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Two-dimensional NMR spectroscopic studies of fenugreek (*Trigonella foenum-graecum* L.) galactomannan without chemical fragmentation

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Abstract

Fenugreek galactomannan, as compared to other gums particularly of guar and locust bean, is less exploited in the food industry. With regard to the structure of fenugreek galactomannan, though reports on galactose substitutional pattern are available, supporting data by NMR correlation studies are scanty. The present investigation deals with the observations made by 2D NMR spectroscopy of fenugreek galactomannan. Sequential extraction of fenugreek flour with cold water, hot water and alkali resulted in different fractions. Purification of alkali extracted polysaccharide B by copper complex precipitation and GPC (TSK gel) fetched two fractions, viz. Fra.1 and Fra.2 with a gal:man ratio of 1:1.04 and 1:1.12, respectively. *Aspergillus niger* β-D-mannanase had no effect on the hydrolysis of Fra.1 and Fra.2. indicating high substitution and interference by galactose stubs. All the assignments of ^{1}H- and ^{13}C- signals could be achieved and verified through various ^{1}H–^{1}H and ^{1}H–^{13}C correlation experiments, viz. DQF-COSY, TOCSY and HSQC. HMBC experiments resulted in correlations of H-1(galα)/C-6(man), H-4(man)/C-1(man) and H-6(man)/C-1(gal). The assignment of anomeric protons and confirmation of (1 → 4) linked mannan back bone with (1 → 6) attachment of gal clarified the structure [4)[α-gal-(1 → 6)]-β-man-(1 →]$_n$. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Fenugreek galactomannan; 2D NMR spectroscopy; Structure

1. Introduction

Fenugreek (*Trigonella foenum-graecum* L.), an annual herb of leguminosae is being used as spice with its seeds and also as vegetable with its plant (leaves). Though it is indigenous to Western Asia and Southern Europe, it is mainly cultivated in India, Pakistan, France, Argentina and North African countries (Shankaracharya & Natarajan, 1972). In the food industry, it is being used as a flavourant and also recommended as a superior food emulsifier (Dea & Morrison, 1975; Garti, Madar, Aserin & Sternheim, 1997). The traditional usage of fenugreek as an aphrodisiac by Egyptians, and along with honey, in the treatment of rickets, diabetes, dyspepsia, tuberculosis, rheumatism, anaemia and constipation and as an expectorant in veterinary practice are recorded (Shankaracharya, Anandaraman & Natarajan, 1973). However, experimental data in support of many of these claims are less except for diabetes where its potential benefit in modulating carbohydrate (controlling plasma glucose) and lipid metabolism has been found by alteration of starch digestion and bile acid absorption (Madar, 1984; Sharma, 1986).

Compared to guar gum and locust bean gum, fenugreek galactomannan is less known and less exploited in the food industry (Dea & Morrison, 1975; Garti et al., 1997). Data on fenugreek mucilage compositional analysis, gum crystallography and ^{13}C NMR observations after partial hydrolysis are reported (Bociek, Izzard, Morrison & Welti, 1981; EI-Mahdy, 1984; Song, Winter & Taravel, 1989). However, integrated approaches of 2D NMR correlations to support the substitutional patterns in fenugreek galactomannan structure are scanty. Therefore, generating more data on fenugreek galactomannan, particularly structural details, may help not only the growing areas of general glycan chemistry but also add value to the source for better applications (Painter, 1982). The present investigation deals mainly with NMR studies of fenugreek galactomannan without chemical fragmentation.

