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# The effect of galactose side units and mannan chain length on the macromolecular characteristics of galactomannans

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ABSTRACT

The degree of substitution and the degree of polymerization of galactomannan from guar were altered enzymatically to study the role of side groups and chain length in the behavior of molecules in solution. Unmodified guar galactomannan was found to contain aggregates, but partial removal of galactose side units significantly decreased this tendency. The chain length seemed to dictate the solubility of the molecules together with the degree of substitution. The sample with a relatively low degree of polymerization and low degree of substitution formed large assemblies but stayed in solution while the solubility of the samples with longer chain length was reduced. To our knowledge, this is the first report on the utilization of endo-1,4-β-D-mannanase and α-D-galactosidase as tools for studying the solution properties of galactomannans.



## 1. Introduction

Galactomannans are plant polysaccharides existing in the endosperm of certain leguminous seeds. The β-(1 → 4)-linked backbone in galactomannans is composed of D-mannopyranosyl (β-D-Man*p*) units with D-galactopyranosyl (α-D-Gal*p*) residues α-(1 → 6)-linked to mannose backbone (Smith, 1948). Galactomannan from guar seeds (Guaran, *Cyamopsis tetragonolobus*), which has commercial significance, is known to have a galactose-to-mannose ratio of 0.50–0.73 and a reported molar mass ($M_W$) ranging from 950,000 to 1,900,000 g/mol depending on the author and analysis method. Other commercially important galactomannans include locust bean gum from carob seeds with a wider range of the galactose-to-mannose ratio from 0.19 to 0.83 (Dea & Morrison, 1975). The distribution of α-D-Gal*p* along the β-D-Man*p* backbone has been under debate, but the recent results of Daas, Schols, and de Jongh (2000) suggest that in guar galactomannan the distribution is blockwise whereas locust bean gum may contain random, blockwise, and ordered distributions. Galactomannans are used widely as industrial thickeners and stabilizers.

The structural diversity of naturally occurring polysaccharides, which could be exploited more efficiently, is extensive. Instead of extracting structurally different polysaccharides from plants, specific enzymatic modifications can be used to produce molecules with varying chemical structure. Recently, much interest has arisen in effective production of enzymes to be used in the large-scale modification of polysaccharides such as mannans (Gurkok, Cekmecelioglu, & Ogel, 2011; Kote, Patil, & Mulimani, 2009). However, most studies have focused more on the hydrolysis of polysaccharides into oligo- and monosaccharides than on specific tailoring of biopolymers (Koltermann, Kettling, Brück, & Rarbach, 2008). In recent investigations (Mikkonen et al., 2007, 2009), galactomannan from guar was modified enzymatically, and samples with varying chain length and degree of substitution were used for film formation and emulsion stabilization studies. Hannuksela, Tenkanen, and Holmbom (2002) studied the sorption of similarly modified galactomannans onto bleach kraft pulp in the subtext of mechanical pulping.

Studies on the macromolecular characterization of guar galactomannan in dilute aqueous solution have been reported by several groups, including viscometry, light scattering, and high-performance size-exclusion chromatography coupled to multi-angle light scattering (HPSEC-MALS) studies (Picout, Ross-Murphy, Errington, & Harding, 2001; Robinson, Ross-Murphy, & Morris, 1982; Wientjes, Duits, Jongschaap, & Mellema, 2000). Galactomannan chains are regarded as coil-like molecules whose macromolecular characteristics, such as Mark-Houwink parameters and chain persistence length, have been confirmed with extensive experimental data (Picout et al., 2001; Picout & Ross-Murphy, 2007). Variation in these characteristics can be found in the literature due to the different extent of solubilization. Polysaccharides, in general, are known not to form molecularly dis-

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persed aqueous solutions easily, but rather have a tendency toward time-dependent aggregation (Burchard, 2005; Kratochvíl, 1987). Picout et al.'s (2001) and Cheng, Brown, and Prud'homme's (2002) viscometric and HPSEC-MALS data indicate the presence of aggregates in native guar galactomannan solutions and that decreasing the chain length reduces the aggregation tendency. Although the macromolecular characterization of galactomannans from various sources and different structures has been accomplished, the role of α-D-Gal*p* side units in the solution conformation and aggregation behavior, however, has not been previously studied. Various studies, on the contrary, have been conducted on the effect on galactomannan structure to the gelling properties (Dea, Clark, & McCleary, 1986; McCleary, 1979; McCleary, Amado, Waibel, & Neukom, 1981).

