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STRUCTURAL INVESTIGATIONS ON TWO HEMICELLULOSIC POLYSACCHARIDES FROM GROUNDNUT (*Arachis hypogea*) SEED ENDOSPERM

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ABSTRACT

A highly branched xylan and a linear, β-D-(1→4)-linked glucomannan are the two hemicellulosic components isolated from the endosperms of groundnut (*Arachis hypogea*). Electrophoretic, sedimentation, and sugar analysis indicate the polysaccharides to be fairly homogeneous. The *O*-methyl derivatives of the polysaccharides were analysed, after reduction and *O*-acetylation, by gas–liquid chromatography and g.l.c.–mass spectrometry. 2,3,4-Tri-*O*-methyl-D-xylose (3.6 mol), 2,3-di-*O*-methyl-D-xylose (21.0 mol), 3-*O*-methyl-D-xylose (2.8 mol), and D-xylose (4.2 mol) were detected in the xylan, whereas 2,3,4,6-tetra-*O*-methyl-D-glucose and/or mannose (1.6 mol), 2,3,6-tri-*O*-methyl-D-mannose (5.6 mol), and 2,3,6-tri-*O*-methyl-D-glucose (21.2 mol) were found in the glucomannan. Periodate and Smith-degradation studies substantiate the results of methylation analysis on the xylan. A glucose:mannose ratio of 3:1 for the glucomannan, however, suggests that this fraction may be an aggregate of true glucomannan and glucan or degraded cellulose.

INTRODUCTION

Oilseeds are mostly exploited as a potential source of oils and very recently they have been increasingly used as a source of protein for animal and human foods[1]. Groundnut (*Arachis hypogea*) is an important oilseed crop and ranks first both in area and in total production in India[2].

There are several reports in recent years on the polysaccharides of oilseeds[3]. Non-digestible polysaccharide constituents (hemicelluloses, cellulose) of human foods are beneficial in providing bulk, binding water and certain toxic substances, and in exerting hypocholesterolemic effects[4]. These carbohydrate constituents may also have some implications in cardiovascular diseases[4]. It becomes advantageous, therefore, for food scientists, dietitians and nutritionists, and food processors, to know the quantitative makeup and nature of carbohydrates of such food materials.

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Previous reports on groundnuts from this laboratory described the isolation and characterisation of low-molecular weight carbohydrates[5,6], starch[7], hemicelluloses[5], and changes in carbohydrate composition during processing and germination[8]. Complete removal of protein from defatted groundnut flour was found impracticable, even after repeated aqueous extractions at pH 9.0 and proteolysis at pH 7.5 with pronase, and hence a detailed study of the flour was considered necessary.

The present communication describes the main structural features of two hemicellulosic components, namely, the xylan and glucomannan-type fractions, from groundnut-seed endosperm.

RESULTS AND DISCUSSION

Defatted groundnut flour (400 g), after removal of protein (aqueous extraction at pH 9.0 followed by proteolysis at pH 7.5 with pronase) and starch (by hydrolysis at pH 4.8 with glucoamylase), yielded a residue (40 g; N × 6.25, 7.2%, ash 5.0%, pentoses 50%, uronic acid 5.8%) that was used for the extraction of hemicelluloses. Treatment with 10% sodium hydroxide (carbonate free, under nitrogen) gave alkali-soluble extracts which, on acidification (to pH 4.5) furnished hemicellulose A (precipitable fraction, 2 g) and hemicellulose B (supernatant fraction, 14 g). Acid hydrolysis of hemicellulose B showed rhamnose, arabinose, xylose, glucose, and galacturonic acid in the molar proportions of 0.50 : 3.6 : 1.3 : 0.8 : 0.6, together with an unidentified component (0.2 mol) and a trace of mannose. The presence of xylose in hemicellulose B is of structural importance, as the earlier reports[9-11] did not claim its presence in groundnut-seed endosperm, although Radhakrishnamurty *et al.*[12] reported the presence of xylose in the polysaccharide(s) of groundnut-seed shells.

