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C(6)-Oxidation Followed by C(5)-Epimerization of Guar Gum Studied by High Field NMR

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Guar gum, a β-D-(1→4)-linked D-mannan with α-D-galactopyranosyl units attached as side groups, was treated with α-galactosidase, an enzyme that splits off the α-D-galactosyl units to obtain a galactomannan with a low galactose content. The galactose-depleted polysaccharide was then selectively oxidized in C(6) position and epimerized using mannuronan C(5)-epimerases, namely AlgE1, AlgE4, AlgE6, and their mixtures, obtaining new pseudo-alginates. In this paper, we report a full high field 1D and 2D NMR study of guar gum as such and of the galactose-depleted, oxidized and epimerized compounds, respectively. From the ^{1}H NMR spectra, the degree of epimerization, the distribution of mannuronic acid (M) and guluronic acid (G) residues and the average G-block length, $N_{G>1}$, were obtained. By means of NMR diffusion experiments, it was also shown that no significant degradation of the polysaccharide occurs as a consequence of the epimerization reactions.

1. Introduction

Alginates are a family of 1→4 linked copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. These polysaccharides are extracted from brown seaweeds and can also be synthesized by some bacteria belonging to the *Azotobacter* and *Pseudonomas* genera.

Alginates are synthesized as mannuronan by varying the amount of M residues in the polymer and then epimerized to G residues by a family of seven mannuronan C(5)-epimerases,[1−7] namely AlgE1−AlgE7. These enzymes transform M residues into G residues; as a result, alginate chains with different composition and structure are obtained. Thus, the epimerized compounds differ significantly in proportion and distribution of G and M residues. Although AlgE4 epimerase forms alginates with MG blocks,[5] AlgE6[3] preferentially introduces stretches of G blocks and AlgE1 introduces stretches of MG and G blocks.[1,2]

The gel forming, water-binding, and immunogenic properties of the obtained polysaccharides depend on the relative amount and sequence distribution of M and G residues. Due to the increasing demand in the industrial and biomedical fields, we have investigated the possibility of using mannuronan C(5)-epimerases on substrates obtained from low cost polysaccharides with a high mannose content. This investigation has been aimed at obtaining new pseudo-alginates finely tuned for the desired applications.

In a previous paper,[8] we reported on the results obtained from the C(6)-oxidation followed by C(5)-epimerization of Konjac Glucomannan. In that case, NMR data showed that AlgE4 introduced only single G residues in the polysaccharidic chain. In the case of AlgE6, the M*G*M triad comprising isolated guluronic acid residues prevails with respect to the M*GG* and G*GG* triads comprising the *GG* diad. As a consequence, despite its tendency to produce G blocks, AlgE6 works on the substrate even if M blocks are so short that in the majority of cases it is unable to introduce more than one G residue.

Here we have investigated the possibility of epimerizing guar gum to obtain new charged derivatives through a selective C(6)-oxidation mediated by stable nitroxide free-radical TEMPO (2,2,6,6-tetramethylpiperidin-1-oxy).[9,10]

Galactomannans, like guar, have the advantage with respect to glucomannan, like Konjac, to have a backbone consisting only of β(1→4)-D-mannose residues to which are bound α-(1→6)-D-galactopyranosyl units at different ratios of Man/Gal depending on the particular galactomannan considered. This ratio for guar gum is reported to be, on the average, equal to 2.

The guar plant[11] is native to NW India and Pakistan where it is of considerable economic importance. The guar pod and seeds have been used both as human food and as animal feedstuffs. The unique, thickening properties of guar gum have found extensive application in industry including mining, pharmaceuticals, and petroleum technology. The already wide application of guar gum in many fields makes this naturally abundant, mannose rich polysaccharide a good

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candidate to obtain a new pseudo-alginate with the gelling, viscosifying, and stabilizing properties of natural alginates.

To increase the number of the mannose residues available to the selective C-(6)-oxidation and epimerization, the side galactose groups of guar gum must be split off by treatment with α-galactosidase.

A high field NMR characterization of guar gum and of the galactose depleted, oxidized, and epimerized derivatives is reported. The obtained epimerized compounds are also characterized in terms of statistical distribution of M and G residues in the polysaccharidic chain. Moreover, the average G block length in the epimerized derivatives, N_G, as obtained from ^{1}H high field NMR spectra is also reported.

2. Experimental Section

2.1. α-D-Galactosidase Treatment of Guar Gum. D-Galactose depleted guar gum was prepared following an optimized procedure: 1 g of guar gum (Sigma) dissolved in 500 mL of 0.1 M acetate buffer (pH = 4.6) was treated with α-D-galactosidase (37.5 U; one unit is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol/min at pH 6.5 and 25 °C) at 35−40 °C for 48 h. During the reaction, due to the decreased solubility of D-galactose depleted guar gum, a polysaccharide precipitate was observed.

The suspension was brought to 95 °C for a short time (6 min) to inactivate the enzyme and then cooled at room temperature.

2.2. D-Galactose Depleted Guar Gum Oxidation. The depleted polysaccharide (1 g) was suspended in 800 mL of distilled water, and TEMPO (2,2,6,6-tetramethylpiperidin-1-oxy) (Sigma) (0.06 g) and NaBr (0.3 g) were added to the reaction mixture. The suspension was cooled overnight at 4 °C. Afterward, cold sodium hypochlorite, previously adjusted to pH 9.4 was added. Without further cooling, the pH of the reaction medium was monitored and, if necessary, 0.5 M NaOH was added to maintain the pH at 9.4. After 1 h, the pH tended to decrease slowly, and then EtOH was added to the reaction mixture to deplete the NaOCl left. Afterward, $NaBH_4$ was added gradually until the reaction mixture became clear.

The reaction medium was left overnight at 4 °C, and then it was neutralized by addition of 4 M HCl. The resulting solution was extensively dialyzed against distilled water and then freeze-dried.

2.3. D-Galactose Depleted and Oxidized Guar Gum Epimerization. Samples of D-galactose depleted oxidized guar gum were dissolved (Cp = 0.1% w/v) in TRIS/HCl 50 mM pH 6.9 buffer, plus $NaClO_4$ 50 mM and $CaCl_2$ 2mM. These samples were epimerized using three different C-5 mannuronan epimerases: AlgE4 (C = 0.005% w/v), AlgE1 (C = 0.004% w/v), AlgE6 (C = 0.01% w/v), and their mixtures such as AlgE1 + AlgE4 + AlgE6, AlgE1 + AlgE6, and AlgE4 + AlgE6 (each enzyme at the concentration previously described). All samples were incubated with the enzyme at 40 °C for 48 h under magnetic stirring. The reaction was stopped by chelation of Ca^{2+} ions with the addition of EDTA until a concentration of 13 mM was reached. The pH was adjusted to 7.0; the protein was removed by heating, and the filtration of the solution was performed using 0.22 μm pore size filters selective for proteins but not for polysaccharides.

Samples were extensively dialyzed against distilled water and recovered by freeze-drying.