In the present study, the chemical structure of galactomannan from guar was modified using specific enzymatic treatments. Enzymatic modifications were accomplished with endo-1,4-β-D-mannanase (EC 3.2.1.78) cleaving bonds in the middle of the chain and α-D-galactosidase (EC 3.2.1.22) acting on the terminal α-D-Gal*p* units. HPSEC with multiple-detection and AsFlFFF (asymmetric flow field-flow fractionation) were used to monitor the molecular characteristics and solution properties of tailored polysaccharides. The special interest was to see the effect of the structure, i.e., the degree of substitution and chain length, on the behavior of polysaccharide solutions.

## 2. Materials and methods

Galactomannan from guar (guar gum, G-4129) was purchased from Sigma (Munich, Germany). Endo-1,4-β-D-mannanase purified from *Trichoderma reesei* (VTT, Finland) (Stålbrand, Siika-aho, Tenkanen, & Viikari, 1993) and α-D-galactosidase (Biogalactosidase, Quest, The Netherlands) were used for enzymatic modification of galactomannan. Water was purified with a Milli-Q-Plus system (Millipore Corp., Billerica, MA, USA). $NaNO_3$ was from Merck (Darmstadt, Germany).

### 2.1. Enzymatic modification of galactomannan

Stepwise enzymatic modification for galactomannan was performed according to Mikkonen et al.'s (2007) and Hannuksela et al.'s (2002) protocols, to achieve a series of samples with four different chain lengths and three different degrees of galactose substitution. The endo-1,4-β-D-mannanase dosages used were 0, 2, 10, or 50 nkat/g of mannan, and the incubation time was 4 h. After the mannanase treatment, the samples were further incubated with α-D-galactosidase (0, 200, or 500 nkat/g) for 5 h. Enzymatic treatments were carried out in ammonium acetate buffer, pH 5. After the enzymatic modification, the samples were freeze-dried, washed with ethanol to remove monosaccharides and short oligosaccharides, and freeze-dried again. The carbohydrate composition of the enzymatically modified galactomannan samples was determined as alditol-acetate derivatives using GC after complete enzymatic hydrolysis (Mikkonen et al., 2007). The sample codes, enzyme dosages, and galactose-to-mannose ratios are summarized in Table 1. The 6Gal-H represents the unmodified galactomannan sample, which was treated similarly as the other samples but without any enzyme addition.

### 2.2. Preparation of galactomannan solutions for HPSEC and AsFlFFF

HPSEC and AsFlFFF analyses of the modified galactomannan samples were accomplished using aqueous eluent (0.1 M $NaNO_3$). The solutions for HPSEC were prepared in 0.1 M $NaNO_3$ with a concentration of 1 mg/ml and dissolved at 4 °C for 4 days. The preparation of the samples for AsFlFFF was similar, except that the concentrations of the low and medium low samples were 2 mg/ml and 1.5 mg/ml for the medium high samples, 1 mg/ml for the 2Gal-H and 4Gal-H samples, and 0.9 mg/ml for the 6Gal-H sample. Before analysis, the samples were filtered with 0.45 μm syringe filters (GHP Acrodisc 13, Pall Corp., Ann Arbor, MI, USA). The d*n*/d*c* value used for the molar mass calculation was 0.150 ml/g (Kapoor, Milas, Taravel, & Rinaudo, 1994).

