An Enzymic Technique for the Quantitation of Galactomannan in Guar Seeds

Dedicated to Prof. Dr. H. Neukom on the occasion of his 60th birthday

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An enzymic technique has been developed for the rapid and accurate quantitation of the galactomannan content of guar seeds and milling fractions. The technique involves the measurement of the galactose component of galactomannans using galactose dehydrogenase. The galactomannans are converted to galactose and manno-oligosaccharides using partially purified enzymes from a commercial preparation and from germinated guar seeds. Simple procedures have been devised for the preparation of these enzymes. Application of the technique to a number of guar varieties gave values for the galactomannan content ranging from 22.7 to 30.8% of seed weight.

Introduction

A number of techniques have been developed for the estimation of the galactomannan content of guar seeds. Of these techniques, the most reliable and accurate involve extraction and purification of the galactomannan, which is then alcohol precipitated, dried and weighed (1–3). However, these methods are laborious and time consuming and thus are not applicable to the routine analysis of large numbers of samples as are derived from guar breeding trials. More rapid semi-quantitative techniques have been developed but these are not sufficiently accurate to distinguish between guar varieties differing by less than 5% in galactomannan content (4, 5). Furthermore, some of these tests do not give a true measure of galactomannan content, but rather, also measure soluble raffinose-series oligosaccharides (5). These oligosaccharides represent up to 5% of total seed weight (3) and may vary between guar varieties.

The aim of the current work was to develop a rapid enzymic technique for the accurate quantitation of the galactomannan content of guar seeds. The technique was designed for use in the analysis of material derived from guar breeding trials and also to allow the quantitation of galactomannan content of seed fractions obtained on industrial milling of guar.

Materials and Methods

Seed material

Guar seeds used in these studies were grown in Australia from lines imported from India and U.S.A. Details of the guar varieties studied are given in Tab.2. Most of the varieties were unestablished experimental lines. Seeds were ground to a fine flour by several passages through a Die Krone laboratory mill KE 02T (Krone-Muchlen and Gerätebau, West Germany). The flours were dried at 40°C in a forced-air oven for 20 h before analysis.

Assay of α-galactosidase and β-mannanase

α-Galactosidase was assayed by incubating enzyme preparation (0.1 ml) with 10 mM p-nitrophenyl α-D-galactopyranoside (0.1 ml) in 100 mM sodium acetate buffer (pH 4.5). One unit of activity is defined as the amount of enzyme which releases 1 μmol of p-nitrophenol from the above substrate in one minute at 40°C and pH 4.5.

β-Mannanase was routinely assayed with Remazol brilliant Blue R-carob galactomannan as substrate (6). Activity on this substrate was converted via a standard curve to units of activity on 0.2% carob galactomannan. One unit of β-mannanase activity is defined as the amount of enzyme which releases one μmol of mannose reducing sugar equivalents from 0.2% carob galactomannan in one minute at 40°C and pH 4.5.

Preparation of β-mannanase from “Cellulase” preparation

Commercial Cellulase preparation (25 g, cat. no C 7502, Sigma Chemical Co. St. Louis, Missouri, U.S.A.) was suspended in Tris/HCl buffer (250 ml, 100 mM, pH 8) and the pH adjusted to 8 by addition of 100 mM NaOH to the stirred enzyme solution. Insoluble material was removed by filtration through glass-fibre filter paper and the solution chilled in ice to 4°C. The solution was then dialyzed against two changes of ice-cold Tris/HCl buffer (5 l, 20 mM, pH 8) for 20 h. Dialysis at higher temperatures or lower pH values results in digestion of the dialysis sac by cellulase. After dialysis, the enzyme solution was added to a bed of DEAE-cellulose (3 cm × 12 cm diameter) in a sintered glass funnel. The DEAE-cellulose was washed with Tris/HCl buffer (1 l,
50 mM, pH 8) and the enzyme was eluted by washing with Tris/HCl buffer (1 l, 50 mM, pH 8) containing 200 mM KCl. To the enzyme solution (1 l), solid (NH₄)₂SO₄ (500 g) was added and the precipitated protein recovered by centrifugation. The brown precipitate was resuspended in 80% (NH₄)₂SO₄ solution (80 ml). This suspension contained 80 U/ml of β-mannanase and the overall recovery of the enzyme was 75%.

**Preparation of Guar Seed α-galactosidase**

Fresh guar seed (cv. Brocks, 1979 harvest, 500 g) was germinated for 4 days at room temperature (22 °C) and then extracted with 0.1 M sodium acetate buffer (2 l, pH 4.5) using a Waring blender. After homogenization the mixture was incubated at 40 °C for 1 h to allow β-mannanase to degrade remaining traces of galactomannan. The slurry was re-blended, filtered through muslin, and then centrifuged (5,000 g, 20 min). The supernatant was then transferred to a tube containing 10% (v/v) acetic acid (4.5-5) by the addition of 10% (v/v) acetic acid adjusted to pH 4.5. The contents of each tube were then transferred to a tube containing 80% (NH₄)₂SO₄ solution (80 ml). This suspension contained 80 U/ml of β-mannanase and the overall recovery of the enzyme was 75%.

