Bio MACROMOLECULES

Biosynthesis and Function of Long Guluronic Acid-Blocks in Alginate Produced by *Azotobacter vinelandii*

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S Supporting Information

ABSTRACT: With the present accessibility of algal raw material, microbial alginates as a source for strong gelling material are evaluated as an alternative for advanced applications. Recently, we have shown that alginate from algal sources all contain a fraction of very long G-blocks (VLG), that is, consecutive sequences of guluronic acid (G) residues of more than 100 residues. By comparing the gelling properties of these materials with in vitro epimerized polymannuronic acid (poly-M) with shorter G-blocks, but comparable with the G-content, we could demonstrate that VLG have a large influence on gelling properties. Hypothesized to function as reinforcement bars, VLG prevents the contraction of the gels during formation (syneresis) and increases the Young's modulus (strength of the gel). Here we report that these VLG structures are also present in alginates from *Azotobacter vinelandii* and that these polymers consequently form stable, low syneretic gels with calcium, comparable in mechanical strength to algal alginates with the similar monomeric composition. The bacterium expresses seven different extracellular mannuronan epimerases (AlgE1-AlgE7), of which only the bifunctional epimerase AlgE1 seems to be able to generate the long G-blocks when acting on poly-M. The data implies evidence for a processive mode of action and the necessity of two catalytic sites to obtain the observed epimerization pattern. Furthermore, poly-M epimerized with AlgE1 in vitro form gels with comparable or higher rigidity and gel strength than gels made from brown seaweed alginate with matching G-content. These findings strengthen the viability of commercial alginate production from microbial sources.
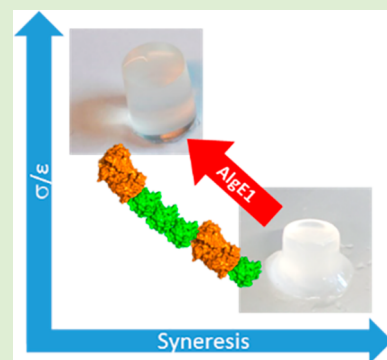
■ INTRODUCTION

Alginate is a collective term for a family of polysaccharides containing $(1 \rightarrow 4)$ linked residues of β-D-mannuronic acid (M) and α-L-guluronic acid (G) in varying ratio and distribution pattern. Alginate is found in brown algae (*Phaeophyceae*) and in some bacteria belonging to the genera *Pseudomonas* and *Azotobacter*. Alginate is the major structural polysaccharide in brown algae providing the plants with strength and flexibility, analogue to the role of cellulose and pectin in terrestrial plants. In bacteria, alginate probably have multiple functions. In *Pseudomonas* sp., alginate acts as a virulence factor mediating the growth in the biofilm state. Although the precise role of alginate in biofilm formation is not yet completely clear,[1] it is believed that by forming a capsule around the pathogen, it suppresses phagocytosis and impedes host immune clearance.[2] In *Azotobacter* sp. alginate is essential for the cyst formation, where an alginate gel forms the protective walls on metabolic dormant cysts. In addition, in the vegetative stage, alginate may serve as a diffusion barrier for oxygen to protect the nitrification system of the bacteria.[3]

Alginate forms ionotropic gels with divalent cations such as calcium where the presence of G-blocks is the main structural feature contributing to gel formation. The mechanism behind gel formation is most easily visualized by analogy with an egg box.[4] In this model, divalent cations (notably Ca^{2+}) are coordinated in the cavities between dimers of guluronate from two opposing alginate chains creating junction zones. Eventually this forms a network, provided that there is an average of more than three G-blocks with a minimum length of 8 units per polymer chain.[5] This model has been considerably refined through theoretical[6] and experimental[7−9] work.

A family of seven secreted mannuronan C-5 epimerases (AlgE1-AlgE7) has been identified in the soil bacterium *Azotobacter vinelandii*.[10−12] These enzymes catalyze the conversion of D-mannuronic acid into L-guluronic acid by inversion of the stereocenter at C-5 and introduce G-residues in the mannuronan chain at the polymer level in alginate either as monomers or in blocks. Although present in all brown algae, algal epimerases have proved difficult to isolate due to denaturation by polyphenols in the extracellular tissue during extraction. However, cDNAs homologous to bacterial epimerases extracted from *Laminaria digitata* are previously reported.[13] Most of the knowledge about mannuronan C-5 epimerases therefore originates from *A. vinelandii* containing
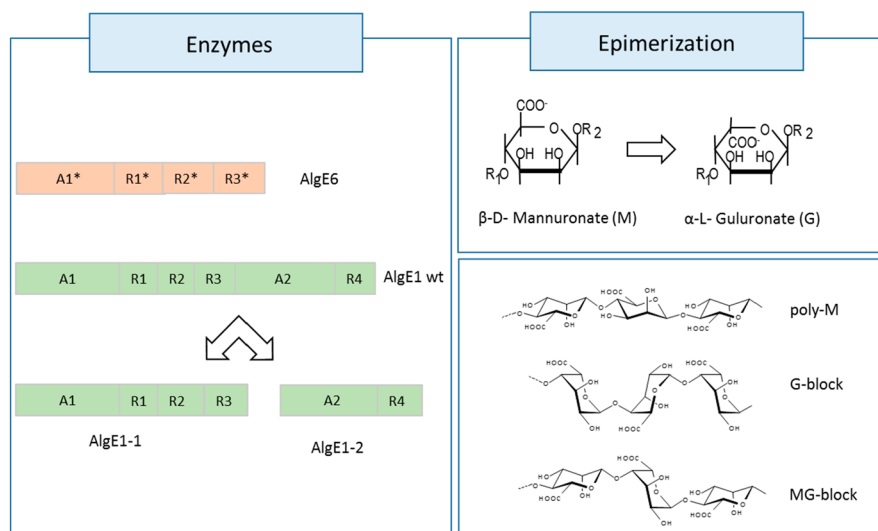
Figure 1. Modular structures of the alginate epimerases AlgE1 and AlgE6. The structure of alginate monomers and the block structures formed during epimerization of poly-M.

71 seven epimerase genes (algE1–algE7), all of which have been
72 cloned and expressed in *Escherichia coli*.[14] The C-5 epimerases
73 are modular enzymes containing repeats of two structural units
74 indicated as A and R where the A module contains the catalytic
75 activity. Figure 1 illustrate the modular structure of AlgE1 and
76 AlgE6 as well as the substrates and the products formed by the
77 epimerases used in this study.

78   Each isoenzyme has a distinctive epimerization pattern and
79 is $[Ca^{2+}]$ optimum.[14,15] Furthermore, it has been shown that
80 the mechanical properties of alginate gels can be modulated by
81 in vitro epimerization of seaweed alginates with the epimerases
82 AlgE1, AlgE4 and AlgE6.[16,17]

83   The main structural difference between algal and bacterial
84 alginates is the presence of O-acetyl groups in the latter where
85 the acetyl groups invariably are linked to the M residues in the
86 C-2 and C-3 position. Since acetylated M-residues are not
87 epimerized the acetyl substitution also controls C-5 epimeriza-
88 tion and indirectly facilitates the reswelling of the cysts.[18]
89 Alginate hydrogels have been extensively studied, see for
90 example ref 19 for a review.