**Table 1**
Sample codes, enzyme dosages, and galactose-to-mannose ratios of enzymatically modified galactomannans adapted from Mikkonen et al. (2007).

| Sample code[a] | Galactosidase/mannanase dosage (nkat/g) | Gal:Man ratio | Molar mass code |
|---|---|---|---|
| 6Gal-H | 0/0 | 0.49 | High |
| 6Gal-MH | 0/2 | 0.52 | Medium high |
| 6Gal-ML | 0/10 | 0.57 | Medium low |
| 6Gal-L | 0/50 | 0.54 | Low |
| 4Gal-H | 200/0 | 0.36 | High |
| 4Gal-MH | 200/2 | 0.37 | Medium high |
| 4Gal-ML | 200/10 | 0.39 | Medium low |
| 4Gal-L | 200/50 | 0.36 | Low |
| 2Gal-H | 500/0 | 0.23 | High |
| 2Gal-MH | 500/2 | 0.23 | Medium high |
| 2Gal-ML | 500/10 | 0.20 | Medium low |
| 2Gal-L | 500/50 | 0.19 | Low |

[a] 6, 4, and 2 denote the approximate number of galactose units per 10 mannose units.

### 2.3. HPSEC analysis

The HPSEC equipment consisted of an integrated autosampler and pump module (GPCmax, Viscotek Corp., Houston, TX, USA), a combined light scattering and viscometric detector (270 Dual Detector, Viscotek Corp.), and a refractive index (RI) detector (VE 3580, Viscotek Corp.). The light scattering detector ($\lambda_0$ = 670 nm) included two scattering angles: 7° (low angle light scattering, LALS) and 90° (right angle light scattering, RALS). Two OHpak SB-806 M HQ columns (8 × 300 mm, exclusion limit $2 \times 10^7$, Showa Denko, Ogimachi, Japan) with an OHpak SB-6 guard column (4.6 mm × 10 mm) were used for separation. The flow rate was 1 ml/min and the injection volume 100 μl.

### 2.4. AsFlFFF analysis

The AsFlFFF experiments were carried out using an AF2000 MT instrument (including software, Postnova Analytics, Landsberg/Lech, Germany) equipped with multi-angle light scattering (MALS, Brookhaven Instruments Corporation, Holtsville, NY, USA) and refractive index (PN 3150, Postnova Analytics) detectors. The MALS detector contains 30 mW laser as the light source operating at $\lambda_0$ = 660 nm with seven scattering angles (35°, 50°, 75°, 90°, 105°, 130°, 145°). Separation occurred in a rectangular channel consisting of a bottom plate with a ceramic frit, a spacer with a thickness of 350 μm, and a top plate with flow outputs. A membrane was placed on top of the ceramic frit. Two membrane materials, regenerated cellulose and polyethersulfone with a cut-off value of 10,000 g/mol (Postnova Analytics), were tested to ensure the best possible fractionation conditions. The pump system included two isocratic pumps and a piston pump for maintaining cross-flow. Exponential decay of cross-flow (exponent 0.2) was used for the separation step starting with a cross-flow of 2 ml/min, and the detector flow was kept constant at 0.5 ml/min during the analysis. Because molecules flow in the channel near the membrane, the excess solvent from the upper part of the channel was pumped to the waste at a flow rate of 0.5 ml/min before the detector outlet to intensify the detec-

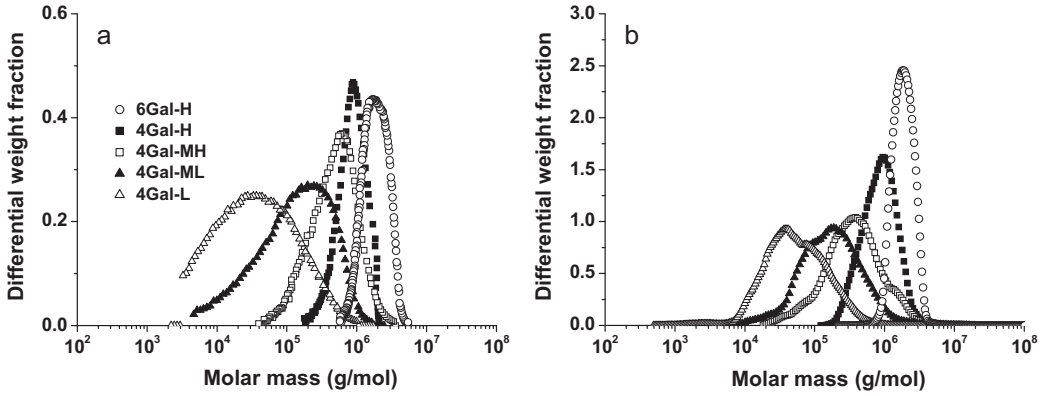


**Fig. 1.** Molar mass distributions for 6Gal-H and 4Gal samples obtained with (a) HPSEC and (b) AsFlFFF. The open circles represent the unmodified 6Gal-H sample, solid squares 4Gal-H, open squares 4Gal-MH, solid triangles 4Gal-ML, and open triangles 4Gal-L sample.

