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Note

Structural characteristics of a mixed linkage β-D-glucan from sorghum (*Sorghum bicolor*)

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Abstract

Hemicellulose B, obtained by alkali extraction of sorghum flour on DEAE-cellulose chromatography gave a water-eluted neutral polysaccharide composed exclusively of D-Glc. Further purification on Sephacryl S-400 gave a major fraction (Fra.II) which was homogeneous by electrophoretic and SEHPLC methods. It had a MW of 3.6×10^4 Da and showed a bathochromic shift (19 nm) in the λ_{max} of its complex with Congo Red. Permethylation and GC–MS analysis revealed (1→4)- and (1→3)-glucosidic linkages in a ratio of 2.3:2 which were corroborated by ^{13}C NMR and CD spectral measurements. The β configuration of the glucan was further confirmed by treatment with β-glucanase and also by its low $[\alpha]_D$ value of +12.5°.

Keywords: Sorghum; Hemicellulose; Mixed linkage β-D-glucan; Structural studies

Sorghum (*Sorghum bicolor* (L.) Moench), commonly referred to as *jowar* in India is a drought resistant grain and forage crop grown in the tropical and semiarid parts of the world [1]. It is an important staple diet in India, Pakistan, central and northern China, and Australia [1,2]. It is rich in glucan-type (α- and β-) polysaccharides, which also includes the mixed linkage β-D-glucan (MLG). Very little information is available on the latter. (1→3)-β-D-Glucans, especially those derived from fungi and seaweeds are shown to exhibit a number of biological response modification (BRM) properties [3,4]. MLGs are primarily localized in the aleurone and subaleurone cell walls [5,6]. Barley and oat β-D-MLGs are the most studied examples. In view of the fact that the Indian diet is primarily cereal based, a detailed study of the chemical and biological properties of such nonstarch and noncellulosic MLGs was considered desirable to appreciate the (un-) known physiological usefulness of consuming cereals as a staple food [7]. The structural details of a homogeneous glucan derived from sorghum hemicellulose B are presented in this communication. The histochemical localization of MLG in the grain as well as some of its biological properties will be published elsewhere.

1. Experimental

Materials.—Authentic variety (SB 905) of sorghum (*S. bicolor*) grains was procured from the

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Agricultural Station, Dharwad. The cleaned seeds were ground in a standard plate mill to 100 mesh. All chemicals used were of analytical reagent grade. Amyloglucosidase (E.C. 3.2.1.3, 10 units mg^{-1} solid), D-glucose oxidase (E.C. 1.11.1.7, 100 units mg^{-1} solid), Triton X-100, laminarin, zymosan, barley and yeast glucans were procured from Sigma Chemical Co., USA.

General methods.—Total sugar, reducing sugar and D-Glc were determined by the modified phenol–H_2SO_4 [8], Nelson–Somogyi [9] and D-Glc oxidase [10] methods, respectively, using D-Glc or maltose as reference compounds. ^{13}C NMR spectra of polysaccharides (in D_2O) were obtained at a probe temperature of 800 °C in a Bruker AMX-400 spectrometer at 100 MHz. The spectra were obtained from 8000 scans in a pulsed FT mode with complete proton decoupling. Tetramethylsilane was the external reference used. Specific rotation $[\alpha]_D$ of the sample (0.5–1% in water) was determined in a Perkin-Elmer model 243 polarimeter at 200 °C. GLC of the alditol acetates was performed as before [11].

MLG isolation.—The sorghum flour was gelatinized by heating an aqueous suspension (10%) for 30 min in a boiling water bath and subjected to amylolysis [12]. The starch-free residue (KI–I_2 negative) was repeatedly extracted with water at boiling temperature and the resulting water-insoluble residue was then extracted with 1 N NaOH at room temperature (2 h under N_2 atmosphere). After centrifugation, the alkaline supernatant was adjusted to pH 4.5 with dil. acetic acid at 4 °C to precipitate out hemicellulose A. Addition of ethanol (3 vol) to the supernatant gave hemicellulose B. An aqueous solution (1%) of the latter was fractionated on DEAE-cellulose (CO_3^{2-}) by eluting sequentially with water, ammonium carbonate (0.5 M) and NaOH (0.2 M) solutions. Fractions (12–14 mL) were monitored for total sugar content. The water-eluted fraction was further fractionated on Sephacryl S-400 column.

Permethylation analysis.—The purified polysaccharide was permethylated by the Hakomori method [13] and the products cleaned by passing through Sep-Pak C18 cartridges [12]. After hydrolysis with formic acid–H_2SO_4, the partially methylated alditol acetates were prepared (using NaB^2H_4 in ^2H_2O) and analyzed by GC–MS [14].

