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Note

Serological and chemical investigations of the anomeric configuration of the sugar units in the D-galacto-D-mannan of fenugreek (*Trigonella foenum-graecum*) seed

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The structures of the D-galacto-D-mannans of leguminous seeds, such as carob seed, guar seed, and fenugreek seed show a backbone of (1→4)-linked D-mannose residues and α-D-galactosyl groups attached to O-6 of the D-mannose residues[1]. Similar findings were made by Mukherjee *et al.*[2] for the D-galacto-D-mannan from the kernel of green palmyra palm nut. The anomeric configurations of the sugar residues in these D-galacto-D-mannans have not been established serologically and chemically, except by isolation and identification of the structures of oligosaccharides by such physical method as specific rotation. In this communication, we report the chromium trioxide oxidation study and precipitin reaction, by different lectins, of the D-galacto-D-mannan fenugreek seed. *Abrus precatorius* and *Arachis hypogaea* lectins were shown to be specific for both α- and β-D-galactopyranosyl residue[3–5], *Abrus* lectin reacting preferentially with the β-D-, and *Arachis* lectin with the α-D form. The Tridacnin lectins, obtained from the bivalve clam *Tridacna maxima* and from the sponge *Axinella polypoides*, and the *Abrus precatorius* lectin were found to possess a specificity for (1→6)-linked residues[6], and reacted with several D-galactans of various origins in the precipitin test[7,8]. The occurrence of a lectin specific for α-D-galactosyl residue, obtained from the seeds of *Bandeiraea simplicifolia*, was reported by Hayes and Goldstein[9]. Recently, two more lectins, specific for α-D-galactopyranosyl residues, one from the seeds of *Artocarpus integrifolia* (Jack fruit) and another from the seeds of *Artocarpus lakoocha* (both plants belonging to the Moraceae family) were isolated and characterized[10]. The specificity of both partially purified lectins was investigated by hemagglutination-inhibition studies; methyl α-D-galactopyranoside and melibiose gave good inhibition, D-galactose showed weak inhibition, and methyl β-D-galactoside and lactose did not inhibit, even at a 0.1M concentration.

Lectins from *A. integrifolia* and *A. lakoocha* gave a precipitin band in Ouchterlony gel plates with fenugreek-seed galactomannan added to the middle well, whereas no such precipitin line was observed with the lectins from *A. polypoides*, *T. maxima*, *A. precatorius*, and *A. hypogaea* (see Fig. 1). These results indicate the presence of a

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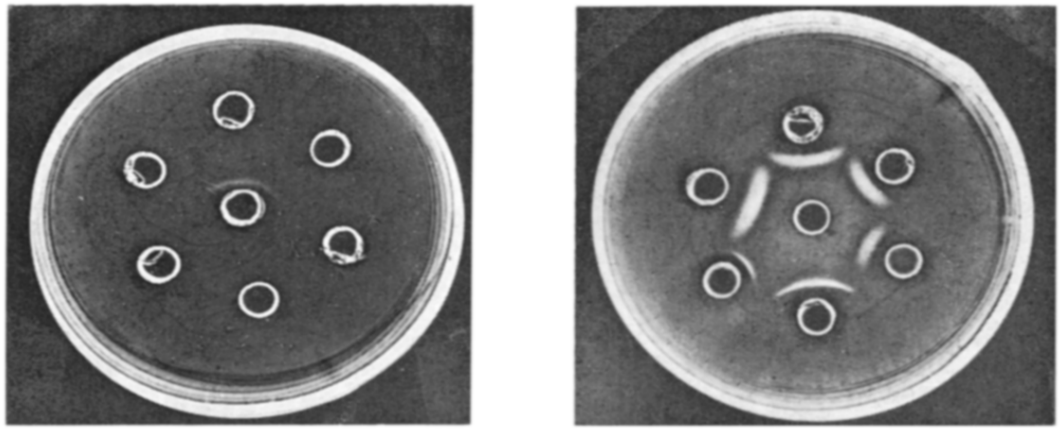

Fig. 1. Precipitin reaction of fenugreek seed D-galacto-D-mannan (in middle well) with various lectins: (1) *Artocarpus integrifolia*, (2) *Artocarpus lakoocha*, (3) *Axinella polypoides*, (4) *Tridacna maxima*, (5) *Abrus precatorius*, and (6) *Arachis hypogaea*. The wells are numbered clockwise from noon (well 1) on. All wells were filled with 1% of test substance (20 μL).

Fig. 2. Precipitin reaction of *A. integrifolia* lectin (in middle well) with various D-galacto-D-mannans from the seeds of: (1) *Sesbania sesban*, (2) carob, (3) palmyra palm, (4) guar, (5) *Poinciana pulcherrima*, and (6) fenugreek. For numbering of wells and conditions, see legend to Fig. 1.

terminal α-D-galactopyranosyl residue in this galactomannan. Lectin treatment of other D-galacto-D-mannans from different seeds with *A. integrifolia* lectin gave an intense line in each case (see Fig. 2), thus confirming the presence of identical, nonreducing, terminal α-D-galactopyranosyl residues as receptors in these galactomannans.

