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Structural features of β-(1→4)-D-galactomannans of plant origin as a probe for β-(1→4)-mannanase polymeric substrate specificity

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ABSTRACT

Statistical modeling was applied for describing structural features of β-(1→4)-D-galactomannans. According to the model suggested theoretical ratios of limiting degrees of locust bean, tara gum and guar gum galactomannan conversions by two β-(1→4)-mannanases of different origin (*Myceliophthora thermophila* and *Trichoderma reesei*) were calculated. Then the enzymes were tested for enzymatic hydrolysis of three considered galactomannans. Experimentally observed results were compared with theoretically calculated ones. It was shown that *T. reesei* β-mannanase attacks sequences of four and more unsubstituted mannopyranosyl residues in a row, while *M. thermophila* β-mannanase is a more specific enzyme and attacks sequences of five and more mannopyranosyl residues in a row. Considered statistical model and approach allows to characterize both galactomannan structures and enzyme requirements for regions of unsubstituted mannose residues for substrate hydrolysis.

1. Introduction

Galactomannans of plant origin are commonly described by their chemical name α-(1→6)-D-galacto-β-(1→4)-D-mannan. In general, they are composed of a linear β-(1→4)-D-mannopyranosyl polymeric backbone to which single α-D-galactopyranosyl residues are attached in a more or less random fashion. In fact, the literature does not make it clear, whether it is a random or a 'block-wise' distribution of galactose along the mannopyranose chain. Galactomannans from various plant sources are reported to have a ratio of mannose/galactose (Man/Gal) between, for example, 1.1 (in *Galega orientalis* and *Medicago falcata*, goat's rue and yellow lucerne, respectively) and 4.0 (in *Ceratonia siliqua*, locust bean) or even 5.0–5.5 (*Styphnolobium japonicum*, Japanese pagoda tree, the tree legumes).[1–4]

In a recent work[5] it was reported that the β-galactomannan from *Cyamopsis tetragonoloba* (guar gum) specifically binds to galectin-1 (gal-1), a member of a sub-family of lectins that bind β-galactosides and, as a group, share significant amino acid sequence conservation in their carbohydrate recognition domain.[6] It was shown that the binding occurs over a relatively large surface area that is located primarily on that side of the protein opposite to its canonical lactose binding site.[7] In a recent study[8] the authors used NMR spectroscopy to investigate binding of gal-1 to a series of α-galactomannans (GMs) with a range of Man/Gal ratios from 1.1 to 4, with the goal of delineating the optimal structural signature for GM binding to gal-1. Results indicated that gal-1 binds GMs preferentially at α-D-galactopyranosyl doublets that are surrounded by regions of unsubstituted Man residues. This work expanded current views of how galectins in general interact with more complex glycans such as galactomannans. In other words, the authors have employed galactomannans with different ratio of Man/Gal as probes to delineate the specificity of binding of galactomannans to galectins, using the NMR spectroscopy galectin-1 resonance intensity broadening as a readout.[8] In our studies the enzymatic rate and the degree of conversion of galactomannans can be employed as a readout, because it is expected that α-D-galactopyranosyl substituents would create steric hindrances for the enzymatic hydrolysis of stretches of unsubstituted mannose residues, forming the backbone of the galactomannans.

2. Materials and methods

2.1. Chemicals and substrates

Chemicals, used for the preparing buffer systems, Somogyi–Nelson, Lowry and SDS–PAGE reagents were from Sigma–Aldrich (St. Luis, MO, USA), Bio-Rad (USA), MP Biomedicals Inc. (Illkirch, France), Reanal (Budapest, Hungary) and Reachim (Russia).

Galactomannans tara gum ferwotar from *Caesalpinia spinose* (Man/Gal = 3.0), guar gum from *Cyamopsis tetragonoloba* (Man/Gal = 1.7) and locust bean gum from *Ceratonia siliqua* (Man/Gal = 4.0) were kindly provided by Henk Schols, Wageningen University.[1]

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2.2. Enzymes

Two homogeneous β-(1→4)-mannanases (β-(1→4)-D-mannan mannanohydrolase, EC 3.2.1.78) were purified to homogeneity from *Myceliophthora thermophila* and *Trichoderma reesei* cultural supernatants by column chromatography: *M. thermophila* β-mannanase, 48 kDa (GH family 5) and *T. reesei* β-mannanase, 52 kDa (GH family 5).

