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Note

Isolation and structural analysis of ajugose from *Vigna mungo* L.

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Abstract—The hexasaccharide ajugose, α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside, generally uncommon in legumes, was detected in the seeds of *Vigna mungo* L. by TLC and paper chromatography. Ajugose was then isolated by silica gel chromatography and its structure was established by acid and enzymatic hydrolysis, fast atom bombardment mass spectrometry and both one- and two-dimensional ^{1}H and ^{13}C NMR techniques.

Keywords: *Vigna mungo* L.; Raffinose family oligosaccharides; Ajugose; Flatulence

Raffinose family oligosaccharides (RFO) rank second to sucrose as the most abundant soluble carbohydrates in plants. They are synthesised and deposited in storage organs, such as seeds and tubers during the maturation process, and are mobilised during the early stages of germination.[1] RFO consist of linear chains of galactopyranosyl residues attached to the glucose moiety of sucrose via an α-(1→6)-galactopyranosidic linkage[2] (Fig. 1). The first member of this series, raffinose, is the main oligosaccharide in most monocotyledon seeds, while its higher homologues, the tetrasaccharide stachyose and the pentasaccharide verbascose, accumulate in the seeds of dicotyledons.[3] RFO have multiple functions in plants, they serve as transport carbohydrates in the phloem,[4] as storage reserves and cryoprotectents in frost-hardy plant organs,[5,6] and may play a role in the acquisition of desiccation tolerance and storability of seeds.[7] RFO content, along with that of other carbohydrates, decreases with higher temperature,[8] and increases during cold acclimatisation in vegetative tissues.[9]

Flatulence is the most common symptom associated with pulse consumption.[10] Abdominal pain and diarrhoea are often experienced by susceptible individuals, especially children, causing pulses to be less readily accepted.[11] The production of flatus in monogastric animals is due to colonic fermentation of carbohydrates that escape digestion and absorption in the stomach

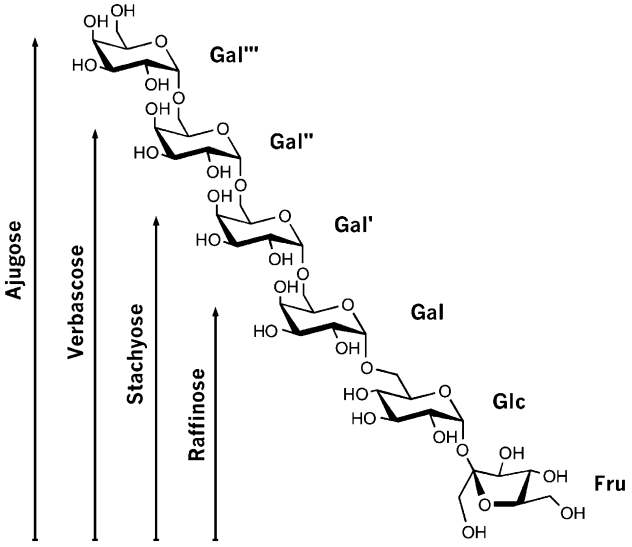

Figure 1. Structural relationship of the raffinose family of oligosaccharides.

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and small intestine. Owing to the absence of an α-galactosidase enzyme capable of hydrolysing the α-(1→6)-galactosidic linkage, these oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria, which result in the production of flatus gases (H_2, CO_2 and small amounts of CH_4). Abdominal rumbling, diarrhea and attendant discomfort are often experienced by pulse consumption.[12–14] Legume researchers, growers, processors and consumers consider flatulence to be the single most important factor that deters people from eating more legume seeds, despite the fact that they are a good source of protein in the diet.[15]

The hexasaccharide ajugose has been identified in roots and leaves of several Lamiaceae.[1] Seeds of some leguminous plants also contain ajugose along with the other RFO, albeit in much smaller quantities.[16] Ajugose was also reported in a few genotypes of peanut.[17–19] Previous work has indicated the occurrence of RFO up to verbascose and there are no reports on the presence of ajugose in black gram (*Vigna mungo* L.) seeds. To the best of our knowledge, this is the first report of structural analysis of ajugose and its presence in black gram. The present work describes the isolation and structural analysis of ajugose from black gram seeds.