Homogeneity criteria.—*Gel filtration*. An aqueous solution (5 mg in 1 mL water) of the polysaccharide was loaded onto a precalibrated (with T-series dextrans of known MW) column of Sephacryl S-400 and eluted with water (18 mL h^{-1}). Fractions (3 mL) were analyzed for total sugar and their approximate MW computed.

Sedimentation behaviour. The sedimentation behaviour of a solution of the glucan (1% in 0.1 M NaCl) at 59 780 rpm was determined in a Beckman model E Spinco analytical ultracentrifuge with phase plate Schlieren optics.

Electrophoresis. Microzone electrophoresis of the dyed (Procion Brilliant Red 2BS) polysaccharide on cellulose acetate membranes was performed in a Beckman microzone cell in ammonium carbonate–NaCl buffer, 0.05 M, pH 9.3) at an applied voltage of ~15O V and 7 mA current for 40 min [15].

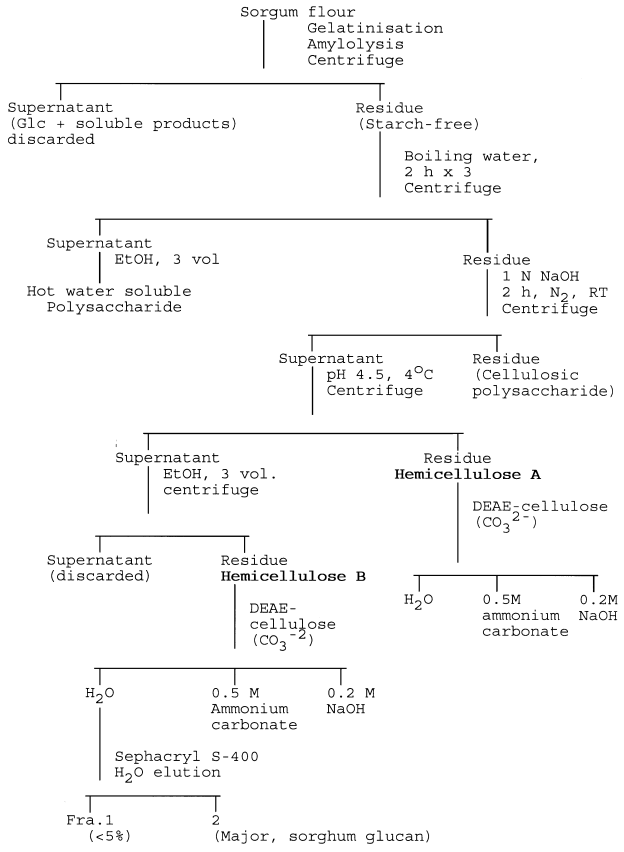
Size exclusion high performance liquid chromatography. SEHPLC on E-linear and E-1000 (Waters Associates, Milford, USA, ss, 3.9 mm i.d.×30 cm) μ-Bondagel columns connected in series with a guard column was performed on a Shimadzu HIC-6A chromatograph as described before [16].

Circular dichroism studies.—The CD spectra of the Congo Red–glucan complex were measured in a Jasco model J-20C automatic recording spectropolarimeter. Samples were scanned at 2 mg mL^{-1} concentration and specific ellipticity $[\theta]$ was calculated by the equation $[\theta] = H \times S/L \times C$, where H is the height of the peak (cm), S is scale sensitivity, L is the path length and C is the concentration (g mL^{-1}).

Helix coil transition (HCT) analysis.—This was done on a polysaccharide solution (10 mg mL^{-1} of 0.001 M NaOH) containing Congo Red (88 μm in 0.001 M NaOH). The λ_{max} was scanned from 400 to 650 nm [17]. Laminarin, carboxymethyl cellulose, barley and yeast glucans and zymosan were the positive controls; xylan, soluble starch and dextran T40 were the negative controls.

Partial purification of a β-*glucanase*.—The commercial enzyme (5 mg mL^{-1} of 0.05 M acetate buffer, pH 5.0) was purified by GPC on Sephacryl S-400 and eluted with the acetate buffer. Fractions (2 mL) were analyzed for protein by measuring the OD at 280 nm.

β-D-*Glucan content*.—*By 'difference' method*. The polysaccharide was acid hydrolysed (5 mg in 1 mL of 2 N H_2SO_4, 6 h at boiling water bath temperature). The total Glc in the neutralized ($BaCO_3$) hydrolyzate was determined by the glucose oxidase method and the content of α-glucan (starch type) was separately determined by digesting the



Scheme 1. Isolation of glucan from sorghum hemicellulose B.

