SOLUBLE DIETARY FIBERS FROM TRIGONELLA FOENUM GRAECUM LINN. SEED AND THEIR STRUCTURES

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ABSTRACT

Soluble dietary fibers of Trigonella foenum graecum Linn. seed were isolated and fractionated into neutral and acidic parts. Structures of both the fractions were determined by sugar analysis and high resolution 2D NMR spectroscopic studies including total correlation (TOCSY) spectroscopy. Main chain of the neutral SDF fraction was found to be a galactomannan composed of mannose and galactose (~1 : 1.5 ratio) having 1,3-α linkages between each other. Terminal mannose residue was linked to galactose residue of the main chain at its 4 position by α-linkage. The acidic SDF was a rhamnogalacturonan, in the main chain, galacturonic acids and rhamnose had 1,4-α- and 1,2-β-linkages, respectively. Terminal β-galactose and β-glucuronic acid were linked to α-galacturonic acid of the main chain at its 3-position.

Key words: Dietary fiber, Trigonella foenum graecum, Galactomannan, Rhamnogalacturonan

INTRODUCTION

Dietary fibers (DF) play an important role in human health management; it reduces the risk of colon cancer, heart disease and risk of gallstone formation, helps to reduce blood glucose and blood cholesterol level and other complications of diabetes like hypertension, atherosclerosis and hyperlipidaemia (Anderson 1986). The fibers are also helpful to control irritable bowel syndrome (Anderson and Tietyen-Clark 1986). The soluble components of DF cause an increase in the viscosity of the stomach contents, thereby retarding gastric emptying. This then affects the rate of digestion and the uptake of nutrients and creates a feeling of satiety (Jacobs 1983, Nyman and Aspinal 1985, Sapuntzakis et al. 2001). This protective effect is associated with a selective decrease in biliary cholesterol (Nyman and Aspinal 1985). Trigonella foenum graecum Linn. (in English Fenugreek) known as methi is an ingredient of mixed-masala (pachphoron) commonly used in Indian sub-continent; it has folkloric use to manage diabetes. The seed powder, its water extract and soluble dietary fibers (SDF) have been reported to possess blood glucose lowering effect on model rats (Ali et al. 1995). Mechanism of action of SDF as an antidiabetic agent has also been reported (Hannan et al. 2003). But chemical structure of SDF has not been studied in detail. In this paper present authors are reporting structure elucidation of SDF of fenugreek seeds which has not been published earlier.

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The structural studies will help to use SDF of Fenugreek seed as a dietary supplement for management of diabetes.

MATERIALS AND METHODS

Good quality fenugreek seeds were purchased from local city shop. The dried seeds (20 g) were suspended in water (250 ml) at room temperature for overnight. The mucilaginous water extract was separated manually by squeezing the wet seeds through a pre-cleaned cloth filter, concentrated (~100 ml) by rotary vacuum evaporator (bath temperature not exceeding 40°C), and was poured into four times of its volume of absolute alcohol. The precipitate was collected by centrifugation, re-dissolved in water (~50 ml), centrifuged again (removed small insoluble fiber, if there is any); the clear supernatant was concentrated and freeze-dried to get mucilage (~3 g).

Protease from Streptomyces caespitosus (purified Type IV, Sigma Chemical Company, USA), and Termamyl 120L (NOVO A/S Copenhagen, Denmark) and amyloglucosidase from Aspergillus niger (Boehringer-Mannheim, Germany) were used for enzymatic degradation of protein and starch, respectively.

Gas chromatographic analysis was carried out by gas chromatograph (Pye Unicum 4500 and Shimadzu 8A) having flame ionization detectors and equipped with quartz capillary columns (25 m length, 0.2 mm i.d.). Temperature programmed was 120 - 210°C, 3°C rise per min (oven). Detector and injector temperatures were set at 230 and 220°C, respectively. Quantification was done by using a recording integrator (LKB 2200).

NMR spectrum was recorded by dissolving the SDF sample in D$_2$O using TSP (Deuterated trimethylsilylsodium propionate) on 600 MHz Bruker NMR machine. 2D NMR spectra was performed with standard TOCSY, relayed COSY, double-relayed COSY, the COSY experiments were performed with (90°-t$_1$-90°-t$_2$) for higher sensitivity.

