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Enzymatic modification of alginates with the mannuronan C-5 epimerase AlgE4 enhances their solubility at low pH

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Abstract

The recent availability of mannuronan C-5 epimerase from *Azotobacter vinelandii* opens up for modifying the sequential structure of alginates in a controlled way. To investigate the effect of an increased amount of alternating sequences on the acid solubility of alginate, different alginates from *Durvillea antarctica*, *Lessonia nigrescens*, *Laminaria hyperborea* and a bacterial mannuronan were epimerized using AlgE4. This enzyme is produced recombinantly in *Escherichia coli* and converts the M blocks into MGM sequences leaving the G-blocks intact. The solubility of the modified alginates was investigated as a function of pH and time.

Keywords: Alginate; C-5 epimerase; Acid solubility

1. Introduction

Alginates are glycuronans extracted from seaweeds or produced by some bacteria. The molecules are linear chains of (1–4)-linked residues of β-D-mannuronic acid (M) and α-L-guluronic acid (G) in different proportion and sequential arrangements. The most common arrangement is that of a block copolymer, in which long, homo-polymeric sequences of ManA residues ('MM-bloc-ks') and similar sequences of GulA residues ('GG'-blocks) are interspersed between sequences of mixed composition ('MG-blocks'). They are highly soluble in water and form gels with cations and the gel-forming capacity correlates with the content and length of the G-blocks.

The pH of the solvent is one essential parameter determining and limiting the solubility of alginates in water because it will determine the presence of electrostatic charges on the uronic acid residues. Earlier experimental and theoretical studies on the solubility of alginate at low pH indicated a complex relation between the chemical composition and monomer sequence of alginate, and the resulting polymer properties, such as chain-extension, conformation in solution, and ion-exchange properties (binding of counter-ions). These factors in turn affect the solubility (acid dissociation constants, intrinsic pK_a), and the gel-forming ability of the particular alginate sample. Additionally, the pH of precipitation was shown to vary for molecules with different molecular weight, and the overall solubility is dependent on the molecular weight distribution of the sample. Other, better controllable factors affecting the solubility are, besides the pH, the ionic strength of the solvent and the type of salt, temperature, and alginate concentration (Cesáro, Delben, & Paoletti, 1990; Haug, 1964; Haug & Larsen, 1963; Myklestad & Haug, 1966).

The solubility of alginate at a given pH can be studied by two, basically different, experimental approaches: either by addition of acid to a neutral alginate solution, leading to precipitation of the insoluble fraction as alginic acid, or by addition of NaOH to an acidic suspension of alginic acid, leading to neutralisation and dissolution of soluble sodium alginate. Haug (1964) used the latter approach to measure titration curves and determine pK_a values for different alginates. As expected from the pK_a values of the monomers (3.38 for ManA and 3.65 for GulA), the polymer pK_a was found to increase with increasing GulA-content. The results reported by Haug and Larsen (1963) on acid solubility of alginate upon addition of acid, were in accordance with the titration curves, i.e. a high-G alginate from *Laminaria hyperborea* stipes precipitated around pH 3.25, while alginates with a lower GulA-content required a lower pH to precipitate. Alginate from *Ascophyllum nodosum*, with a high content of alternating MG-sequences, was found to have the highest solubility at low

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pH. The alginate samples used in these studies, however, were isolated from different seaweed species, and since the molecular weight distributions were not characterised and may vary between the species, the results are not directly comparable. By treating industrial grade alginate samples with the recombinantly produced epimerase AlgE4, we produced samples that mainly differ in the content of alternating MG-sequences, while the other properties, especially the content and length of G-blocks and molecular weight, were largely left unchanged.