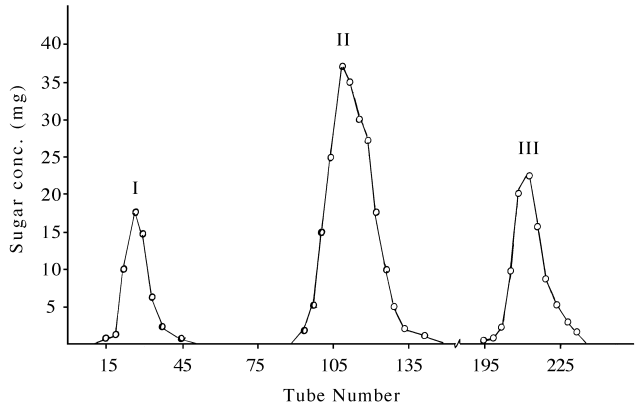


Fig. 1. Fractionation of hemicellulose B on DEAE-cellulose (CO_3^{2-}) and elution with water (I); ammonium carbonate (0.5 M) (II); sodium hydroxide (0.2 M) (III).

polysaccharide with amyloglucosidase followed by glucose oxidase assay [12]. The difference between the two values represented the β-glucan content [18,19].

Enzymatic method. To a solution of glucan (2 mL, sodium acetate buffer, 0.1 M, pH 5.0) was added partially purified β-D-glucanase (0.12 mg) and incubated at 55 °C for 1 h. Enzyme action was stopped by heat inactivation and the digest was centrifuged and the reducing sugar released into the supernatant was determined [20].

2. Results and discussion

Alkali extraction of the starch-free residue of sorghum furnished hemicelluloses A and B (Scheme 1) in varying yields. Of the three fractions obtained on DEAE-cellulose chromatography (Fig. 1) of hemicellulose B, the water-eluted polysaccharide (Fra.I) showed a relatively high total sugar value of ~95%, all of which was essentially composed of Glc (Table 1). In HCT analysis, Fra.I showed a significant bathochromic shift of 13.6 nm, whereas Fra.II and III contained relatively more protein and both were highly pigmented.

Fra.I was further resolved on Sephacryl S-400 into a major fraction that was homogenous by cellulose membrane electrophoresis, sedimentation and SEHPLC analyses. Acid hydrolysis followed by both TGO assay and GLC of the derived alditol acetates revealed the polysaccharide to be a glucan. The MW determined by GPC was found to be 3.6×10^4 Da. The MW of the isolated glucan preparation depends upon several factors, such as extraction temperature, the nature of the starting material and fractionation methodology adopted. For example, in barley, a wide range of MW values, ranging from 2×10^4 to 2×10^7 Da has been reported [21].

The solubility in water of the sorghum glucan was found to be affected slightly upon frequent freezing and thawing cycles. Such solubility behavioural changes of MLG induced by different processing treatments have been reported, and even treatments like repeated centrifugation lead to

Table 1
Composition % of fractions derived from sorghum hemicellulose B

Eluent	Yield	Total sugars	Protein	Sugars identified	Congo Red shift (nm)
Water	0.04	95.0	3.4	Glc and Xyl (< 1%)	13.8
Ammonium carbonate	0.89	72.8	11.8	Xylose	0.2
Sodium hydroxide	0.31	85.0	15.0	Glc and Xyl (1:1.2, % ratio)	Nil

gelation of the polysaccharide [22]. The presence of (1→3)-linkages, which causes chain flexibility and leads to extended straight ribbon configuration, would also influence the solubility behaviour [23].

Polysaccharides existing in an ordered conformation, preferably by (1→3)-linked glucose moieties, form a complex with Congo Red in dilute NaOH solution as denoted by a shift in the λ_{max} [24]. As seen from Fig. 2 and Table 2, a significant bathochromic shift of ~19 nm was observed for this glucan in comparison to shifts of 10.5 and 16.5 nm observed for laminarin and barley glucan complexes, respectively. The very low shift of 0.5 nm observed for zymosan complex was attributed to its extremely low solubility in 0.001 N NaOH. No shift was observed for any of the non-(1→3)-linked polysaccharides used as negative controls. Circular dichroism studies of the sorghum glucan complex with Congo Red (Fig. 3) gave a positive band at 430 nm and a negative band at 525 nm in comparison with 430 and 530 nm bands seen for laminarin, which is exclusively composed of β-(1→3)-linkages. These CD spectral data were similar to those observed with curdlan-type polysaccharide 13140, which is a bacterial β-(1→3)-D-glucan [25]. A slightly lower value for the negative band of sorghum glucan could probably be attributed to the interspersed (1→4)-linkages in it. The low positive specific rotation $[\alpha]_D$ +12.5° (C, 1% in H_2O) also substantiated a β-configuration in sorghum glucan. ^{1}H-Decoupled ^{13}C FT NMR spectrum of laminarin (Fig. 4b) showed six signals at 105.11, 86.82, 78.19, 75.83, 70.69 and 63.28 ppm corresponding to C-1, C-3 (substituted), C-5, C-2, C-4 and C-6 nuclei, respectively. Out of the eight signals observed in the corresponding spectrum (Fig. 4a) of sorghum glucan, six were almost identical to those of laminarin with slight downfield chemical shifts of $\delta < 2$ ppm. Two additional signals observed in this spectrum at 81.0 and 76.5 ppm could be assigned to C-4 of the glucosidic linkages (C-4 substituted) and C-3 (unsubstituted) nuclei, respectively. Anomeric signals were well separated from signals of other nuclei. These observations gave evidence for the mixed linkage (1→3, 1→4) pattern of sorghum β-D-glucan. This