Fractionation of hemicellulose B with water on DEAE-cellulose (borate form) yielded a fraction (A) in 14% yield from hemicellulose B (Table I). Fractionation of A on Sephadex G-75 furnished two neutral polysaccharide fractions, A_1 (39.5%) and A_2 (57.1%). Rechromatography of these fractions on Sephadex G-75 gave two major fractions, A'_1 and A'_2 in yields of 4.0 and 7.0% (based on total hemicellulose B), respectively. Sugar analysis by g.l.c. of the alditol acetates[13] from A'_1 and A'_2 showed that the former fraction contained 4.1 : 1.4 of glucose and mannose, together with small proportions of xylose and arabinose, whereas the latter fraction has xylose as the major constituent (~87.2%), together with glucose and mannose (~12%). The polysaccharides A'_1 and A'_2 were designated as glucomannan and xylan-type polymers, respectively. Table II summarises the composition and some of the properties of the hemicellulose B and the two neutral fractions.

The two neutral polysaccharides gave single peaks on sedimentation, but the peaks were slightly asymmetrical at the ascending side, indicating some polymer contaminants. However, thin-layer, high-voltage electrophoresis revealed a single band for both of the polysaccharides. From the quantitative sugar analysis, it was observed that contamination from the associating polysaccharides was ~12.1% for the xylan, and ~10% in the glucomannan-type fraction. An acidic xylan of

TABLE I

CHEMICAL COMPOSITION[a] OF DEAE-CELLULOSE FRACTIONS FROM GROUNDNUT HEMICELLULOSE B

Composition	*I*	*II*	*III*	*IV*	*V*	*VI*
Yield (%)[b]	14.0	0.50	0.62	0.74	52.3	33.8
$[\alpha]_D$[c]	−50°	−65°	−74°	−85°	−84°	−103°
Moisture	2.82	4.51	5.42	4.91	4.83	5.22
Crude protein (N × 6.25)	0.02	0.06	0.02	0.04	0.06	0.02
Total carbohydrate	89.41	87.82	90.23	88.62	92.0	91.23
Sugars detected[d]						
Galacturonic acid	—	0.5	1.0	0.5	5.0	4.0
Glucose	8.0	2.8	3.0	4.0	2.0	5.5
Mannose	4.0	—	—	—	1.0	—
Arabinose	0.5	3.0	3.5	2.0	10.0	10.0
Xylose	5.0	4.0	2.0	1.5	4.5	3.0
Rhamnose	—	—	—	—	0.5	1.0

[a]Figures constitute the average of triplicate experiments in all instances. [b]The percentage of each fraction is represented on the basis of hemicellulose B. [c]Polysaccharide solution (0.5%) in 4% alkali at 28°. [d]The values are the approximate sugar proportions.

TABLE II

PERCENTAGE YIELDS AND CARBOHYDRATE COMPOSITION OF HEMICELLULOSE B, GLUCOMANNAN, AND XYLAN

Composition	*Hemicellulose B*	*Glucomannan*	*Xylan*
Yield (%)	(100)	4.0	7.0
$[\alpha]_D$[a]	−38°	−48°	−80°
Total carbohydrate	80.2	98.5	99.4
Sugars detected[b]			
Rhamnose	0.5	—	—
Arabinose	3.6	0.1	—
Xylose	1.3	1.0	6.6
Mannose	trace	1.4	0.5
Galacturonic acid	0.6	—	—
Glucose	0.8	4.1	0.3
Unidentified component	0.2	—	—

[a]Solution (0.5%) in 4% aqueous alkali at 28°. [b]The values are the molar proportions as analysed by g.l.c. of the alditol acetates on a 3% ECNSS-M column at 165°.

comparable purity (80%) has been isolated from defatted rapeseed (*Brassica campestris*)[14]. Here, the contaminating impurities are fucoamyloid (7%) and pectic polysaccharide (14%) which, in spite of repeated fractionations, could not be removed.

However, it was observed that the ratio of glucose to mannose was found to be constant (~3:1) whenever the fractionation of hemicellulose B was carried out for the isolation of the glucomannan, but the proportions of other sugars varied each time, thus suggesting their origin from the contaminating pentoglycan fraction(s).

TABLE III

METHYLATION ANALYSIS OF GROUNDNUT POLYSACCHARIDES

Peak No.	T_R[a]	*Sugars*	*Molar ratio*	*Mode of linkage*
Permethylated xylan				
1[b]	0.65	2,3,4-Tri-*O*-methyl-D-xylose	3.6	D-Xyl*p*-(1→
2	1.54	2,3-Di-*O*-methyl-D-xylose	21.0	→4)-D-Xyl*p*-(1→
3	2.92	3-*O*-Methyl-D-xylose	2.8	→2,4)-D-Xyl*p*-(1→
4	3.16	D-Xylose	4.2	→2,3,4)-D-Xyl*p*-(1→
Permethylated glucomannan				
1	1.00	2,3,4,6-Tetra-*O*-methyl-D-glucose and/or D-mannose	1.6	D-Glc*p*- and/or D-Man*p*-(1→
2	2.20	2,3,6-Tri-*O*-methyl-D-mannose	5.6	→4)-D-Man*p*-(→1
3	2.50	2,3,6-Tri-*O*-methyl-D-glucose	21.2	→4)-D-Glc*p*-(1→

[a]Retention time (min) with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 3% ECNSS-M column at 165°. [b]The quantitation of this volatile sugar-derivative is uncertain.