The specificity of the reaction of *A. integrifolia* and *A. lakoocha* lectins with α-D-galactopyranosyl residues was confirmed by the lack of a precipitin reaction with a D-galactan obtained from the albumen gland of the snail *Achatina fulica* (see Fig.

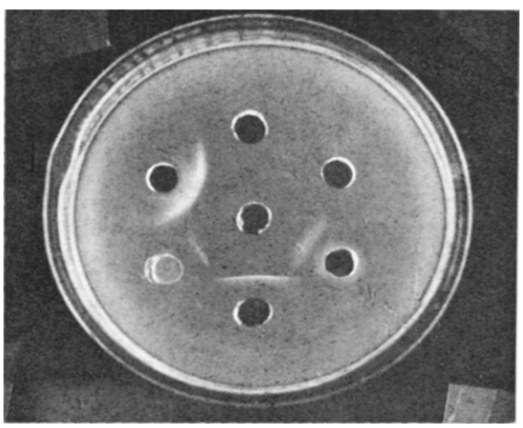

Fig. 3. Precipitin reaction of *Achatina fulica* snail D-galactan with various lectins: (1) *A. integrifolia*, (2) *A. lakoocha*, (3) *A. polypoides*, (4) *T. maxima*, (5) *A. precatorius*, and (6) *A. hypogaea*. For numbering of wells and conditions, see legend to Fig. 1.

TABLE I

STABILITY OF SUGAR COMPONENTS OF ACETYLATED D-GALACTO-D-MANNAN IN CHROMIUM TRIOXIDE OXIDATION[a]

Time (h)	*Mannose*	*Galactose*	myo-*Inositol*[b]
0	48.5	38.5	10
1	8.5	28.7	10
2	2.1	14.7	10
3	1.2	12.2	10

[a]The sugar components were analyzed by g.l.c. in a 3% ECNSS-M column at 190°. [b]As standard.

3). This polysaccharide is composed of β-(1→3)- and β-(1→6)-linked D-galactose units, as established by methylation, periodate oxidation, and chromium trioxide oxidation studies[10]. All four anti-β-D-galactopyranosyl lectins precipitated *A. fulica* D-galactan (see Fig. 3).

The anomeric configuration of the sugar units of fenugreek-seed galactomannan was confirmed by chromium trioxide oxidation[11–13]. In this degradation, the D-mannose residues were degraded at a rate faster than that of the D-galactose residues (see Table I), suggesting that all the D-mannose residues are β-linked and the D-galactose residues, present as nonreducing end groups, are α-linked. These results are in agreement with those obtained earlier[1].

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer Model 241 MC polarimeter. I.r. spectra were recorded with a Beckman IR-20A spectrophotometer. Descending paper chromatography (p.c.) was performed on Whatman No. 1 papers for qualitative analysis. The following solvent systems (v/v) were used: (*A*) 4:1:5 (upper phase) 1-butanol–acetic acid–water[14], and (*B*) 9:2:2 ethyl acetate–acetic acid–water[15]. Chromatograms were developed with the alkaline silver nitrate reagent[16]. Evaporations were conducted in a rotary evaporator below 40° (bath temperature). Gel filtration was performed with Sephadex LH-20 and G-100, both elutions being monitored with a differential refractometer (Model R-403) and polarimetrically. G.l.c. was performed with a Hewlett–Packard Model 5730 A gas chromatograph, fitted with a flame-ionization detector and a glass column (1.83 m × 6 mm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–200 mesh), and (*b*) 3% of OV-225 on Gas Chrom Q (100–200 mesh). Electrophoresis was conducted on a silica-coated glass plate, subsequently sprayed with 30% sulfuric acid for identification.

Isolation of D-*galacto*-D-*mannans.* — The pulverized seeds (10 g) of fenugreek were extracted with cold water (500 mL) overnight. The supernatant solution obtained after centrifugation of the slurry was added to ethanol (1.5 vol.). The precipi-

tate collected by centrifugation was freed of ethanol and redissolved in water. The process was repeated, and the precipitate was centrifuged off, triturated with acetone, and dried (P_2O_5); yield, 4.5 g. A portion of the polysaccharide (100 mg) was dissolved in ammonium hydrogencarbonate buffer (pH 8.0), and the solution was applied to a column (80 × 2.5 cm) of Sephadex G-100, which was eluted with the same buffer. The major portion of the material was eluted as a single fraction, and the solution was freeze-dried to yield 70 mg of polysaccharide, which moved as a single component in electrophoresis (16 V/cm) in phosphate buffer (pH 7.0), for 90 min; $[\alpha]_D^{26}$ +68.5° (*c* 0.2, water). The D-galacto-D-mannan from the seeds of *Poinciana pulcherrima* was obtained by the same method and was found to be homogeneous by electrophoresis. The D-galacto-D-mannan from the seeds of guar was obtained according to the method of Heyne and Whistler[17]. The D-galacto-D-mannan of palmyra palm nut was a kind gift of Dr. A. K. Mukherjee of this department, and the D-galacto-D-mannans of carob seed and *Sesbania sesban* seeds were generously supplied by Dr. Nilima Banerjee and Dr. S. B. Bhattacharya of the Indian Institute of Experimental Medicine, Calcutta.

