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Note

Structural features of acidic xylans isolated from red gram (*Cajanus Cajan*) husk

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Red gram (*Cajanus cajan*) is a legume commonly consumed in tropical countries, usually after dehusking as *dhal*. Industries have developed in these countries for dehusking red gram seeds and other legumes. Different varieties of red gram that are grown, have differences in dehusking quality that cause problems for these industries[1]. It is well established that such differences in milling characteristics are due to differences in adherence of husk to cotyledon, and non-starchy polysaccharides are implicated in this process[1–3]. This prompted a systematic study of the chemical nature and physical properties of non-starchy polysaccharides of red gram isolated from different parts of the seeds, in varieties that have differences in dehusking characteristics. The present paper deals with structures of glucuronoxylans isolated from a difficult-milling variety of red gram.

The cold- and hot water-soluble polysaccharides and hemicellulose A from red gram (Mysore-red variety, difficult-milling) husk contained mainly arabinose and xylose, along with small amounts of glucuronic acid. All of the fractions were highly pigmented and posed problems during purification. They were subjected to different fractionations, namely, alcohol precipitation[4], acid precipitation[5], Cetavlon precipitation[6], alkali precipitation[6], Soxhlet extraction, Me_2SO extraction[7], trichloroacetic acid (10%) extraction[8], cellulase treatment[9], DEAE-cellulose column chromatography[10]; none of these yielded colorless, pure polysaccharide fractions. The fractions could, however, be purified by precipitating with Fehling's reagent[11,12]. These precipitates were colorless and rich in carbohydrate (Table I), while the supernatants contained all the pigments and mainly arabinose. Electrophoresis of the dyed polysaccharides[13] indicated them to be homogeneous, and this was also confirmed by h.p.l.c. and by gel-permeation chromatography. The molecular weight of these polysaccharides, estimated on a Sephacryl S-300

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TABLE I

CHEMICAL COMPOSITION (%) OF NATIVE AND PURIFIED GLUCURONOXYLANS

Fraction	*Yield*	*Total sugar*	*Uronic acid*	$[\alpha]_D^a$	*Sugars identified*					
					Rha/ Fuc	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Cold-water-soluble	Native (100)	34.0	6.0	N.d.[b]	14.8	55.6	26.7		2.9	
Polysaccharide	Purified 32.0	100.0	9.0	−37°		3.0	93.6		1.9	1.5
Hot-water-soluble	Native (100)	51.6	7.2	N.d.[b]	6.4	54.9	34.7		4.0	
polysaccharide	Purified 40.0	100.0	19.0	−29°		4.7	95.3			
Hemicellulose A	Native (100)	98.0	5.8	N.d.[b]	3.2	1.0	94.6		0.1	1.0
	Purified 35.0	100.0	12.0	N.d.[b]		4.8	91.2			3.9

[a]Measured in water. [b]Not determined.

column[14] was 50 000, 79 400, and 13 000 for acidic xylans isolated from cold- and hot-water-soluble polysaccharides, and hemicellulose A, respectively.

These acidic xylans were methylated by the Hakomori method[15] and, after derivatization, were subjected to g.l.c. and g.l.c.–m.s. analysis (Table II). All fractions contained mainly, 2,3-Me_2-Xyl (86.1–92.0) and 3-Me-Xyl (7.1–13.0), with small amounts of 2,3,4-Me_3-Xyl. These polysaccharides were then reduced with $NaBH_4$ through their carbodiimide derivatives[16]. The reduced polysaccharides were then methylated, derivatised, and analysed by g.l.c. and g.l.c.–m.s. (Table II). The presence of 2,3,4,6-Me_4-Glc was indicative of terminal, carboxyl-reduced glucuronic acid residues. 2,3,4-Me_3-Xyl was not detected.