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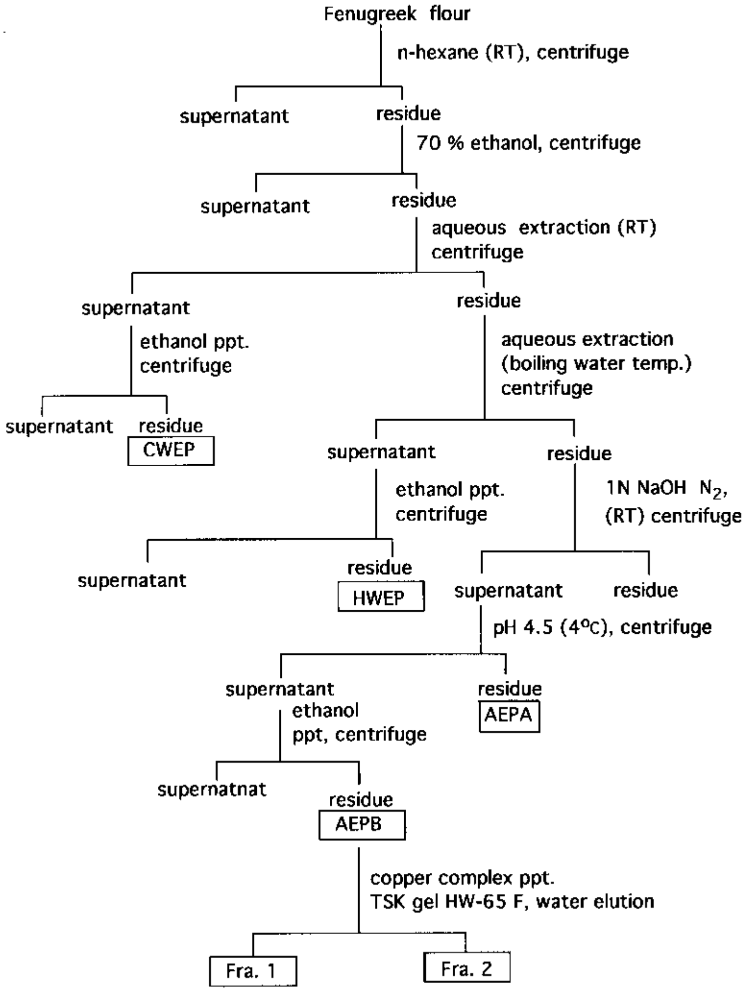

Fig. 1. Scheme of extraction and purification of fenugreek polysaccharides. CWEP—cold water extracted polysaccharide; HWEP—hot water extracted polysaccharide; AEPA—alkali extracted polysacchride A; AEPB—alkali extracted polysaccharide B.

2. Materials and methods

2.1. Materials

Fenugreek (*Trigonella foenum-graecum* L.) was procured from the local market at Mysore, Karnataka India. Standard sugars and other important chemicals were from Sigma Co. (USA); WAKO Pure chemical Industries Ltd., and Nacalai Tesque Inc. (Japan); Enzymes: α-galactosidase (3.2.1.22) and β-galactose dehydrogenase (1.1.1.48) from Sigma (USA); *Aspergillus niger* β-mannanase (3.2.1.78) from Megazyme, (Australia); β-NAD from Kohjin Co. (Japan).

2.2. General analytical methods

Total carbohydrates were estimated by the modified phenol–H_2SO_4 method (Rao & Pattabiraman, 1989); galactose estimation was done as per the procedure of McCleary (1994); protein estimation was done by using the DC protein assay kit, (BIO-RAD, Hercules, CA, USA); acid hydrolysis of polysaccharides was performed using either H_2SO_4 (2N) or TFA (2M); neutralized hydrolysates were used for TLC analysis using TLC aluminium sheets (20 × 20 cm) precoated with silica gel 60 (Merck Co., Germany); sugars were separated by running in the solvent system of 1-propanol, ethanol and water (7:1:2); chromatograms were developed by spraying with a anisaldehyde–H_2SO_4 reagent (Stahl, 1967); gel permeation chromatography was performed using pre-swollen TSK gel (HW-65F, TOSOH Corp., Japan) in a column (43 × 510 mm) and water was the eluent at a flow rate of 1.3 ml/min.; fractions (20 ml/tube) were collected and analysed for total sugars. Centrifugations were done at 8000 rpm.

2.3. ^1H, ^{13}C, and 2D NMR spectroscopy

Experiments were carried out with a Bruker DRX 600 spectrometer, operated at 600.13 and 150.91 MHz for ^1H and ^{13}C, respectively. Studies included ^1H, ^{13}C, Total Correlation Spectroscopy (TOCSY), Double-Quantum Filtered COSY (DQF-COSY), Heteronuclear Single Quantum

Table 1
Yield and compositional analysis of fenugreek polysaccharides

	Cold water extracted polysaccharide	Hot water extracted polysaccharide	Alkali extracted polysaccharide	
			A	B
Yield (%)	10.83	18.81	07.48	11.47
Total sugars (%)	85.30	91.00	12.33	86.66
Protein (%)	10.00	05.10	ND[a]	12.09
TLC profile	Man, gal and glc (traces)	Man, gal and glc (traces)	ND	Man, gal and glc (traces)
Copper complex precipitation				
	Cold water extracted polysaccharide	Alkali extracted polysaccharide B		
Yield (%)	7.36	3.30		
Total sugars (%)	95.00	100.00		
Protein (%)	2.40	0		
GPC (TSK gel HW-65F) fractionation of AEPB				
	Fra.1	Fra.2		
Yield (%)	52.20	47.77		

[a] Not detected.

Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC). Polysaccharide samples (9 mg) were dissolved in D_2O (0.5 ml) and analyses were performed at a probe temperature of 343 K using a 5-mm QNP(X(^{13}C, ^{15}N, ^{31}P)/^1H; *Z*- gradient)) probehead. The internal standard used was *t*-BuOH (1H: 1.23 ppm, ^{13}C: 31.3 ppm). All NMR data were reported in ppm (δ) down field from Me_4Si.

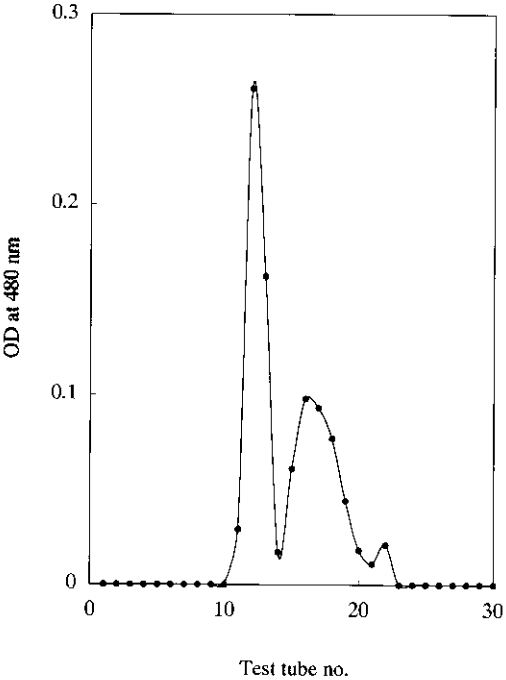

Fig. 2. GPC profile of the copper-complex precipitated AEPB fraction on the TSK gel (HW-65F).

2.4. Extraction and purification of fenugreek polysaccharides

Fenugreek flour (100 gm) was extracted with *n*-hexane at room temperature (1 h × 2) and centrifuged. The residue was treated with 70% ethanol (1 *h* × 2) and centrifuged. The residue obtained was thoroughly extracted (×4) with water at an ambient temperature (27°C) for 1 h each, and centrifuged. From the supernatant, the cold water extracted polysaccharide (CWEP) was precipitated using three volume of ethanol. The residue was subjected to aqueous extraction (×4) at boiling water temperature (1 h). After centrifugation, from the supernatant, the hot water extracted polysaccharide (HWEP) was precipitated by ethanol. The above residue was subjected to alkaline extraction (NaOH 1 N, under N_2, 1 h). After centrifugation, the supernatant was adjusted to pH 4.5 with dil.acetic acid at 4°C to get the alkali extracted polysaccharide A (AEPA). By adding ethanol to the supernatant, the alkali extracted polysaccharide B (AEPB) was precipitated. All the extracted polysaccharides were dialysed against water and lyophilized. The above polysaccharides were further subjected to purification by copper complex precipitation (Jones & Stoodley, 1965). The aqueous solution of the copper-complex purified AEPB was further purified by GPC on a TSK gel (HW-65F).

3. Results and discussion

3.1. Extraction and purification of fenugreek polysaccharides

A sequential pattern of extraction (Fig. 1), using cold water (at ambient temperature), hot water and alkali, was

Table 2
Ratio of galactose and mannose in AEPB fractions

Hydrolysis	Fraction used	TLC profile	Gal (%)	Man (%)(TS[a]-gal)	Gal:man
TFA	Fra.1	Gal and man	49	51	1:1.04
	Fra.2	Gal and man	47	53	1:1.12
A. niger	Fra.1	No hydrolysis			
β-D-mannanase	Fra.2	No hydrolysis			

[a] Total sugars.

adopted to extract all the polysaccharides extractable at different conditions of temperature and pH (Ramesh & Tharanathan, 1999). As shown in Table 1, the yield of HWEP was maximum to an extent of 18.81%. The minimum yield was 7.48% of AEPA. A water-soluble mucilage of 18% has been reported in fenugreek (Shankaracharya & Natarajan, 1972). In Egyptian fenugreek it was found to be 33% (Hassan & Boctor, 1962). NaOH extractions of gum is well known (Dea & Morrison, 1975). Bardalaye and Nordin (1977) isolated a galactomannan from *A. niger* by the alkali extraction method as it facilitated dissolution. D-galactose side chains have been found to play a very important role in determining the ease with which galactomannan can be dissolved (Dea & Morrison, 1975; Garti et al., 1997). The AEPA was slightly brownish and it was only partially soluble in water. The AEPB fraction contained a maximum of 12.09% protein.