tor signals. Similar conditions were used for arabinoxylans in our recent study (Pitkänen, Tenkanen, & Tuomainen, 2011). The RI and MALS detectors were calibrated according to instructions from Postnova Analytics (Postnova Analytics, 2009) using the bovine serum albumin and polystyrene sodium sulfonate standards. The Berry equation was used for the molar mass and size calculations, and the injection volume was 50 μl.

## 3. Results and discussion

### 3.1. The effect of endo-1,4-β-D-mannanase treatment on the polydispersity of galactomannans

The commercial galactomannan from guar was treated with endo-1,4-β-D-mannanase and further with α-D-galactosidase to form the series of samples with different chain lengths and degrees of substitution. The chain length-reducing effect of mannanase is evident accompanied by increased polydispersity when higher enzyme dosages were used (Fig. 1). A similar increase in polydispersity after endo-1,4-β-mannanase treatment was previously observed by Cheng, Brown, and Prud'homme (2002), who also presented three possible mechanisms for it. First, the distance between the enzyme molecules compared to the size of the galactomannan molecules in solution is long due to the low concentration of the enzyme. Thus, the chains that are close to the enzyme are most probably hydrolyzed. The uneven distribution pattern of the α-D-Gal*p* side groups can also affect the action of the mannanase. The enzyme might have higher activity on the non-substituted regions on the mannan backbone. The third explanation lies in pathways for the enzymatic degradation of polymers. If the degradation occurs in the single chain attack pathway, the enzyme remains in a complex with the substrate molecule until it has hydrolyzed all the possible bonds in the chain. In the case of all above-mentioned mechanisms, however, shorter chains form, and thus, the molar mass distribution broadens.

### 3.2. Comparison of separation methods in the galactomannan characterization

Enzymatically modified galactomannan samples were analyzed with HPSEC and AsFlFFF to see the possible effect of the separation method on the results. Native galactomannans are reported to have high molar mass in the range of $2 \times 10^6$ g/mol (Dea & Morrison, 1975) and large size due to a fairly extended chain conformation. Picout et al. (2001) reported an $R_g$ (radius of gyration) of 133 nm for the commercially available native galactomannan sample from guar. Because such a large size, we also analyzed the samples with AsFlFFF, which is suitable for separation of large polymers and particles. The separation was not, however, affected by the method as seen in the similar molar mass distributions (some lower molar mass material went through the membrane and thus was not seen in the distribution of AsFlFFF) presented for the unmodified 6Gal-H sample and the 4Gal samples (Fig. 1), and both HPSEC and AsFlFFF can be considered applicable for separating galactomannans. One concern in the AsFlFFF analysis was the low recovery values compared with HPSEC. The recovery values calculated from the RI signal in AsFlFFF were below 60% for all the galactomannan samples using regenerated cellulose and polyethersulfone membrane whereas the recoveries in HPSEC were above 60% for all samples except for the mainly insoluble 2Gal samples (Table 2). As the membrane material in AsFlFFF had no effect on the results, the data presented in this paper are from the analyses accomplished using the regenerated cellulose membrane.

### 3.3. Solution properties of galactomannans with enzymatically altered degree of substitution and chain length

The weight-average molar masses, intrinsic viscosities, and recovery values obtained from the HPSEC analyses are summarized in Table 2. The recovery values indicate that the samples with only two α-D-Gal*p* units per 10 β-D-Man*p* units are mostly insoluble. An exception to this is the 2Gal-L sample with the shortest chain length. Although most of the sample is in solution, the sample contains aggregates seen as a sharp pre-peak in the light-scattering signal (Fig. 2a, chromatogram of 6Gal-L presented for comparison in Fig. 2b) and as the higher $M_w$ value when compared to the other low-molar mass samples (Table 2). The samples with higher α-D-Gal*p* content (the 6Gal and 4Gal samples) can be regarded as soluble in water containing 0.1 M $NaNO_3$. In general, if the different