The isolated mucilage (200 mg) was suspended in phosphate buffer (pH 0.1 M), treated with protease (0.5 mg), heated in a thermostatic shaker (60°C, 3 hrs) and protein (Weterlund et al. 1991) free mucilage was collected by dialysis and freeze-drying (192 mg). The protein free material was again suspended in acetate buffer (0.1 M, pH 5.0), treated with termamyl (2 µl), heated in a thermostatic shaker (1 hr, 60°C), cooled, amyloglucosidase (2 µl) was added to the mixture and shaken again (16 h). The enzyme-treated mixture was dialyzed (48 hrs), concentrated, centrifuged and the starch free material (Solomonsson et al. 1984) was collected by freeze drying (SDF, 163 mg).

SDF of fenugreek (160 mg) was suspended in water, NaOH (1M) was added dropwise to reach pH 12 and kept at 0°C for 2 hrs, neutralized with 10% acetic acid, dialyzed and freeze-dried to get de-esterified (Bitter and Muir 1962) SDF (158 mg).
Fractionation of soluble dietary fiber of fenugreek (150 mg) was done by ion-exchange column chromatography using DEAE-Sepharose CL-6B gel column and acetate buffer (0.1 M, pH 6.5) as mobile phase. The fractions were collected in test tubes by an automatic fraction collector (Pharmacia Frac-100; flow rate: 1 ml/min was maintained by a Peristaltic pump). The neutral fraction was eluted with acetate buffer and monitored by phenol sulphuric acid test whereas the acidic fraction was eluted with 1 M NaCl in acetate buffer and monitored by carbazole test. The neutral and acidic fractions were collected separately by dialysis followed by freeze-drying and 26 mg of neutral and 32 mg acidic fractions were obtained. Uronic acid contents of acidic SDF were determined by taking a part (2 mg) which gave positive carbazole test (14%) with respect to certified carbazole.

Carboxylic functional group of uronic was reduced into alcoholic group, by carbazole method with reference to standard glucuronic acid. The acidic fraction (10 mg) was dissolved in water (1 ml) in a small beaker and an electrode of pH meter was immersed in the solution (pH meter was calibrated with buffers of pH 5 and 7), diethyl sodium carbamidemide (EDC, 5 mg in 2 ml water) solution was added to the SDF solution dropwise maintaining pH 4.5 by adding NaOH (0.1M) simultaneously also dropwise. The solution was kept at pH 4.5 for 1 hr and NaBH₄ solution (10 mg in 2 ml water) was added to the solution by maintaining pH 7.0 by adding HCl (1M) simultaneously dropwise. Evolution of gas vapor was controlled by addition of 1 - 2 drops 1-octanol. The solution was kept in that condition for 2 hrs, dialyzed and freeze dried to get uronic acid reduced neural SDF (Taylor and Conrad 1972).

The neutral fraction (5 mg) was hydrolyzed with 2 M TFA (1 ml, 2 hrs, 120°C), evaporated to dryness with added rectified spirit, re-dissolved in water (3 ml), reduced with NaBH₄ (2 mg, 2 hrs), the reduced material was acidified with Dowex H⁺ and filtered. The filtrate was evaporated to dryness with added methanol (to make absolute water free). The reduced sugars (converted into sugar alcohols) were converted into their corresponding alditol acetates with acetic anhydride in dry pyridine (1 ml; 1 : 1, 20 min, 96°C) and were analyzed by GC-FID (Sawardeker et al. 1965). Mannose (54.66%), galactose (41.34%) and rhamnose (4%) were the three sugars indentified in the neutral part of SDF. Similarly acidic and the carboxyl reduced acidic fractions were hydrolyzed, reduced and converted into their alditol acetates and analyzed by GC-FID. Rhamnose (54%), galactose (39%) and arabinose (7%) were found to be present in the acidic fraction and rhamnose (35%), galactose (45%), glucose (15%) and arabinose (5%) were identified and quantified in the carboxyl reduced acidic fraction.