The observed traces of glucose in TLC (Table 1) of acid hydrolysates of extracts were expected, as glucan can be co-precipitated with galactomannan (Bardalaye & Nordin, 1977). Further purification, particularly of AEPB by copper complex precipitation, resulted in a pure galactomannan fraction which upon GPC (TSK gel) fetched two major fractions, Fra.1 and Fra.2. Fra.1 was homogeneous as reflected by its sharp peak, while Fra.2 was slightly heterogeneous with its little broad peak (Fig. 2). However, their composition remained the same as revealed by TLC (Table 2). Fra.1 and Fra.2 contained galactose and mannose in the ratio of 1:1.04 and 1:1.12, respectively. The difference in the ratios of gal and man could be due to chain cleavage by copper complex precipitation (Dea & Morrison, 1975). The ratio obtained is in agreement with earlier reports, viz. 1:1.05 (Dea & Morrison, 1975); 1:1.5 and 1:1.1 (Edwards, Scott, Gidley & Reid, 1992); 1:1.12 and 1:1.08 (Bociek et al., 1981); 1:1 and 1:2 (Bock & Thogersen, 1982; Garti et al., 1997). Song et al. (1989) reported a G/M ratio of 0.93 and found fenugreek galactomannan to be physically more regular, exhibiting markedly less loss of crystallinity upon drying when compared to locust, guar or taro gums. The proportional variations of galactose to mannose units in seed galactomannan have been attributed to: the presence of differing ratios of galactosyl to mannosyl transferases between species and also post-deposition modifications (Edwards, Bulpin, Dea & Reid, 1989; Edwards et al., 1992); metabolic degradation (Dea & Morrison, 1975); varietal or environmental factors (Reid, 1985) and method of extraction (Reid & Meier, 1970). Sequential purification steps involving both copper complex precipitation and GPC, employed in the present work, were advantageous in obtaining the quantitative recovery of galactomannan fractions probably of different chain length.

3.2. Studies with *A. niger* β-D-*mannanase*

A. niger β-D-mannanase had no effect on the hydrolysis of Fra.1 and Fra.2 indicating the high substitution and interference by galactose stubs (Table 2). McCleary and Matheson (1983) observed a decrease in the amount of hydrolysis with an increase in galactose substitution. It has also been noted that β-D-mannanase (EC.3.2.1.78) generally can only depolymerize their substrates to oligasaccharides having a minimum degree of polymerization (d.p.) of 2 or 3. Galactomannans having a man:gal ratio close to 1:1 are practically unaffected by β-D-mannanase (Dea & Morrison 1975).

3.3. NMR studies

Integrated approaches of 2D NMR correlations are being applied for sequencing of oligosaccharides as well as polysaccharides (Cassels, Fales, London, Charlson & Van Halbeek, 1990; Pfeffer, Valentine & Parish, 1979). In the present investigation, due to the simplicity of the fenugreek galactomannan structure no chemical fragmentation was carried out before NMR analysis. Ikuta, Shibata, Kanehiko, Kobayashi, Suzuki and Ogawa (1997) reported characterization of galactomannan of *Candida lypolitica* by various correlation NMR experiments without chemical fragmentation of polysaccharides and they also confirmed the results with acetolysis experiments. Complete structural characterization of the polysaccharide from *Streptococcus sanguis* J22 reported by Abeygunawardana and Bush (1990) is also based almost exclusively on NMR methods without methylation analysis. The phase sensitive DQF-COSY (Fig. 3(A) and (B)) was used in the determination of 1H assignments. It has several advantages over conventional COSY in terms of, pure absorption line shapes, reduced diagonal intensity and antiphase components (Abeygunawardana & Bush, 1990). 1H assignments were verified by TOCSY. The signals of ^{13}C, were assigned by correlating plots obtained by HSQC (Fig. 4). HSQC was carried out in view of its discrete cross peaks even for strong

