Overall Size of Mannuronan C5-Epimerases Influences Their Ability to Epimerize Modified Alginates and Alginate gels

Annalucia Stanisci[a], Olav A. Aarstad[a], Anne Tøndervik[b], Håvard Sletta[b], Lene B. Dypås[c], Gudmund Skjåk-Bræk[a] and Finn L. Aachmann [a*]

[a]Department of Biotechnology, NOBIPOL, NTNU Norwegian University of Science and Technology, Trondheim, Norway

[b] SINTEF Materials and Chemistry, Department of Biotechnology and Nanomedicine, Trondheim, Norway

[c] Department of Molecular Biology, Norwegian Institute of Public Health, Oslo, Norway

Abstract

A family of seven mannuronan C5-epimerases (AlgE1-AlgE7) produced by *Azotobacter vinelandii* is able to convert β-D-mannuronate (M) to its epimer α-L-guluronate (G) in alginates. Even sharing high sequence homology at the amino acid level, they produce distinctive epimerization patterns. The introduction of new G-blocks into the polymer by *in vitro* epimerization is a strategy to improve the mechanical properties of alginates as biomaterial. However, epimerization is hampered when the substrate is modified or in the gelled state. Here it is presented how native and engineered epimerases of varying size perform on steric hindered alginate substrates (modified or as hydrogels). Reducing the size of the epimerases enables the epimerization of otherwise inaccessible regions in the alginate polymer. Even though the reduction of the size affects the productive binding of epimerases to the substrate, and hence their activity, the smaller epimerases could more freely diffuse into calcium-alginate hydrogel and epimerize it.

Keywords

Alginate, mannuronan C5-epimerases, chemo-enzymatic strategies, alginate hydrogels, chemically modified alginates

*Corresponding author:

Finn L. Aachmann, NTNU, Department of Biotechnology, Sem Sælands veg 6/8, N-7491 Trondheim, Norway, E-mail: finn.l.aachmann@ntnu.no, Phone: +47 73 59 33 17, Fax: +47 73 59 12 83

INTRODUCTION

Alginate is an industrially important biopolymer due to its viscosifying and gelling properties (Matricardi, Alhaique, & Coviello, 2015; Onsøyen, 1996; Skjåk Bræk & Draget, 2012). It is present as the main structural component of brown seaweeds (Painter, 1983) and is also naturally synthesized by bacteria belonging to the genera *Azotobacter* and *Pseudomonas* (Gorin & Spencer, 1966; Govan, Fyfe, & Jarman, 1981; Linker & Jones, 1966). In seaweeds as well as in bacteria, the alginates are initially produced as a linear polymer of 1,4-linked β-D-mannuronic acid (M) (Pindar & Bucke, 1975). This polymer is then modified by the action of C5-mannuronan epimerases which are able to convert M-residues to its C5 epimer α-L-guluronic acid (G) (Haug & Larsen, 1971; Larsen & Haug, 1971a, 1971b).

Azotobacter vinelandii encodes a family of seven extracellular and active mannuronan C5-epimerases which are of biological importance for the formation of a specific life stage, known as cyst (Høidal, Glærum Svanem, Gimmestad & Valla, 2000; Sadoff, 1975). The seven secreted epimerases (AlgE1-AlgE7) are all modular proteins composed of one or two A-modules and from one to seven R-modules (Ertesvåg et al., 1995; Svanem, Skjåk-Bræk, Ertesvåg & Valla, 1999). The A-modules contain the catalytic site, while the non-catalytic R-modules have varying affinity for different alginate structures, thus affecting the degree of processivity displayed by a given epimerase i.e. the R-module from AlgE4 binds stronger to alginate substrates than the R-modules from AlgE6 (Buchinger et al., 2014). Furthermore, the R-modules are also responsible for modulating the epimerase activity probably by reducing the level of calcium needed for full enzyme activity (Ertesvåg & Valla, 1999b). It has been shown that the A-module is able to epimerize even without the R-module, but the presence of the R-module increases the activity rate ten-fold (Ertesvåg et al., 1999b). All the epimerases of the AlgE family are highly homologous but each of them produces specific epimerization patterns which give different properties to the alginate chains. Except for AlgE4, which produces nearly exclusively MG-blocks, all the other C5-mannuronan epimerases are G-block forming. However, the products differ in G-content and mean G-block length, even at comparable degrees of epimerization (Ertesvåg, Høidal, Schjerven, Svanem & Valla, 1999a). All the mannuronan C5-epimerases have differences in substrate specificity and concentration of calcium ions needed for full activity. AlgE4, for example, acts processively by sliding along the alginate chain and epimerizing every second residue, generating alternating MG-sequences (Campa et al., 2004). The enzyme requires a hexameric mannuronan oligomer as minimum polymer length to begin the epimerization (Hartmann, Holm, Johansen, Skjåk-Bræk & Stokke, 2002). Similarly, AlgE6 is able to introduce G-blocks in a processive manner but it needs a minimum of eight residues to make a productive binding (Holtan, Bruheim & Skjåk-Bræk, 2006).