¹H NMR spectra neutral (Fig. 1) and acidic SDF fractions (Fig. 3) were recorded and signals were found at 5.00 (unresolved) and 4.10 - 3.70 ppm for neutral fraction and acidic SDF fraction gave signals at 5.75, (J 1.2 Hz), 5.30 (unresolved), 5.15 (J₁₂ ~1.2 Hz), 5.08 (unresolved), 4.60 (J 1.3 Hz), 4.4 (J 4.3 Hz), 4.25 - 3.30 and 1.25 ppm. H-H
TOCSY spectra neutral (Fig. 2) and acidic SDF (Fig. 4) fractions were recorded and spectral data are given in Tables 1 and 2.

### Table 1. Total correlation spectroscopic data of neutral SDF of fenugreek seeds.

<table>
<thead>
<tr>
<th>Galactopyranose (Galp) residue (ppm)</th>
<th>Mannopyranose (manp) residue (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1 (5.00) → H-2 (3.97)</td>
<td>H-1 (5.00) → H-2 (3.80)</td>
</tr>
<tr>
<td>H-2 (3.97) → H-3 (3.70)</td>
<td>H-2 (3.80) → H-3 (4.10)</td>
</tr>
<tr>
<td>H-3 (3.70) → H-4 (3.92)</td>
<td>H-3 (4.10) → H-4 (3.70)</td>
</tr>
<tr>
<td>H-1 (5.00) → H-3 man (4.10)</td>
<td>H-1 (5.00) → H-4 gal (3.70)</td>
</tr>
<tr>
<td>H-4 (3.92) → H-1 man (5.00)</td>
<td>H-3 (4.10) → H-1 gal (5.00)</td>
</tr>
</tbody>
</table>

### Table 2. H-H TOCSY NMR spectroscopic data of acidic SDF of fenugreek seeds.

<table>
<thead>
<tr>
<th>α-galacturonic acid (GalpA) residue (ppm)</th>
<th>Linked α-GalpA residue (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1 (5.08) → H-2 (4.10)</td>
<td>H-1 (5.08) → H-2 (3.75)</td>
</tr>
<tr>
<td>H-2 (4.10) → H-3 (3.97)</td>
<td>H-2 (3.75) → H-3 (3.45)</td>
</tr>
<tr>
<td>H-3 (3.97) → H-4 (3.75)</td>
<td>H-3 (3.45) → H-1 galp(4.60)</td>
</tr>
<tr>
<td>H-4 (3.75) → H-1 rha (5.75)</td>
<td></td>
</tr>
<tr>
<td>H-3 (3.97) → H-1 glcpA (4.40)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>β-Galp residue (ppm)</th>
<th>β-Rhamnopyranose (Rhap) residue (ppm)</th>
<th>β-Glucuronic acid (GlcA) residue (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1 (4.60) → H-3 galpA (3.45)</td>
<td>H-1 (5.75) → H-4galpA (3.75)</td>
<td>H-1 (4.40) → H-3 galpA (3.97)</td>
</tr>
<tr>
<td>H-2 (3.75) → H-1 galpA (5.08)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

Mannose (54.66%) and galactose (41.34%), the two major neutral sugars and small amount of rhamnose (4.0%) were found to be present in the SDF fraction of fenugreek seeds.

The 1H-NMR spectrum (Figs 1 - 4) of neutral fraction of SDF had one anomic signal at 5.00 ppm (Table 1). But in 2D NMR the single peak showed correlations with two sugar residues.

In the total correlation spectroscopy (TOCSY) of neutral SDF (Fig. 1 and Table 1), the anomic proton, H-1 of galactose at 5.00 ppm coupled with ring proton H-2 of galactose at 3.97 ppm, H-2 coupled with H-3 of galactose at 3.70 ppm. H-3 of galactose at 3.70 ppm coupled with H-4 of galactose at 3.92 ppm. The anomic proton H-1 of mannose at 5.00 ppm coupled with H-2 of mannose at 3.80 ppm, H-2 of mannose at 3.80 ppm coupled with H-3 of mannose at 4.10 ppm. H-3 of mannose at 4.10 ppm also coupled with proton H-4 of mannose at 3.70 ppm.
H-1 of galactose at 5.00 ppm had cross peak with H-3 of mannose at 4.10 ppm and H-1 of mannose at 5.00 ppm coupled with H-4 of galactose at 3.92 ppm indicated galactose and mannose are linked with each other. The cross peak proved that galactose and mannose had 1,3-α-linkages. Cross peak between H-1 of mannose with H-4 of galactose, showed that there was a side chain of α-mannose at the 4-position of galactose.