**Extraction of galactomannan**

Finely milled whole guar-seed flour (1 g) or commercial guar flour (0.5 g) was accurately weighed into polypropylene centrifuge tubes (250 × 1100 mm, 50 ml) and an aliquot (25 ml) of ethanolic water (80% w/v) was added. The tubes were thoroughly stirred on a Syltron Thermolyne Maxi Mix test-tube stirrer, incubated at 80 °C for 30 min, and then centrifuged (2,000 g, 10 min) in a Tosco M. S. E. bench centrifuge. The supernatant solution was discarded and the pellet resuspended in 25 ml of 80% ethanol in water, mixed with a spatula and incubated for 15 min at 85 °C, centrifuged (2,000 g) and the supernatant discarded. This step was repeated and the pellet was suspended in NaOH solution (15 ml, 2%) incubated at 85 °C for 15 min (to remove ethanol) and then at 100 °C for 30 min. Special care is required in this step. The seed galactomannan swells rapidly to form a gel "plug" which can be extruded from the top of the tube as remaining traces of ethanol boil off. This can be avoided by frequently stirring the tubes with a spatula over the first 5 minutes of incubation at 100 °C. (The contents of the tubes were then blended using a Janke and Kunkel Ultra turrax (30 seconds, 80% of maximum setting).) The pH of each sample was adjusted to 4.5-5 by the addition of 10% (v/v) acetic acid (2.5 ml) and the shaft of the Ultra turrax was rinsed with a minimum volume of water from a wash bottle. To each sample, an aliquot (50 µl, 4.0 U) of the β-mannanase preparation was added, and the tubes capped and incubated at 40 °C overnight. The contents of each tube were then transferred to a beaker (140 ml volume), blended with an Ultra turrax (30 seconds, 80% of max. setting), transferred to a volumetric flask (250 ml) and the volume adjusted to the mark after addition of a few drops of ethylenediaminetetraacetic acid (EDTA) (2,000 g, 10 min) and aliquots (0.2 ml) taken for α-galactosidase treatment and galactose determination.

**Determination of galactose content of samples (ref. 7)**

To aliquots (0.2 ml) of the guar extract was added 100 mM sodium acetate buffer (0.2 ml, pH 4.5) and guar α-galactosidase preparation (20 µl, 2,3 U), and the solutions incubated at 40 °C for 1 h. The solutions were adjusted to pH 8.6 by the addition of 200 mM Tris/HCl buffer (2.5 ml, pH 8.6). An aliquot (0.1 ml) of NAD (0.1 g/10 ml) was added and the absorbance at 340 nm measured. β-D-Galactose dehydrogenase (10 µl, 5 mg/ml, cat. no. 104981, Boehringer Mannheim GmbH, West Germany) was added to each tube, the tubes incubated at 35 °C for 1 h and the absorbance at 340 nm measured. Duplicate galactose standards (40 and 80 µg) were included with each set of unknowns. To allow for possible reversion by α-galactosidase, the galactose standards were also treated with this enzyme. Galactomannan content of samples was estimated from the measured galactose values and the known galactose content of guar galactomannan (i.e. galactose/mannose = 38:62).

**Extraction of α-galactosidase**

This suspension termed guar α-galactosidase preparation, contained 116 U/ml of α-galactosidase (assayed on p-nitrophenyl α-D-galactopyranoside, 10 mM, pH 4.5) and 12.5 U/ml of β-mannanase (assayed using RBB-carob galactomannan (6) and converted to U/ml on carob galactomannan, 0.2% w/v, pH 4.5). The overall recovery of α-galactosidase was 80%.

**Results and Discussion**

In the development of the current technique for the quantitation of the galactomannan content of guar seeds, it was essential to ensure that the procedures used gave complete and reproducible extraction of galactomannan. It was also necessary to demonstrate that the α-galactosidase used gave quantitative and reproducible release of galactose and that reversion reactions catalysed by this enzyme were minimized and allowed for. Furthermore, galactomannan should be the only source of the galactose being analysed, and finally, if the technique is to be applicable to the quantitation of galactomannan in seeds of a wide range of guar varieties, it is essential that the galactose/mannose ratios of these galactomannans are relatively constant.

Guar seeds contain significant, and possibly varying, quantities of oligosaccharides containing (1-6)-α-linked p-galactose. The oligosaccharides are susceptible to the action of α-galactosidase (3) and thus are potentially a major source of error in the current assay procedure for galactomannan, via galactose quantitation. However, this problem has been circumvented by preliminary extraction of the guar flour with aqueous ethanol to remove these oligosaccharides. The amount of oligosaccharides removed in three successive washings of the flour was 75%, 20% and 4.5% respectively. The recovered flour contained an insignificant amount of oligosaccharide (less than 0.5% of the original amount).