**Table 2**
Weight-average molar masses ($M_w$), intrinsic viscosities ([$\eta$]), and sample recoveries obtained with HPSEC for enzymatically modified galactomannans.

| | $M_w \times 10^{-5}$ (g/mol) | [$\eta$] (ml/g) | Recovery % |
|---|---|---|---|
| 6Gal-H | 18.4 | 1050 | 65 |
| 6Gal-MH | 8.56 | 600 | 78 |
| 6Gal-ML | 3.45 | 300 | 103 |
| 6Gal-L | 1.00 | 110 | 86 |
| 4Gal-H | 9.33 | 890 | 70 |
| 4Gal-MH | 5.96 | 610 | 73 |
| 4Gal-ML | 2.41 | 310 | 86 |
| 4Gal-L | 0.83 | 130 | 79 |
| 2Gal-H | 3.76 | 510 | 32 |
| 2Gal-MH | 2.26 | 340 | 31 |
| 2Gal-ML | 1.30 | 180 | 38 |
| 2Gal-L | 2.17 | 120 | 68 |

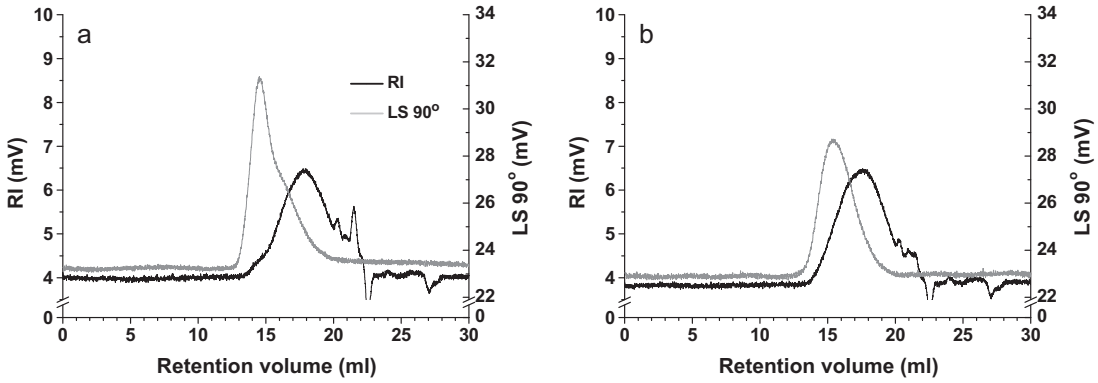


**Fig. 2.** Overlay of refractive index and light scattering (90°) signals for (a) 2Gal-L and (b) 6Gal-L samples obtained with HPSEC.

chain lengths are compared, the samples with the highest degree of polymerization have the lowest recovery values. Thus, the chain length seems to influence the solubility of galactomannans. The remarkable difference in molar mass between unmodified sample (6Gal-H) and the sample with no mannanase treatment and only slight α-galactosidase treatment (4Gal-H) (Fig. 1, Table 2) cannot be caused by the mass loss of removed α-D-Gal*p* units in the 4Gal-H sample, which is around 9% when calculated from the amount of released α-D-Gal*p*. The 6Gal-H sample most probably contains some aggregated molecules. Cheng et al. (2002) found that the Huggins coefficient, describing polymer-polymer interactions, for native galactomannan from guar was significantly higher than for acid/enzymatically degraded galactomannan, indicating additional interactions between galactomannan chains. In addition to viscometric studies, Gittings et al. (2000) investigated guar solutions with ultra-small-angle light scattering and observed the presence of aggregates. According to our data, partial removal of α-D-Gal*p* side groups (4Gal series) seemed to reduce the formation of these intermolecular complexes. In fact, the effect of side groups on the aggregation can be considered more significant than the chain length. The average intrinsic viscosity of 6Gal-MH and 4Gal-MH are identical being approximately 600 ml/g, but the $M_w$ of 6Gal-MH is higher compared with that of 4Gal-MH, indicating the presence of aggregates in the 6Gal-MH sample in addition to the unmodified 6Gal-H sample (Table 2). A significant difference between the molar mass of 6Gal-H and 4Gal-H can also be seen in the molar mass distributions (Fig. 1). As a whole, the intrinsic viscosity data is consistent, and the values are not largely affected by the presence of aggregates as discussed by Robinson et al. (1982) and more recently Picout et al. (2001).