2.4. NMR Experiments. The samples, ≈2 mg, were dissolved in 700 μL of D_2O or of a phosphate buffered (pD = 7) D_2O solution, 0.01 M NaCl.

^{1}H and ^{13}C NMR experiments were performed on a Bruker AVANCE AQS 600 spectrometer operating at 600.13 and 150.92 MHz, respectively, with a Bruker inverse multinuclear z-gradient probehead. Since at room temperature the ^{1}H spectra appear broad and poorly resolved, spectra were recorded at 343 K; at this temperature, in fact, all resonances appear sufficiently resolved to be assigned. When necessary, to avoid some signal overlapping, the spectra were also run at 333 K.

In all ^{1}H spectra, a soft presaturation of the HOD residual signal was performed.[12]

^{1}H and ^{13}C assignments were obtained using ^{1}H−^{1}H COSY,[13] ^{1}H−^{1}H TOCSY,[14] and ^{1}H−^{13}C HMQC[15] experiments. All 2D experiments were acquired using a time domain of 1024 data points in the F2 dimension and 512 data points in the F1 dimension, the recycle delay was 2s; TOCSY experiments were acquired with a spin-lock duration of 80 ms. The HMQC experiments were performed using a coupling constant $^1J_{C-H}$ = 150 Hz. The number of scans was optimized for obtaining a good signal/noise ratio.

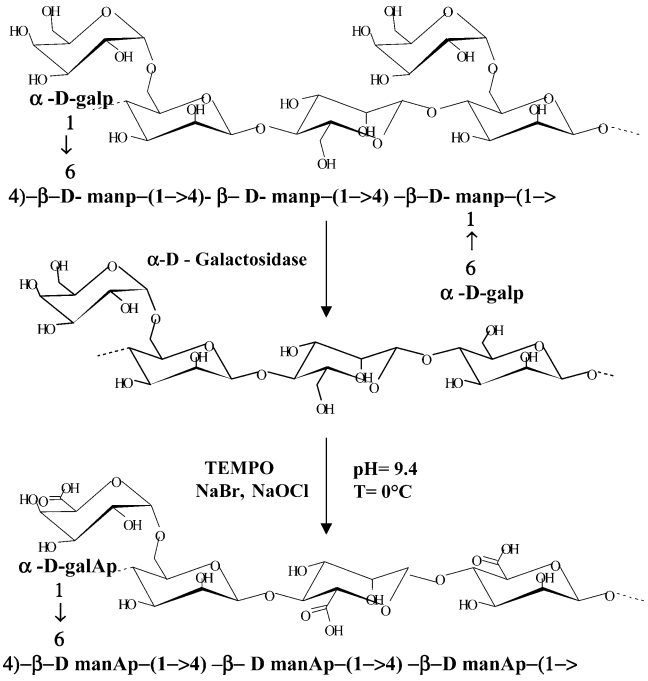
TOCSY and HMQC experiments were processed in the phase-sensitive mode (TPPI) with 512 × 512 data points, whereas COSY experiments were processed in the magnitude mode using 512 × 512 data points.

Chemical shifts of ^{1}H and ^{13}C spectra are reported in ppm with respect to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as an internal standard.

^{1}H and ^{1}H-detected DOSY experiments[16] were performed using a Bruker z-gradient probehead capable of producing gradients in the z direction with a strength of 55 G cm^{-1}. To avoid any overheating of the probehead and any possible convection effect, the diffusion experiments were recorded at 300 K. At this temperature, even though some resonances in the ^{1}H spectrum overlap, accurate diffusion coefficients can be measured. The stimulated echo sequence with bipolar gradients and a longitudinal eddy current delay was used.[17] Typically, the strength of the gradient pulses with a duration of 1.5 ms was logarithmically incremented in 32 steps, with a diffusion time of 550 ms and a longitudinal eddy current of 25 ms. After Fourier transformation and baseline correction, the diffusion dimension was processed by means of the DOSY subroutine of the Bruker Xwinnmr software package (version 3.5).

The deconvolution of the ^{1}H NMR spectra was performed using the SHAPE2000 (version 2.1) software package.[18] Applying the deconvolution program, the areas I_i of peaks resonating in the 4.4−5.1 ppm spectral region are obtained along with errors ΔI_i. It is worth to note that the obtained errors are always well within 4−5% of the nominal I_i values. Therefore, the error on the area of each resonance is

Scheme 1. Sketch (from top to bottom) of Native Guar Gum; Depleted Guar Gum; and Depleted and C(6)-Oxidized Guar Gum



satisfactory small. Since it is not possible to obtain errors on the calculated molar fractions, on the doublet and triplet frequencies, and on the average block lengths, straightway from the deconvolution program, the propagation error theory has been applied. However, as is well-known, this theory may overestimate the errors. As a consequence, errors reported in Table 7 are overestimated.

In our notation, gal = galactose unit; Gal = galacturonic acid unit; m = mannose unit; m* = branched mannose unit; M = mannuronic acid unit; G = guluronic acid unit.

3. Results

3.1. Guar Gum. Guar gum is a β-D-(1→4)-linked D-mannan with α-D-galactopyranosyl units attached as side groups, see Scheme 1A. The ^{1}H NMR spectrum of a sample of guar gum solubilized in a phosphate buffered D_2O solution at pD = 7, is shown in Figure 1a. Resonances belonging to the α-D-galactopyranosyl units (gal) are sharp and well resolved, whereas resonances due to the mannose (m) backbone appear rather broad.

The mannose/galactose ratio obtained integrating the resonances due to the anomeric protons of m (4.73 ppm) and gal units (5.02 ppm) is about 1.4 corresponding to an apparent percentage of galactopyranosyl units of ≈42%.

The ^{1}H spectral assignment, reported in Table 1, was obtained by means of ^{1}H−^{1}H COSY and ^{1}H−^{1}H TOCSY experiments (data not shown). In the same table, the assignment of the ^{13}C spectrum as obtained by means of an HMQC experiment is also reported, see Figure 2a.

It is worth noting that in the 2D experiments the cross-peaks due to mannose units (m*) branched to α-D-galactosyl units are well distinguishable from resonances due to non branched mannose units (m); in particular, the cross-peaks due to the methylenes m6 and m6′ are observed at 3.75 ppm

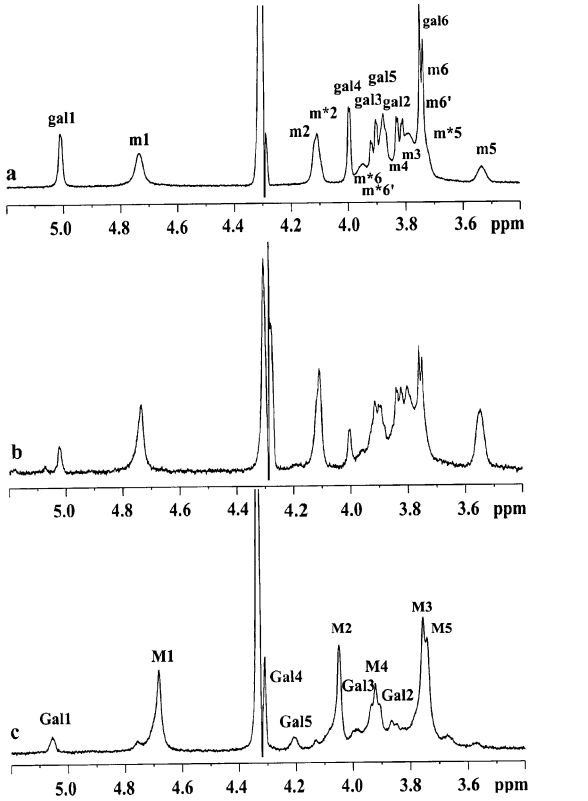


Figure 1. 600.13 MHz 1H NMR spectra in phosphate buffered solutions at 343 K of (a) guar gum, (b) depleted guar gum, (c) depleted and C(6)-oxidized guar gum.