To obtain further information regarding the substitution of glucuronic acid to xylose, purified fractions from cold and hot-water-soluble polysaccharides were methylated[15] and subjected to selective degradation[17] with base–sodium methoxide or methylsulfinyl carbanion. The degraded products were then ethylated with ethyl iodide and then conventionally derivatised. It was observed that the amount of 3-*O*-methylxylose diminished and a new peak 2-*O*-ethyl-3-*O*-methylxylose was identified (Table II). The latter peak had characteristic fragments of *m/z* 189 and 132. These results indicated that the terminal glucuronic acid residues were linked to O-2 of xylose. However, quantitative conversion was not observed, as also reported earlier[17].

Periodate oxidation[18] of these polysaccharides with $NaIO_4$ consumed ~1.0 mol of periodate (range 1.0–1.2) and a small amount of formic acid was liberated. On CrO_3 oxidation[19], glucuronic acid was the only surviving sugar, indicating glucuronic acid in α-glycosidic linkage and xylose in β-glycosidic linkage. Smith end-group analysis of the resulting oxopolysaccharides yielded mainly glycerol.

^{13}C-N.m.r. analysis of the glucuronoxylan isolated from the hot-water-soluble polysaccharide showed well resolved signals (Fig. 1). Correlation of the signals with a possible structure (one of the many possibilities, Fig. 2) is presented in Table III. Signals were assigned on the basis of methylation data and from published

TABLE II

METHYLATION ANALYSIS (%) OF ACIDIC XYLANS-NATIVE, REDUCED, AND DEGRADED

Fraction	*2,3,4-Me_3-Xyl (0.6)*[a]	*2,3,4,6-Me_4-Glc (1.0)*[a]	*2Et-3Me-Xyl (1.1)*[a]	*2,3-Me_2-Xyl (1.2)*[a]	*3-Me-Xyl (2.0)*[a]
A. Cold-water-soluble					
Native	0.9			86.1	13.0
Carboxyl reduced		6.7		74.6	18.7
Degraded by MeO^-	1.7		4.5	89.2	4.7
Degraded by $CH_3SOCH_2^-$	1.6		2.2	91.3	4.8
B. Hot-water-soluble					
Native	1.0			89.4	8.6
Carboxyl-reduced		16.5		57.1	26.4
Degraded by MeO^-	3.6		1.6	78.6	16.3
Degraded by $CH_3SOCH_2^-$	2.8		3.4	85.2	8.5
C. Hemicellulose A					
Native	0.9			92.0	7.1
Carboxyl-reduced		10.1		78.8	11.1

[a] R_T in comparison to 2,3,4,6-Me_4-Glc on a column of OV-225.

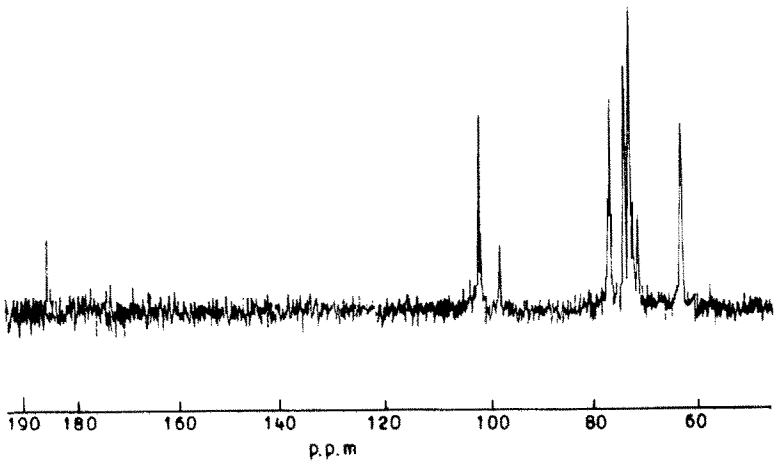

Fig. 1. ^{13}C-n.m.r. spectrum of glucuronoxylan isolated from hot water-soluble polysaccharide.