The close association of glucomannan, xylan, and microfibrillar cellulose in softwoods has been extensively investigated[15,16]. The glucomannan is located deeper inside the framework of the cellulose microfibrils than is the xylan, and is thus difficult to purify. It is also obvious that, as the glucomannan is structurally more similar to cellulose than the hemicelluloses, a glucomannan–cellulose association is quite likely. This inference is well documented in earlier investigations on glucomannans isolated from various sources[17–19], wherein pentoglycan and cellulose contaminations have invariably been observed.

The highly negative $[\alpha]_D$ values (−80 and −48°, respectively) for the xylan and glucomannan indicated predominantly β-D links in both polymers. The molecular weight of the xylan was found by gel chromatography to be 31,000, whereas that of glucomannan was 50,000 ±1,000. Literature value[20,21] for the molecular weight of plant xylans are 10–20,000.

The polysaccharides were permethylated[22], and the fully methylated products were subjected to hydrolysis, reduction (NaB^2H_4 in ^2H_2O), and acetylation. The identities and proportion of the sugars in each fraction were determined by combined g.l.c.–m.s. and by the more-sensitive technique of single-ion mass fragmentography[24]. The quantitative results are presented in Table III.

Methylation analysis showed that the xylan has a highly branched structure, with a (1→4)-linked backbone of D-xylopyranose residues having branch-points at O-2 (9.4%, identification of 3-*O*-methyl-D-xylose), and at O-2 and O-3 (15.6%, identification of free xylose), respectively. The identification of 3-*O*-methylxylose was confirmed by mass-fragmentographic scanning for the ion pairs at *m/e* 189/190, 129/130, and 87/88. The absence in the spectrum of a primary fragment at *m/e* 118 excluded the presence of any 2-*O*-methylxylose. The identity of 2,3,4-tri-*O*-methyl-D-xylose revealed the presence of D-xylose at non-reducing terminals. Discrepancies

between the terminal and branching xylitol acetates, however, might arise from the high volatility of 2,3,4-tri-*O*-methylxylose[25], and hence the quantitation of this derivative (Table III) seems to be doubtful.

Similar methylation studies on the glucomannan indicated linear chains of (1→4)-linked D-glucose and D-mannose residues having D-glucose and/or mannose at the non-reducing end. However, from Table III, it is evident that the proportion of tetra-*O*-methylhexitol acetates is fairly high in comparison with tri-*O*-methylhexitol acetates.

High branching in the xylan was also indicated by periodate oxidation and Smith-degradation studies. Glycerol was the major product, together with intact xylose and traces of ethylene glycol. Formation of these is in accordance with (1→4)-, and (1→2)-, and (1→3)-glycosidic linkages in the xylan. The periodate consumption and liberation of formic acid were 1.14 and 0.288 mol per sugar residue, in good agreement with the calculated values of 1.12 and 0.30 mol per sugar residue, respectively.

Parallel studies on glucomannan yielded mainly erythritol, as expected from a linear, (1→4)-linked hexoglycan. The hydrolysate contained no free hexoses, indicating the absence of branch points in the molecule.

From the apparent molecular weight (~31,000) of the xylan, it may be concluded that the macromolecule consists of about 7 repeating units, each unit in turn, being composed of 39 sugar residues of which 11 are terminal non-reducing D-xylose residues and 21 nonterminal, (1→4)-linked D-xylose residues. There are also 7 D-xylose residues involved in branching, including three through O-3 and four through both O-2 and O-3. Further, detailed studies are needed before a definite structure can be given for the xylan.