Table 1. ^{1}H and ^{13}C NMR Assignments of Guar Gum in a Buffered Solution (pD = 7) at T = 343 K[a]

mannose residue			galactose residue		
	^{1}H	^{13}C		^{1}H	^{13}C
m1	4.73	102.1	gal1	5.02	100.8
m2	4.11	71.9	gal2	3.82	70.3
m*2	4.12	71.9			
m3	3.78	73.4	gal3	3.91	71.3
m4	3.85	78.8	gal4	4.00	71.3
m5	3.53	77.0	gal5	3.88	73.1
m*5	3.73	75.3			
m6, m6′	3.75	62.9	gal6, gal6′	3.75	62.9
m*6, m*6′	3.81	68.5			
	3.96				

[a] Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. ^{13}C NMR assignment has been obtained by means of 2D HMQC experiments.

in the ^{1}H dimension and at 62.9 ppm in the ^{13}C dimension, whereas the cross-peaks due to the methylenes m*6 and m*6′ are observed at 3.81 and 3.96 ppm in the ^{1}H dimension and at 68.5 ppm in the ^{13}C dimension, see Figure 2a.

Cross-peaks due to m5 and m*5 are observed at 3.53 and 3.73 ppm in the ^{1}H dimension and at 77.0 and 75.3 in the ^{13}C dimension. Cross-peaks due to m2 and m*2 are resolved in the ^{1}H dimension being respectively observed at 4.11 and 4.12 ppm, whereas a single cross-peak is observed at 71.9 ppm in the ^{13}C dimension.

3.2. α-D-Galactose Depleted Guar Gum. The presence of α-D-galactosyl side groups at O6 of some D-mannosyl residues impairs both the C(6)-oxidation and the C(5)-epimerization of the residues. In fact, possibly due to the

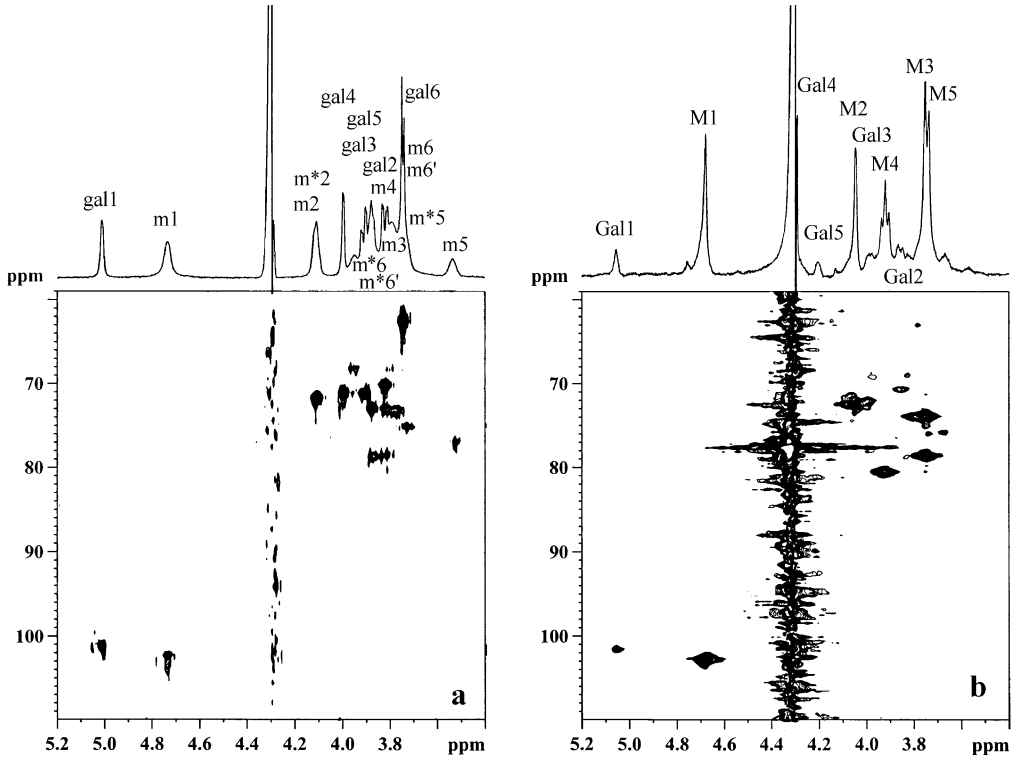


Figure 2. HMQC experiments at $T = 343$ K performed on (a) guar gum and (b) depleted and C(6)-oxidized guar gum.

steric hindrance exerted by the α-D-galactosyl side groups, the degree of epimerization obtained starting from a non-depleted Guar sample is rather low.

Guar gum was treated with α-galactosidase. The α-galactosidase splits off the α-D-galactosyl units to obtain a galactose-depleted guar gum with a galactosyl units content as low as possible, see Scheme 1B.

The ^{1}H spectrum of galactose-depleted guar gum is shown in Figure 1b. In this spectrum, all resonances due to the α-D-galactosyl units are much weaker than the corresponding ones shown in the spectrum of Figure 1a. The mannose/galactose ratio is about 8.6, with a percentage of galactosyl units reduced to $\approx$ 12%.

3.3. C(6)-Oxidized Guar Gum. The C(6)-oxidation mediated by the stable nitroxide free-radical TEMPO transforms mannose and galactose residues in the corresponding acids, namely, mannuronic and galacturonic acids, see Scheme 1C.

The ^{1}H spectrum of a depleted and C(6)-oxidized sample of guar gum is shown in Figure 1c, whereas the HMQC 2D map is reported in Figure 2b. Note that, due to the C(6)-oxidation, the cross-peaks due to methylene carbons belonging to m and gal units observed at 62.9 ppm are much weaker than the corresponding ones in the HMQC map of the native guar gum sample, see Figure 2a. Cross-peaks due to methylene carbons of m* units are still well observable at 68.5 ppm, see Figure 2b, since methylene groups of m* units do not undergo C(6)-oxidation.