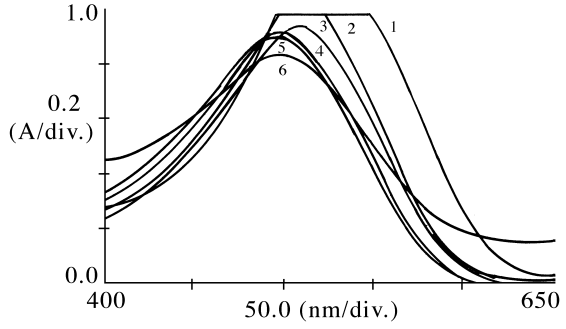


Fig. 2. Absorption spectra of Congo Red complex, with β-linked polysaccharides: 1, sorghum glucan; 2, barley glucan; 3, laminarin; 4, carboxymethylcellulose; 5, Congo Red; 6, yeast glucan.

Table 2
Shift in the λ_{max} of Congo Red complex with sorghum glucan and other polysaccharides

Compound/complex	λ_{max}	Congo Red shift (nm)
Congo Red	497.0	—
Congo Red + sorghum glucan	516.0	19
Congo Red + barley glucan	513.5	16.5
Congo Red + laminarin	507.5	10.5
Congo Red + zymosan	496.5	0.5
Congo Red + CMC[a]	496.0	−1.0
Congo Red + xylan	495.0	−2.0
Congo Red + soluble starch	497.0	0
Congo Red + dextran T-40	497.0	0

[a] CMC-carboxymethylcellulose.

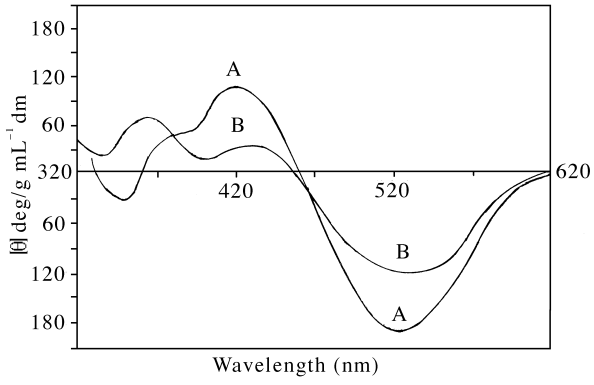


Fig. 3. CD spectra of Congo Red complex with sorghum glucan (A) and laminarin (B).

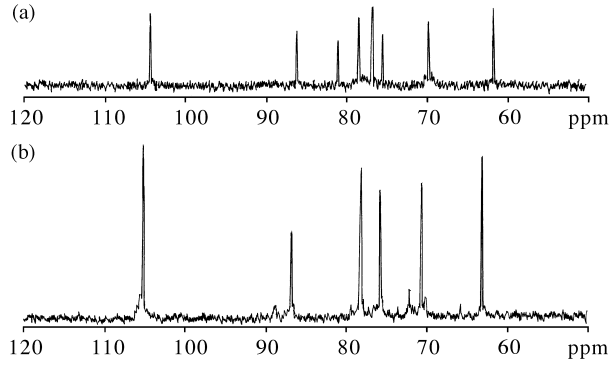


Fig. 4. ^{13}C NMR spectra of sorghum glucan (a) and laminarin (b).

Table 3
Permethylated alditol acetates identified (GC-MS) in sorghum glucan and laminarin

Methyl ether	R_t [a]	Mode of linkage	Approximate percentage	
			Sorghum glucan	Laminarin
2,3,4,6-Me_4–	1.00	Glcp-(1→	Trace	Trace
2,4,6-Me_3–	1.33	→3)-Glcp-(1→	46.5	100
2,3,6-Me_3–	1.52	→4)-Glcp-(1→	53.5	—
2,4-Me_2–	1.85	→6,3)-Glcp-(1→	—	—

[a] R_t with respect to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-glucose.

linkage profile was further confirmed by permethylation followed by GC–MS analysis (Table 3). The β-D-glucan derived from hemicellulose A of sorghum is reported to contain (1→3)- and (1→4)-linked glucose residues in the ratio of 3:2 [26]. In the present investigation the β-D-glucan was derived from the alkali- and acid-soluble hemicellulose B fraction. In the water-soluble barley glucan, these two linkages are present in the ratio of 3:7, respectively [27].

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