91   As natural resources of strong gelling alginates extracted
92 from brown seaweed are becoming limited, there has been an
93 increasing interest in microbial alginates.[20,21] One key question
94 is to establish whether it is possible to produce microbial
95 alginates with properties resembling those of the strong gelling
96 alginates extracted from *Laminaria hyperborea* stipe. In a
97 previous paper we reported that gels made from in vitro
98 epimerized poly-M lacks the fraction of very long G-blocks that
99 are ever-present in brown seaweed alginates and thereby
100 display an unusually low Young's modulus and a high degree of
101 syneresis.[22]

102   In this study, we compare the G-block distributions and gel
103 properties of *A. vinelandii* alginate with in vitro epimerized
104 poly-M and brown seaweed alginate. Data on the epimerization
105 pattern obtained by epimerization with full length AlgE1, as
106 well as its individually active G-block forming A-modules is
107 also presented.

■ MATERIALS AND METHODS

109   **Epimerases and Lyases.** The alginate epimerases and lyases used
110 are described in Table 1. AlgE1 and AlgE6 were expressed in *E. coli*

111 strain SURE and partially purified by ion-exchange chromatography
112 on Q-Sepharose FF, as previously described by Svanem et al.[12]

Table 1. Origins and Properties of Enzymes Used in This Study

enzyme	source/host	comment	provider/ref
AlgE1	*E. coli* SURE	For in vitro epimerization. Modules: A1R1R2R3A2R4	12
AlgE1-1	*E. coli* T7 Express	For in vitro epimerization. Modules A1R1R2R3	15
AlgE1-2	*E. coli* T7 Express	For in vitro epimerization. Modules A2R4	15
AlgE4	*E. coli* T7 Express	For in vitro epimerization (pHH4 in ref 12)	15
AlgE6	*E. coli* SURE	For in vitro epimerization	12
M- lyase	*Haliotis tuberculata*	M-lyase for degradation of epimerized alginate	24
AlyA	*Klebsiella pneumoniae*	G- lyase for degradation of epimerized alginate	25

113   AlgE4 was cloned into the pTYB1 vector system, expressed in T7
114 Express cells and further purified based on a protocol from Impact-
115 CN System (New England Biolabs, Inc.). In short, the cultures
116 incubated at 30 °C overnight in LB medium supplied with 100 μg/
117 mL ampicillin. For enzyme expression, strains (1% final concen-
118 tration) were grown in 2 LB supplied with 100 μg/mL ampicillin in
119 baffled shake flasks at 30 °C until $OD_{600nm} \sim 0.8–1.0$ was reached.
120 Then the cultures were cooled for 5 min on ice and then induced for
121 protein expression with isopropyl β-D-thiogalactopyranoside (IPTG)
122 to a final concentration of 1 mM. Expression continued overnight at
123 16 °C before harvesting the cells by centrifugation (Sorvall, 5500 g, 5
124 min, 4 °C). For preparation of enzyme extracts, the cells were
125 resuspended in 25 mL of HEPES lysis buffer (20 mM HEPES pH 6.9
126 with 5 mM $CaCl_2$, 500 mM NaCl, 0.05% Triton X-100), disrupted by
127 sonication and centrifuged for at least 45 min at 23000 g. The
128 supernatant was filtered (0.22 μm) and the supernatant with the
129 enzyme fraction purified using Fast Protein Liquid Chromatography
130 (FPLC; ÄKTA FPLC system, GE Healthcare). Protein extracts were
131 applied to a 20 mL Chitin Resin column, pre-equilibrated with
132 HEPES running buffer (20 mM HEPES pH 6.9 with 5 mM $CaCl_2$,
133 500 mM NaCl). Washing of the column was with HEPES running
134 buffer. Finally, to cleave off the intein tag and release target protein,
135 the column was washed with running buffer containing 50 mM DTT

Table 2. Origins and Key Properties of Alginates Used in This Study[a]

source	F_G	F_{GG}	F_{GM}	F_{GGG}	$N_{G>1}$	M_w (Da)	provider/ref	comments
Azotobacter vinelandii	0.42	0.29	0.130	0.28	25	1.2×10^5	in house	5% acetyl
Azotobacter vinelandii	0.37	0.27	0.10	0.26	27	1.3×10^5	in house	deacetylated
Azotobacter vinelandii	0.5	0.48	0.05	0.47	42	1.4×10^5	in house	14.5% acetyl
Pseudomonas fluorescens	n.d	n.d	n.d	n.d	n.d	3.4×10^5	[26]	Poly-M
Poly-M	0.46	n.d	0.46	n.d	n.d	1.4×10^5	[27]	AlgE4 + Poly-M, in vitro epimerized
Poly-M	0.21	0.19	0.01	n.d	n.d	2.1×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.30	0.26	0.05	n.d	n.d	2.0×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.43	0.36	0.07	0.35	57	2.0×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.51	0.44	0.08	0.43	64	2.1×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.59	0.50	0.08	0.50	70	2.1×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.69	0.60	0.09	0.59	71	1.9×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.80	0.71	0.09	0.69	48	1.9×10^5	this work	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.51	0.41	0.09	0.39	16	3.0×10^5	[22]	AlgE1 + Poly-M series, in vitro epimerized
Poly-M	0.68	0.59	0.09	0.55	17	2.9×10^5	[22]	AlgE6 + Poly-M, in vitro epimerized
Laminaria hyperborea	0.67	0.56	0.11	0.52	13	2.0×10^5	DuPont, Sandvika	alginate extracted from stipe. characterized, used in gel experiments
Durvillea potatorium	0.32	0.20	0.12	0.16	6	1.6×10^5	DuPont, Sandvika	characterized, used in gel experiments
Macrocystis pyriferia	0.41	0.21	0.20	0.17	5	1.8×10^5	Sigma-Aldrich	characterized, used in gel experiments

[a]F_G denotes the fraction of guluronic acid. Fractions of diads and triads are indicated with two and three letters, respectively. Degree of acetylation (%) is defined as $([Ac]/([M] + [G])) \times 100$.

(freshly made). The column was left overnight in room temperature before elution. To identify active fractions we used SDS-PAGE followed by microassay.[23] The active fractions were dialyzed against 5 mM HEPES pH 6.9, 5 mM CaCl$_2$, and freeze-dried for storage. Protein concentration were estimated with NanoDrop prior to end-point epimerization.

Plasmids pHE37 and pHE56[15] in *E. coli* strains T7 Express were used for recombinant production of AlgE1−1 and AlgE1−2 respectively. The cultures were grown at 30 °C in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplied with 100 μg/mL ampicillin. For enzyme expression, strains (1% final concentration) were grown in 2 × LB; (20 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl) supplied with 100 μg/mL ampicillin in baffled shake flasks at 30 °C until OD$_{600nm}$ ∼ 0.8−1.2 was reached. Cultures were induced for protein expression with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Growth was continued overnight at 16 °C before harvesting the cells by centrifugation (Sorvall, 5000 g, 6 min, 4 °C). For preparation of enzyme extracts, the cells were disrupted by sonication in 25 mL of MOPS buffer (40 mM MOPS pH 6.9 with 5 mM CaCl$_2$) and centrifuged for at least 45 min at 23000 g. The supernatant was filtered (0.22 μm) and the enzymes purified using Fast Protein Liquid Chromatography (FPLC; ÄKTA FPLC system, GE Healthcare). AlgE1-1 and AlgE1-2 extracts were applied to a 5 mL HiTrap Q HP column (GE Healthcare), respectively, and the recombinant proteins were eluted using a stepwise NaCl gradient (0 to 1 M) of 40 mM MOPS pH 6.9 with 5 mM CaCl$_2$. Fractions were analyzed for epimerase activity by using a previously reported assay.[23] The total protein content was estimated by NanoDrop.