The HMQC map of the C(6)-oxidized sample along with the ^{1}H−^{1}H COSY and ^{1}H−^{1}H TOCSY experiments, data not shown, allow both the proton and carbon resonances to be assigned to mannuronic acid units (M), see Table 2.

However, due to both the high degree of depletion and the high degree of oxidation, very weak cross-peaks due to Gal units and to residual m* units are observed in the HMQC map shown in Figure 2b, making the ^{13}C assignment of these residues rather uncertain.

Table 2. ^{1}H and ^{13}C NMR Assignments of C(6)-Oxidized Guar Gum in a Buffered Solution (pD = 7) at $T = 343$ K;[a] Sample with a High Degree of Depletion and a High Degree of Oxidation

mannuronic acid residue			galacturonic acid residue		
	^{1}H	^{13}C		^{1}H	^{13}C
M1	4.68	102.3	Gal1	5.05	101.0
M2	4.05	72.3	Gal2	3.86	70.5
M3	3.75	73.7	Gal3	3.98	71.8
M4	3.92	80.4	Gal4	4.32	72.9
M5	3.75	78.5	Gal5	4.21	74.5

[a] Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. ^{13}C NMR assignment has been obtained by means of 2D HMQC experiments.

Moreover, the proton resonance due to Gal4 underlies the HOD residual signal. Therefore, to confirm the assignment, we performed 1D and 2D experiments at $T = 333$ K on a purposely chosen sample with both a low degree of depletion and a rather low degree of oxidation. The HMQC 2D map performed on this sample shows intense cross-peaks due to Gal and m* residues. Moreover, at this temperature, the HOD residual signal shifts downfield allowing the clear observation of the resonance due to Gal4.

The HMQC map and the corresponding ^{1}H spectrum are shown in Figure 3, along with the resonances assignment. The full spectral assignment[19] of M, gal, and m* residues of this sample is reported in Table 3.

3.4. Determination of the Degree of Oxidation. The degree of oxidation can be quantitatively evaluated by integrating the ^{1}H spectrum of the oxidized sample; in fact, the degree of oxidation can be obtained integrating all resonances due to the anomeric protons with respect to all

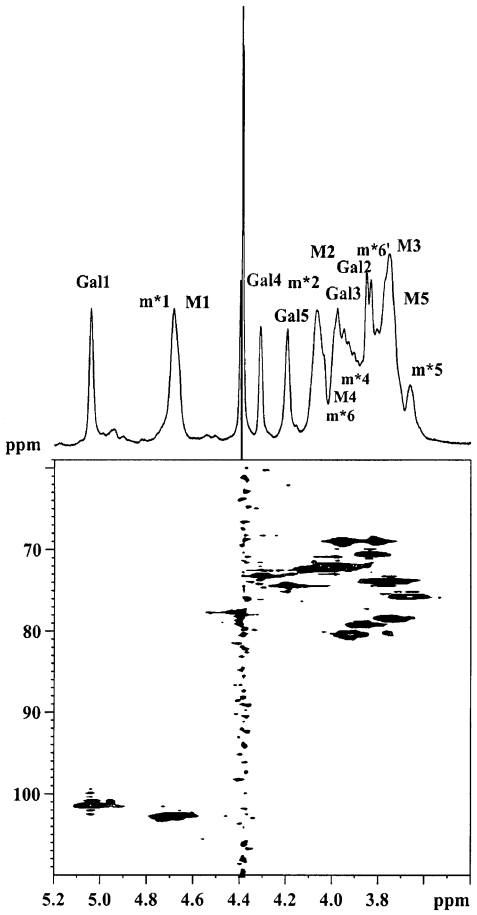


Figure 3. HMQC experiment performed at 333 K on a purposely chosen sample with both a low degree of depletion and a low degree of oxidation.

Table 3. ^{1}H and ^{13}C NMR Assignments of C(6)-Oxidized Guar Gum in a Buffered Solution (pD = 7) at $T = 333$ K;[a] Sample with a Low Degree of Depletion and a Low Degree of Oxidation

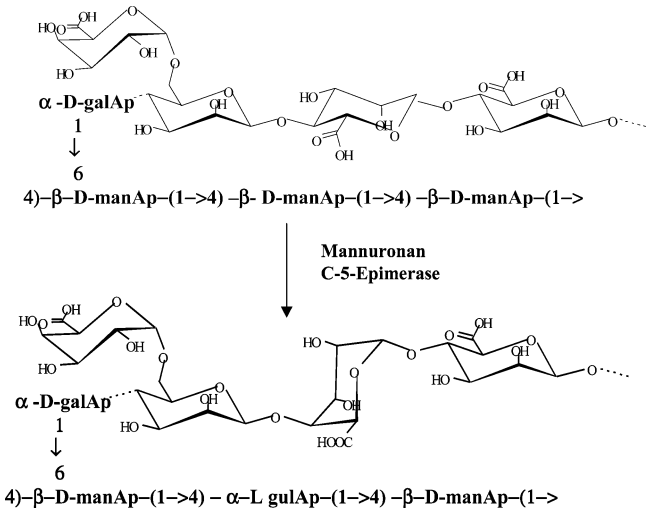
mannuronic acid residue			galacturonic acid residue			branched mannose residue		
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C
M1	4.66	102.3	Gal1	5.04	101.0	m*1	4.69	102.3
M2	4.03	72.1	Gal2	3.84	70.4	m*2	4.07	72.1
M3	3.76	73.7	Gal3	3.99	71.6	m*3		73.7
M4	3.93	80.1	Gal4	4.31	73.0	m*4	3.87	78.9
M5	3.75	78.2	Gal5	4.19	74.2	m*5	3.66	75.5
						m*6, m*6′	3.96	68.8
							3.82	

[a] Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. ^{13}C NMR assignment has been obtained by means of 2D HMQC experiments.

other resonances due to non anomeric protons. In this case, the ^{1}H spectrum was performed at 333 K since, at this temperature, no protons underlie the HOD signal.

In a non oxidized sample, seven non exchangeable protons belong to each monomeric unit; in this case, if the area of the resonances due to the anomeric protons is equal to 1, the integral of all signals due to the nonanomeric protons is equal to 6. In a fully oxidized sample the integral of all resonances due to all non anomeric protons must be equal to 4. In the case of the C(6)-oxidized sample shown in Figure 1c, the value of the integral of the non anomeric protons is 4.4. As a consequence, the percentage of oxidation of the sample with both a high degree of oxidation and a high degree of depletion is about 81%.

Scheme 2. Sketch (from top to bottom) of Depleted, C(6)-oxidized Guar; depleted, C(6)-oxidized, C(5)-epimerized Guar



3.5. C(5)-Epimerized−C(6)-Oxidized Guar Gum. NMR is useful in monitoring the enzymatic epimerization process. In fact, it allows us to quantitatively establish the transformation of D-mannuronic acid into L-guluronic acid residues obtaining the M and G residues distribution in terms of diads and triads and the average G-block length $N_{G>1}$.

