Use of ¹³C-NMR in structural elucidation of polysaccharides: case of locust bean gum.

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introduction

Locust bean gum (LBG) is an additive (E410) used mainly in the food industry for its rheological, texturing and gelling properties. LBG comes from the endosperm of the seeds of the carob tree (*Ceratonia sliliqua L.*). The endosperm is composed of reserve polysaccharides (hemicelluloses) called galactomannans. Galactomannans consist of a β-(1→4) D-mannopyranosyl backbone substituted to varying degrees in α-(1→6) with single D-galactopyranosyl residues (Fig.2) (McCleary, 1980). This basic structure is the same for all galactomannans, however, three elements may vary: the **degree of substitution** of galactose on mannosyls residues, the **molecular sizes** and the **distribution pattern** of galactosyl substituents along the main chain of mannans (Gillet *et al.*, 2014[a]).

Crude locust bean gum (CLBG) was purified and fractionated into two parts : the first was obtained by solubilization in water at 25 °C (**GM25**) and the second consisted in a further extraction at 80 °C on the residual impoverished fraction (**GM80**). These Fractions have different physical properties in aqueous solutions (Gillet *et al.*, 2014[b]). The rheological behaviors of **GM25** and **GM80** were evaluated and those properties where related to the fine structure of galactomannanes revealed by [¹³C]-NMR analysis of these two fractions.

Rheological properties

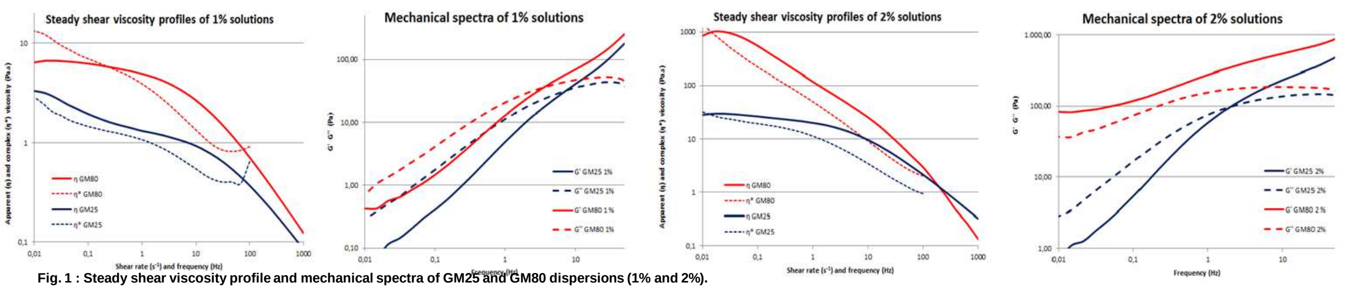

Fig. 1 : Steady shear viscosity profile and mechanical spectra of GM25 and GM80 dispersions (1% and 2%).

The fractions studied showed a shear thinning behavior on the steady shear viscosity profile (1% or 2% w/v). This means that the apparent viscosity (η) decreased as the shear rate increased (Fig. 1). The shear thinning effect is much stronger for the **GM80** fraction than for **GM25**. The mechanical spectra of **GM25** and **GM80** at 1% is typical of viscoelastic fluids generated by an entangled network. The **GM80** fraction had a much stronger viscoelastic behavior, meaning that it quickly had an elastic (G') dominance behavior generated by entanglements and hyper-entanglements which dissociate less easily. On the mechanical spectra at 2%, **GM80** showed a weak gel behavior while **GM25** still remained a viscoelastic fluid. Thus, the **GM80** fraction generated a denser and tougher network, characterized by stronger intermolecular interactions and many more hyperentanglements. This can only be explained by differences in the fine structure.

Structural analysis

The **degree of substitution** and the **distribution pattern** of galactosyl substituents can be estimated by [¹³C]-NMR analysis (Gillet *et al.*, 2014[c]).

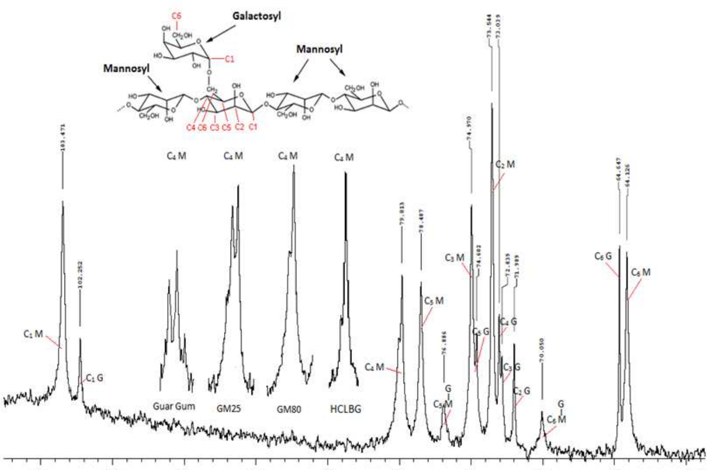

Fig.2 : [¹³C]-NMR spectra of different galactomannans: GM25, GM80, HCLBG and guar gum. Insert : focus on mannosyl fourth carbon (C_4).