Xylans of plant origin appear to fall into four classes. The first comprises homoxylans containing xylose residues only, such as the xylans of esparto grass[26] and tobacco stalk[27]. The former xylan has one branch per molecule, and the latter is essentially a β-(1→4)-linked, linear polymer with no branching. Such a xylan having an unbranched straight chain, is very rare in Nature. The second group consists of arabinoxylans[28] (present in the flour and grain of cereals) having a (1→4)-linked backbone of β-D-xylopyranose residues with (1→3)-linked L-arabinofuranosyl side-chains. The relative proportions of these side-chains vary within very wide limits, probably according to the botanical origin of the polysaccharide. The third group consists of xylans containing (1→2)-linked 4-*O*-methylglucosyluronic acid side-chains, and they are thus 4-*O*-methylglucuronoxylans[29]. The hemicellulose fraction isolated from softwoods represents the fourth group; the xylan backbone has both L-arabinosyl and 4-*O*-methylglucosyluronic acid side-chains, and they may thus be designated as arabino(4-*O*-methylglucurono)xylans[30]. The xylan from groundnut belongs to none of these groups and probably represents a separate class of highly branched, plant xylans.

The glucomannan fraction isolated from groundnut, although fairly pure (~90%), contained more glucose than mannose. The possibility thus exists that the

preparation may also have contained a glucan and/or degraded cellulose. Such types of polymer–polymer associations are encountered in seed mucilages (for example, mustard-seed mucilage[31]) and plant hemicelluloses[32]. However, glucomannans containing more glucose than mannose have also been reported, for example, in the core polysaccharide of *Ocimum basilicum* seed mucilage (glucose : mannose ratio of 10 : 3)[33]; sun hemp fibre (1.2 : 1)[34]; and the glucomannan (one fraction) from Western hemlock (2 : 1)[35]. Glucomannans are generally reported[36] as containing more mannose than glucose, the usual ratio being 3 : 1.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Hilger triple-shape polarimeter at 28 ± 2° and, unless otherwise mentioned, are equilibrium values. All solutions were evaporated under diminished pressure below 35°.

Paper chromatography was performed on Whatman Nos. 1 and 3 MM papers with the solvent systems, (*A*) 10:4:3 1-butanol–pyridine–water; (*B*) 7:1:2 1-propanol–ethanol–water; and (*C*) 8:2:1 ethyl acetate–pyridine–water.

T.l.c. was performed on Polygram-Sil G Silica gel precoated plastic sheets (Machery Nagel Co., Germany) with (*D*) 3:1:1 1-butanol–ethanol–water. Electrophoresis was conducted with Whatman No. 3 MM papers or Polygram-Sil G Silica gel precoated sheets for 1–2 h at a voltage of 40 V/cm with 0.05M borate buffer, pH 9.2 or 10.0. Sugars were detected on chromatograms and electrophoretograms with *p*-anisidine hydrochloride[37] and ammoniacal silver nitrate[38] reagents. Polysaccharide bands on electrophoretograms were detected by charring with 5% sulphuric acid in ethanol.

G.l.c. of partially methylated alditol acetates was performed by the method of Björndal *et al.*[25] with a Varian Aerograph Model 1530 B equipped with a flame-ionization detector and fitted with a glass column (0.32 × 152 cm) packed with 3% of ECNSS-M on Gas Chrom Q (100–200 mesh) at a column temperature of 170°. The flow rate for nitrogen was 28 mL/min. The retention times (T_R) of alditol acetates are given relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. For combined g.l.c.–m.s.[23] of the partially methylated alditol acetates, a Finnigan quadrupole model 3200 spectrometer coupled to a model 6000 data and graphic output system was used. A U-shaped glass column (0.2 × 150 cm) filled with 3% ECNSS-M on Gas Chrom Q (100–200 mesh) at a temperature of 170° and a helium flow rate of 20 mL/min was used. The spectra were taken at 70-eV electron energy in the mass range 35–400, with an integration time of 7 msec/scan.

Total sugar, pentose, and uronic acid were determined by phenol–sulphuric acid[39], orcinol[40], and carbazole[41] methods, respectively. Glucose was estimated in polysaccharides hydrolysates by the procedure of Dahlqvist[42]. Protein was determined by the micro Kjeldahl method.

Groundnut seeds (*Arachis hypogea*, Red netal) obtained from a local market (1974), were defatted and homogenised in a Waring blendor to 60 mesh size.

Preparation of polysaccharides. — Hemicelluloses were isolated from defatted groundnut flour as described previously[5].