The mannuronan C(5)-epimerases introduce guluronic acid (G) residues in the polysaccharidic chain by C(5)-epimerization of some unbranched mannuronic acid (M) residues, see Scheme 2B. Depending on the specific epimerase, the epimerized products may differ significantly both in the content and in the distribution of G residues.

Galactose-depleted C(6)-oxidized guar gum samples were treated with different epimerases AlgE1, AlgE4, and AlgE6 and their mixtures to obtain the corresponding uronans namely DOGuarE1, DOGuarE4, DOGuarE6, DOGuarE1+E6, DOGuarE4+E6, and DOGuarE1+E4+E6, with a various content and distribution of G residues. As previously mentioned, AlgE4 forms alginates with MG blocks, whereas AlgE6 preferentially introduces stretches of G blocks into the polymer, and AlgE1 makes stretches of MG and G blocks.[1−7]

For characterizing the epimerized compounds obtained from guar gum, 1D and 2D NMR experiments have been performed.

The ^{1}H NMR spectrum of a sample of guar epimerized with AlgE4 in a phosphate buffered solution is shown in Figure 4a.

The C(5)-epimerization induces a ring inversion; in fact, the axial proton of C(1) of some M units becomes equatorial, the equatorial proton on C(2) becomes axial, and so on.

The assignment of the NMR signals due to diads and triads has been performed according to the results obtained in the case of alginates.[20,21] The full spin system due to the MGM triad is well observable, see Table 4. The ^{1}H spectrum shows diads sensitivity; in fact, the resonance at 4.68 ppm due to the anomeric proton of M units having an M unit as a first neighbor, *M*-1M, is well distinguishable from the resonance

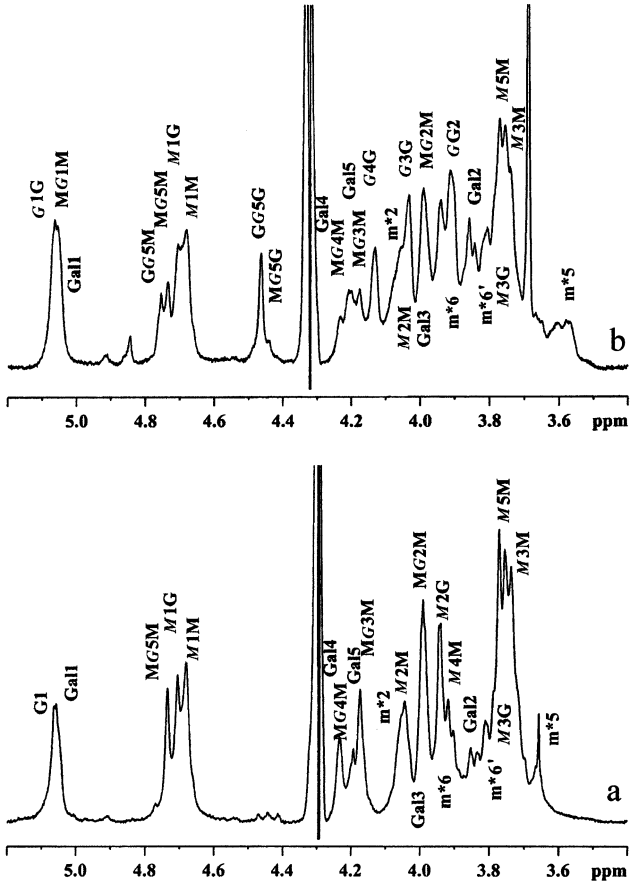


Figure 4. 600.13 ^{1}H NMR spectra in phosphate buffered solutions at 343 K of (a) a sample epimerized with AlgE4 (DOGuarE4) and (b) a sample epimerized with an AlgE4 + E6 mixture (DOGuarE4 + E6). The resonance assignments are also reported.

at 4.71 ppm due to the anomeric proton of M units having a G unit as a first neighbor, *M*1G. Moreover, *M*2M and *M*2G diads are well distinguishable being at 4.05 and 3.95 ppm, respectively. Resonances due to Gal residues as well as those due to m* residues are also observable, see Table 4.

The ^{13}C spectral assignment of DOGuarE4 has been performed using an HMQC 2D map, see Figure 5a. It is worth noting that the diads sensitivity is observed on the anomeric carbon of M units having an M unit or a G unit as a first neighbor. Again, the diads sensitivity is clearly observed, see Table 4.

Since AlgE4 introduces only MG blocks into the polysaccharidic chain, spectra of samples epimerized with AlgE4 show a minor multiplicity with respect to the spectra of samples epimerized with the other enzymes or their mixtures. In the latter case, in fact, resonances due to diads and triads of G blocks also appear in the spectrum.

The ^{1}H spectrum of a sample of DOGuarE4+E6 solubilized in a phosphate buffered solution is shown in Figure 4b, and the assignment is reported in Table 5. The spectrum shows the presence of both G blocks and stretches of MG blocks. In fact, besides the resonances due to the M*G*M triad, the *G*G diad is also well observable, being *G*1G at 5.06 ppm, *G*2G at 3.91 ppm, *G*3G at 4.03 ppm, *G*4G at 4.13 ppm, and *G*5G at 4.46 ppm. Again, diads sensitivity is also observed in the case of *M*1M and *M*1G diads observed at 4.68 and 4.71 ppm, respectively and in the case of *M*2M and *M*2G diads observed at 4.05 and 3.95 ppm, respectively.

The assignment of residual galacturonic acid units and branched mannose units is also reported in Table 5.

The ^{13}C spectral assignment has been performed using an HMQC 2D map; the ^{13}C spectrum shows diads and triads sensitivity, see Table 5. Note that, since the ^{1}H resonance due to Gal4 lies near to the residual HOD signal, its chemical shift can only be assigned by performing an HMQC experiment.

We also performed 1D and 2D experiments on a DOGuarE4+E6 sample solubilized in un-buffered D_2O. In this case, the residual HOD signal is slightly shifted downfield allowing the straightforward assignment of the ^{1}H chemical shift of Gal4. Moreover, in D_2O, the resolution in the ^{13}C dimension allows the ^{13}C chemical shift of both M*G*5G and G*G*5M triads to be assigned, whereas in the case of the sample solubilized in a phosphate buffered solution, the ^{13}C chemical shifts of the G*G*5G and M*G*5G triads overlap. As a consequence, we also give the ^{1}H and ^{13}C assignment of DOGuarE4+E6 in D_2O, see Table 6. The HMQC experiment is shown in Figure 5b along with the ^{1}H NMR spectrum.

3.6. Spectral Deconvolution. The presence of resonances due to G*G*5M, M*G*5M, G*G*5G, and M*G*5G triads and to *G*5G, *M*1M, and *M*1G diads allows a quantitative measurement of the enzymatic activity. However, due to the overlapping of ^{1}H resonances in the spectra of epimerized guar gum samples, to evaluate the signal areas, a full spectral deconvolution is mandatory.