The presence of G-blocks in the alginate polymer is correlated to its potential to form hydrogels in presence of divalent cations, such as calcium. Indeed, in the so called “egg-box model”, calcium ions are responsible for coordinating strong inter-chain interactions between stretches of G-blocks, forming junction zones that hold the polymer in the gel state (Grant, Morris, Rees, Smith & Thom, 1973). The gelling property of the alginate polymer is utilized in different industrial and biomedical processes. As biomaterial, alginate hydrogels can be used for cell encapsulation, where alginate is applied for entrapping the cells, to provide a scaffold for regeneration of different tissues, or as immune barrier. Clearly, the mechanical and chemical properties of the polymer used are of crucial importance for the final material properties. It is therefore desirable to be able to fine tune alginate structures by chemical and/or enzymatic modification for specific applications. Potential applications have been investigated by using chemically modified alginates. Periodate oxidized alginates, for example, are more susceptible to degradation, promoting the hydrolysis of alginate in aqueous solution with a controlled degradation rate (Bouhadir et al., 2001). These properties make such modified alginates more suitable for tissue

engineering since the polymer could be easily cleared from the body in a controlled manner. Therefore, still keeping some gelling properties, oxidized alginates could have a potential for being used as cell transplantation vehicles. Neither periodate oxidized nor bacterial *O*-acetylated residues in alginates are substrates for the C5-epimerases (Kristiansen, Potthast & Christensen, 2010), although it is not clear to which extent these moieties interfere with the epimerization reaction of neighboring non-modified M-residues. For alginate hydrogels, the presence of junction zones as well as the pore size of the gel matrix can create steric hindrance for the introduction of G-blocks by epimerization.

In the present work, we aimed at investigating whether the size of mannuronan C5-epimerases is influencing on the accessibility of alginate substrates that could be sterically hindered in different ways. This knowledge will be important for designing *in vitro* epimerization strategies in tailoring of specific alginate biomaterials. We therefore report on the epimerization properties of four epimerases differing in size on periodate oxidized mannuronan, *O*-acetylated alginates as well as alginates constituting a hydrogel matrix. AlgE1 (two A-modules, four R-modules) and AlgE6 (one A-module, three R-modules) were used as example of large enzymes, while the hybrid enzyme AlgE64 represents the minimum construct of full-length epimerase, having one A-module from AlgE6 combined with the stronger alginate binding R-module from AlgE4. AlgE6A, finally, is the smallest active epimerase constituted by the sole A-module from AlgE6.

MATERIALS AND METHODS

Bacterial strains, Growth conditions, and DNA manipulations

The mannuronan C5-epimerases AlgE1, AlgE6, AlgE64 and AlgE6A were produced by fermentation of recombinant bacterial strains. *Escherichia coli* strain DH5α (Bethesda Research Laboratories) was used as general cloning host, whereas RV308 (ATCC 31608) and the T7-based strains BL21 (DE3) (New England BioLabs) were used for the expression of the proteins. BL21 (DE3) strain carries the T7 expression system which is used with the IMPACT^{TM} system for inducible recombinant protein expression and purification in *E. coli.*

Bacteria were routinely grown at 37 °C in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or on LB agar (LB medium supplemented with 20 g/L agar). The media were supplied with 100 µg/mL ampicillin when appropriate. For enzyme expression, strains were grown in double-strength LB medium (2 X LB; yeast extract, 10 g/L; tryptone, 20 g/L; and NaCl, 10 g/L) supplied with 2 mM $CaCl_2$, pH 7. Cultures were induced for protein expression with isopropyl β-D-thiogalactopyranoside (IPTG) or *m*-toluate for a final concentration of 1 mM.

Standard recombinant DNA procedures were performed as described previously (Sambrook & Russell, 2001). Plasmids were purified by the WizardPlus SV Minipreps DNA purification system (Promega). Transformations of *E. coli* strains were performed according to RbCl transformation protocol (New England BioLabs).