Hannuksela et al. (2002) analyzed a similar, enzymatically produced galactomannan set using size-exclusion chromatography, but their results regarding $M_w$ values are somewhat lower than the values we present here, especially for the samples with the highest molar mass (high and medium high samples). One explanation for the difference in results could be the filtration of samples with 0.2 μm filters whereas we used filters with 0.45-μm pores.

The effect of the α-galactosidase treatment on the intrinsic viscosity–molar mass relationship is presented as double-logarithmic Mark-Houwink plots (Fig. 3, average intrinsic viscosities in Table 2). A separate line is plotted for the sample set containing 6 and 4 α-D-Gal*p* side groups per 10 β-D-Man*p* units to find out if the substituents affect the molecular density of the polysaccharide. The 2Gal series was excluded from the plot due to the partial insolubility of the 2Gal-H, 2Gal-MH, and 2Gal-ML samples and the aggregation of the 2Gal-L sample. The 6Gal samples seem to adopt slightly denser conformation in solution than the 4Gal samples, but this difference in the intrinsic viscosities is more likely due to the presence of aggregates in the 6Gal samples rather than the effect of side units.

To obtain a more realistic idea of the effect of side units on the solution conformation of galactomannans, the double-logarithmic radius-molar mass relationships of 6Gal-MH and 4Gal-MH were compared to the corresponding relationships of 6Gal-ML and 4Gal-ML with shorter chain lengths (Fig. 4). As discussed earlier in this paper, 6Gal-MH with a higher degree of substitution than 4Gal-MH is most probably partially aggregated. Therefore, the apparent difference between the plots of the 6Gal-MH and 4Gal-MH results from the compact aggregates that reduce the $R_g$ values of 6Gal-MH (Fig. 4a). Because the extent of aggregation seems to be lower in the samples with shorter chain length (Cheng et al., 2002), the data for the medium low (ML) samples can be considered the most reliable for investigating the effect of the substitution. When the radius-molar mass plots of 6Gal-ML and 4Gal-ML were compared, the $R_g$ values for both samples increased similarly as the molar mass increased (Fig. 4b).

### 3.4. Problems related to the macromolecular characterization of galactomannans

The variation among the light scattering/HPSEC data found in the literature for galactomannans is mainly due to the difficulty in dissolving these molecules. The results of this study supported the earlier findings of Picout et al. (2001) and Cheng et al. (2002), who concluded that native galactomannan from guar is prone to forming aggregates in aqueous solution. Often,

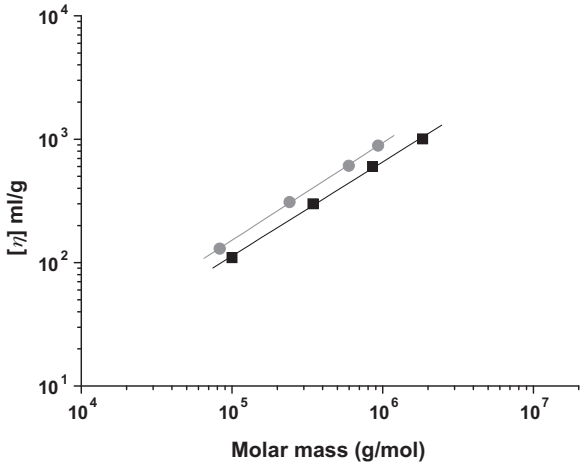


**Fig. 3.** Mark-Houwink plots for galactomannans with six (black squares) and four (gray circles) α-D-Gal*p* side units per ten β-D-Man*p* units.