TLC separation of extracts of black gram gave five spots when sprayed with α-naphthol. The spots appeared with a characteristic blue colour, suggesting the presence of fructose unit.[20] None of the spots gave the characteristic yellow colour when sprayed with *p*-anisidine phthalic acid reagent,[1] indicating that the oligosaccharides were non-reducing. Sucrose, raffinose and stachyose in the extract were identified by comparing with the R_f values of standards. The presence of verbascose was confirmed by comparison of the black gram oligosaccharides with that of the red gram.[21]

A similar pattern of separation was observed when the oligosaccharides were subjected to paper chromatography. For the identification of the fifth spot, R_f values were used to calculate the partition function $\log \alpha'$ defined by $\log \alpha' = \log(R_f/1 - R_f)$ as described by Feingold et al.[22] The values were plotted against the number of hexose units, yielding a straight line (Fig. 2). Because $\log \alpha'$ of a linear polymer can be expected to be the sum of increments contributed by the tail term, the head term and the mid term of the polymer.[22] These results suggested that the fifth spot represents the ajugose, the oligosaccharide of the raffinose family with a degree of polymerisation of six. Ajugose has been reported to have a low mobility on paper and thin layer chromatograms.[19]

The RFO were isolated by column chromatography. Fractions 12–48 were found to contain RFO, as determined by the method of Tanaka et al.[23] TLC was used to monitor the separated oligosaccharide in each fraction. Ajugose eluted in the fractions 44–47. The purity of ajugose was checked with HPLC (data not shown). Acid hydrolysis of ajugose in 0.5 M H_2SO_4 for 3 h

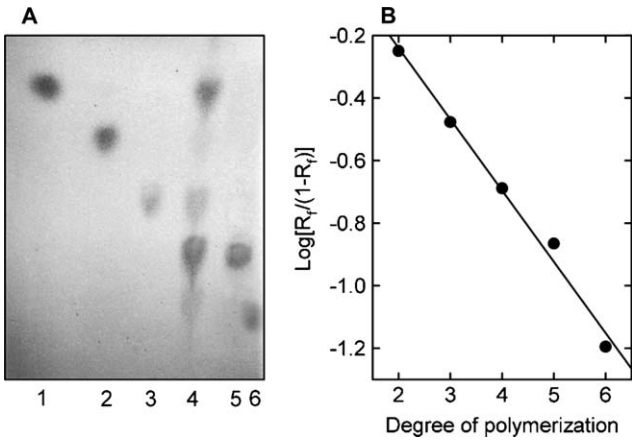

Figure 2. (A) Paper chromatographic pattern of separation of oligosaccharides from black gram sprayed with α-naphthol: (1) sucrose standard; (2) raffinose standard; (3) stachyose standard; (4) black gram extract; (5) verbascose isolated from black gram; (6) ajugose isolated from black gram. (B) Plot of papergram mobilities of the oligosaccharides in black gram extracts versus their degree of polymerisation.

yielded fructose, glucose and galactose in the ratio of 1:1:4. On partial hydrolysis, the oligosaccharide yielded verbascose (trace), raffinose, glucose, galactose and fructose. Incubation with α-galactosidase from *Aspergillus oryzae* revealed the presence of verbascose, stachyose, raffinose and sucrose (faint spots) along with galactose.

Fast atom bombardment mass spectrometry (FABMS) gave a molecular ion *m*/*z* at 1013, inclusive of a sodium ion. Because this compound contains hexose units in a sequential order, there is a cleavage of the bond between oxygen and the carbon of the hexoses to felicitate the fragmentation of a single hexose unit. This is indicated by the sequential loss of hexose units giving rise to fragment ions at *m*/*z* 851, 689, 527 and 365, respectively. Retention of fructose moiety in the molecule is indicated by the peak at *m*/*z* 165.