2. Experimental

2.1. Materials

Industrial-grade alginates from the brown algae *Durvillea antarctica* (Protanal LF 120, No. 645034) and *Lessonia nigrescens* (Protanal LF 120, No. 907788) and L. *hyperborea* stipes (LF10/60) were provided by FMC Biopolymer AS, Drammen, Norway. Mannuronan was produced from an epimerase-negative mutant ($AlgG^-$) of *Pseudomonas fluorescence* (Gimmestad, Sletta, Ertesvåg, Bakkevig, Jain, & Skjåk-Bræk, 2003), grown on agar plates, and was deacetylated by mild alkaline hydrolysis as described earlier (Ertesvåg & Skjåk-Bræk, 1999). The mannuronan C-5 epimerases AlgE4 with molecular mass of 57.7 kDa was produced by fermentation of the recombinant *E. coli* strains JM 105 (Campa, Holtan, Nilsen, Bjerkan, Stokke & Skjåk-Bræk, 2004; Høydal, Ertesvåg, Stokke, Skjåk-Bræk., & Valla, 1999). The enzyme was partly purified by ion-exchange chromatography on Q-Sepharose FF (Pharmacia, Uppsala, Sweden) and by hydrophobic-interaction chromatography on Phenyl Sepharose FF (Pharmacia). The activity of the enzyme was assayed by measuring the release of tritium to water, when ^{3}H-5-labeled mannuronan was incubated with the enzymes. (Ertesvåg & Skjåk-Bræk, 1999). The mannuronan and the other alginate samples were epimerised with AlgE4, as described by (Ertesvåg & Skjåk-Bræk, 1999) using 5 mg enzyme/g of alginate in 25 mM MOPS, pH 6.9 and 2 mM $CaCl_2$.

The change in composition was determined by ^{1}H and ^{13}C NMR (Grasdalen, Larsen, & Smidsrød, 1979; 1981; Hartmann, Holm, Johansen, Skjåk-Bræk, Stokke, 2002) spectroscopy and the intrinsic viscosity of the aqueous polymer solutions was determined by a Schott-Geräte apparatus with automatic dilution, using an Ubbelohde capillary ($\varnothing = 0.53$ mm) at 25 °C and an added salt concentration of 0.1 M NaCl.

2.2. Solubility measurements

A solution of 10 mg/ml alginate in 0.1 M NaCl was distributed in 1-ml portions into microcentrifuge tubes. Different volumes (0–1 ml) of 0.1 M NaCl were added, followed by addition of 0.1 M HCl (1–0 ml) to obtain a total sample volume of 2.0 ml, resulting in a 5 mg/ml alginate solution with an ionic strength of 0.1 M. To reach pH values below 1.5, HCl-concentrations of 0.2, 0.5, and 1 M were used, and the ionic strength was not corrected. Preliminary experiments with non-epimerized alginate from *L. nigrescens* showed no increase of precipitation after 20 min (data not shown). We therefore decided to leave the samples undisturbed at room temperature (22 °C) for 30 min only, to avoid possible hydrolysis, which would result in polymer degradation and an increase in soluble material. After 30 min, pH was measured, and the samples were centrifuged for 20 min at 23,000*g*. The alginate concentration in the supernatant was determined in triplicate by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.3. Gelling kinetics

For this study, mannuronan from *P. fluorescens* fully epimerized with AlgE4 was used. HCl (0.1 M) was added to the alginate solutions to reach pH 1.3, yielding alginate concentrations of 10 and 1.33 mg/ml. After mixing with the acid, each solution was immediately transferred to the rheometer and the oscillation measurements were started within 1 min. The experiments were performed on a stress-controlled Reologica Stress-Tech rheometer (Reologica Instruments AB, Lund, Sweden), with a serrated plate-plate measuring geometry ($d = 40$ mm), gap $= 1.00$ mm, with $T =$ 20 °C, and a frequency of 1 Hz. Shear stresses were 3.00 Pa at 10 mg/ml alginate concentration and 0.03 Pa at 1.33 mg/ml alginate. G' and G'' were recorded every 100 s for approximately 70 h. A low viscosity (10 mPa) silicon oil was used to seal the sample to avoid evaporation during long-time measurements. Stress and frequency sweeps were recorded to check that the measurements were performed in the linear visco-elastic region of the gel.

3. Results and discussion

3.1. Epimerization

The mannuronan C-5 epimerase AlgE4 acts by converting mannuronic acid into G residues in the polymer chain as illustrated in Fig. 1. NMR analysis demonstrated that all

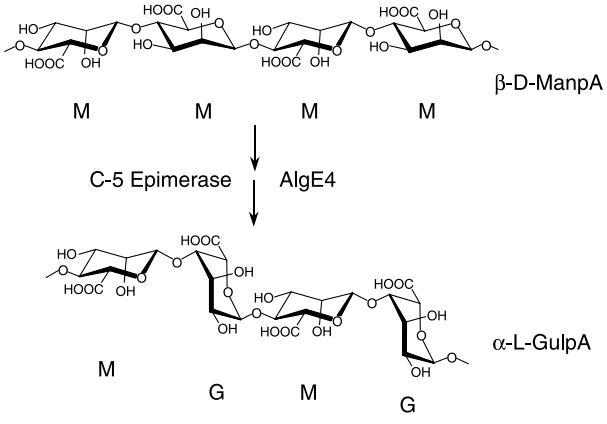


Fig. 1. Mannuronan segment (a) and the change in structure following epimerisation with AlgE4.