Table 4. ^{1}H and ^{13}C NMR Assignments of C(6)-Oxidized and C(5)-Epimerized Guar Gum in a Buffered Solution (pD = 7) at T = 343 K;[a] Epimerization Performed with AlgE4 C(5)-Epimerase

mannuronic acid residue			guluronic acid residue			galacturonic acid residue			branched mannose residue		
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C
*M*1M	4.68	102.3	M*G*1M	5.07	101.8	Gal1	5.05	101.1	m*1	4.69	
*M*1G	4.71	103.4									
*M*2M	4.05	72.3	M*G*2M	3.99	67.1	Gal2	3.86	70.5	m*2	4.06	
*M*2G	3.95	72.9									
M3	3.73	73.8	M*G*3M	4.17	71.6	Gal3	3.99	71.8	m*3	3.75	
			M*G*4M	4.23	82.1	Gal4	4.32	73.1	m*4	3.88	79.1
*M*4M	3.92	80.4									
*M*5	3.76	78.3	M*G*5M	4.73	69.1	Gal5	4.20	74.3	m*5	3.67	75.6
									m*6, m*6′	3.97	68.9

[a] Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. ^{13}C NMR assignment has been obtained by means of 2D HMQC experiments.

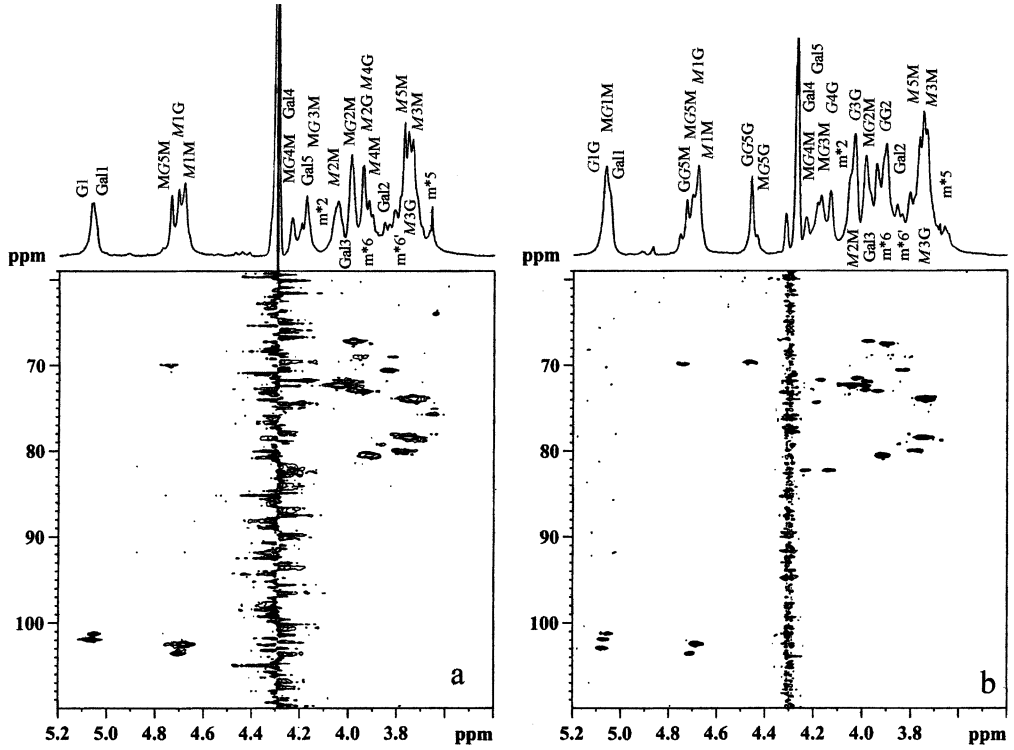


Figure 5. HMQC experiments at $T = 343$ K performed on (a) a sample epimerized with AlgE4 (DOGuarE4) and solubilized in a phosphate buffered solution (pD = 7) and (b) a sample epimerized with an AlgE4 + E6 mixture (DOGuarE4 + E6) and solubilized in D_2O (b).

Table 5. ^{1}H and ^{13}C NMR Assignments of C(6)-Oxidized and C(5)-Epimerized Guar in a Buffered Solution (pD = 7) at $T = 343$ K;[a] Epimerization Performed with the AlgE4 + E6 Mixture

mannuronic acid residue			guluronic acid residue			galacturonic acid residue			branched mannose residue		
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C
*M*1M	4.68	102.2	M*G*1M	5.05	102.8	Gal1	5.04	101.1	m*1	4.76	102.2
*M*1G	4.71	103.4	*G*1G	5.06	102.8						
*M*2M	4.05	72.1	M*G*2M	3.99	67.0	Gal2	3.85	70.46	m*2	4.13	72.1
*M*2G	3.95	72.1	*G*2G	3.91	67.3						
*M*3M	3.76	73.6	M*G*3M	4.17	71.6	Gal3	3.99	71.9	m*3		
			*G*3G	4.03	71.3						
*M*4M	3.93	80.3	M*G*4M	4.23	82.2	Gal4	4.32	73.0	m*4	3.84	
			*G*4G	4.13	82.1						
*M*5M	3.75	78.3	M*G*5M	4.73	69.7	Gal5	4.20	74.2	m*5	3.57	77.3
			G*G*5G	4.46	69.4						
			M*G*5G	4.44	69.4				m*6, m*6′	3.83	68.9
			G*G*5M	4.76	69.4					3.97	

[a] Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. ^{13}C NMR assignment has been obtained by means of 2D HMQC experiments.

A full deconvolution of the 4.4−5.1 ppm region of the ^{1}H spectrum of all epimerized derivatives has been performed. From the deconvolution, the area of all signals resonating in this range of frequency has been obtained.

In Figure 6, the 4.4−5.1 ppm region of ^{1}H spectra of DOGuarE1 (a), DOGuarE4 (b), DOGuarE6 (c), DOGuarE1+E4+E6 (d), DOGuaE4+E6 (e), and DOGuarE1+E6 (f) are shown; the deconvoluted stectra have been superimposed on the experimental ones.

Since AlgE4 introduces MG blocks in the polysaccharidic chain, the resonance due to M*G*5M triads is observed, see Figure 4a; the mole fraction of L-guluronic acid residues can be calculated according to $F_G = I_G/I_{TOT}$, and the mole fraction of mannuronic acid residues can be calculated according to[20,21] $F_M = I_M/I_{TOT}$, being $I_G = I_{MG5M}$; $I_M = I_{M1M} + I_{M1G}$; $I_{TOT} = I_M + I_G$ and I = area of the resonance as obtained from the deconvolution procedure.

In the case of Guar epimerized with AlgE1, AlgE6, AlgE4 + E6, AlgE1 + AlgE6, and AlgE1 + E4 + E6, also G blocks occur in the polysaccharidic chain. Therefore, besides the resonance due to M*G*5M triads, also resonances due to G*G*5G, G*G*5M, and M*G*5G triads are observed, see Figure 4b. Under these circumstances, the area due to resonances of L-guluronic acid residues is $I_G = I_{MG5M} + I_{GG5M} + I_{GG5G} + I_{MG5G}$. Using this I_G value, the mole fraction of L-guluronic acid residues can be calculated according to $F_G = I_G/I_{TOT}$.