Two problems associated with the quantitative extraction of guar galactomannan are the relatively slow rate of hydration and the high viscosity of the resulting solution. In the current procedure, rapid hydration was achieved by incubating the flour in 2% sodium hydroxide at 100 °C for 30 min. The viscosity problem was overcome by incubating the neutralised (pH 4.5-5) slurries with β-mannanase, an endo-depolymerase (12).
When the described extraction and assay procedure was applied to samples of finely milled, oven dried guar galactomannan, the results shown in Fig. 1 were obtained. It is evident that for pure galactomannan the technique is very reproducible and gives a quantitative measure of galactomannan over a wide range of values.

The level of α-galactosidase used in the assay procedure is in sufficient excess to ensure complete hydrolysis of all (1→6)-α-D-linked galactosyl residues. However, α-galactosidase, like most other carbohydrate hydrolases, also catalyses the reverse reactions. The significance of these reverse reactions was greatly reduced by keeping the galactose concentrations to a minimum. Under these conditions, reaction resulted in less than a 3% decrease in galactose levels, and this was allowed for by also incubating the galactose standards with α-galactosidase.

Another potential source of galactose in the described assay procedure is from hydrolysis of other galactose-containing polysaccharides, e.g., galactans and arabino-galactans. These polysaccharides are found in significant quantities in legume seed cotyledons in the middle lamella and primary cell walls. The galactose in these polymers is α-1,6-linked and in theory could be cleaved by β-galactosidase which is present in the currently employed, partially purified enzyme preparations. However, results shown in Tab. 1 indicate that release of galactose from polysaccharides other than galactomannan is insignificant. When guar flour was diluted with soybean flour (essentially devoid of galactomannan), the galactose values for the ethanol washed, enzymically hydrolysed flour were directly proportional to the galactomannan content of the flour mixture, i.e., the galactomannan content of the flour mixtures calculated as a percentage of the guar flour was essentially constant (28.7±0.3%).

Tab. 1 Quantitation of galactomannan in guar-seed flour in the presence of soybean seed flour

<table>
<thead>
<tr>
<th>Guar seed flour mg</th>
<th>Soybean seed flour mg</th>
<th>Galactosea content mg</th>
<th>Galactomannanb content mg</th>
<th>Galactomannanc content of guar flour %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1000</td>
<td>0.3±0.05</td>
<td>0.8±0.1d</td>
<td>28.3±0.7</td>
</tr>
<tr>
<td>200</td>
<td>800</td>
<td>21.5±0.5</td>
<td>56.6±1.3</td>
<td>28.4±0.4</td>
</tr>
<tr>
<td>400</td>
<td>600</td>
<td>43.1±0.6</td>
<td>113.4±1.5</td>
<td>28.9±0.5</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>55.0±1.0</td>
<td>144.7±2.5</td>
<td>29.0±0.2</td>
</tr>
<tr>
<td>600</td>
<td>400</td>
<td>66.0±0.5</td>
<td>173.7±1.3</td>
<td>29.8±0.3</td>
</tr>
<tr>
<td>800</td>
<td>200</td>
<td>87.5±0.8</td>
<td>230.3±2.0</td>
<td>29.0±0.2</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>111.0±0.8</td>
<td>289.5±2.2</td>
<td>29.0±0.2</td>
</tr>
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</table>

a Determined with galactose dehydrogenase after treatment of samples with guar α-galactosidase.
b Calculated using the determined galactose/mannose ratio (i.e. 38:62).
c Galactomannan content calculated as a percentage of the guar flour in the mixture.
d Soybean seeds contain a trace of galactomannan.

The galactomannan used was highly purified and had a galactose/mannose ratio of 38:62. Samples were solubilised and analysed in duplicate.

obtained using the partially purified enzymes in the standard procedure. The electrophoretically pure β-mannanase was prepared by substrate affinity chromatography on glucomannan-AH-Sepharose 4B (12), and guar α-galactosidase by affinity chromatography on N-e-aminocaproyl-α-d-galactopyranosylamine-Sepharose 4B (13, 14).

Seeds of a number of varieties of guar have been analysed for galactomannan content using the currently described procedure. The results are summarised in Tab. 2. The galactomannans from several of the varieties have been purified and the galactose/mannose ratio found to be essentially constant (i.e. 38.0±0.9%, Tab. 2).