data[20–23]. Anomeric signals for the main chain (A) and branched (B) xylose were at 102.3 and 102.0 p.p.m., respectively, characteristic of β-linked xylopyranose residues. The signal at 98.5 p.p.m. was assigned to C-1 of α-linked glucuronic acid[23]. Absence of a signal around 60.0 p.p.m. confirmed the absence of 4-*O*-methylglucuronic acid. Chemical shifts of C-2–C-5 of sugars A and B were mostly identical except for C-2 of sugar B, which is attributable to substitution at this position by glucuronic acid. Chemical shifts for glucuronic acid (C) residues were characteristic, with C-6 at low field (185.5 p.p.m.), possibly because of its substitution by an unidentified metal ion; this was not investigated further. An attached-proton test[24–26] of the polymer gave a negative signal at 63.6 p.p.m. that was assigned to C-5 of xylose (Fig. 3).

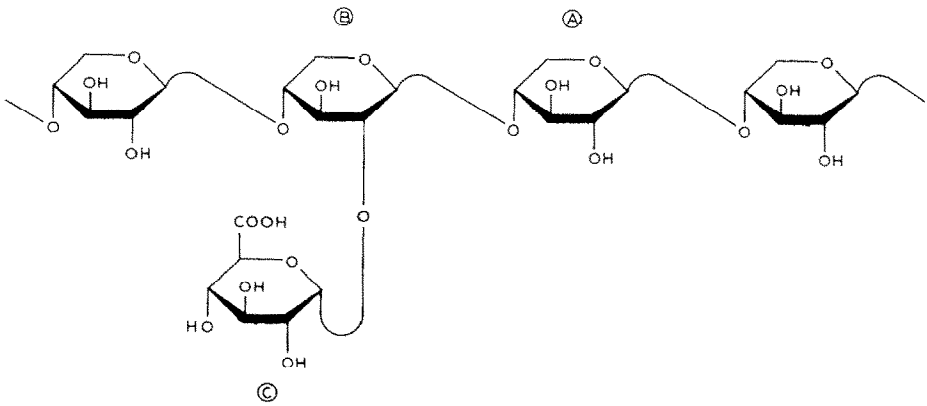

Fig. 2. Partial (one of the probable) structure of glucuronoxylan [identification of ^{13}C-n.m.r. signals (Fig. 1) as shown in Table III].

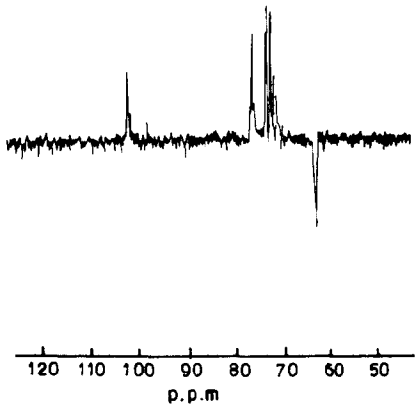

Fig. 3. Attached-proton test spectrum of glucuronoxylan isolated from the hot water-soluble polysaccharide.

Native hemicellulose A was used for oligosaccharide fragment-analysis without further purification. Of the many methods tried 0.25M trifluoroacetic acid for 45 min at 100° was found the best and was used for bulk hydrolysis. Separation of neutral and acidic oligosaccharides was achieved on a column of Dowex-2 and further purification into individual oligosaccharides was achieved by preparative p.c. in solvent *B* for neutral oligosaccharides and in solvent *C* for acidic oligosaccharides. Oligosaccharides were tested for purity by p.c. in different solvents; final purification was on Biogel P-2 column. Four neutral and two acidic oligosaccharides were obtained, in pure form in good amounts, and these were taken for further studies.