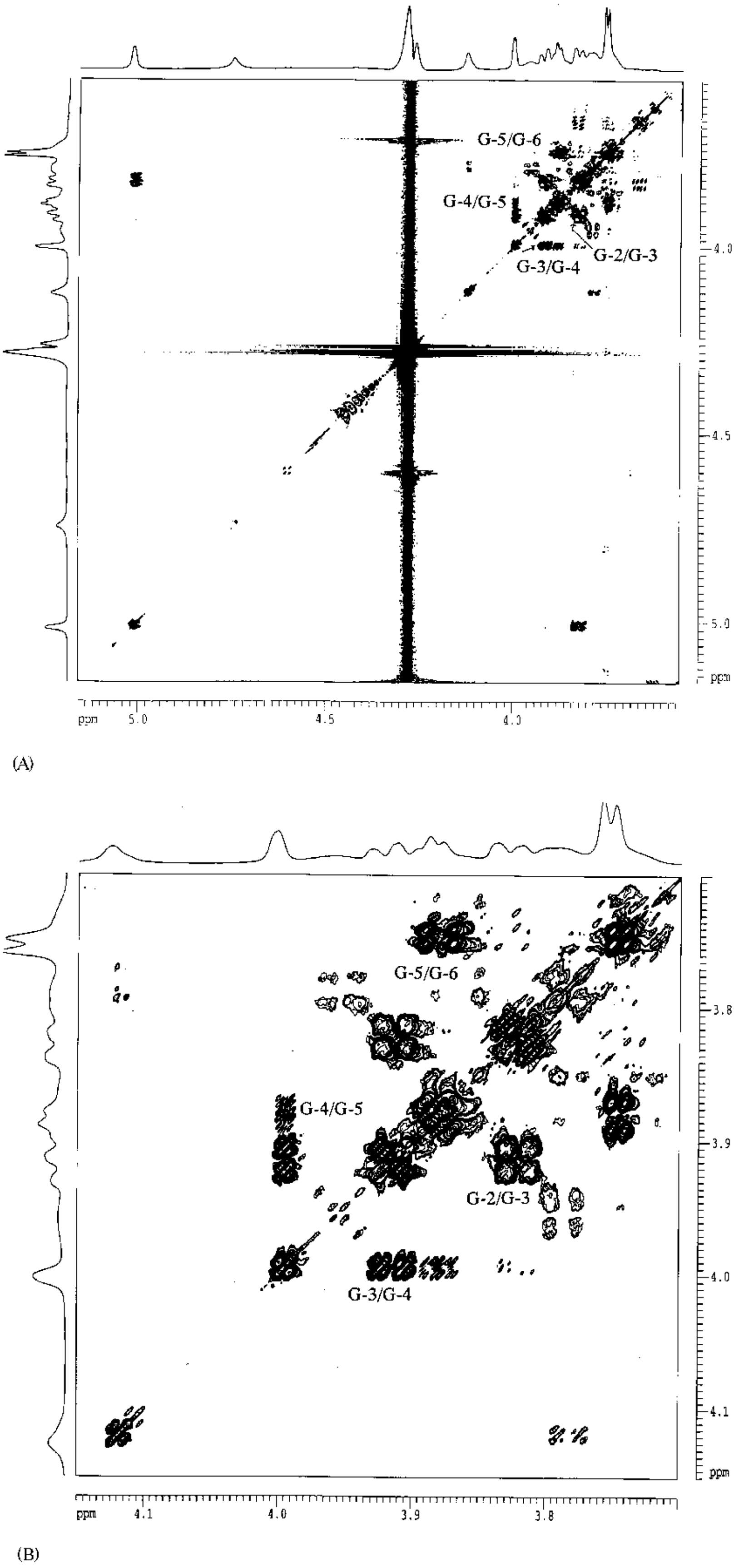

Fig. 3. (A) and (B)^{1}H–^{1}H DQF COSY spectra of Fra.1.