The galactomannan content of the guar seeds analysed in the current study ranged from 22.6 to 30.8%. There appeared to be no correlation between gum content and seed weight or shape. However, seed shape does affect the ease with which...
Taliangar Type 2.86 of the residues with 2% sodium hydroxide yielded extraction procedures the galactomannan contents of the was thus considered not to be galactomannan. Using these ant exhaustively extracted (in duplicate) with warm water and the galactomannan was extracted with water (17). Extraction then with hot 2% and but this material had a galactose content of only higher gum contents in some guar varieties measured both galactomannan and soluble oligosaccharides. Thus, in conclusion a specific and very reproducible assay procedure has been developed for the quantitation of the galactomannan content of guar seeds. The technique is sufficiently rapid to allow the analysis of 20–40 samples in a two day period, and thus should find application in industry and in guar breeding programmes.

References
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<table>
<thead>
<tr>
<th>Guar Variety</th>
<th>Seed weight g/100 seeds</th>
<th>Galactomannan content %</th>
<th>Galactose Content of galactomannans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pusa Naubahar</td>
<td>3.86</td>
<td>22.7 ± 0.1</td>
<td>36.8</td>
</tr>
<tr>
<td>I.C. 9203</td>
<td>1.95</td>
<td>26.8 ± 0.2</td>
<td>36.0</td>
</tr>
<tr>
<td>C.P. 31095</td>
<td>1.98</td>
<td>24.8 ± 0.2</td>
<td>36.2</td>
</tr>
<tr>
<td>Groller</td>
<td>3.55</td>
<td>25.3 ± 0.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>C.P. 177</td>
<td>3.50</td>
<td>27.0 ± 0.3</td>
<td>38.7</td>
</tr>
<tr>
<td>B19-1-55</td>
<td>2.60</td>
<td>27.7 ± 0.3</td>
<td>37.5</td>
</tr>
<tr>
<td>Brooks</td>
<td>3.08</td>
<td>27.5 ± 0.2</td>
<td>39.3</td>
</tr>
<tr>
<td>CP27 Type 2</td>
<td>2.56</td>
<td>27.0b</td>
<td>37.0</td>
</tr>
<tr>
<td>CP39 Type 1</td>
<td>3.08</td>
<td>26.4</td>
<td>38.6</td>
</tr>
<tr>
<td>CP39 Type 2</td>
<td>3.11</td>
<td>24.1</td>
<td>37.5</td>
</tr>
<tr>
<td>CP66 Type 1</td>
<td>2.78</td>
<td>25.1</td>
<td>38.9</td>
</tr>
<tr>
<td>CP303 Type 1</td>
<td>3.01</td>
<td>30.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>IC9007/P2</td>
<td>3.15</td>
<td>27.7</td>
<td>n.d.</td>
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<tr>
<td>IC9031 Type 1</td>
<td>3.18</td>
<td>26.8</td>
<td>38.1</td>
</tr>
<tr>
<td>IC9224/P3 Type 2</td>
<td>3.03</td>
<td>27.9</td>
<td>n.d.</td>
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<tr>
<td>PLS101 Type 1</td>
<td>4.97</td>
<td>28.6</td>
<td>39.2</td>
</tr>
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<td>MSS1 Type 1</td>
<td>2.57</td>
<td>27.9</td>
<td>38.3</td>
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<tr>
<td>MSS2</td>
<td>3.06</td>
<td>28.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>MSS3</td>
<td>3.05</td>
<td>28.3</td>
<td>n.d.</td>
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<tr>
<td>ST326</td>
<td>3.10</td>
<td>27.0</td>
<td>n.d.</td>
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<tr>
<td>NC70 Type 2</td>
<td>2.96</td>
<td>27.7</td>
<td>39.7</td>
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<tr>
<td>Kinman</td>
<td>2.58</td>
<td>28.0</td>
<td>n.d.</td>
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<td>Punjab</td>
<td>2.98</td>
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<tr>
<td>Taliangar Type 1a</td>
<td>2.86</td>
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<td>Taliangar Type 2</td>
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<td>Pusa Mosami Type 1</td>
<td>4.35</td>
<td>30.0</td>
<td>39.4</td>
</tr>
<tr>
<td>Katherine local</td>
<td>3.12</td>
<td>30.8</td>
<td>38.5</td>
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</table>

* Duplicate extractions and determinations; b Single extractions and determinations; n.d. not determined.

Flours of the first five guar varieties shown in Tab. 2 were 23.0 ± 2.0%, 25.5 ± 2.0%, 26.0 ± 2.0%, 25.1 ± 2.0%, and 27.5 ± 2.0% respectively. These values correlated reasonably well with those obtained using the enzymic technique, however quantitation of gum by conventional extraction and precipitation procedures lacked the accuracy of the enzymic technique. The reasons for this are considered to be that the polysaccharide recovered in conventional quantitation procedures is variously contaminated with protein and other polysaccharides (particularly arabinogalactans). Also, the recovered polysaccharide may contain varying quantities of water, depending on how carefully it was dried.