The ^{13}C NMR spectrum of the compound presented 28 signals, 25 of them being of similar intensity and the remaining signals two to four times as strong, apparently due to overlap of resonances. Carbon chemical shift values were very similar to those reported for ajugose tentatively identified from seeds of *Mimosa scabrella*.[24] A ^{1}H–^{13}C correlation spectrum (HSQC) was used to link the carbon signals to the corresponding proton resonances. Five anomeric carbons (94.98, 100.63, 100.66, 100.89 and 101.22 ppm) were apparent. By spin–echo Fourier transform spectroscopy (SEFT), one signal attributable to a quaternary carbon (C-2 of the fructose residue at 106.68 ppm) and seven signals originating from CH_2 groups were identified (Fig. 3A). Taking the quaternary carbon as a starting point, the carbon and proton signals of the fructose and the glucose residue were assigned using heteronuclear multiple bond correlation (HMBC). Most of the connectivities of the glucose residue could also be traced in the COSY

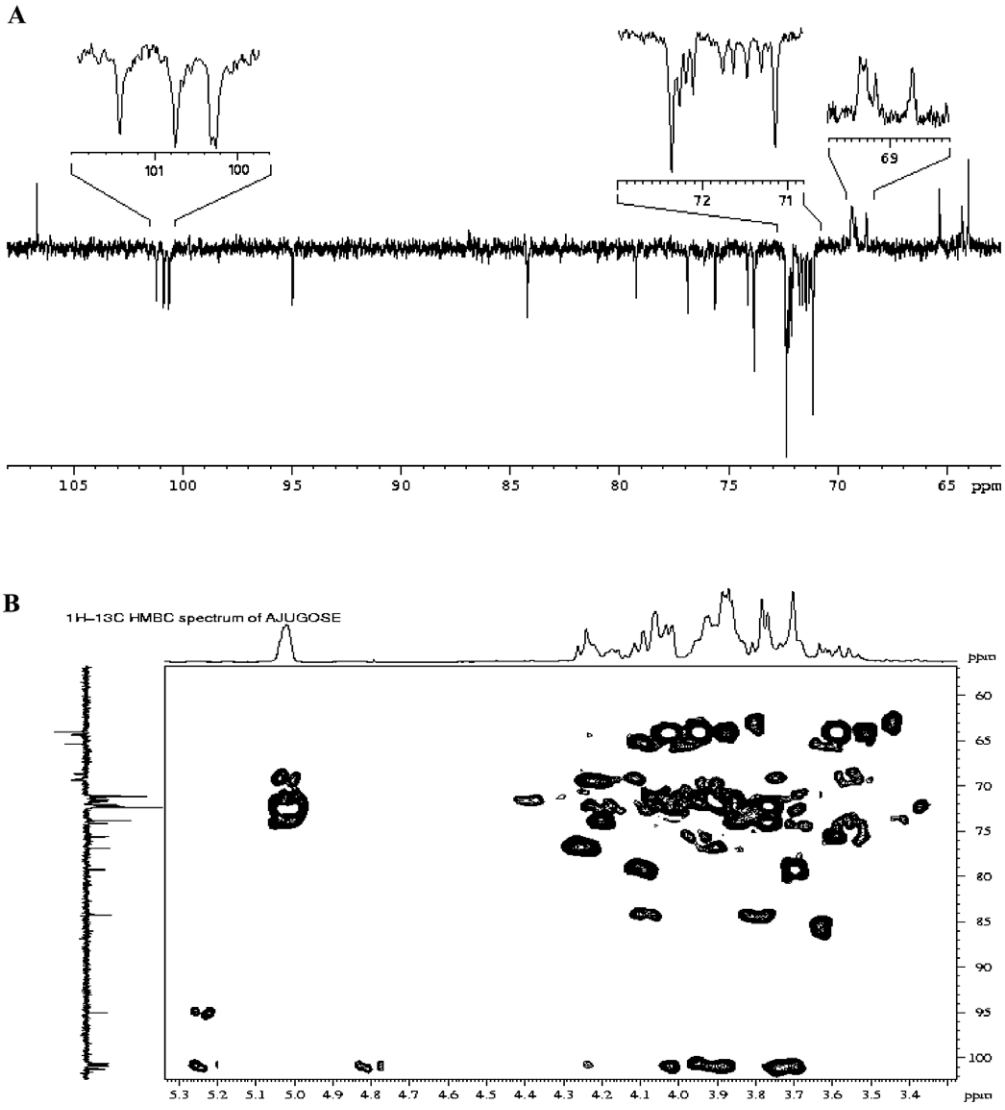

Figure 3. (A) ^{13}C (SEFT) NMR spectrum of the oligosaccharide purified from black gram. (B) ^{1}H–^{13}C (HMBC) NMR spectrum of the oligosaccharide purified from black gram.