An alginate M-lyase specific toward M-M and G-M linkages was purified from *Haliotis tuberculata* according to Boyen et al.[24] The G-lyase AlyA from *K. pneumoniae*, specific toward G−G and G−M linkages was produced as previously described.[25] The specific activity (U/mL) on poly-M and G-block (0.5 mg/mL) was determined as the initial slope (Abs$_{230 nm}$/min) of the absorbance−time curve measured on a UV-spectrophotometer.

Alginates. Poly-M ($F_G = 0.0$, $M_w = 275$ kDa) was produced by cultivation of an epimerase negative AlgG mutant of *Pseudomonas fluorescens*.[26]

Poly-MG ($F_G = 0.46$, $F_{GG} = 0.0$, $M_w = 210$ kDa) was made by in vitro epimerization of poly-M with AlgE4, as previously described.[27]

Sodium alginate from *L. hyperborea* stipe and *Durvillea potatorium* were provided by DuPont (Sandvika, Norway). Sodium alginate from *Macrocystis pyriferia* was purchased from Sigma-Aldrich. Sequence parameters and molecular weight of the seaweed alginates previously reported[25] are included in Table 2.

Three *A. vinelandii* alginates previously produced at our department following the protocol of Skjåk-Bræk et al.[28] were reanalysed with respect to F_G, M_w, and degree of acetylation and used in this study.

Prior to analysis of G-block length, the *A. vinelandii* alginates were deacetylated in 0.1 M NaOH at room temperature for 30 min, dialyzed and freeze-dried. Molecular weight and sequence information based on SEC-MALS and NMR was obtained as described later in this section and are shown in Table 2.

In Vitro Epimerization. Poly-M (2.5 mg/mL) or Poly-MG (2.5 mg/mL) was epimerized with native AlgE1, AlgE4, or AlgE6, respectively. The enzyme and substrate mixtures were incubated at 37 °C in 50 mM MOPS buffer, pH 6.9 with 2.5 mM CaCl$_2$ and 40 mM NaCl.

In the case of AlgE1-1 and AlgE1-2 (the separately expressed, catalytically active parts of AlgE1), poly-M and poly-MG was epimerized at 25 °C in 50 mM MOPS, pH 6.9 with 4 mM CaCl$_2$ and 75 mM NaCl.

The epimerization reaction reached its end point for both AlgE4 and AlgE6. For poly-M epimerized with full length native AlgE1, a withdrawal of aliquotes at given times enabled comparison of alginate sequence data with a previously made AlgE6 series. For all epimerization experiments the reaction was quenched by calcium chelation with EDTA (10 mM) followed by denaturation at 95 °C for 5 min. The epimerised samples were dialyzed against 50 mM NaCl and deionized water, and finally freeze-dried.

Lyase Degradation. Alginate samples (1−5 mg/mL) were dissolved in 200 mM ammonium acetate with 50 mM NaCl and pH adjusted between 7.2−7.3). Degradation of alginate samples with M-lyase from *Haliotis tuberculata* was performed by adding of 0.016 U of enzyme/mg substrate followed by incubation at 30 °C for 24 h.[25] The solution was heated for 5 min at 95 °C to avoid further degradation of the alginate sample.

Preparation of Ca-Alginate Gels and Force-Deformation Measurements. Gel cylinders were made by mixing alginate solutions with a dispersion of CaCO$_3$ (15 mM, particle size 4 μm) and a freshly made solution of D-glucono-δ-lactone (30 mM), as previously described.[17] The solution was poured into tissue culture plates (16/18) and allowed to cure for 24 h before saturation in a solution of 50 mM CaCl$_2$ and 200 mM NaCl.

Syneresis (%) was determined as 100× fractional weight reduction with respect to initial well volume, assuming neglectable change in
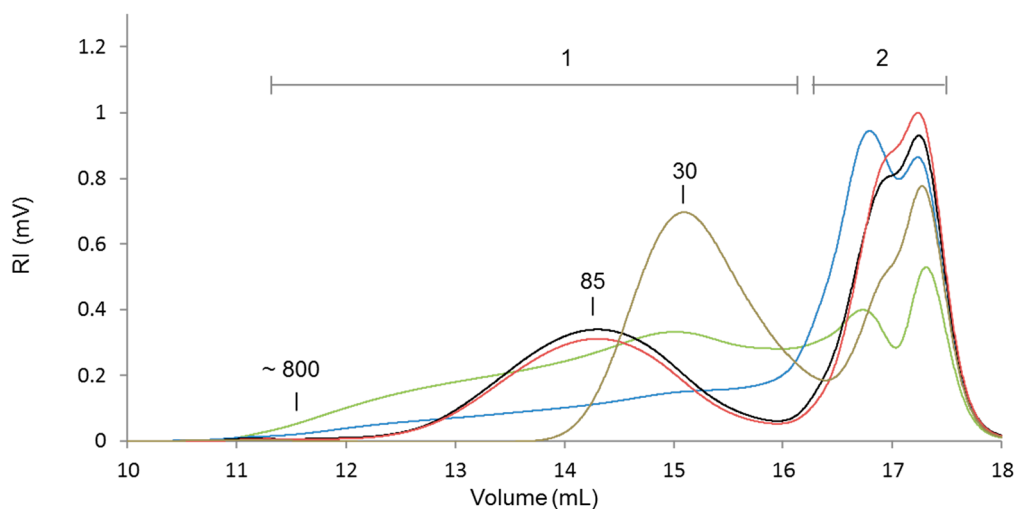
Figure 2. SEC-MALS analysis of chain length distribution of G-blocks in (green line) *L. hyperborea* (F_G = 0.67), (blue line) *M. pyrifera* (F_G = 0.41), (red line) *A. vinelandii* (F_G = 0.37), (black line) *A. vinelandii* (F_G = 0.42), and (gray line) poly-M epimerized with AlgE6 (F_G = 0.51). Region 1 represents the G-block distribution. Chain length at maximum peak height calculated from the M_w−volume regression line is indicated in the chromatogram. Oligomer degradation products (DP < 10) and salts elutes in region 2.