Neutral oligosaccharide I, contained only xylose. Its $[\alpha]_D$ in water was −28° and it had $R_{maltose}$ 1.08 in solvent *B*. It was reduced with $NaBD_4$, and methylated[15], and used for g.l.c. and g.l.c.–m.s. analysis without hydrolysis. The $T_{maltose}$ of this oligosaccharide on OV-225 was 0.58. A fragment, *m/z* 175, and its secondary fragments *m/z* 143 and 111 derived from the elimination of methanol were charac-

TABLE III

ASSIGNMENT OF ^{13}C-N.M.R. SIGNALS OF ACIDIC XYLAN ISOLATED FROM HOT WATER-SOLUBLE POLYSACCHARIDE

Sugar residues[a]	*Chemical shifts in p.p.m.*[b]					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
A	102.3	73.4	74.4	77.1	63.6	
B	102.0	77.3	73.1	76.9	63.4	
C	98.5	72.6	72.9	77.5	71.8	185.5

[a]Refers to sugar residues as shown in Fig. 2. [b]In p.p.m. relative to the singal for Me_4Si.

teristic for xylose at the non-reducing end. Fragment *m/z* 192 was characteristic for methylated xylose at the reducing end. Its characteristic J_1 fragment *m/z* 252 (192 + 60) was also observed. A diagnostic fragment for (1→4)-linkage, *m/z* 134, was also present. Thus neutral oligosaccharide I was identified as β-Xyl-(1→4)-Xyl.

Neutral oligosaccharides II, III, and IV were reduced successively with $NaBD_4$, methylated, hydrolysed, and derivatized for g.l.c. and g.l.c.–m.s. analysis. They gave different proportions of 1,2,3,5-Me_4-Xyl, 2,3,4-Me_3-Xyl, and 2,3-Me_2-Xyl (Table IV) indicating them to be linear, (1→4)-linked xylose oligosaccharides. On the basis of their $[\alpha]_D$ (Table IV) they are assigned the β-linkage.

Acidic oligosaccharides I and II contained xylose and glucuronic acid in the approximate ratio of 4:1 and 5:1, respectively. They were reduced with $NaBH_4$ through their carbodiimide derivatives, methylated, and derivatized conventionally for g.l.c. and g.l.c.–m.s. analysis. Components were identified as 1,2,3,5-Me_4-Xyl, 2,3,4-Me_3-Xyl, 2,3-Me_2-Xyl, 3-Me-Xyl and 2,3,4,6-Me_4-Glc, and were in different proportions (Table IV). The $[\alpha]_D$ for acidic oligosaccharides I and II were +57.0° and +50.0° respectively. These results indicate acidic oligosaccharide I to have four xylose residues linearly linked by β-(1→4)-linkages and one of the central xylose residues is branched by glucuronic acid in α-(1→2) linkage. Acidic oligosaccharide II has one more xylose residue in β-(1→4) linkage in the main chain.

Structures of all those oligosaccharides were in accord with the methylation analysis data of the native and reduced hemicellulose A fraction. Structures of the acidic polysaccharides isolated from cold- and hot-water-soluble polysaccharides (oligosaccharide analysis was not done for these polysaccharides) were also of the same type, but had differences in the uronic acid content and the molecular weight. The ^{13}C-n.m.r. analysis of the hot-water-soluble polysaccharide gave results in agreement with other data. Hence, all these acidic polysaccharides were of the glucuronoxylan-type, having xylose in the backbone in β-(1→4) linkage and having singly substituted glucuronic acid residues in α-(1→2) linkages at the branch points. 4-*O*-Methylglucuronic acid was absent (no ^{13}C-n.m.r. signal near 60.0 p.p.m.) and no comparable sugar was observed on p.c. analysis of the hydrolysates of any of these polysaccharides or oligosaccharides.