spectrum, which provided little additional structural information due to the superposition of six ring systems. By comparison with the completely assigned ^{1}H and ^{13}C spectrum of stachyose, the tetrasaccharide of the RFOs,[25] the highest field signal (64.90 ppm) was assigned to C-6 of the terminal non-reducing galactose residue. The remaining signals from the terminal galactose residue were established by HMBC (Fig. 3B). The proton and carbon resonances of these three ring systems were in excellent agreement with the corresponding values of those of stachyose.[25] Careful examination of HMBC connectivities allowed the identification of several positions of the internal glycosyl residues (Table 1). The chemical shift values of the three secondary carbons corresponding to C-6 of these moieties, resonating closely together at 69.17, 69.31 and 69.39 ppm, respectively, compare favourably with that of C-6 of the internal galactose of stachyose and support the presence of three internal moieties substituted at C-6. Although an unambiguous assignment of the remaining signals was not possible due to strong overlap of signals, all the above findings lead to the conclusion that the isolated compound is ajugose.

The identity of sucrose, raffinose and stachyose in the black gram extract was further confirmed by HPLC, comparing the retention time of peaks in the sample with that of peaks of standards. Taken together, our results indicate the presence of the entire family of raffinose oligosaccharides, up to a degree of polymerisation of six, in black gram seeds. Ajugose content in black gram is comparatively higher than raffinose and stachyose, which can be clearly seen by TLC. The higher oligosaccharide, ajugose, induces more flatulence than the lower degree of polymerisation oligosaccharides.

Table 1. ^{1}H and ^{13}C NMR chemical shift values for ajugose

Pos.	Fru		Glc		Gal		Gal′		Gal″		Gal‴	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	3.67	64.32	5.43	94.98	4.99	101.22	4.99	100.63	4.99	100.66	4.99	100.89
2	NP[a]	106.67	3.56	73.83	3.84	71.14[b]	3.85	71.14	3.83	71.31	3.80	71.14
3	4.22	79.24	3.75	75.62	3.85[a]	72.41[b]	3.85[b]	72.41[b]	3.85[b]	72.41[b]	3.91	72.28
4	4.06	76.88	3.51	72.36	3.89[a]	72.37[b]	3.89[b]	72.37[b]	4.03[b]	72.20[b]	3.99	72.11
5	3.89	84.22	4.07	74.14	4.14	71.78	4.20	71.65	4.12	71.49	4.00	73.84
6a	3.78	65.35	3.66	68.69	3.73	69.31	3.64	69.17	3.67	69.39	3.75	64.90
6b	3.84		4.06		3.84		3.92		3.89			

[a] NP, not present.
[b] Tentative assignments.

There are several processing methods including soaking, cooking, malting and dehulling that could reduce oligosaccharide content of legumes.[13,26] There are few reports of the use of α-galactosidase from plants and microorganisms to reduce oligosaccharides present in legume flours and soymilk.[27–29] Plant breeding could be an alternative approach to the reduction of flatus activity, but the fact that oligosaccharides are suggested to play a role in defense mechanism against diseases[30] in seed viability and cold acclimatisation suggest that their elimination from the plant itself could adversely affect the growth and yield.

1. Experimental

1.1. Plant material, extraction of oligosaccharides

Black gram seeds were obtained from the Agriculture Research Station, Gulbarga, India. The seeds were passed through a screen to remove dust and foreign particles. Seed material was milled to flour and passed through a 400 μm sieve. The flour (5 g) was added to an Erlenmeyer flask containing 70% EtOH (50 mL, v/v) and placed on an orbital shaker at 130 rpm for 13 h at room temperature (35 ± 1 °C). The contents were filtered through Whatman No. 1 filter paper, the filtrate was concentrated in a rotary vacuum evaporator and the residue was dissolved in distilled water (5 mL).