GM25 and **GM80** were comparatively studied by 600 MHz [¹³C]-NMR. **Guar gum** and hydrolyzed crude locust bean gum (**HCLBG**) were also analyzed to obtain a comparative control in the analysis. Mannosyls/Galactosyls (M/G) ratios can be determined by considering the intensities of C_1 mannose and galactose signals in [¹³C]-NMR spectra (Fig. 2). This method provides results relatively close to those obtained by GC-FID analysis (Table 1). Spectra also showed that resonance from C_4 of D-mannose residues were split, in evident dependence upon the nearest-neighbor probabilities ("diad frequencies") of D-galactosyl groups along the mannan chains. Diad frequencies were obtained by integrating C_4(Man) peak areas. F_{11}, F_{21}/F_{12} and F_{22} gave respectively the proportions of di-, mono- or non-substituted mannose pairs. The percentages of total lateral substituents obtained by C_4(Man) peak analysis [F_{11} + (F_{21} or F_{12})/2] were fairly well correlated with M/G ratios.

Table 1 : Fine structural characterization of different galactomannans

Structural characterization	Guar	GM25	GM80	HCLBG
M/G ratio (GC-FID)	1.51 ± 0.01	2.85 ± 0.01	3.84 ± 0.01	5.98 ± 0.01
M/G ratio ([¹³C]-NMR)	1.70 ± 0.02	2.98 ± 0.04	3.50 ± 0.01	6.55 ± 0.04
Degree of blockiness (%)	40.00 ± 1.23	70.00 ± 2.04	83.00 ± 2.47	96.00 ± 3.15
F11 diad frequencies (%)	41.28 ± 1.25	12.35 ± 1.17	15.65 ± 0.28	6.13 ± 0.18
F12 or F21 diad frequencies (%)	46.53 ± 0.54	35.52 ± 0.95	30.35 ± 0.59	21.01 ± 1.49
F22 diad frequencies (%)	12.19 ± 0.71	52.10 ± 0.20	54.00 ± 0.31	72.85 ± 1.66

Thus, **guar gum** has a high proportion of di-substituted (F11) or mono-substituted (F_{12}/F_{21}) di-mannosyls and very few pairs without substituents. This makes sense since guar gum consists of a large proportion of side galactosyls residues (M/G = 1.5). Therefore consecutive "smooth" mannosyl residues remain limited. This gum has a fairly homogeneous profile. **GM25** and **GM80** fractions present similar profiles (Fig. 2), different from guar gum. It indicates a much larger proportion of non-substituted di-mannosyls, explained by higher M/G ratios than guar gum. However, **GM80** fraction has a higher proportion of F_{11} and F_{22} than **GM25** while its M/G ratio is more important. This clearly indicates that the distribution of galactosyls on the mannose main chain does not respond to a simple statistical law. **HCLBG** has a [¹³C]-NMR spectrum with the same morphology as **GM25** and **GM80** fractions. However the proportion of non-substituted pairs and M/g ratio are much more important. Splitting of the C_6 substituted D-mannose resonance provides, therefore the basis for determining the next-nearest-neighbor probabilities (triad frequencies). However, the spectrum is often not sufficiently resolved to accurately quantify and interpret the results.

The study of the degree of blockiness is focused on α-D-galactopyranosyl side-chain groups distribution. It was determined by measurement of *O*-acetyl-*O*-methyl-D-mannitol derivatives by GC-MS, which gives a characteristic of the distribution pattern in the parent galactomannan (Baker & Whistler, 1975). Results of these analyses confirm the trend observed (Table 1).

The **molecular sizes** were determined by HPSEC-MALLS analysis (Table 2). M_w value of **GM80** fraction (910 kDa) was higher than **GM25** fraction (760 kDa), so did the degree of polymerization (DP).

Table 2 : Molecular sizes data.

	Phosphate buffer	
	GM25	GM80
M_w (kDa)	761.1 ± 9.3	911.3 ± 54.2
M_n (kDa)	655.9 ± 13.9	897.7 ± 67.2
DP_w [a]	4698 ± 57	5625 ± 335
DP_n [a]	4049 ± 86	5541 ± 415
PI	1.16 ± 0.03	1.02 ± 0.02
$(Rg)_w$	91.7 ± 1.7	106.9 ± 4.2
$(Rg)_n$	98.2 ± 2.2	109.5 ± 3.5

[a] Equivalent hexose units.

Conclusions

The fractionation process generated two fractions with different structures : the **GM25** fraction consisted of galactomannans composed of shorter chains, richer in galactosyl, which were distributed at 70% inside "block" structures (grouped in "hairy" regions); The **GM80** fraction consisted of longer galactomannans less substituted in galactosyls, although these were concentrated to 83% as substituted blocks (also grouped in "hairy" regions). So, the average structure of **GM80** may have generated larger intra-chain, inter-chain and inter-molecular interactions, resulting in a stronger network when in aqueous solution. According to our data of the structural analysis, interaction areas may be the non-substituted blocks on the main chain. Small structural differences therefore generated very different physical behaviors, such as the transition form a viscoelastic behavior to a jellified structure.

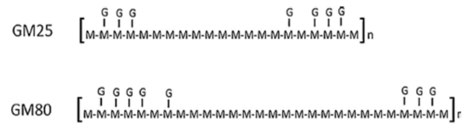

Fig. 3 : Plausible schematic representation of GM25 and GM80 fractions structures

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