223 density. Force/deformation curves recorded at 22 °C using a TA-XT2
224 texture analyzer with a P/35 probe and a compression rate of 0.1
225 mm/s. Youngs modulus ($E = (F/A)/\Delta l/l$) was calculated from the
226 initial slope of the curve (0.1−0.3 mm). For comparison of
227 mechanical strengths of gels with different degrees of syneresis, the
228 final alginate concentration was calculated, and E was recalculated
229 using the semiempirical relationship:[29]

$$E = E_{measured} \times (C_{initial}/C_{final})^2$$

230 **HPAEC-PAD.** Lyase degraded alginates (25 μL, 0.05−1 mg/mL)
231 were analyzed on an ICS-5000+ system (Thermo Scientific) with
232 IonPac AG4A guard and AS4A main columns using 0.1 M NaOH for
233 the mobile phase and sodium acetate as eluent as previously
234 described.[25]
235 Poly-MG (F_G = 0.46 and poly-M (F_G = 0.00) partially degraded by
236 *H. tuberculata* M-lyase where used as standards in order to identify
237 some of the shorter degradation products, whereas poly-G (F_G >
238 0.97) partially degraded by a G-lyase from *K. pneumoniae*[25] were
239 compared with the longest oligomers in the lysates.
240 **Size Exclusion Chromatography (SEC).** Lyase degraded
241 alginates (10−50 mg) were eluted with 0.1 M ammonium acetate
242 on three serially connected Superdex 30 columns (2.6 × 60 cm)
243 connected to a Shimadzu RID-6A detector. Fractions were pooled,
244 dialyzed, and freeze-dried.[30]
245 **¹H-NMR Spectroscopy.** To reduce viscosity, high M_w samples
246 were partially degraded by two-step acid hydrolysis prior to NMR
247 analysis. The sample was first hydrolyzed at 95 °C, pH 5.6 for 60 min
248 and then for 50 min at pH 3.8 to give a final DPn in the range 30−50.
249 Samples (5−10 mg) were dissolved in 600 μL of D_2O with TSP, 3-
250 (trimethylsilyl)-propionic-2.2.3.3-d4 acid sodium salt, (1%, 5 μL) as
251 an internal standard and triethylenetetraamine-hexaacetate (0.3 M, 20
252 μL) as a calcium chelator. ¹H NMR spectra were recorded on a
253 BRUKER AVIIIHD 400 MHz equipped with 5 mm SmartProbe at 82
254 °C. Signal assignment and data processing was done as previously
255 described.[31,32] The fraction of O-acetylated mannuronic acid was
256 determined in a separate experiment according to Skjåk-Bræk et al.,[28]
257 whereas the monad, diad, and triad frequencies were measured on
258 deacetylated samples.
259 For the time-resolved NMR analysis of epimerization reactions, a
260 stock solution of 22 mg/mL ¹³C-1-enriched poly-M (average DPn ∼
261 50) in 5 mM MOPS, pH 6.9, with 75 mM NaCl in 99.9% D_2O was
262 prepared. Purified enzyme fractions from ion exchange chromatog-
263 raphy were subject to buffer exchange and concentrated (final
264 concentration around 2.3 mg/mL) by spin columns (VivaSpin,

265 Sartorius Stedim Biotech) with molecular cutoff 10 kDa. Samples
266 were washed with 5 mM MOPS, pH 6.9, with 75 mM NaCl and 27.5
267 mM $CaCl_2$ in 99.9% D_2O. Protein concentrations were determined
268 with a NanoDrop ND-1000 to ensure similar enzyme concentration
269 in the epimerization reaction. A total of 500 μL of ¹³C-1-enriched
270 poly-M stock solution was preheated in the NMR instrument and 1D
271 proton and carbon spectra were recorded to ensure that the sample
272 has not undergone any degradation or contamination prior to the
273 time-resolved NMR experiment. A total of 50 μL of enzyme solution
274 was added to preheated substrate and mixed by inverting the sample
275 2−3 times. The sample was immediately inserted to the preheated
276 NMR instrument and the experiment was started. The recorded
277 spectrum is a pseudo-2D type experiment recording a 1D carbon
278 NMR spectrum every 10 min with in total 128 time points. The
279 recorded 1D carbon spectrum (using inverse-gated proton decou-
280 pling) contains 8K data points and has a spectral width of 80 ppm, 32
281 scans with a 30° flip angle, and relaxation delay of 1.1 s (total
282 recording time of 60 s). The spectra were recorded using TopSpin
283 1.3, 2.1, and 3.2 software (Bruker BioSpin) and processed and
284 analyzed with TopSpin 3.5 pl7 software (Bruker BioSpin).
285 **SEC-MALS.** Samples (1−5 mg/mL, 100−200 μL, 0.2 μm filtered)
286 were analyzed on a HPLC system with serially connected TSK 6000,
287 5000, and 2500 (PWXL) size exclusion columns (tosoh Bioscience
288 LLC) using 0.15 M $NaNO_3$ and 0.01 M EDTA, pH 6.0, as elution
289 buffer. The column outlet was connected to a Dawn Helios II
290 multiangle laser light scattering photometer (Wyatt, U.S.A.) and an
291 Optilab T-rEX differential refractometer. To collect and process data
292 ASTRA 6.1 software was used.

RESULTS AND DISCUSSION

293

Alginates from Seaweeds Have a Broad G-Block Distribution Compared to Alginate Produced by *A. vinelandii*. In order to compare the G-block distributions in
296 alginates from different sources, two samples from *A. vinelandii*,
297 two algal polymers from *L. hyperborea* and *M. pyrifera*,
298 respectively, and an AlgE6 epimerized mannuronan were
299 degraded with a M-lyase cleaving M−M and G−M linkages.
300 The distributions of G-blocks were analyzed by SEC-MALS,
301 and results are shown in Figure 2. The irregular parts of the
302 chromatograms, starting from about 16.5 mL, contain shorter,
303 compositionally heterogeneous degradation products. A
304 striking difference in G-block distribution was observed
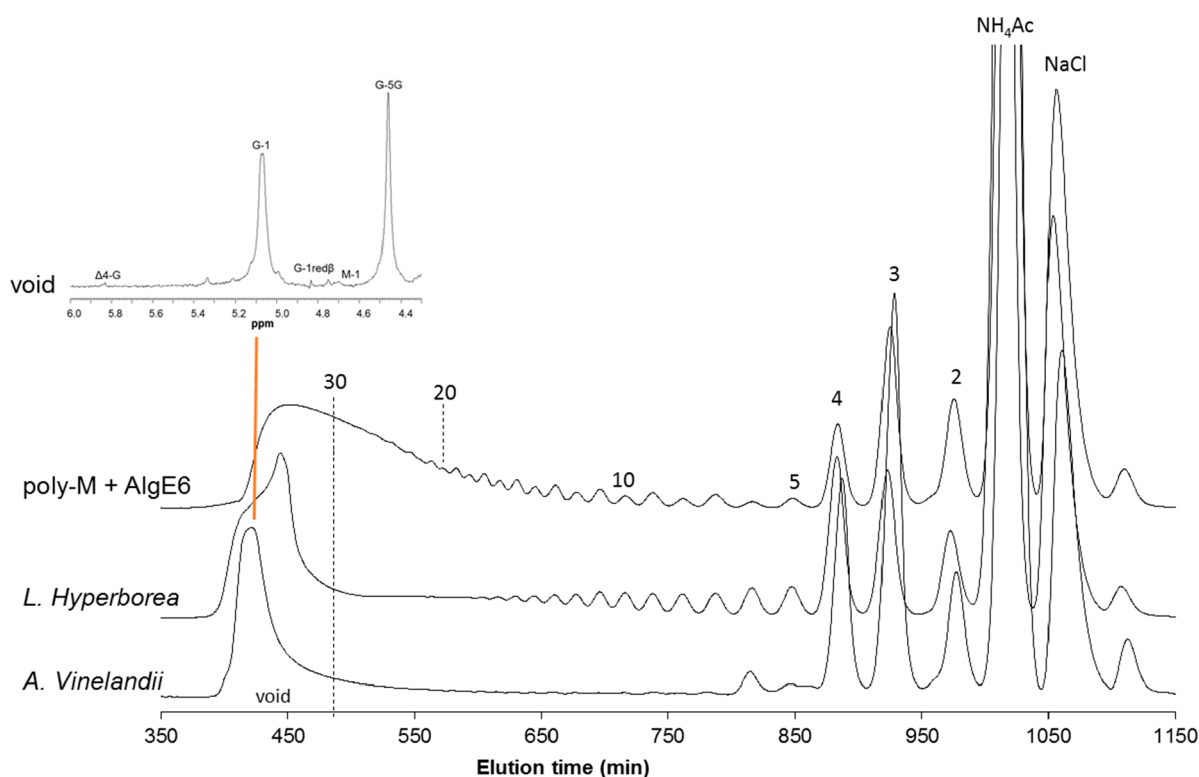305 between seaweed alginates and alginates from *A. vinelandii*.