Epimerase AlgE6 (defined as residues 1-853 from AlgE6) and the gene coding for AlgE6A (residues 1-385 from AlgE6 full length sequence) were synthesized *de novo* (GenScript, Piscataway, USA). The DNA sequences corresponding to AlgE6 and AlgE6A were cloned into pTYB1 (IMPACT-CN system, New England BioLabs) using *Nde*I and *Xho*I sites. The hybrid enzyme AlgE64 is a synthetic DNA

sequence encoding the A-module from AlgE6 (residues 1-385) combined with the R-module from AlgE4 (145 residues) (GenScript, Piscataway, USA). The gene coding for AlgE64 was inserted in pMV23 (Bakke et al., 2009) as an *Nde*I-*Not*I fragment, generating pJB–AlgE64. AlgE1 was expressed from pJB658, where the coding region of AlgE1 was inserted as 2994 gene fragment (encoding for residues 1-1403).

Production of Alginate Substrates

Sodium alginate from *Laminaria hyperborea* leaves with fractional amount of G (F_G) = 0.46 was obtained from FMC Biopolymer. High-molecular-weight mannuronan (PolyM), $F_G = 0.00$ was isolated from an epimerase-negative strain of *Pseudomonas fluorescens* (Gimmestad et al., 2003). Periodate oxidized alginates were produced with oxidation degrees ranging from 2% to 8%, as described elsewhere (Kristiansen et al., 2009). The samples were reduced with sodium borohydride, dialyzed and freeze dried before use. Oxidation causes a slight degradation of the polymer chain (Balakrishnan, Lesieur, Labarre & Jayakrishnan, 2005; Kristiansen et al., 2010), however this will have neglectable influence on the epimerization. A native acetylated alginate (17% acetylation; $F_G = 0.10$) was produced from *Pseudomonas aeruginosa* strain DE 127 (Skjåk-Bræk, Grasdalen & Larsen, 1986).

Calcium-alginate beads prepared with alginates from *L. hyperborea* leaves (1% w/v) were made with the use of an electrostatic bead generator following a procedure previously described (Strand, Gåserød, Kulseng, Espevik & Skjåk-Bræk, 2002). This approach allowed obtaining alginate droplets ranging from ~250 to 1000 µm in diameter.

Internally set, homogeneous calcium-alginate gel cylinders prepared with alginates from *L. hyperborea* leaves (1% w/v) were made in 24-well tissue culture plates (16/18 mm, Costar, Cambridge, MA), as described previously (Draget, Østgaard & Smidsrød, 1990). A buffer containing 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.9, 75 mM NaCl, 15 mM $CaCl_2$, was used for saturation of the calcium binding sites after gelling incubation.

Protein Expression and Purification and Epimerase Activity Assay

Epimerase expressing strains (from overnight cultures, 1% final concentration) were grown in 500 mL of 2 X LB medium supplied with 2 mM $CaCl_2$ and 200 µg/mL ampicillin, pH 7.0, in 3 L baffled shake flasks at 30 °C until OD_{600nm} ~0.8-1.2 was reached. Cultures for AlgE6 and AlgE6A production were induced for protein expression using 1 mM IPTG, while AlgE1 and AlgE64 cultures were induced with 1 mM *m*-toluate. Growth was continued overnight at 16 °C before harvesting the cells by centrifugation. For preparation of enzyme extracts, the cells were sonicated in 10-20 mL of 40 mM MOPS pH 6.9 with 5 mM $CaCl_2$, and then centrifuged for 45 min at 23000*g*. The supernatant was filtered (0.22 µm) and the enzymes were purified using Fast Protein Liquid Chromatography (FPLC) (ÄKTA FPLC system – GE Healthcare). AlgE1 and AlgE64 extracts were applied on a 5 mL HiTrap Q HP column (GE Healthcare), and proteins were eluted using a stepwise NaCl gradient (0 to 1 M) in the same buffer as stated above. Extracts for pTYB1-AlgE6 and pTYB1-AlgE6A, which are expressed using the IMPACT™ system, were applied on a 20 mL column prepared with Chitin Resin (New England Biolabs), and purified according to the product protocol (IMPACT™ manual, 2014). Fractions were analyzed for epimerase activity by using a previously reported assay (Tøndervik et al., 2013) and the total protein content was measured by SDS-PAGE and ImageLab™ Software.