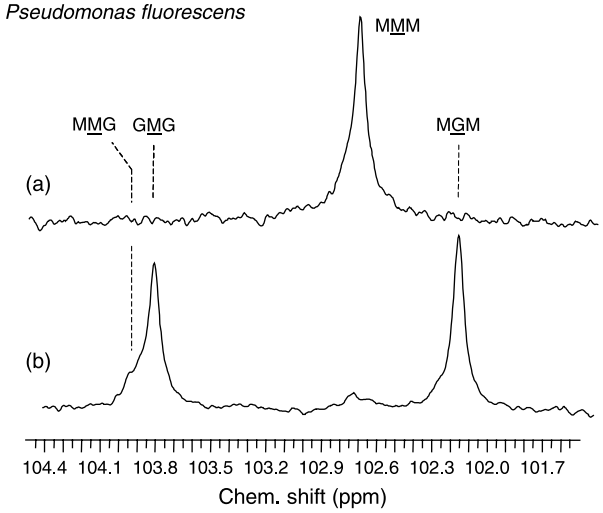
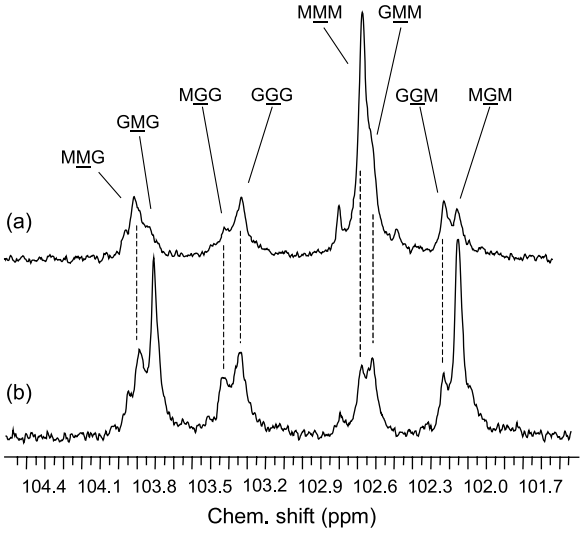


Fig. 2. Anomeric region of the ^{13}C NMR spectra (75 MHz) of mannuronan (a) native, and (b) epimerised with AlgE4:



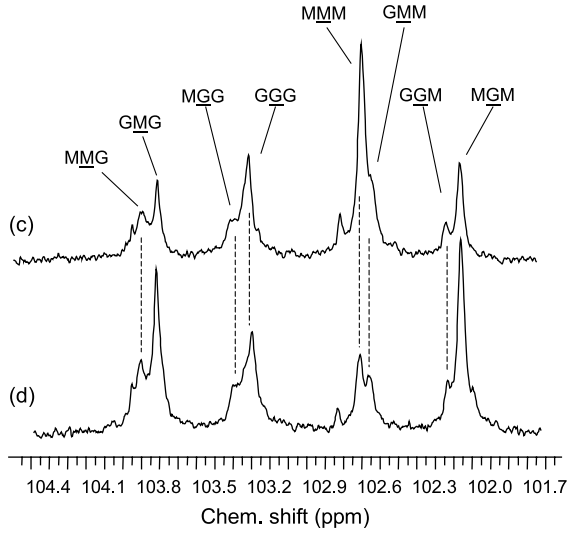


Fig. 3. Anomeric region of the ^{13}C NMR spectra (125 MHz) of seaweed alginates (a) and (b) native and epimerised alginate from *Durvillea anatarctica,* respectively, (c) and (d) native and epimerises alginate from *Lessonia nigrescens*, respectively.

the samples were highly epimerized after enzymes treatment. The anomeric regions of the ^{13}C NMR spectra are shown in Figs. 2 and 3, and the calculated chemical composition and sequential parameters of the samples are shown in Table 1. For all samples the epimerisation led mainly to an increment in F_{MGM} at the expense of the MMM sequences as is visualised in the NMR spectra (Figs. 2 and 3). The G-blocks represented by F_{GG} and F_{GGG} remained almost constant even at high degrees of conversion. This is clearly demonstrated for the mannuronan, which is converted into a poly-alternating structure (F_G=0.47; F_{GG}=0, Fig. 2) and thus virtually represents, in addition to mannuronan itself, a compositionally homogeneous alginate with MG as repeating unit.