Therefore, from the 1H-NMR and TOCSY spectral data and comparing with the earlier published reports (Kapoor and Mukherjee 1969, Kapoor and Mukherjee 1971) it was concluded that neutral SDF of fenugreek was a galactomannan; the main chain consisted of α-1,3-linked galactopyranose and α-1,3-linked manno.pyranose residues. Terminal mannosepyranose residue was attached to galactose in the chain at its 4 position also by α linkage (Partial structure 1).
In H-H total correlation spectrum (TOCSY) of acidic SDF of fenugreek was very complex (Fig. 4). Assignment of the signals is given in Table 2. The signal at 5.08 (major, un-resolved) showed correlations with rhamnose, galacturonic acid, galactose and rhamnose sugar residues. Anomeric proton of galacturonic acid, H-1 proton (major signal, unresolved) at 5.08 coupled with H-2 proton at 4.10 ppm. H-2 coupled with H-3 at 3.97 ppm, H-3 at 3.97 ppm also coupled with H-4 at 3.75 ppm of same residue. In addition H-4 proton of galacturonic acid at 3.75 ppm had cross peak with anomeric proton H-1 of rhamnose at 5.75 ppm having coupling constant ~1.2 Hz indicated that galacturonic was in the main chain and rhamnose residues were attached with glucuronic acid at its 4-position β-linkage. Besides H-3 proton of galacturonic acid at 3.97 ppm had another cross peak with anomeric proton H-1 of at 4.40 ppm which showed that glucuronic acid attached to the main chain at its 3-position by β-linkage. Anomeric proton, H-1 (5.08 ppm; another residue) coupled with its H-2 proton at 3.75 ppm, H-2 coupled with H-3 proton at 3.45 ppm. It also showed cross peak with H-1 of galactose at 4.60 ppm. Therefore, it is assumed that galactose was also attached to galacturonic at its 3-position by β-linkage.

Anomeric proton of rhamnose (H-1) at 5.75 ppm coupled with H-2 proton at 3.75 ppm. H-2 had cross peak with H-4 proton of galacturonic acid at 3.75 ppm which showed that rhamnose was also present in the main chain galactose by (1-2) by β-linkage.

Small signals of arabinose showed cross peaks i.e. correlations. H-1 at 5.30 ppm coupled with H-2 at 3.85 ppm. Due to complex spectral cross peaks it was not possible to assign all the protons of five sugar residues.

On the basis of spectroscopic studies and comparing with reported data (Nahar et al. 1994, Rahman et al. 2007, Rahman et al. 2005, Selvendran et al. 1987), it was concluded that the acidic SDF was a rhamnogalacturonan, the main chain of the polysaccharide composed of 1,4-α-galacturonic acid and 1,2-β-rhamnopyranose sugar residues. Terminal β-galactose and β-glucuronic acid residues were attached to α-galacturonic residues at its 3 position. Small amount of arabinofuranose residue found in the sugar analysis might be an associated arabinan (partial structure).
Fenugreek seeds and its SDF fraction showed significant serum glucose lowering effect on type 2 diabetic model rates ($p < 0.05$). Mechanism of action of SDF was studied on gut level using sucrose as control (Ali et al. 1995) which showed delayed emptying glucose load at the upper GIT (gastrointestinal tract). The effect is much better than existing available drug acarbose. Hypoglycemic activities of Fenugreek seed might be due to neutral and acidic fractions of SDF.

ACKNOWLEDGEMENTS

Thanks are due to Prof. A K Azad Khan, Prof. Liaquat Ali and Prof. Begum Rokeya of BIRDEM for their kind interest on the structures of SDF fractions of fenugreek. Authors are grateful to Late Professor Lennart Kenne, Swedish University of Agricultural Sciences for his kind support to record high resolution NMR spectra of SDF fractions. International Science Programme (ISP), University of Uppsala, Sweden is thanked for financial support.

REFERENCES


*(Received revised manuscript on 25 December, 2013)*