Epimerase activity assay

The epimerase activity for AlgE1, AlgE6, AlgE64 and AlgE6A was determined by a spectrophotometric assay developed by the authors of this paper. The assay is not previously published although the same principle was used in a high throughput screen for mutant epimerases (Tøndervik et al., 2013). For activity determination, polyM was used as substrate, meaning that all G residues present after incubation with epimerases are due to enzymatic activity. Epimerase-containing samples (up to 50 µL) diluted in a buffer composed of 20 mM MOPS, 3.6 mM $CaCl_2$ and 100 mM NaCl were mixed with polyM (1 mg/mL) dissolved in the same buffer to a total volume of 200 µl and incubated at 37 ºC for 16-18 h. 10 µL of alginate lyase AlyA with activity 1 U/mL (as defined in Tøndervik et al, 2010) was then added and incubation continued at 25 ºC for 4 h. AlyA cleaves both G-M and G-G linkages introduced by the epimerases. The absorbance at 230 nm (A_{230}) was recorded before (T_0) and after (T_1) the addition of AlyA. Lyase degradation at the indicated linkages introduces unsaturated uronic acid residues that can be quantified by A_{230}, and the epimerase activity (i.e. introduction of G-residues into polyM) in the samples is thus proportional to the ΔA_{230} (T_1-T_0) obtained. One unit of epimerase activity is defined as the amount of enzyme resulting in ΔA_{230}=1 under the described conditions.

End-Point Epimerization

Calcium Dependency - End point epimerization for calcium dependency experiment was performed by using polyM 0.25% (w/v) in 50 mM MOPS pH 6.9 with 75 mM NaCl buffer supplied with different $CaCl_2$ concentration ranging from 1 to 8 mM. Epimerases were added in similar amounts measured by activity (in U) to ensure that the results could be directly compared. The mixtures were kept at 37 °C for 48 h and the epimerization was stopped by adding EDTA to a final concentration of 4 mM. The samples were purified by dialysis against 50 mM NaCl and finally against deionized water. The alginate samples were partially depolymerized by two-step acid hydrolysis prior to NMR analysis (see below).

Epimerization of oxidized/reduced polyM and acetylated alginate – The same procedure as described above was followed for the epimerization of oxidized/reduced polyM and acetylated alginate samples except for a final concentration of 4 mM $CaCl_2$ in the buffer. Prior to NMR analysis acetylated samples were subjected to de-acetylation by adding 0.1 M NaOH in the mixture, followed by incubation for 20 min at room temperature under magnetic stirring and then neutralization with HCl.

Epimerization of calcium-alginate gel beads - Epimerization was performed in 50 mM MOPS pH 6.9 with 75 mM NaCl, 5 mM $CaCl_2$. 10 mg of alginate (in the form of gel beads) were used for each epimerization reaction at 37 °C for 48 h in oscillating water bath. The epimerization reaction was terminated and the beads dissolved with 50 mM EDTA pH 8.0. The samples were dialyzed against 50 mM NaCl with 5 mM EDTA first, 50 mM NaCl and finally deionized water.

Epimerization of calcium-alginate gel cylinders - Epimerization was conducted in 50 mM MOPS pH 6.9, 75 mM NaCl, 5 mM $CaCl_2$, and three parallels were made for each enzyme. After incubation with epimerases (37 °C, 48 h in oscillating water bath), the gel cylinders were dissected in a core (diameter 8 mm) and external part (diameter outer circle 16 mm), and analyzed separately. A 3 mm slice was removed from the top and the bottom of each cylinder to avoid overestimation of the epimerized sample more exposed to the buffer. All the sections were dissolved in 100 mM EDTA and dialyzed against 10 mM MOPS (pH 7), 50 mM EDTA, 75 mM NaCl first, 50 mM NaCl, and finally deionized water.

^{1}H-NMR spectroscopy

All the epimerized samples were subjected to two-step acid hydrolysis, which ensures an homogenous depolymerization of alginate, prior to NMR analysis (Ertesvåg & Skjåk-Bræk, 1999). The pH of the

epimerized samples was adjusted to 5.6 followed by incubation at 95 °C for 1 h. The samples were then cooled, pH adjusted to 3.8 and incubated at 95 °C for 50 min (30 min for oxidized/reduced samples) followed by neutralization and freeze drying.