294
295
296

302 f2

Figure 3. SEC chromatogram showing a comparison of chain length from M-lyase treated poly-M + AlgE6 (F_G = 0.68), *L. hyperborea* (F_G = 0.67) and *A. vinelandii* (F_G = 0.42). Samples prepared by size exclusion chromatography (column material: superdex 30). Chain length is indicated above the chromatograms. The ^{1}H NMR spectrum of the void fraction from *A. vinelandii* alginate confirms the compositional purity of the sample.

In the seaweed samples, the G block length differs largely in the alginate sequence, as there is no clear maximum. This in contrast to the *A. vinelandii* samples where a distinct peak with G-block lengths around 85 was observed. The pattern with a narrow distribution of G-block lengths are also found in Poly-M. This despite of a higher degree of epimerization and total G content in the Poly-M in vitro epimerized with AlgE6 compared to the *A. vinelandii* alginates.

The broad distribution of G-blocks observed in the seaweed samples compared to the bacterial alginates presumably reflects their higher compositional heterogeneity. The *M. pyrifera* alginate was extracted from whole plants, while the *L. hyperborea* alginate used in this study was extracted from stipes. In both cases it is reasonable to assume that the M/G composition and, hence, the G-block length in alginate from the newly formed tissue in the leaves (*M. pyrifera*) or in the core (*L. hyperborea*) is lower than in alginate located in the older tissues.[33,34] It is worth to notice that the longest G-blocks in the *L. hyperborea* sample has a DP of about twice the average DP_n of the nondegraded sample. The possibility that a small fraction of the alginate chains in stipes from *L. hyperborea* are present as essentially pure G-blocks can therefore not be excluded.

The number and mode of action of the mannuronan C-5 epimerases expressed in brown seaweed and *A. vinelandii* is likely to contribute to the observed differences. Little is known about the former, but six different cDNAs with homology to bacterial mannuronan C-5-epimerases has been isolated from *L. digitata*,[13] indicating that a family of epimerase isoenzymes is required also in brown seaweed in order to produce alginates with the needed properties. Finally, the environment in which

the epimerases are secreted could have an influence on the efficacy of the epimerases. Epimerization of poly-M with AlgE6 and alginate production in *A. vinelandii* were carried out in vitro, whereas a restricted network in *L. hyperborea* in vivo might render a fraction of the mannuronic acid residues unavailable for epimerization.

To verify compositional purity of the high M_w fractions showed in Figure 2 the M-lysates were fractionated on preparative SEC columns, dialyzed, and freeze-dried. Superdex 30 was used as separation material in the SEC system.

Figure 3 shows the difference in G distribution between alginate from the three sources utilized in this study. The G residues in bacterial alginate from *A. vinelandii* are organized almost exclusively in long G-blocks (DP > 50) as there are no detectable G-oligomers between the void and the hexamers. This, in contrast to the alginate extracted from the macro algae *L. hyperborea* as well as the AlgE6 in vitro epimerised material, which has a broad distribution of G block length. The ^{1}H NMR spectra included in Figure 3 show that all void fractions (DP > 50) were essentially pure G-blocks with F_G > 0.97, as there will be 1−3 M-residues on the reducing and nonreducing end of the blocks as a consequence of the cleavage pattern of the M-lyase.[25] Oligomers with DP 2−4 present in all chromatograms were confirmed to be degradation products of M- and MG-blocks based on an HPAEC-PAD analysis of the same samples (Figure S1 in the Supporting Information section).

The chromatograms show that the G-residues in the bacterial alginates ^{1}H NMR analysis (Table 2) also confirms the existence of these extreme block structures in the bacterial alginate as the molar fraction F_{GGM} representing the G-block
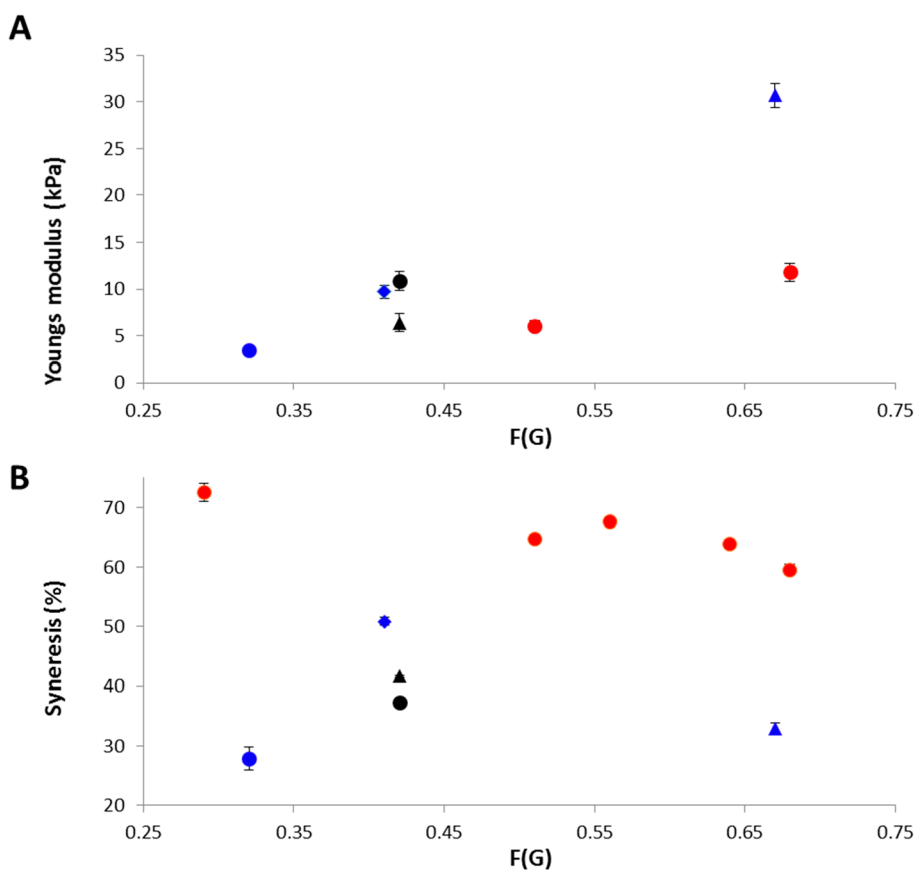
Figure 4. Youngs modulus (A) and syneresis (B) of alginate gels from *A. vinelandii* compared to brown seaweed alginate gels: black diamond, *A. vinelandii*; black circle, *A. vinelandii* deacetylated; blue diamond, *L. hyperborea*; blue tilted square, *M. pyrifera*; blue circle, *D. potatorium*; red circle, poly-M + AlgE6. SD indicated by error bars, 6−9 replicas. Composition of alginate materials was determined by NMR and can be found in Table 2 (poly-M epimerized with AlgE6[22] added for comparison).