All of the epimerized polymers display a slight decrease in intrinsic viscosity with respect to the starting materials (see Table 1). Whether this is due to a slight degradation or results from a less extended modified polymer following the introduction of the more flexible alternating sequences, or both, is at present not clear.

3.2. Solubility

The acid precipitation curves of the starting materials (Fig. 4) show the precipitation of *D. antarctica* and *L. nigrescens* alginates in a relatively broad pH-range, between pH 3.5 and 2.0, with the mid-point of the precipitation-curves at a pH of about 2.6–2.7. After epimerization of the samples, the pH of precipitation did not change significantly. However, epimerisation had a distinct effect on the proportion of material that was not precipitated below the pH of precipitation after 30 min equilibration time. For the modified alginate from *D. antarctica* more than 40% was still in solution at pH 1 while the material from *Lessonia* only 20% remained in solution. This difference is probably due to the higher content of G-blocks in the alginate from *Lessonia*. For the high G alginate sample from *L. hyperborea* (data not shown) epimerisation did not have any impact on acid solubility probably due to the long G-block in the starting material. The mannuronan sample from *P. fluorescence* precipitates in a much narrower pH-range, and at a slightly higher pH of about 2.8–2.9.

The effect on solubility was, however, most extreme with the fully epimerised mannuronan, which did not precipitate at all at any pH after 30 min (Fig. 4). However, when the time of equilibration was increased, the solutions of this sample became increasingly viscous, and eventually, within several days, formed a clear, transparent gel, occupying the entire volume of the samples. After 6 days storage at 4 °C the gels were centrifuged (compressed), and the alginate concentration in the supernatant was found to be about 0.1 mg/ml, i.e. about 97.5% of the alginate was precipitated.

We have not measured the pK_a values for the epimerised samples, but since the pK_a for GulA is slightly higher than for

Table 1
Table1 Composition, sequential data and intrinsic viscosity ([η]) of the alginates used in this study (molar fractions from ^{1}H NMR spectra)

	F_G	F_M	F_{GG}	F_{GM} F_{MG}	F_{MM}	F_{GGM} F_{MGG}	F_{MGM}	F_{GGG}	$N_{G>1}$[a]	[η] (ml/g)
L. nigrescens	0.41	0.59	0.22	0.19	0.40	0.05	0.14	0.17	5.6	812
Epimerized *L. n.*	0.56	0.44	0.25	0.31	0.13	0.06	0.25	0.19	5.1	640
Change	0.15	−0.15	0.03	0.12	−0.27	0.01	0.11	0.02	−0.5	−172
D. antarctica	0.32	0.68	0.16	0.17	0.51	0.05	0.12	0.11	4.3	782
Epimerized *D. a.*	0.52	0.48	0.18	0.34	0.14	0.09	0.25	0.09	3.0	659
Change	0.20	−0.20	0.02	0.17	−0.37	0.04	0.13	−0.02	−1.3	−123
L. hyperborea	0.67	0.33	0.55	0.11	0.22	0.04	0.07	0.51	14.0	746
Epimerized L.h	0.74	0.26	0.58	0.16	0.10	0.05	0.11	0.52	11.8	569
Change	0.07	−0.07	0.03	0.05	−0.12	0.01	0.04	0.01	−2.2	−177
P. fluorescens[b]	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	2821
Epimer. *P. f.*[b]	0.46	0.54	0.00	0.46	0.08	0.00	0.46	0.00	0.00	1624
Change	0.46	−0.46	0.00	0.46	−0.92	0.00	0.46	0.00	0.00	-1197

[a] Average number of GulA residues in G-blocks longer than one unit.
[b] Epimerase-negative mutant.

ManA, epimerisation would lead to a slight increment in pK_a. The increased solubility upon epimerisation is therefore not a simple pK_a effect but either owing to the higher flexibility of the poly-MG sequences (Smidsrød, Glover, & Whittington, 1973) or to some geometric restrictions in the association of the modified polymers.

3.3. Gelling kinetics

To monitor the slow processes of acid gel formation of an $(MG)_n$-rich alginate, two dynamic oscillation measurements were carried out, similar to the experiment shown in Fig. 5. Fig. 5(a) shows the sol–gel transition of epimerized mannuronan (10 mg/ml) at pH 1.3. The gelling occurs rapidly, indicated by a sharp drop in phase angle between the first and second measurement, from 61.2° after 4 s to 3.1° after 104 s (data not shown). The gel strength (storage modulus G') initially increased to 200 Pa within 1 h, then increased slowly, reaching 300 Pa after about 6.5 h, and 400 Pa after 17 h. Unexpectedly, about 22 h after addition of the acid and the formation of the initial acid gel, the increase in G' accelerated and entered into a linear slope, reaching 1000 Pa after 31 h and 6000 Pa after 68 h.