Freeze-dried samples (6-8 mg) were dissolved in 600 µL D_2O. 3-(Trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP) (Aldrich, Milwaukee, WI) in D_2O (2%, 5 µL) was added as the internal standard for the chemical shift, and triethylenetetra-amine hexa-acetate (TTHA) (Sigma-Aldrich) was added as calcium chelator (0.3 M, 20 µL). ^{1}H-NMR spectra were recorded on BRUKER AVIIIHD 400 MHz equipped with 5 mm SmartProbe or with BRUKER Avance DPX 300 MHz equipped with 5 mm QNP (C/H) probe or BRUKER Avance DPX 400 MHz equipped with 5 mm z-gradient DUL (C/H) probe). For determination of alginate block composition the 1D ^{1}H spectra were recorded at 90°C. The spectra were recorded using TopSpin 1.3, 2.1, 3.2 software (Bruker BioSpin) and processed and analyzed with TopSpin 3.0 software (Bruker BioSpin).

Analysis of G-block length

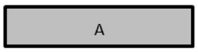
In order to compare the epimerization patterns introduced by the epimerases on different substrates, epimerized periodate oxidized mannuronate and acetylated samples were degraded by *Haliotis tuberculata* alginate lyase which cleaves M-M and M-G linkages. The lysates (1mg/mL) were thereafter analyzed by HPAEC-PAD as previously described (Aarstad, Tøndervik, Sletta & Skjåk-Bræk, 2012). Apart from the oligomers with DP< 4, which stems from degraded M- and MG-blocks, the chromatograms represent the chain-length distribution of G-blocks (Supplementary Material Fig S3).

RESULTS AND DISCUSSION

Previous studies on enzymatic introduction of G-blocks in chemically modified alginates and hydrogels (Sandvig et al., 2015) did not consider how the accessibility of non-epimerized regions (M/MG blocks) is related to the size of the epimerases and the actual G-blocks formation. Here, selected substrates and genetically engineered epimerases are used to explore the possibilities of reducing the size of the enzymes in order to obtain enzymes suitable for developing improved alginate-based biomaterials.

The size and shape of the epimerases were elucidated by Buchinger *et. al* (Buchinger et al., 2014). AlgE6 (A-R-R-R) has an elongated shape of 180 Å, while AlgE64 (A_6-R_4), which is comparable in size to AlgE4 (A-R), has a maximum length around 100 Å. AlgE6A (A) measures 65 Å representing the minimal active epimerase (Tab. 1). Attempts on making even smaller epimerases by reducing the β-helix from the C-terminal end of the A-module from AlgE6 ($AlgE6A_{1\text{-}272}$ comprised of AA 1-272, $AlgE6A_{1\text{-}295}$ comprised of AA 1-295 and $AlgE6A_{1\text{-}336}$ comprised of AA 1-336) resulted in non-functional enzymes (data not shown). This indicates that the A-module is the minimal size for an active epimerase, even though the active site is located in proximity of the N-terminus (Rozeboom et al., 2008). Lack of activity for the truncated epimerases may be caused by misfolding or by their inability to bind the substrate. Generally, β-helix proteins fold sequence-wise (from N-terminus to C-terminus) and previous studies have shown a clear correlation between substrate-binding and introduction of G-residues (Buchinger et al., 2014). Based on SAXS data in Buchinger *et. al.,* AlgE1 should conserve an elongated shape of approximately 280 Å, and was included in this study as an example of a large epimerase being approximately 4 times the size of AlgE6A.

Tab 1. Modular structure, size (overall) and molecular weight of the mannuronan C5-epimerases AlgE1, AlgE6, the A-module AlgE6A, and the hybrid enzyme AlgE64 constituted by the A-module from AlgE6 and the R-module from AlgE4. Patterns and gray tones indicate similarities or differences between the modules.

Enzyme	size[a]	Modular structure	Mw (kDa)
AlgE1	~280 Å[b]		147.2
AlgE6	~180 Å		90.2
AlgE64	~100 Å[b]		55.4
AlgE6A	~65 Å		40.0

[a](Buchinger, Knudsen et al. 2014). [b]The overall size of AlgE1 and AlgE64 are deduced from SAXS data in Buchinger et al. 2014. The calculated molecular weights are determined by use of ProtParam (Gasteiger E., 2005)