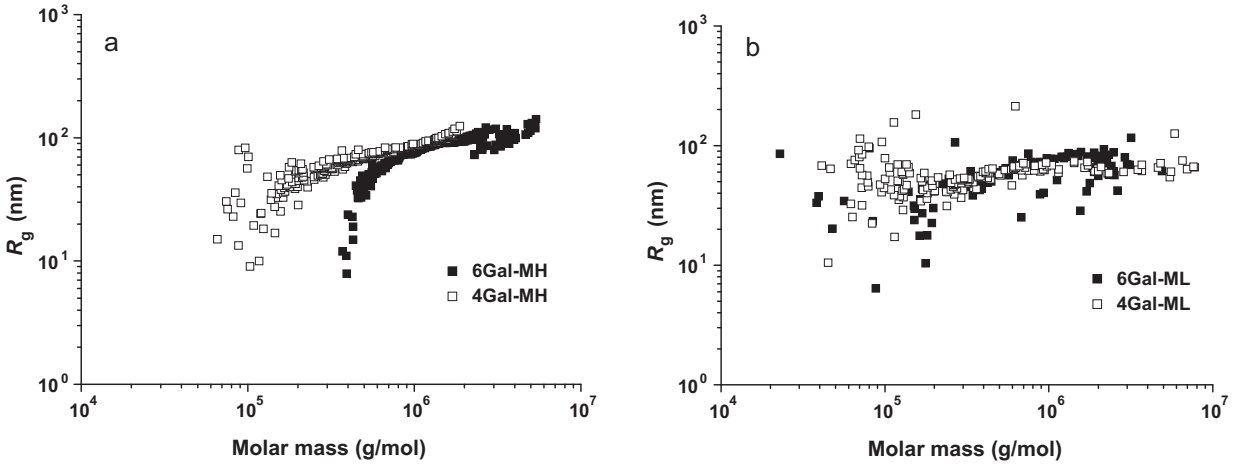


**Fig. 4.** Comparison of the radius of the gyration–molar mass relationships for the 6Gal-MH (solid squares) and 4Gal-MH (open squares) samples (a) and for the 6Gal-ML (filled squares) and 4Gal-ML (open squares) samples (b). Size and molar data was obtained with AsFlFFF.

observing these assemblies can be quite difficult even when a separation method, such as HPSEC or AsFlFFF, is used on-line with light scattering and/or viscometric detection. As seen in this study, the aggregation of the unmodified galactomannan sample was not unambiguously revealed by the HPSEC and AsFlFFF analyses, but only after the enzymatically modified samples were characterized. Detecting aggregates is also complicated by the solubility of galactomannans solely in aqueous solutions and not, e.g., for DMSO or DMAc, which are commonly used for polysaccharides. In the case of other (1 → 4)-linked polysaccharides, such as arabinoxylans, the presence of aggregates in aqueous solution was confirmed with the HPSEC analysis in organic solvent, namely in DMSO (Pitkänen, Virkki, Tenkanen, & Tuomainen, 2009). When the selection of solvents is limited, another option is to develop solubilization methods to prevent chain entanglements from forming. Picout et al. (2001) used pressure cell treatments for galactomannan samples to achieve solution without aggregates. However, the temperatures, pressures, and times for such treatments should be carefully selected to avoid possible chain degradation.

In addition to compact, initial aggregates present in the unmodified guar galactomannan sample, as a result of the extensive treatments with endo-1,4-β-D-mannanase and α-D-galactosidase, the galactomannan chains formed large intermolecular assemblies that can be seen in the HPSEC chromatogram of the 2Gal-L sample (Fig. 2a). Thus, the formation and form of aggregates are affected by the chemical structure of galactomannans.

## 4. Conclusions

The set of galactomannan samples with an enzymatically altered degree of polymerization and degree of substitution were characterized using HPSEC and AsFlFFF with multiple detection. Both separation methods were found to be applicable for galactomannans, and the data obtained with the two methods were consistent. The partial removal of α-D-Gal*p* side groups decreased the determined molar mass drastically, which was attributed to the aggregation of the unmodified sample and further disaggregation when the degree of substitution was moderately lowered. The decreased tendency for aggregate formation of the partially debranched guar galactomannan sample was discovered for the first time in this study. An extensive debranching of galactomannans caused insolubility of samples with higher chain lengths and assembly formation of the sample with the lowest chain length. Due to the aggregation tendency of galactomannan, the molar masses reported in various studies for native guar galactomannan are mostly overestimated.

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