The cultural supernatant (*M. thermophila* or *T. reesei*) was centrifuged to precipitate all insoluble components. The crude enzyme solution containing 100 mg of protein was subsequently loaded onto a Source 15Q anion exchange column 160 × 5 mm (Pharmacia, Sweden) equilibrated in buffer A (20 mM Bis–Tris–HCl pH 6.8) with a flow rate of 5 ml/min using an FPLC chromatographic system (Pharmacia, Sweden). Before the elution of β-mannanase the column was washed with buffer A to remove unbound protein. The β-mannanase was eluted off the column with a linear gradient from 0 to 1 M of NaCl in buffer A. Fractions which contained β-mannanase activity were combined, mixed with saturated $(NH_4)_2SO_4$ to a final concentration of 1.7 M and subjected to a hydrophobic chromatography on Source 15ISO 13 × 80 mm column (Pharmacia, Sweden) equilibrated in 50 mM sodium acetate (NaOAc) pH 5.0 1.7 M $(NH_4)_2SO_4$ with a flow rate of 4 ml/min using the FPLC chromatographic system (Pharmacia, Sweden). Fractions containing β-mannanase were desalted using Econo-System liquid chromatography (Bio-Rad, USA) on a Bio-Gel P4 12 × 460 mm column and NaOAc buffer with a flow rate of 1 ml/min. The desalted β-mannanase sample was further purified by chromatofocusing using the FPLC system again with a Mono P column 5 × 20 mm (Pharmacia, Sweden) equilibrated in 25 mM imidazole-HCl pH 7.4. Elution of β-mannanase was carried out using a Polybuffer 74-HCl pH 4.0 (Amersham Biosciences, USA) with a flow rate of 0.5 ml/min. β-Mannanase containing fraction was dialyzed for 16 h against 50 mM NaOAc pH 5.0 to remove the Polybuffer and the sample obtained was used for all further experiments described. The protein composition of the fractions obtained and the purity of the enzymes were assessed by SDS–PAGE.

It is very important for the approach considered to provide the absence of other galactomannan-degrading enzymes from *M. thermophila* and *T. reesei* (β-mannosidase (β-D-mannopyranoside hydrolase, EC 3.2.1.25), α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22)) in β-mannanase purified samples. To confirm the absence of β-mannosidase and α-galactosidase final products of galactomannan hydrolysis by β-mannanases were analyzed by HPLC (Agilent 1100 chromatography workstation with 1312A binary pump and 1367A micro-plate automated liquid sampler, ESA Coulochem III electrochemical detector, 5040 electrochemical cell with gold electrode, Dionex CarboPak PA100 4 × 250 mm analytical column with PA100 precolumn, USA) and oligosaccharide peaks were observed. Then *M. thermophila* β-mannosidase and α-galactosidase (the enzymes were isolated and purified in our laboratory previously) were added to final products of galactomannans hydrolysis and products obtained were analyzed again. Two sets of HPLC-profiles significantly distinguished from each other. Oligosaccharide peaks decreased gradually, whereas low-molecular product peaks arose (mannose, galactose). Data obtained evidenced the absence of β-mannosidase and α-galactosidase in β-mannanase samples.

2.3. Enzyme activity

β-Mannanase specific activities toward locust bean, tara gum and guar gum galactomannans (5 mg/ml) were determined by measuring the release of reducing sugars (RS) using the modified Somogyi–Nelson method.[9,10] All activities were expressed in units. One unit of activity was defined as the amount of enzyme catalyzing the liberation of 1 μmol of the product/min under the given conditions. Specific activities were related to 1 mg of enzyme protein (Table 1).

Table 1
β-Mannanase specific activities toward locust bean, tara gum and guar gum galactomannans (pH 5.0, 0.1 M sodium acetate buffer, 50 °C)

Substrate	Specific activity (U/mg)	
	β-Mannanase *M. thermophila*	β-Mannanase *T. reesei*
Locust bean GM	76	25
Guar gum GM	52	23
Tara gum GM	56	19

2.4. Polysaccharides hydrolysis

Extended enzyme treatment of different galactomannans by β-mannanases from *M. thermophila* and *T. reesei* was carried out in 0.1 M Na-acetate buffer pH 5.0 at 50 °C. The reaction mixture contained 5 mg/ml of galactomannan and β-mannanase in the concentration of 0.048 mg/ml. During the hydrolysis the aliquots were taken from the reaction mixture, boiled in water bath for 10 min, centrifuged and assayed for reducing sugars (RS) content. The RS release was evaluated by modified Somogyi–Nelson method.[9,10]

It is very important for the approach considered to provide the exhaustive hydrolysis of galactomannans by β-mannanases. To prove the completion of the reaction additional aliquot of enzyme was added after 22 h and product quantity was monitored. In all cases adding of enzyme did not lead to increase in product quantity, which evidenced the exhaustive hydrolysis of galactomannans by β-mannanases.

2.5. Protein determination

Protein concentration was determinated by Lowry's method[11] using bovine serum albumin as a standard as well as spectrophotometrically at 280 nm (A_{280}) and calculated using the molar absorbance of the enzyme. The molar absorbance of each enzyme was calculated on the bases of the amino acid composition using the ProtParam program (http://au.expasy.org/tools/protparam.html). Both methods gave comparable results, the first one was applied for cultural supernatant protein determination and the second one was applied for β-mannanase concentration determination in purified samples.

3. Results and discussion

3.1. Statistical modeling

A galactomannan with a ratio of Man/Gal = 2.0 does not mean that the polymer is homogeneous, with one Gal residue exactly positioned every two Man residues throughout the entire polysaccharide. For any polymer like this, there will be a distribution of the substituents (α-D-galactopyranosyl residues) with regions where each Man will have a linked Gal, as well as regions where backbone Man residues will have no linked Gals (i.e., unsubstituted mannan sequences). Based on principles of combinatorial mathematics, this distribution can be statistically modeled and compared with experimental data, in this particular case with results of the enzymatic hydrolysis of the galactomannans. For a random distribution, the statistical probability of having $(Gal/Man)_m$ clusters along the mannan backbone is given by:

$$P[(Gal/Man)_m] = q^2 \times (1-q)^m$$