Fractionation of hemicellulose B on DEAE-cellulose. — Hemicellulose B (10 g) was fractionated on a large column (8.0 × 45.0 cm) of DEAE-cellulose (borate form). The column was washed successively with distilled water (3 L), borate solution (0.05 and 0.10M; 2.5 L each), and 0.05, 0.10, and 0.4M sodium hydroxide solution (3 L each). A flow rate of 60 mL/h was maintained, 25-mL fractions were collected, and the total carbohydrate in individual fractions was determined. Fractions were pooled separately and successively dialysed (twice) against running tap-water and distilled water, concentrated to low volume, and lyophilised. Thus, water, 0.05 and 0.10M sodium tetraborate; and 0.05, 0.10, and 0.4M sodium hydroxide yielded I (A), II, III, IV, V, and VI fractions, respectively. The per cent yield and gross composition of these DEAE-Cellulose fractions are detailed in Table II.

Acid hydrolysis of the water-eluted fraction (A) revealed glucose, xylose, and mannose in the molar proportions of 8:5:4; traces of arabinose were also detected.

Purification of A *on Sephadex G-75.* — The polysaccharide (1.4 g) was dissolved in 0.1M sodium chloride (20 mL) and loaded onto a column (3.5 × 55.0 cm) of Sephadex G-75 that was washed with 0.1M sodium chloride. The flow rate was 20 mL/h and 5-mL fractions were collected. Appropriate fractions, A_1 and A_2, were pooled separately, dialysed against two changes of distilled water (2 L) overnight, evaporated, and lyophilised. Hydrolysis of fraction A_1 (0.55 g) gave glucose, mannose, and xylose in 6:2:1.4 ratio. Traces of arabinose was also noted on paper chromatograms. Hydrolysis of A_2 (0.80 g) gave mainly xylose plus small amounts of glucose and mannose. Refractionation on Sephadex G-75 furnished A'_1 and A'_2 which, based on their sugar-analysis patterns, were designated as glucomannan and xylan-type polymers.

Analysis of groundnut polysaccharides. — Table II gives the general composition of polysaccharides from groundnut. Sedimentation analysis of a 1.0% solution in in 0.1M sodium chloride at 59,500 r.p.m., and thin-layer electrophoresis, showed a major symmetrical peak with a very small shoulder. Sugar analysis of the polysaccharides was performed by g.l.c. of the alditol acetates by the method of Sawardeker *et al.*[13]. Polysaccharides (2 mg) were hydrolysed with 0.1M hydrochloric acid (200 μL) in a sealed tube for 48 h at 100°. The hydrolysate was made neutral by passage through a small column of Amberlite IRA-410, and the product reduced with sodium borohydride (10 mg) overnight at room temperature. After removal of the excess of borohydride, the alditols were acetylated and analysed by g.l.c. The molar ratios in the xylan were 6.6:0.5:0.3 xylose:glucose:mannose. The glucomannan had glucose and mannose in molar ratios of 4.1:1.4, together with arabinose and xylose (10%).

Molecular-weight determination. — The molecular weights of the polysaccharides were determined by gel chromatography, using dextrans (Sigma Chem. Co.) of known molecular weights.

Permethylation analysis. — Polysaccharides (2–4 mg) were permethylated,

twice, by the method of Hakomori[22]. The resulting, fully methylated samples were purified by passing them through Sephadex LH-20. Permethylation was complete, as OH absorption was absent from the i.r. spectrum. The samples were treated at 90° with 0.5M sulphuric acid in acetic acid (mild acetolysis) in sealed tubes. After 16 h, an equal volume of water was added and hydrolysis continued for a further 5 h at 90°. The resulting, partially methylated sugars were reduced with NaB^2H_4 (in ^2H_2O) and acetylated. The derived alditol acetates were analysed by g.l.c. and g.l.c.–m.s.[23].

Periodate oxidation. — Polysaccharides (200 mg) were dissolved in water (50 mL) and oxidised with 0.1M sodium metaperiodate (50 mL) for 110 h at 5° in the dark. Periodate consumption and liberation of formic acid were determined on aliquots by the methods of Fleury and Lange[43] and Brown *et al.*[44]. The results were: periodate reduced, 1.14 and 1.06; formic acid produced, 0.288 and 0.042 mol per sugar residue for the xylan and glucomannan, respectively.

Smith degradation. — The foregoing periodate-oxidised polysaccharides were reduced overnight with sodium borohydride and then hydrolysed with sulphuric acid (0.25M, 100°, 15 h). Examination of the neutralised (barium carbonate) hydrolysates by paper chromatography showed mainly xylose and glycerol, together with traces of ethylene glycol from the xylan, and erythritol from the glucomannan.

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