A similar behaviour was observed for the sol–gel transition in the second experiment with an alginate concentration of 1.33 mg/ml (Fig. 5(b)), albeit at much lower gel strength. The initial gelling required more time and was followed by a period of slow increase in G', and a rapid increase after 14 h. After 27 h G' levelled off at about 80 Pa, and decreased slowly, probably due to the onset of syneresis at this low concentration. Applying a $G' \propto c^2$ relationship for dependence of the modulus with respect to concentration, the 85 Pa maximum in Fig. 5(b) (1.33 mg/ml) correlates to 4800 Pa with respect to the gel in Fig. 5(a) (1.0 mg/ml). Accepting 85 Pa as a levelling-off value for 1.33 mg/ml implies less optimal network structuring, probably due to a less optimal network connectivity as this concentration is approaching the critical overlap concentration for gelling.

In any case, a restructuring of the initial gel took place after a 12–24 h lag phase, depending on the alginate concentration and in both cases a substantial increase in gel strength was observed. The mechanism leading to the formation of the initial and secondary gels, and the gel-structures are not yet completely understood. It has, however, been shown (Draget, Skjåk-Bræk, and Smidsrød, 1994; Draget, Smidsrød and Skjåk-Bræk, 2002) that GG-blocks play a crucial role in the formation and stabilisation of the alginic acid gel, whereas the alternating MGM-structure actually perturbs such gel formation formed by alginates with an ordinary (i.e. as found in nature) sequential occurrence of the two monomers. The present poly-alternating alginate therefore seems to represent a complete new gelling system. In a recent study where the local structure of alginic acid gels was studied by small-angle X-ray scattering (SAXS) and linked to macroscopic gel properties (Draget, Stokke, Yuguchi, Urakawa, & Kajiwara, 2003), it was found that for alginates with a high G content, acidic network formation proceeds through an initial phase consisting of quasi-ordered junction zone (3–4 laterally associated chains). Subsequent assembling of linked junction zones, supporting a continuous network formation, takes place in the second stage forming larger domains. A lowering of the G content led to domains composed of more loosely packed and shorter junction zones. In any case, similar suprastructures were obtained at equilibrium indicating similar mechanisms to be involved. In the case of the present poly-alternating MGM sample, it is fair to anticipate that it will also follow a two-stage mechanism for acid gel formation as is indeed suggested by the results presented in Fig. 5. The observed and dramatically increased delay for the MGM sample must be due to the fact that although being homo-polymeric, the inherent time constant for building larger structures supporting an increased network connectivity is substantially larger compared to MM- and GG-blocks. Such an increased time constant must, in turn, be due to the irregularity of