Pure acidic xylans and heteroxylans have been isolated from different plant sources, including acidic xylans from apple wood[27], cherry wood[28], birch wood[29], leaf fibres of pineapple[30,31], *Sansevieria trisaciata*[32], jute fibre[33], and from *Graminaceace*[34]. The outer pericarp of wheat kernel also contains a branched acidic xylan. The majority of these reported acidic xylans contain glucuronic acid and 4-*O*-methylglucuronic acid. Acidic xylans substituted only with glucuronic acid have been identified from the stalk of *Cyperus papyrus*[35], soybean husk[36], corn husk[37], and groundnut shell[38]. Neutral, linear xylans are reported from guar seed husk[14], esparto grass[39], *Chetangium fastigiatum*[21] (red algae), and *Rhodochorton floridulum*[40] and these are rare in nature. Interestingly acidic xylans, substituted with glucuronic acid alone, have been identified thus far only in the husk portion (covering of the seeds). It is tempting to speculate that the presence of such poly-

TABLE IV

CHARACTERISTICS OF THE OLIGOSACCHARIDES OBTAINED AFTER PARTIAL ACID HYDROLYSIS OF HEMICELLULOSE A

Oligosaccharide	R_{Mal}[a]	$[\alpha]_D$[b]	*Ratios of permethylated sugar derivatives*				
			1,2,3,5-Me_4-Xyl	*2,3,4-Me_3-Xyl*	*2,3-Me_2-Xyl*	*3-Me-Xyl*	*2,3,4,6-Me_4-Glc*
Neutral	0.72	−45.8°	1	1	1		
	0.55	−55.0°	1	1	2		
	0.29	−69.0°	1	1	3		
	R_{GlcA}[c]						
Acidic I	0.38	+57.0°	1	1	1	1	1
II	0.15	+50.0°	1	1	2	1	1

[a]Mobility in p.c. with respect to maltose in solvent B. [b]Measured in water. [c]Mobility in p.c. with respect to glucuronic acid in solvent *C*.

saccharides in only this part of the seeds (the husk) may have important physiological functions, one of which may be to protect the seeds from desiccation.

EXPERIMENTAL

Materials. — Red gram (*Cajanus cajan*), Mysore-red variety, was purchased from the local market. The seeds were conditioned and milled[3] in an abrasive mill to separate husk, cotyledon, and the intermediate fraction (uppermost layer of the cotyledon). The husk used in the present study was powdered and sieved to 60 μm size.

Isolation of polysaccharides[41]. — The husk was extracted three times with 1:1 (v/v) $CHCl_3$–MeOH, petroleum ether, and 70% EtOH and all these extracts were rejected as they were highly colored and low in carbohydrate content. The insoluble residue was extracted three times with cold and hot water (95°) to afford cold- and hot-water-soluble polysaccharides, respectively. The insoluble residue was extracted three times with 0.5% ammonium oxalate at room temp. and at 80° to furnish cold- and hot-ammonium oxalate extractable pectins, respectively, and with hot EDTA (80°) to give EDTA-soluble polysaccharides. The insoluble residue was then extracted with 10% NaOH to afford hemicelluloses as described by Whistler and Feather[42], and the precipitate obtained with AcOH was hemicellulose A.

General methods. — Evaporations were performed under diminished pressure at <40°. The polysaccharide fractions were dried either by lyophilization or by the solvent-exchange method (alcohol–ether). The polysaccharides were hydrolysed either with H_2SO_4 (72%, solubilization at ice-cold temperature followed by dilution to 10% acid concentration) for 8 h at 100°, or 2M CF_3CO_2H, for 6–8 h at 100°, or M CF_3CO_2H for 4–5 h at 100°. The total carbohydrate content was estimated[41] by phenol–H_2SO_4 and uronic acid by the carbazole method[41]. The sugars were identified[41] by p.c. on Whatman No. 1 or No. 3 paper, using either (*A*) 6:4:3 BuOH–pyridine–water, (*B*) 7:1:2 PrOH–EtOH–water, or (*C*) 5:5:1:3 EtOAc–pyridine–AcOH–water and spraying with $AgNO_3$–NaOH or aniline–phthalate reagents. Sugars were identified by g.l.c. as their alditol acetates on a column of OV-225. Methylated, unhydrolysed oligosaccharides were analyzed on a 10% SE-30 column. G.l.c.–m.s. analysis was performed either on a Hewlett–Packard model 5995 or Varian MAT 311 instrument. ^{13}C-N.m.r. spectra were recorded with a Bruker WM 300 spectrometer, 20-mg samples and D_2O as solvent; the standard used was sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$) pentanoate. Specific rotations were measured at 0.5–1.0% in water with a Perkin–Elmer model 243 polarimeter. For electrophoresis on cellulose acetate membranes[13], the polysaccharides were dyed with Procion dye, and acetate buffer (pH 4.8, 0.05M) was used. Gel-permeation chromatography was performed on a Sephacryl S-300 column using 0.1M NaCl as the eluent. H.p.l.c. of the polysaccharides was performed on Waters Associates liquid chromatography (Milford, MA, U.S.A.) using a μ-Bondagel E-linear column equipped with a 6000 Å pump, U6K injector, and R

