexose residue The oxopolysaccharide was dialysed and reduced with sodium borohydride The polyalcohol was hydrolysed and examined by paper chromatography (irrigants *1* and *3*); erythritol, glycerol and traces of hexose were detected A sample of the hydrolysate was acetylated, and g l c (column *B*) of the products revealed components with retention times identical to those of glycerol triacetate and erythritol tetra-acetate, in the molar ratio 1 0 5 8

Methylation of the galactoglucomannan — A sample (100 mg) of the galactoglucomannan was methylated three times by the method of Haworth[10] and once by the method of Hakomori[11]. The product was extracted with light petroleum, and the residue (90 mg), which was soluble in chloroform, showed no hydroxyl absorption in its i r. spectrum A sample of the methylated material was methanolysed and examined by g l c (columns *A* and *B*), another sample of the material was hydrolysed, and the derived glycitol acetates were also examined by g l c (column *B*) The hydrolysate was examined by paper chromatography and t.l c. (irrigants *5*, *6*, and *7*). The following sugars were identified 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, 2,3-di-*O*-methylglucose, 2,3,4,6-tetra-*O*-methylmannose, 2,3,6-tri-*O*-methylmannose, 2,3-di-*O*-methylmannose, and 2,3,4,6-tetra-*O*-methylgalactose

Partial, acid hydrolysis of the galactoglucomannan. — A sample (150 mg) of the hemicellulose was treated with 25mM oxalic acid for 6 h at 100°; only galactose was released The insoluble residue was then heated with 0.1M sulphuric acid for 6 h at 100° in a sealed tube. The neutralized hydrolysate was shaken with activated charcoal (Norite A) and filtered The aqueous phase contained only monosaccharides and was discarded The charcoal was then washed on a filter with 1%, 10%, and 50% aqueous ethanol solutions Paper chromatography showed that the first eluate contained mainly monosaccharides and the other two, which were combined, contained mainly oligosaccharides These oligosaccharides were purified by paper chromatography (irrigants *2* and *4*) to give seven components (Table I) They were

TABLE I

IDENTIFICATION OF THE OLIGOSACCHARIDES PRODUCED ON ACID HYDROLYSIS OF THE GALACTOGLUCOMANNAN

Component	*Products on acid hydrolysis*	*Products on acid hydrolysis after reduction*[a]	*Products on treatment with β-D-glucosidase*	*Proposed identity*
1	Glucose and mannose	Glucose mannitol (ratio 1 1)	Glucose and mannose	4-*O*-β-D Glucopyranosyl-D-mannose
2	Glucose	Glucose glucitol (ratio 1 1)	Glucose	4-*O*-β-D-Glucopyranosyl-D-glucose
3	Mannose	Mannose mannitol (ratio 1 1)	Component 3	4-*O*-β-D-Mannopyranosyl-D-mannose
4	Glucose and mannose	Mannose glucitol (ratio 1 1)	Component 4	4-*O* β-D-Mannopyranosyl-D glucose
5	Mannose	Mannose mannitol (ratio 2 1)	Component 5	β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man
6	Glucose	Glucose glucitol (ratio 2 1)	Glucose	Cellotriose
7	Mannose	Mannose mannitol (ratio 3 1)	Component 7	β-D-Man*p* (1→4)-β-D-Man*p* (1→4) β D-Man*p*-(1→4) D-Man

[a]Ratios determined by g l c. of the derived acetates

identified by direct comparison on paper chromatograms (irrigants *2*, *3*, and *4*), by examination of the hydrolysis products of the oligosaccharides and the corresponding reduced oligosaccharides, and by enzymic hydrolysis with the β-D-glucosidase from almond emulsin

Enzymic hydrolysis of the galactoglucomannan — A sample of the polysaccharide was incubated with an α-D-galactosidase, isolated from the seeds of lentil (*Lens esculanta* Mnch), at pH 5 in phosphate–citrate buffer contained in a dialysis sac After 24 h, the diffusible material was examined by paper chromatography (irrigant *1*), and by g l c (column *B*) of the reduced and acetylated material, only galactose was detected

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