Hydrolysis and characterization of structures. — The polysaccharides from fenugreek seed and *Poinciana pulcherrima* seed were each hydrolyzed with 0.5M sulfuric acid, in a sealed tube, for 16 h in a boiling-water bath. Each hydrolyzate, after the usual treatments, was examined by p.c. in solvents (*A*) and (*B*), and revealed the presence of galactose and mannose. Both hydrolyzates were also examined by g.l.c. of the alditol acetates with *myo*-inositol as the internal standard. The peaks corresponding to galactose and mannose were obtained in a ratio of 1:1.25 in the case of fenugreek-seed galactomannan, and 1:2 in the case of galactomannan of *Poinciana pulcherrima* seed. The galactomannan of fenugreek seed was methylated according to the method of Hakomori[18] and the resulting methylated sugars quantitatively determined by g.l.c.; our methylation results fully corroborate those obtained earlier[19].

Isolation of lectins. — The seeds (100 g) of *A. integrifolia* (jack fruit) and *A. lakoocha* each were homogenized in a blender with 0.9% sodium chloride (1 L), and the slurry was stirred overnight at 4°. The supernatant solution obtained after centrifugation in a Sorvall RC-5B refrigerated centrifuge at 18000 r.p.m. was fractionated by ammonium sulfate precipitation. The fraction (60 mg) obtained at 40–70% of ammonium sulfate in the case of *A. integrifolia* was dissolved in phosphate-buffered saline (PBS), pH 7.2, and added to a fetuin-conjugated-Sepharose column. After the column had been washed with PBS, the lectin was eluted with a glycine buffer (0.5M glycine hydrochloride in 0.5M sodium chloride), pH 3.0. The fractions were monitored by measuring the absorbance at 280 nm, and by hemagglutination assay. The combined fractions were dialyzed against distilled water and lyophilized; yield, 5 mg. The lectin moved as a single band in polyacrylamide-gel electrophoresis at pH 8.9. *A. lakoocha* lectin was used in precipitin reaction in partially purified form, as the 40–70% ammonium sulfate fraction. Tridacnin lectin was obtained by courtesy of Prof. G. Uhlenbruck, Cologne, F.R.G., and that from *A. polypoides* from Dr.

Hagen Bretting, Hamburg, F.R.G. Peanut (*Arachis hypogaea*) lectin was purchased from Serva, Heidelberg, F.R.G. *A. precatorius* lectin was isolated on a Con A–Sepharose column as described earlier[3]. *A. fulica* snail D-galactan was isolated as previously described[20].

Serological test. — The precipitin reactions were carried out in agar gel plates coated with 1% agarose (Serva) as described earlier[21]. All lectins and test substances used were at a concentration of 1% in 0.9% sodium chloride. Hemagglutination–inhibition tests were carried out with 25-μL volumes of two-fold serial dilutions of test sugar solutions, mixed with an equal volume of four hemagglutinating doses of lectin. The mixtures were kept for 1 h at room temperature, and then a 2% suspension of erythrocytes (25 μL) was added to each test. The mixture was kept for another hour, and the maximum dilution (titer) of sugar solution showing hemagglutination–inhibition was recorded.

Chromium trioxide oxidation. — To a mixture of fenugreek-seed galactomannan (10 mg) and *myo*-inositol (1.2 mg, internal standard) in formamide (3.0 mL) were added pyridine (2.5 mL) and acetic anhydride (1.5 mL) with stirring, at room temperature, overnight. The mixture was evaporated, and the residue extracted with chloroform (25 mL). The extract was washed with water (3 × 30 mL), dried (Na_2SO_4), and evaporated. The residue was reacetylated in the same way. The acetylated product was oxidized with chromium trioxide (500 mg) in glacial acetic acid (5 mL) with constant stirring at 50°. Aliquots were taken out at time intervals and immediately diluted with water, and subsequently extracted with chloroform (2 × 20 mL). The combined chloroform extract was washed thoroughly with water, and evaporated to dryness. The residue was deacetylated with sodium methoxide, and the solution decationized with Dowex 50 (H^+) ion-exchange resin and evaporated. The residue was hydrolyzed with 0.5M sulfuric acid for 16 h at 100°. The hydrolyzate was derivatized into alditol acetates and analyzed by g.l.c. The results are reported in Table I.

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