The three doublet frequencies F_{G5G}, F_{M1G}, and F_{M1M} and the four triplet frequencies F_{GG5M}, F_{MG5M}, F_{GG5G}, and F_{MG5G} can be calculated, being $F_{M1M} = I_{M1M}/I_{TOT}$; $F_{M1G} =$

Table 6. ^{1}H and ^{13}C NMR Assignments of C(6)-Oxidized and C(5)-Epimerized Guar in D_2O at $T = 343$ K;[a] Epimerization Performed with the AlgE4 + E6 Mixture

mannuronic acid residue			guluronic acid residue			galacturonic acid residue		
	^{1}H	^{13}C		^{1}H	^{13}C		^{1}H	^{13}C
*M*1M	4.68	102.3	M*G*1M	5.06	101.8	Gal1	5.05	101.1
*M*1G	4.70	103.4	*G*1G	5.07	102.8			
*M*2M	4.05	72.2	M*G*2M	3.98	67.1	Gal2	3.84	70.5
*M*2G	3.94	73.0	*G*2G	3.92	67.4			
*M*3M	3.74	73.8	M*G*3M	4.18	71.6	Gal3	4.00	72.3
			*G*3G	4.04	71.4			
*M*4M	3.92	80.5	M*G*4M	4.24	82.1	Gal4	4.32	73.1
			*G*4G	4.14	82.1			
*M*5M	3.75	78.3	M*G*5M	4.74	69.8	Gal5	4.19	74.3
			G*G*5G	4.47	69.5			
			M*G*5G	4.44	69.6			
			G*G*5M	4.76	69.7			

[a] Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. ^{13}C NMR assignment has been obtained by means of 2D HMQC experiments.

I_{M1G}/I_{TOT}; $F_{G5G} = I_{G5G}/I_{TOT}$ and $F_{MG5M} = I_{MG5M}/I_{TOT}$; $F_{GG5M} = I_{GG5M}/I_{TOT}$ $F_{GG5G} = I_{GG5G}/I_{TOT}$; $F_{MG5G} = I_{MG5G}/I_{TOT}$.

The average length of G blocks has also been calculated, being $N_{G>1} = (F_G - F_{MG5M})/F_{GG5M}$.

All data are reported in Table 7.

The highest mole fraction of L-guluronic acid residues is obtained epimerizing with AlgE1, being $F_G = 0.58$, whereas the lowest value is obtained epimerizing with AlgE4 being $F_G = 0.24$. The average block length $N_{G>1}$ ranges between 4.5 and 7.0, with the lowest value obtained epimerizing with the AlgE1 + AlgE4 + AlgE6 mixture ($N_{G>1} = 4.5$) and the highest value obtained epimerizing with the AlgE4 + AlgE6 mixture ($N_{G>1} \cong 7.0$).

3.7. Evaluation of Degradation in Epimerized Guar Gum Samples Using NMR Diffusion Measurements. As known from the literature,[22] molecular self-diffusion can be encoded into NMR datasets by means of pulsed-gradients of magnetic field (PFG-NMR). Diffusion-ordered NMR spectroscopy (DOSY)[16] is a particularly convenient way of displaying the molecular self-diffusion information, organized in a bidimensional array with the NMR spectrum on one dimension and the self-diffusion coefficient on the other one. DOSY has been used for the analysis of a variety of mixtures,[23] for the characterization of aggregates,[24] for the study of intermolecular interactions,[25,26] for the optimization of the dialysis process of hyaluronic acid derivatives,[27] and for the molecular weight determination of uncharged polysaccharides.[28]

As is well-known, an epimerization reaction can alter the polysaccharidic chain and lower the molecular weight of the polysaccharide, thereby compromising its physical properties. In this paper, we have used NMR diffusion measurements in order to check on the possible occurrence of degradation of the polysaccharidic chain due to the epimerization reaction.

The 2D maps of the DOSY experiments recorded on the C(6)-oxidized guar gum sample before and after epimerization with AlgE4+AlgE6 (DOGuarE4+E6) are shown in Figure 7, parts A and B, respectively.

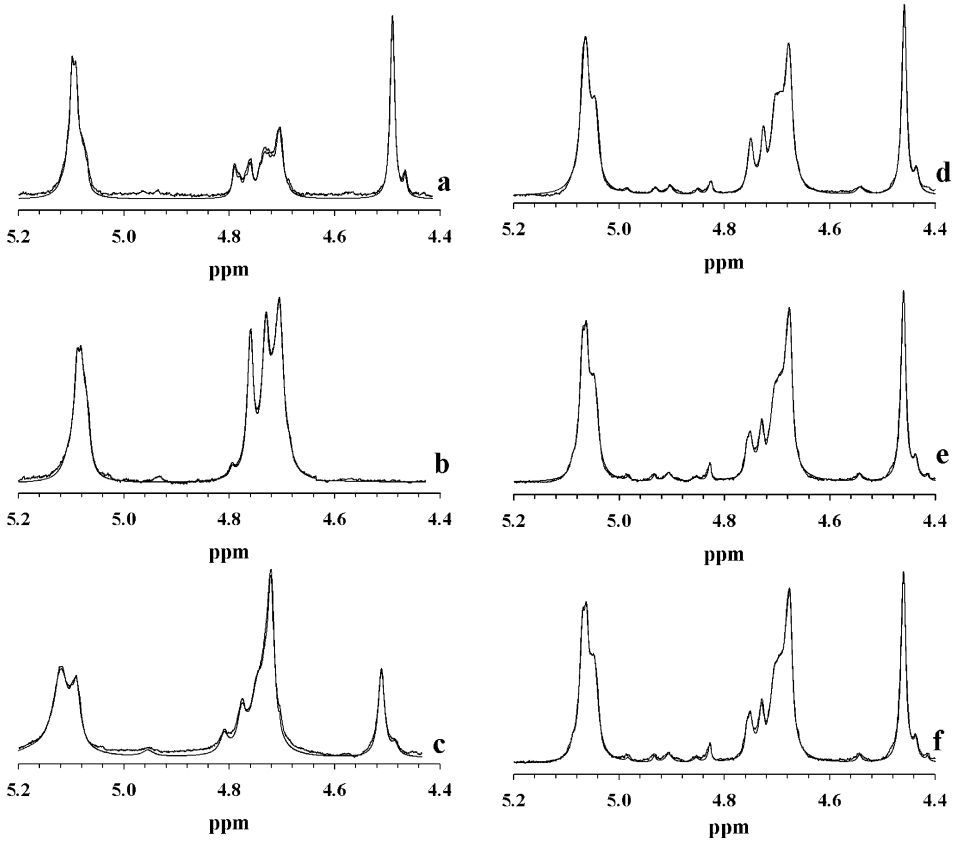


Figure 6. Expansion of the 4.4−5.1 ppm region of the ^{1}H NMR spectra of depleted, C(6)-oxidized, C(5)-epimerized guar samples epimerized with various enzymes: DOGuarE1 (a); DOGuarE4 (b); DOGuarE6 (c); DOGuarE1 + E4 + E6 (d); DOGuarE4 + E6 (e); DOGuarE1 + E6 (f). The deconvoluted spectra have been superimposed on the experimental ones.