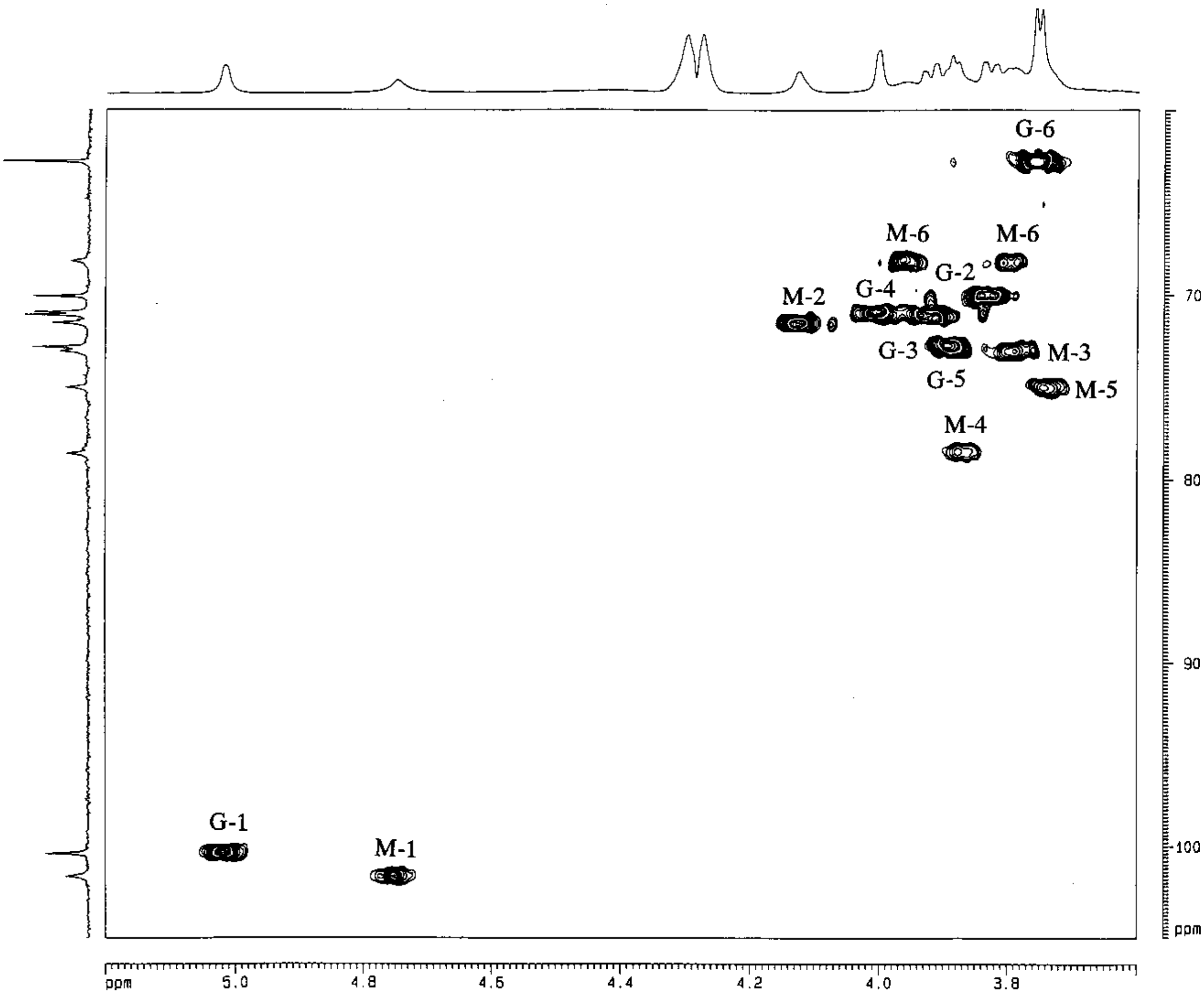

Fig. 4. ^{1}H-^{13}C HSQC spectrum of Fra.1.

coupled 1H resonances (Abeygunawardana & Bush, 1990). Results of both 1D and 2D ^{1}H and ^{13}C NMR studies gave a complete and reliable assignment of ^{1}H and ^{13}C signals. The chemical shifts (ppm) are recorded in Table 3. The assignment of the 1H resonances of mannose and also galactose as being either due to α or β was also compared with available data (Bock & Thogersen, 1982). The resonance of the anomeric protons is well separated. However, two signals at H-6 of galactose are found to overlap as indicated by the single HSQC cross peak and its intensity in the 1H spectrum. The spectral regions of the anomeric carbons (100.20 and 101.80 ppm) and methylene carbons (62.80 and 68.00 ppm) of both gal and man are well documented. The H-1 region of Fra. 2, and the relative intensity of these peaks in the ^{13}C NMR spectrum were found to be in agreement with the observed ratio of gal and man, 1: 1.12 obtained for fra.2, by chemical analysis. All the signals obtained were assignable and no unassignable signals were encountered.