1.2. TLC and paper chromatography

TLC was performed with 0.2 mm thick plates coated with cellulose-G (Acme Synthetics, India). TLC plate was developed with the solvent system *n*-PrOH/EtOAc/H_2O (6:1:3). Fructose-containing oligosaccharides were detected by spraying with 1% α-naphthol (90 mL of 95% ethanol containing 10% orthophosphoric acid).[20] The *p*-anisidine phthalic acid reagent (1.25% *p*-anisidine and 1.75% phthalic acid in 90% ethanol) was used to detect the presence of reducing sugars. Similarly, paper chromatography was performed using Whatman No. 1 filter paper (46 × 57 cm). Extracts were spotted next to spots of authentic sugars. *n*-BuOH/EtOAc/HOAc/H_2O (40:30:25:40, v/v) was used as a solvent system for developing the chromatogram.

1.3. Isolation of ajugose

Black gram powder (100 g) was defatted with chilled acetone (1:6, w/v), followed by chilled hexane (1:6, w/v). The defatted powder (50 g) was added to a flask containing 70% EtOH (500 mL) and was placed on an orbital shaker at 130 rpm for 13 h at room temperature (35 ± 1 °C). The extract was filtered and the filtrate was concentrated in an evaporator (Heidolph, Germany) below 50 °C. The oligosaccharides were purified by extraction with *n*-BuOH/H_2O (1:1, v/v), using a separating funnel. The water phase was separated and lyophilised (Heto-Drywinner, Denmark). The lyophilisate obtained (3.5 g) was dissolved in 60% CH_3OH (15 mL) and loaded onto a column (1 m long and 2.5 cm in diameter) containing Silica Gel 60G (Merck). Oligosaccharides were eluted from the column with *n*-PrOH/EtOAc/H_2O (6:1:3). Fractions (20 mL) were collected and assayed for the presence of sugars by the method of Tanaka et al.[23] The oligosaccharides in each fraction were monitored by TLC. The fractions containing only ajugose were combined and concentrated using a vacuum evaporator. Purity of the ajugose was checked with HPLC.

1.4. Hydrolysis of ajugose by acid and enzymatic methods

Acid hydrolysis was carried out at 100 °C in 0.5 M H_2SO_4 for 3 h. Partial hydrolysis was done with 0.25 M H_2SO_4. For enzymatic hydrolysis, ajugose was incubated with α-galactosidase isolated from *A. oryzae*, in 0.2 M acetate buffer, pH 4.8 at 37 °C for 12 h.

1.5. HPLC analysis

HPLC analysis was performed with a LC 10ATVP pump (Shimadzu Corporation, Japan) equipped with a refractive index detector. Sample injection was via a Rheodyne injector equipped with a 20 μL sample loop.

Carbohydrates were separated on a Phenomenex Bondclone 10 μm CHO column (300 × 3.9 mm). The mobile phase consisted of CH_3CN–H_2O (70:30, v/v) and the flow rate was kept at 1 mL/min. Chromatographic data were collected and plotted using Class-VP 6.1 software. Standards (fructose, glucose, galactose, sucrose, raffinose and stachyose) were from Sigma Chemicals (USA).

1.6. FABMS analysis

FABMS were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The sample (dissolved in $CHCl_3/CH_3OH/CH_3CN/H_2O$) was introduced into the ESI source through a syringe pump at the rate of 5 μL/min. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. Spectra were collected in 6 s scans.

1.7. NMR spectroscopy

One-dimensional ^{1}H, one-dimensional ^{13}C (SEFT), DQF-COSY, HSQC and HMBC NMR spectra were recorded at 25 °C in D_2O using a Bruker Biospin Avance-400 spectrometer operating at 100.62 (^{13}C) and 400.13 (^{1}H) MHz. Chemical shifts values were expressed relative to sodium trimethylsilyl propionate as an internal standard.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.04.043.

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