401 refractive-index detector. The flow rate was 1.5 mL/min and fraction size was 0.4 mL. Polysaccharide (5 mg) was dissolved in 7:3 (v/v) MeCN–H_2O (0.5 mL) and was eluted with the same solvent mixture.

Fehling's precipitation[11,12]. — The polysaccharide (1 g) was dissolved in NaOH (5%, 75 mL) with stirring (4 h) and was centrifuged. To the supernatant solution, Fehling's reagent was added and the resulted precipitate was purified as described[11,12].

Methylation analysis[15]. — Polysaccharides (5 mg) in 0.5 mL Me_2SO were methylated using sodium methylsulfinyl carbanion (0.5 mL, 2M) and methyl iodide (1 mL), essentially as described by Hakomori. Methylated products were purified on a Sep-Pak C_{18} cartridge. The purified, methylated polysaccharides were hydrolysed with formic acid (90°, 2 mL) for 2 h at 100°, followed by CF_3CO_2H (2M, 2 mL) for 6 h at 100° or for 1 h at 121° in sealed tubes. The hydrolysates were reduced with $NaBD_4$ and acetylated for g.l.c. and g.l.c.–m.s. analysis.

Carboxyl reduction of acidic polysaccharides. — This was effected by the method of Taylor and Conrad[16] using 1-cyclohexyl-2-(4-methyl morpholinoethyl)-carbodiimide. The reduced polysaccharides were methylated by the Hakomori method[15].

Carboxyl reduction[43] *of acidic oligosaccharides using carbodiimide–$NaBH_4$.* — Acidic oligosaccharide (~5 mg) was used and quantities of all other reagents taken for the reaction were corrected accordingly. After reduction, MeOH (2 mL) was evaporated 4 times from the mixture and a 1:1 MeOH–H_2O solution of the residue was passed through a column of Amberlite IRA-45. The effluent was lyophilised.

Degradation of acidic polysaccharides[17]. — This was performed using sodium methoxide or methylsulfinyl carbanion, essentially as described by Lindberg *et al.*[17].

Periodate oxidation[18]. — Polysaccharides (10 mg) were dissolved in water (5 mL) and aq. $NaIO_4$ (5 mL, 20 mM)was added and the solution was kept at 4° in dark. Periodate consumption was monitored by the method of Fleury and Lange[18] and formic acid liberation by the method of Brown *et al.*[44].

Chromium trioxide oxidation[19]. — This was performed for the native polysaccharides as described by Hoffman *et al.*[19].

Smith end-group analysis. — After periodate consumption became constant, the reaction was stopped by adding ethylene glycol in water (50%, 2 mL), and the solution was dialysed. The dialysate was concentrated and $NaBH_4$ (50 mg) was added to the solution, which was then kept for 8–10 h at room temperature. The excess of $NaBH_4$ was decomposed and the solution was dialysed and lyophilized. The resulting polyalcohol was hydrolysed with CF_3CO_2H (M, 2 mL) for 6 h at 100° and the hydrolysates used for p.c. and g.l.c. analysis.

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