AlgE64 and AlgE6A are less affected by high calcium concentrations than AlgE6

Enzymatic activity of C5-mannuronan epimerases have previously been shown to be dependent on calcium ions, which are essential both for the A-module activity and the R-module structural stability (Aachmann et al., 2006; Rozeboom et al., 2008). It is also demonstrated that the concentration of calcium does affect the amount and distribution of guluronic acid in the polymer chain (Ertesvåg, Høidal, Skjåk-Bræk & Valla, 1998; Ertesvåg et al., 1999b; Ofstad & Larsen, 1981). Epimerization is self-limiting in two ways since the G blocks will bind calcium cooperatively making the polymer less accessible for the epimerases, and also depleting the free calcium available to the epimerases. Calcium dependency for the epimerases AlgE6, AlgE64 and AlgE6A was determined using end-point epimerization of poly mannuronic acid at different calcium concentrations (1 mM - 8 mM) as it has been shown that enzymatic activity markedly decrease below 1 mM (Skjåk-Bræk & Larsen, 1985). Fig 1 reveals that the highest degree of epimerization is achieved at 2 mM for all three enzymes. The same trends are found when GG-dyads are considered (Fig S1 and S2 Supplementary materials). The degree of epimerization was not tested for calcium concentration lower than 1 mM as epimerization is a calcium-dependence reaction (Ertesvåg, Doseth, Larsen, Skjåk-Bræk & Valla, 1994). The trend in epimerization is similar for all the enzymes; however, both AlgE64 and AlgE6A seem to be more active at higher calcium concentrations then AlgE6. At calcium concentration ranging between 3 mM and 4 mM, gelling formation commences and the newly formed G-blocks arisen from the epimerization reaction can form junction zones that render the substrate less accessible. As both AlgE64 and AlgE6A are smaller they might be less affected by the formation of the gel network than AlgE6.

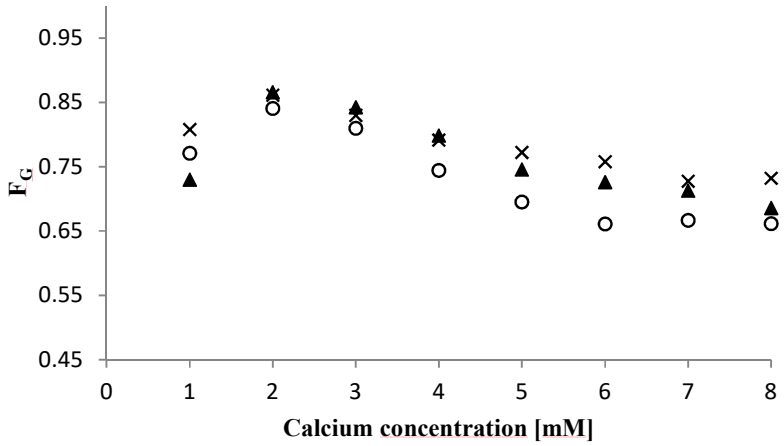


Fig 1. Fraction of G residues (F_G) introduced in polyM by the epimerases (○) AlgE6, (×) AlgE64 and (▲) AlgE6A at different concentrations of calcium. Monad fractions F_G, where G denotes α-L-GulA, were determined from ^{1}H-NMR spectra.

Reducing the size of AlgE6 influences the epimerization of modified alginates in solution

Partial oxidation (Kristiansen et al., 2009) as well as acetylation (Skjåk-Bræk, 1985) have previously been shown to hinder the epimerization of alginate. However, it is not clear to which extent these modified moieties block the accessibility of epimerases to the non-epimerized flanking regions (M-/MG-blocks) in the substrate. To understand how the size of the epimerases influences their ability to epimerize modified alginates, four different oxidized samples and one acetylated substrate were examined. The three AlgE6 based epimerases were able to epimerize all the chemically modified substrates as shown in Fig 2. A clear correlation between the G-content of the epimerized samples and the degree of oxidation was found, i.e. the level of epimerization decreased with increasing degree of substrate oxidation for all enzymes. However, AlgE6 is apparently much more affected by the presence of modified residues than the smaller AlgE64 and AlgE6A indicating that these epimerases display better accessibility to the hindered substrates. Moreover, the A-module from AlgE6 seems to be more affected than AlgE64 at higher degree of oxidation. This can be a result of AlgE6A's advantage of being small in size is overshadowed by the reduction of the substrate binding surface. Consequently, it can result in too few productive binding events, which subsequently lower the overall introduction of G-residues into the modified alginate. As it was observed for the alginate with highest degree of oxidation (8%). AlgE64 R-module thus increasing the affinity to the substrate results in an improved G-formation ability.