369 terminating signal and thus the number of G-blocks, is much
370 lower (0.01), and the average G-block length $N_{G>1} = (F_G - F_{MGM})/F_{GGM} = 25$, much higher for the bacterial alginate
372 samples than for the alginate originating from seaweed.[25]
373 **G-Block Length Strongly Affect Gel Strength in**
374 **Alginate Gels.** To compare the gel strength of *A. vinelandii*
375 alginate with brown seaweed alginates, homogeneous, and
376 calcium saturated alginate gels made from 1% (w/v) solutions
377 was analyzed. The mechanical properties of the gels were
378 compared in terms of rigidity, measured as Young's modulus,
379 and syneresis after calcium saturation as shown in Figure 4.
380 The bacterial alginates form gels with mechanical properties
381 similar to seaweed alginates of comparable compositions. Also
382 their syneretic behavior is similar to the algal samples,
383 somewhat higher than *L. hyperborea*, but significantly lower
384 than *M. pyrifera* alginate. The degree of syneresis after calcium
385 saturation is influenced by several factors, such as G-block
386 length, number of MG-blocks, number of junction zones, and
387 degree of acetylation (or other substituents), as well as
388 molecular weight. Although qualitative effects of single factors
389 are known, there is at present no available model able to
390 predict Young's modulus and syneresis based on composition
391 and molecular weight. Neither is syneresis a function of
392 Young's modulus, as demonstrated for *L. hyperborea* and *D.*
393 *potatorum* (Figure 4). There is a large variation in measured
394 Young's modulus for alginate gels, albeit a similar degree of
395 syneresis in the samples.

396 VLG is an important factor since alginate gels made from
397 poly-M in vitro epimerized with AlgE6 in the range of 29−68%
398 G all has a syneresis between 60 and 70%, despite a low
399 amount of alternating sequences,[22] while gels made from
400 deacetylated *A. vinelandii* alginate has a syneresis below 40%.
401 Altogether, the differences in G-block distribution (Figure 3)
402 and syneresis (Figure 4B) between AlgE6 epimerized poly-M
403 and *A. vinelandii* alginates shows that the latter contains a
404 fraction of VLG. This results in a different organization of the
405 hydrogel network than the short to intermediate length G-
406 blocks found in AlgE6 epimerized poly-M.
407 As expected the presence of O-acetyl groups impairs gel
408 formation, giving weaker gels.[35] The presence of VLG in *A.*
409 *vinelandii* alginate suggest that either one or a combination of
410 the 7 mannuronan C-5 epimerases encoded by the bacteria is
411 able to generate these homogeneous G-blocks also on partially
412 acetylated poly-M.
413 From previous in vitro studies, we have shown that the most
414 effective G-block forming enzymes, such as AlgE6 and their
415 engineered versions,[25] are incapable of generating VLG.
416 However, indications of longer G-blocks was observed from
417 experiments with AlgE1.[19] We therefore decided to further
418 explore the epimerase AlgE1 that in contrast to AlgE6 contains
419 two catalytic modules where each of the modules were
420 expected to act differently. When expressed separately AlgE1−
421 1 (A1R1R2R3) is mainly forming G-blocks, either by
422 elongating existing G-blocks by converting an M neighboring
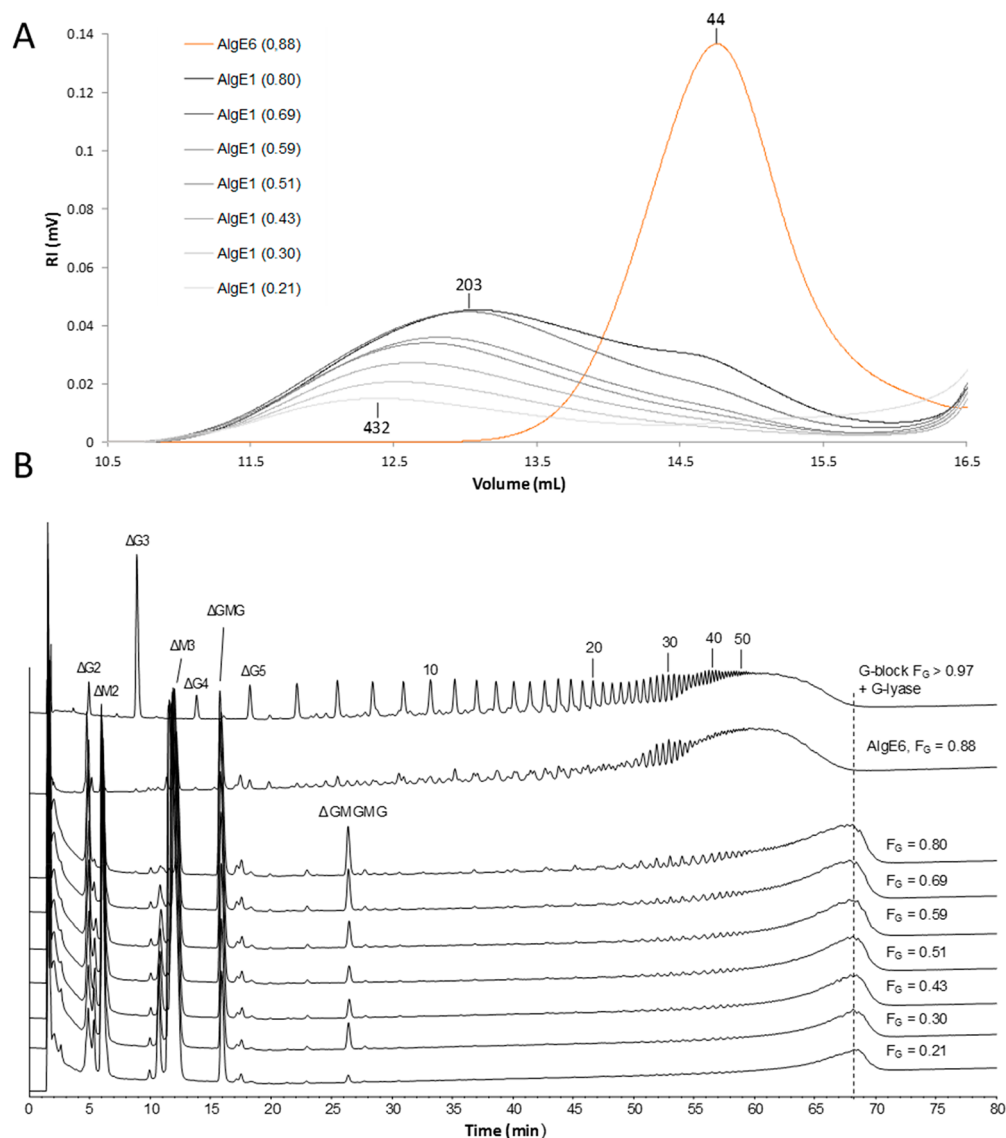423 to a G or by condensation of G-blocks, epimerizing an M