The position of C-6 of the β-D-mannopyranosyl unit was of special interest because of its potential information regarding the substitution patterns. This resonance was observed at δ 68.00 ppm which is for β-D-mannopyranosyl branched at HO-6. This is a clear evidence for β-D-mannosyl substitutions. Further, the assignment given for the carbon resonances of the D-galactose residues agrees well with the reported spectrum of monomeric α-D-galactose pyranose (Dorman & Roberts, 1970). Grasdalen and Painter (1980) and Kapoor, Taravel, Joseleau, Milas, Chanzy and Rinaudo (1998) reported a splitting of C-4 of β-D-mannopyranosyl residue in galactomannan arising from nearest neighbour probabilities. However, no splitting of C-4 was observed in the present investigation and C-4 resembled that of clover seeds (man:gal = 52:42) which is most highly substituted with D-galactose residues. The absence of other resonances assignable for ^{1}H or ^{13}C of β-D-mannopyranosyl unbranched at HO-6, indicates the contiguous substitution of D-mannosyl residues. Our results are in agreement with observations made in ^{13}C NMR studies with legume-seed galactomannan

Table 3
^{1}H and ^{13}C chemical shifts (ppm) of Fra.1

Position	^{1}H	^{13}C
Gal		
1	5.02	100.20
2	3.83	70.00
3	3.92	70.90
4	4.00	70.00
5	3.90	72.65
6	3.76[a]	62.80
Man		
1	4.75	101.80
2	4.13	71.50
3	3.79	73.00
4	3.87	78.30
5	3.74	75.00
6	3.96 3.80	68.00

[a] Two signals overlapped.

by Manzi, Cerezo and Shoolery (1986). However, no resonance assignable for the β-D-mannosyl residue unbranched at O-6 was seen. The chemical shifts of both ^{1}H and ^{13}C are in good agreement with those reported by Davis, Hoffmann, Russell and Debet (1995) for substituted mannose residues. Bociek et al. (1981) have reported the ^{13}C NMR observations of fenugreek galactomannan after partial hydrolysis. Accordingly a low field shift of +5.9 ppm was observed in the δ value of C-6 of 6-O substituted β-D-mannose (δ 69.2 ppm) over that of the unsubstituted one (δ 63.3 ppm). In our study the observed δ value for C-6 of the β-D-mannosyl residue was 68.00 indicating its O-6 substitution. Fig. 5 shows the HMBC spectrum used for sequencing. The relevant long range C–H connectivities of the constituent glycosyl residues are clear. Accordingly, the H-1(gal) at δ 5.02 is coupled to the ^{13}C resonance at δ 68.00 (C-6 of β-man) while the man H-4 at δ 3.87 is coupled to the ^{13}C resonance at δ 101.8 (C-1 of β-man). This is also supported by another cross peak at δ 3.80 (H-6 of β-man) which is coupled to the ^{13}C resonance at δ 100.2 (C-1 of α-gal). Thus