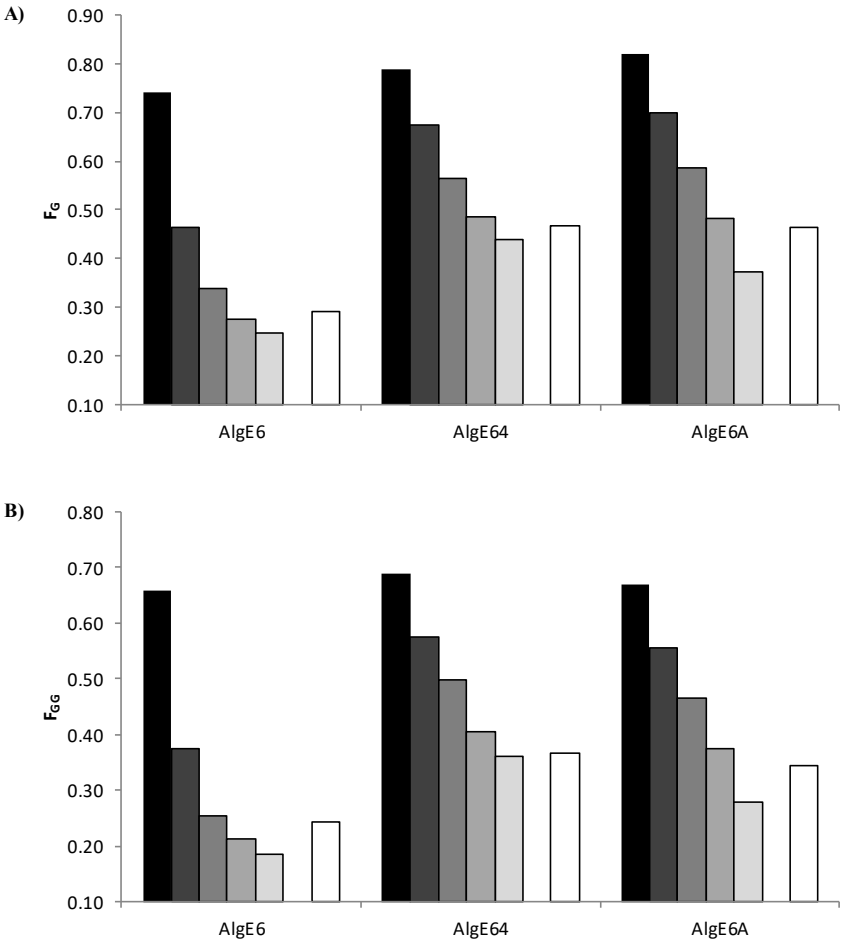


Fig 2. Epimerization of oxidized and acetylated alginate samples by AlgE6, AlgE64 and AlgE6A. Monads (G content) (A) and dyads (GG content) (B) are calculated from ^{1}H-NMR spectra. Oxidized/reduced substrates with different degree of oxidation (■, 2%; ■ 4%; ■ 6%; □ 8%) were used. Starting acetylated alginate substrate showed 17% acetylation and F_G= 0.01 (□). PolyM is epimerized as control (■).

AlgE6, AlgE64 and AlgE6A were further found to be active on alginate from *P. aeruginosa* with 17% acetylation (Fig. 2). Indeed, despite the number of modified residues maximum double as high for the acetylated sample compared to the samples with 8% oxidation degree, they show similar degrees of epimerization (F_G: 0.29-0.47 for acetylated samples; F_G: 0.25-0.44 for oxidized samples). Since substituted residues cannot be epimerized and there is a critical minimal number of monomers required for the productive binding of the epimerases, the distribution of modified residues is expected to influence the degree of epimerization. By using a theoretical approach the chain length distribution of M blocks between modified M residues, assuming that the modification is random, can be described with a Kuhn model:

$$W_n = n(\alpha_0 + b)^2 \cdot (1 - (\alpha_0 + b))^{n-1} \quad (1)$$

Where W_n is the weight fraction of a M-oligomer with chain length n, α_0 is the degree of scission of the starting material (defined as M_0/M_n where M_0 and M_n is monomer weight and number average weight of the sample respectively). Finally, b is the fraction of modified M units.

The yield is given by $W_n \cdot (1-b)$ to correct for fraction of the substrate which is no longer available for epimerization. The distributions for periodate oxidized M and acetylated samples are shown in the Supplementary material (Fig S4, S5).

As an example, the weight fraction of M blocks with n>10 is 0.68 for the periodate oxidized M 8% sample but only 0.14 for the 17% acetylated sample if the acetyl groups were randomly distributed. For the other extreme, assuming all acetyl groups located together as one block in each chain, $W_{>10} = 0.75$. Based on the calculations above it is suggested that the acetyl groups display a non-random distribution.