Table 7. Mannuronic and Guluronic Acid Molar Fractions (F_M, F_G), Doublet Frequencies (F_{M1M}, F_{M1G}, F_{G5G}), Triplet Frequencies (F_{MG5M}, F_{GG5M}, F_{GG5G}, F_{MG5G}), and Average G-Blocks Length N_G

sample	F_M	F_G	F_{M1M}	F_{M1G}	F_{G5G}
DOGuarE6	0.66 ± 0.04	0.34 ± 0.02	0.38 ± 0.02	0.28 ± 0.02	0.20 ± 0.01
DOGuarE4	0.76 ± 0.04	0.24 ± 0.01	0.45 ± 0.02	0.31 ± 0.01	
DOGuarE4 + E6	0.55 ± 0.03	0.45 ± 0.02	0.41 ± 0.02	0.14 ± 0.01	0.26 ± 0.01
DOGuarE1	0.42 ± 0.02	0.58 ± 0.03	0.27 ± 0.01	0.152 ± 0.007	0.41 ± 0.02
DOGuarE1 + E6	0.57 ± 0.03	0.43 ± 0.02	0.25 ± 0.01	0.32 ± 0.02	0.29 ± 0.01
DOGuarE1 + E4 + E6	0.55 ± 0.03	0.46 ± 0.02	0.29 ± 0.01	0.26 ± 0.01	0.29 ± 0.01

sample	F_{MG5M}	F_{GG5M}	F_{GG5G}	F_{MG5G}	N_G
DOGuarE6	0.096 ± 0.006	0.049 ± 0.003	0.18 ± 0.01	0.021 ± 0.001	5.0 ± 0.3
DOGuarE4	0.24 ± 0.01				
DOGuarE4 + E6	0.145 ± 0.007	0.044 ± 0.002	0.24 ± 0.01	0.020 ± 0.001	7.0 ± 0.3
DOGuarE1	0.086 ± 0.004	0.082 ± 0.004	0.39 ± 0.02	0.026 ± 0.001	6.0 ± 0.4
DOGuarE1 + E6	0.062 ± 0.003	0.078 ± 0.004	0.26 ± 0.01	0.021 ± 0.001	4.7 ± 0.2
DOGuarE1 + E4 + E6	0.077 ± 0.004	0.084 ± 0.004	0.27 ± 0.01	0.026 ± 0.001	4.5 ± 0.2

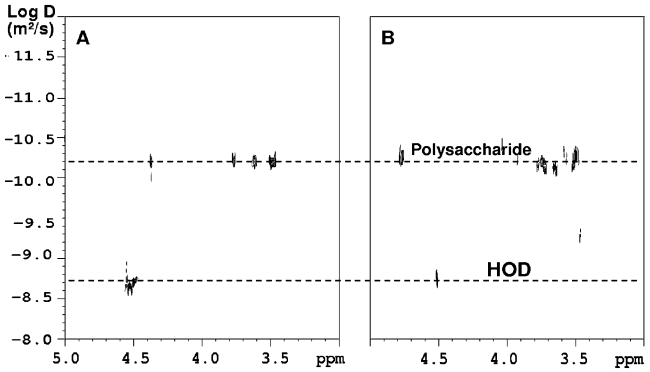


Figure 7. ^{1}H detected 2D DOSY of (A) depleted, C(6)-oxidized guar and (B) depleted, C(6)-oxidized, C(5) epimerized guar sample (DOGuarE4 + E6).

These 2D maps show clearly that the self-diffusion coefficient of the polysaccharide before and after epimerization remains the same within experimental errors, being $6.6 \pm 0.2 \times 10^{-11}$ m^2 s^{-1} and $6.4 \pm 0.2 \times 10^{-11}$ m^2 s^{-1}, respectively. It is worth noting that, due to the presence of charges in the polysaccharidic chains, at pD = 7, the water is a good solvent for both the C(6)-oxidized and the C(6)-oxidized, C(5)-epimerized guar samples. Therefore, provided that the viscosity of both solutions is the same, the diffusion coefficient measured in the oxidized guar and in the epimerized derivative solutions is tightly related to the molecular size of the measured samples; thus, any variation observed in the diffusion coefficient measured before and after the epimerization must reflect a change in the molecular size of the polysaccharides which, in turn, implies a variation in their molecular weight.

In our case, no significant difference can be observed in the diffusion coefficient of the polysaccharide in both solutions. Therefore, NMR diffusion experiments allow us to state safely that no significant degradation, i.e., lowering of the molecular weight, occurs during the epimerization reaction.

4. Conclusions

By combining C(6)-regioselective chemical oxidation with enzymatic modification using α-D-galactosidase followed by different mannuronan C(5)-epimerases, we have obtained a new family of polyuronides with an alginate like backbone substituted randomly on the ManA residues with GalA.

The C(6)-oxidized and galactose depleted guar gum acts as a substrate for all of the three different epimerases. Although the degree of epimerisation is lower than the one observed with pure mannuronan or alginates,[3−6] the nonrandom epimerization pattern which has been attributed to their processive mode of action[2−7] is basically the same; AlgE4 introduces only single G residues, whereas AlgE1 and AlgE6 also form G-blocks of various length.

The highest conversion (58% G) was obtained with the single enzyme AlgE1. This is a bifunctional enzyme comprising two separate catalytic modules in the same polypeptide chain,[2] one with the capacity to introduce single G, similar to AlgE4, and the other one with preference for G block formation. This enzyme can also attack an M flanked by two G residues, thus converting alternating sequences into G-blocks. A similar but slightly lower epimerisation was obtained by combining AlgE1 and AlgE6 ($F_G = 0.43$); AlgE1, AlgE4, and AlgE6 ($F_G = 0.46$); and AlgE4 and AlgE6 ($F_G = 0.45$). The latter enzyme is characterized by forming very long G-blocks in alginates either by stepwise elongation of existing blocks or by block condensation (conversion of an M flanked by two G). In the latter case, alternating sequences can effectively be converted into blocks which is reflected by the high G-block length ($N_{G>1}$) of this sample.

Although all of the enzymes acted on oxidized guar gum, a significant fraction of the M residues were apparently not accessible for epimerization. In the case of AlgE4, taking into account that only every second M can be attached, less than 50% of the M residues are epimerized. The content of galacturonic acid of 12% indicates that not only the substituted residues but probably also flanking residues are protected. As a processive enzyme, AlgE4 slides along the polymer chain carrying out many epimerizations before the enzyme polymer complex dissociates. Processive enzymes recognize more than one residue and normally bind to a multitude of adjacent sites distributed along the polymer substrate.[29] This could explain why even low degrees of substitution can impair the enzyme substrate interaction.

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