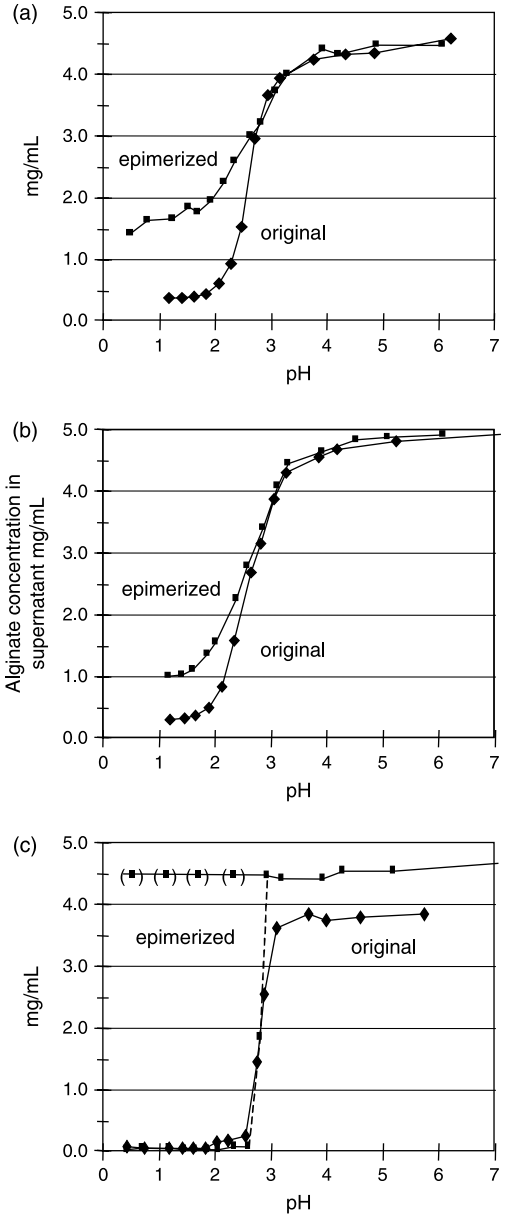


Fig. 4. Acid solubility of different alginates (a, b) and mannuronan (c) before and after epimerization with the C-5-epimerase AlgE4. (a) *Durvillea antarctica*, waiting tme 30 min. (b) *Lessonina nigrescens,* waiting time 30 min. (c) *Pseudomonas aeruginosa,* waiting time: ♦ 30 min, (■) 30 min no visible precipitate, ■ 6 days.

the glycosidic bonds (alternating equatorial–axial) compared to MM-blocks (di-equatorial) and GG-blocks (di-axial).

These experiments show that the processes involved in the acid precipitation of $(MG)_n$-rich alginate are slow and equilibrium is not reached within 3 days. In conclusion, the standard methods for measuring acid-solubility seem inapplicable for high-molecular-weight $(MG)_n$-rich alginate samples, such as those used in this study, since the fundamental requirement for an equilibrium situation is not fulfilled. Therefore, the simple precipitation experiment carried out for the seaweed alginates and their epimerised derivatives is not suitable for $(MG)_n$-rich material. In rheological studies, care must be taken to verify whether the initial gel is the final

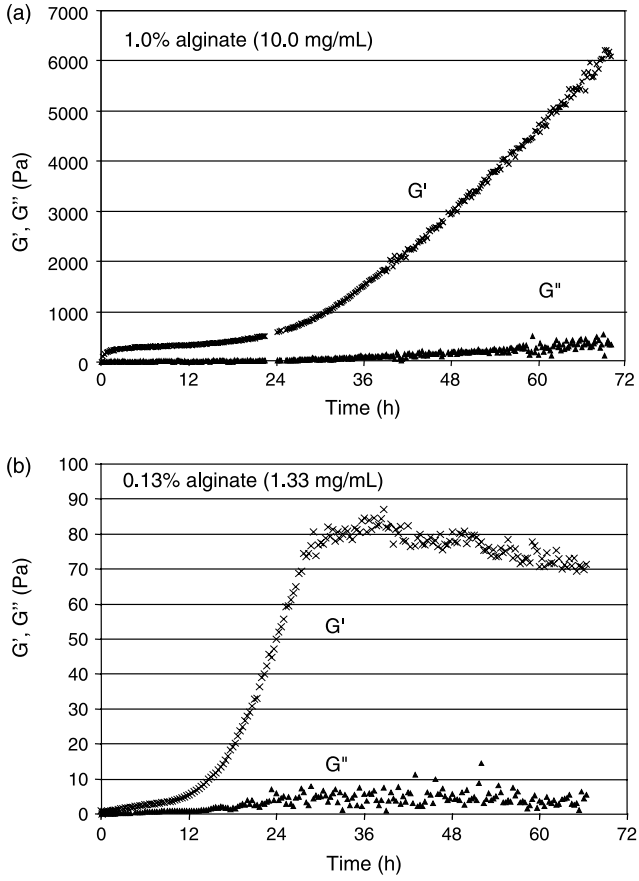


Fig. 5. Sol–gel transition of epimerized *P. fluorescens* alginate from Na-salt to alginic acid at pH 1.3. G': storage modulus (elastic component); G'' loss modulus (viscous component).

product, or whether restructuring and formation of a secondary gel occurs after a period of fairly constant G'.

4. Conclusion

These results clearly show that the gelling of MG-rich alginate with acid is kinetically slower compared to native alginates. A theory for this behaviour based on structural considerations with alternating equatorial/axial linkages has been presented. A practical consequence of these results could be that controlling the fraction of MG-sequences in the polymer might regulate the gelling kinetics of alginic acid gels. The content of MG may also show to be important in the release of drugs based on alginate as carrier. Also, the common method of acid precipitation of alginate to terminate an epimerization, or as a purification step in alginate isolation, cannot be used for MG-rich material.

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