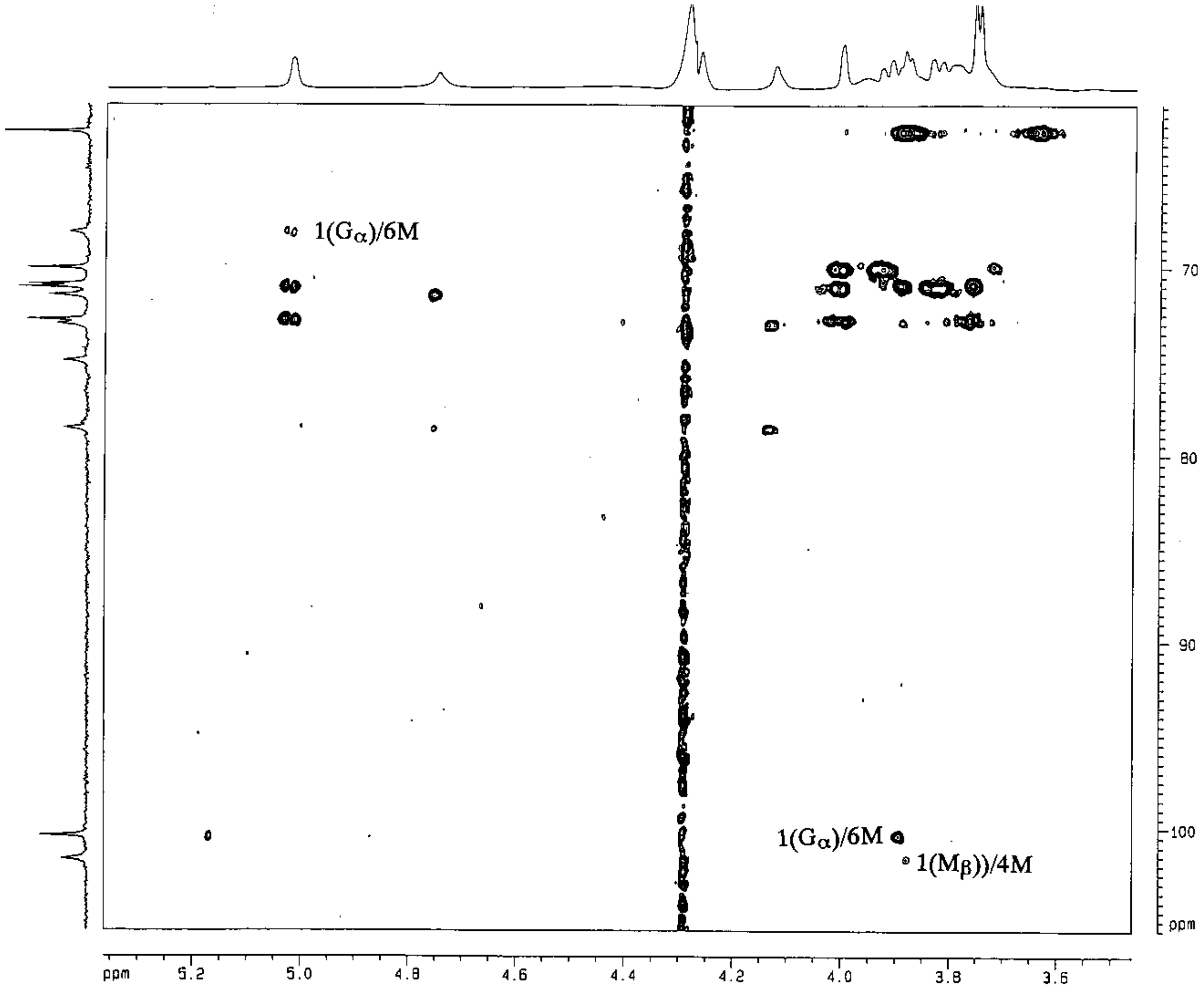

Fig. 5. ^{1}H-^{13}C HMBC spectrum of Fra.1. The labelling pattern is as follows: proton no.(residue)/carbon no.(residue).

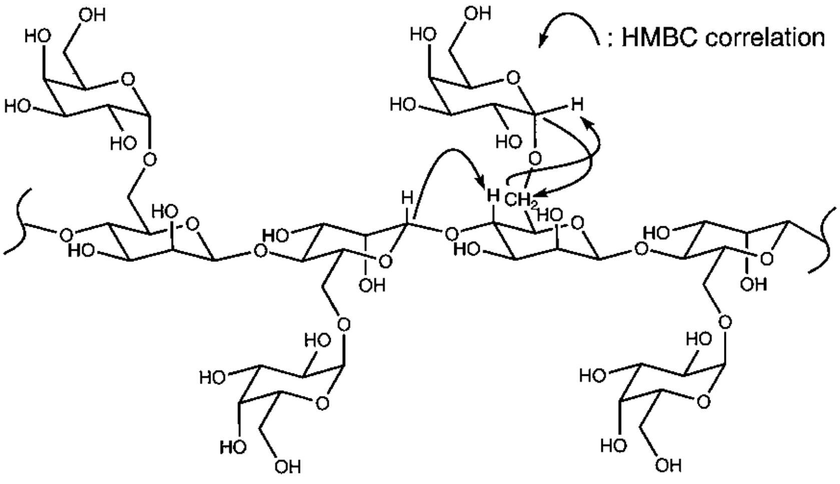

Fig. 6. Repeating unit of fenugreek galactomannan.

anomeric inter-residue linkage, β-man-(1 → 4)-β-man and α-gal-(1 → 6)-β-man (Fig. 6) are clear.

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