Figure 5. Distinct pattern of VLG formed by AlgE1, even at low degree of epimerization (A) Average block length in a series of poly-M epimerized with AlgE1, visualized from SEC-MALS data. AlgE1 epimerized series in tones of gray starting from F_G 0.21 (bright gray) and ending with F_G 0.80 (black). Poly-M epimerized to F_G 0.88 by AlgE6 in red as comparison. DPn calculated from M_w − mL fit indicated for F_G 0.88 (AlgE6), F_G 0.80 (AlgE1), and F_G 0.21 (AlgE1). (B) Overlaid HPAEC-PAD chromatograms of the AlgE1 epimerized poly-M series degraded by M-lyase (from bottom). G-block partially degraded by AlyA and AlgE6 epimerized poly-M, F_G 0.88 degraded by M-lyase included for comparison. Degradation products and chain length of G-oligomers indicated. Dotted line included to emphasize the VLG fraction.

flanked by two G residues. AlgE1−2 (A2R4) can only epimerize an M adjacent to another M, thus, forming poly alternating structures,[15] see Supporting Information.

Analysis of G Block Distribution in AlgE1 Epimerized Poly-M. Poly-M was treated with AlgE1 to obtain polymers with degrees of epimerization ranging from 20−80%. The samples were degraded with an M-specific lyase and the G-block distribution in lysates was analyzed by SEC-MALS (Figure 5A) and HPAEC-PAD (Figure 5B). AlgE6 epimerized poly-M (F_G = 0.88) and lysate of poly-G (F_G > 0.97) were used as controls. AlgE1 introduces significantly longer VLG than AlgE6. Even at low degrees of conversion, AlgE1 forms VLGs. These are outside the separation range of the HPAEC-PAD column, but the SEC-MALS analysis clearly shows a trend were the maximum height of the RI chromatogram shifts toward shorter block lengths for higher degrees of epimeriza-tion. For the highest degrees of epimerization, a shoulder of G-blocks with DPn around 50 appears. We interpret this as a result of substrate depletion, leaving only short to intermediate M-blocks for the epimerase to act upon and that the epimerase lack the ability to efficiently epimerize short M sequences flanked by G-blocks.

Both A-Modules of AlgE1 are Acting Simultaneously on the Polymer Chain. To study the epimerization kinetics of AlgE1, time-resolved NMR was recorded using ^{13}C-1-enriched poly-M. This allows for following the progress of epimerization as a function of time by recording consecutive ^{13}C NMR spectra. As seen in Figure 6, the initial rate of generating MGM (102.3 ppm) and GGG (103.7 ppm) are similar, which indicate that both formation of alternating structure and fill-in of Gs' in alternating structure, takes place plausibly at the same time on the same polymer chain. Over
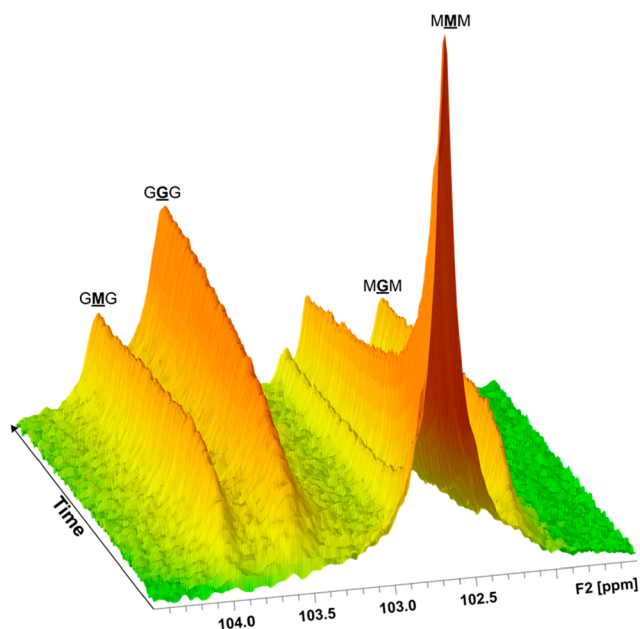
Figure 6. Continuously recorded NMR spectra showing epimerization of ^{13}C-labeled poly-M with AlgE1. Substrate (20 mg/mL) and AlgE1 were mixed and immediately inserted into the NMR instrument before recording of spectra every 10 min. Reactions were performed in 5 mM MOPS, pH 6.9 with 75 mM NaCl and 2 mM CaCl$_2$ recorded at 25 °C. The position of some triads in the spectra is indicated, and the M or G moiety giving rise to the signal is underlined. The black arrow indicates the increasing reaction time.

GGM at 102.8 ppm, indicating that predominantly elongation of existing G blocks is dominating and cause the VLG to be formed. It is interesting that both A-modules seems to be active at the same time on the polymer chain and that the A2-module of AlgE1 epimerizes from M to G for every second sugar unit in the chain.

Coordinated Action of Both A-Modules in AlgE1 is Required to Generate VLG. To further investigate the mode of action of AlgE1 and to get a better understanding of how AlgE1 acts on the polymer chain to form G-blocks, the two catalytic sites together with their respective R modules was separately expressed. The G-block-forming module AlgE1−1 was used to epimerize both poly-M and poly-MG. The epimerized polymers were subsequently degraded with an M-lyase and the alginate sequence found by HPAEC-PAD analysis. From the chromatograms given in Figure 7, it is evident that AlgE1−1 is unable to form the VLG when acting on poly-M. When poly-MG was used as substrate longer G-blocks were formed but still significantly shorter than G-blocks formed by the full-length AlgE1 enzyme.

These observations imply that only full length AlgE1 has the capacity to generate VLG, as found in alginate from *A. vinelandii*. The initial formation of long G blocks and its dependence on an intact full-length enzyme supports a processive mode of action where the two catalytic modules of AlgE1 act in tandem on the same polymer chain.

After the initial random attack, the enzyme presumably slides along the polymer chain, as shown previously for AlgE4.[32] The A2 module of AlgE1 is then creating stretches of alternating structure (poly-MG), which subsequently serve as substrate for the AlgE1-A1 module. For both A-modules the processes only require that every second M-residue is

time, the increase in GMG peak levels off while the G-block formation persists. There is no detectable signal from the
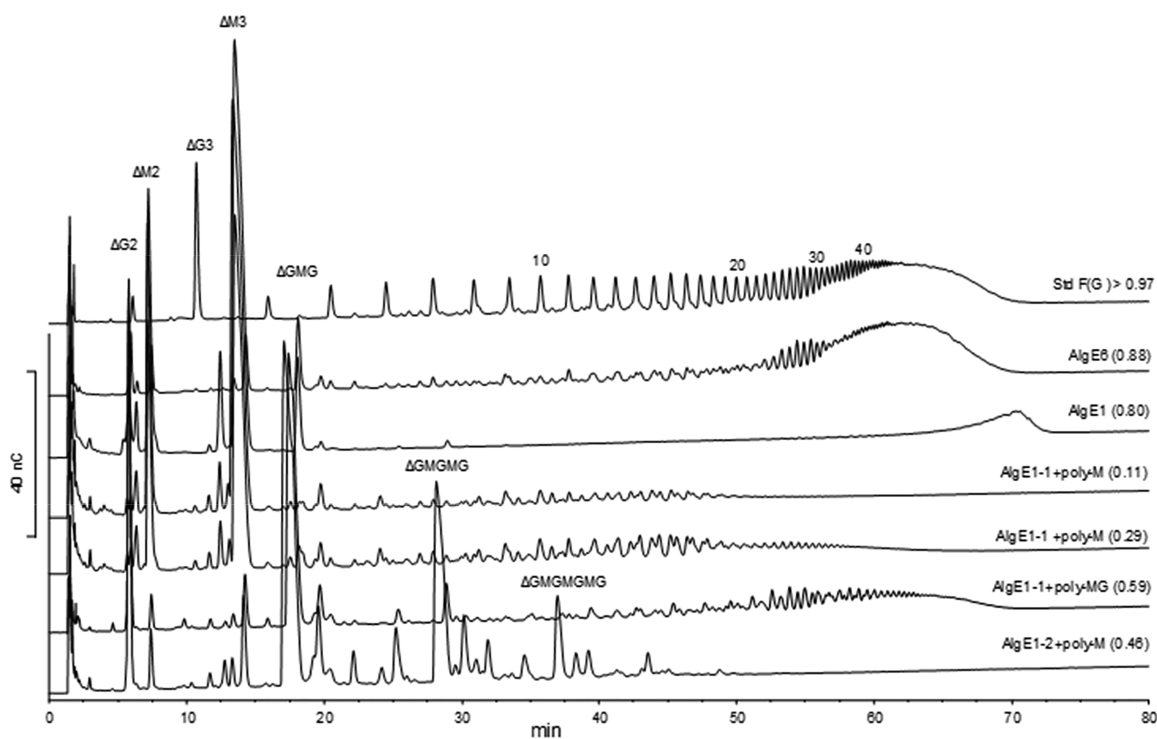

Figure 7. Overlaid HPAEC-PAD chromatograms of AlgE1 epimerized poly-M and poly-MG degraded with M-lyase. AlgE1−1 and AlgE1−2 modules, lysate of G-block, and AlgE6 epimerized poly-M are included for comparison. F(G) after epimerization are indicated in the parentheses. Chain length and end products are indicated next to the chromatograms.

epimerized, allowing the enzyme to slide along the polymer substrate without rotation. The direction of processive propagation was previously described for the poly-MG generating epimerase AlgE4. When acting on poly-M oligomers the residues close to the nonreducing end is epimerised first, suggesting that the epimerases work toward the reducing end of the substrate. The degree of processivity, that is the number of epimerizations before the enzyme is detached from the polymer substrate was found to be on average 12 for AlgE4,[32] which implies that the enzyme moves along the polymer chain for 24 residues. In the present study the VLG generated (DP > 100), even for samples with a low degree of epimerization indicate a much higher degree of processivity for full-length AlgE1.

Gelling Properties of Poly-M Epimerized by AlgE1. When comparing gels made from poly-M epimerized with AlgE1 and AlgE6 to alginate gels from *L. hyperborea*, there is a striking difference between the AlgE1 and AlgE6 epimerized material even with the same degree of epimerization, shown in Figure 8. The former gives comparable or even stronger gels

Figure 8. (A) Youngs modulus (kPa) and (B) syneresis (%) of alginate gels made from poly-M epimerized with AlgE1 (blue diamond) compared with alginate gels from *L. hyperborea* stipe (red circle) and gels from poly-M epimerized with AlgE6 (black triangle). All gels were cured in 50 mM $CaCl_2$ + 200 mM NaCl for 24 h before measurement. SD indicated by error bars, 6−9 replicas.

than can be obtained from algal material, while the AlgE6 converted material gives highly syneretic gels with a lower Young modulus. Since the main structural difference between these two materials is the size distribution of G-blocks (Figures 2 and 5), this confirms the influence of VLG upon both strength and volume stability of calcium alginate gels.

■ CONCLUDING REMARKS

The in vivo produced alginates from *A. vinelandii* in this work possess long G-blocks with a narrower block size distribution than in commercial algal alginates. The size of the longest G-blocks is somewhat shorter than those found in brown algae, but still long enough to form strong and low syneretic gels with

calcium. The C-5 epimerase AlgE1 expressed by *A. vinelandii* have the capacity initially to generate VLG > 100 in microbially produced poly-M. In an in vivo situation, the polymer substrate would be partly acetylated, and since the epimerases only convert nonacetylated M residues, this might explain the somewhat shorter G-blocks in the *A. vinelandii* samples than we could expect from our in vitro results.[18]

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.8b01796.

> Figure S1: Overlaid HPAEC PAD chromatograms showing G-block distributions of alginates from seaweed, *Azotobacter vinelandii* and in vitro epimerized poly-M. Figure S2: Graphics illustrating the importance of AlgE1 in microbial alginate production (PDF)

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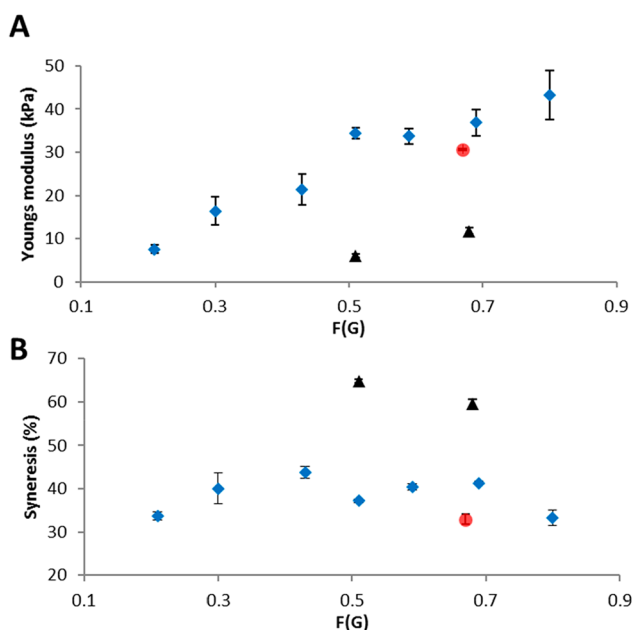The authors declare no competing financial interest.

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