Two catalytic active modules improve the performance of larger epimerases

AlgE1 is larger than AlgE6 and has two catalytic active modules (A_1 and A_2). AlgE1 has previously been found to form very long G-blocks (>100) compared to AlgE6 which form shorter G-blocks (~40) when polyM substrate is epimerized, and it was therefore interesting to compare the activity of these two enzymes on modified substrates (Aarstad, Strand, Klepp-Andersen & Skjåk-Bræk, 2013). Surprisingly, although being substantially larger than AlgE6, AlgE1 is able to epimerize all modified alginate substrates to higher G-content than AlgE6 (Fig 3). This might be due to AlgE1 being a more effective G-block former than AlgE6 suggesting that AlgE1 preferentially elongates the existing G-blocks while AlgE6 works in a more random fashion (Paper in preparation). Another explanation is that the size of AlgE1 is large enough for the enzyme to pass over a modified residue without having to dissociate from the alginate due to the overall supposedly larger substrate binding area. Accordingly, when AlgE1 is compared to AlgE64 and AlgE6A, no significant differences are found for the ability of epimerization of modified substrates by AlgE1, even though it is much bigger than the other two enzymes.

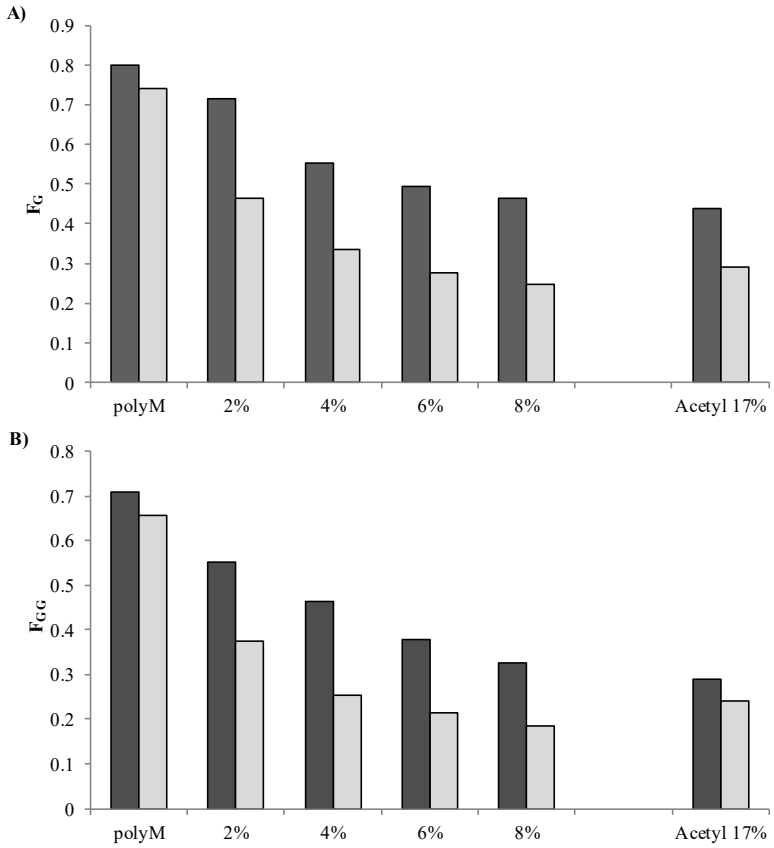


Fig 3. Epimerization of oxidized and acetylated alginate samples by AlgE1 (■) and AlgE6 (□). Monads (A) and dyads (B) are calculated from ^{1}H-NMR spectra. Oxidized/reduced substrates with different degree of oxidation (2%, 4%, 6%, 8%) were used. Starting acetylated alginate substrate (pattern fill) showed 17% acetylation and F_G= 0.10.

Small epimerases AlgE64 and AlgE6A can easily diffuse into and epimerize a preformed gel matrix

AlgE1, AlgE6, AlgE64 and AlgE6A were tested on internally gelled high-M calcium-alginate cylinders (1% w/v), using the method described by Draget *et al.* (Draget et al., 1990), in order to study how their size influences their action in a polymer network. Fig 4 shows the profile of the G-content in the epimerized calcium-alginate cylinders. As illustrated in Fig 4, the fraction of guluronic acid in the gel matrix (F_G) increases after treatment with all four enzymes. AlgE1, AlgE6 and AlgE64 show a gradient in the G-content which decreases from the outer wall towards the core of the cylinder, while AlgE6A gives the same degree of epimerization across the whole gel cylinder. GG-dyads content also follows the same trend (data not shown).

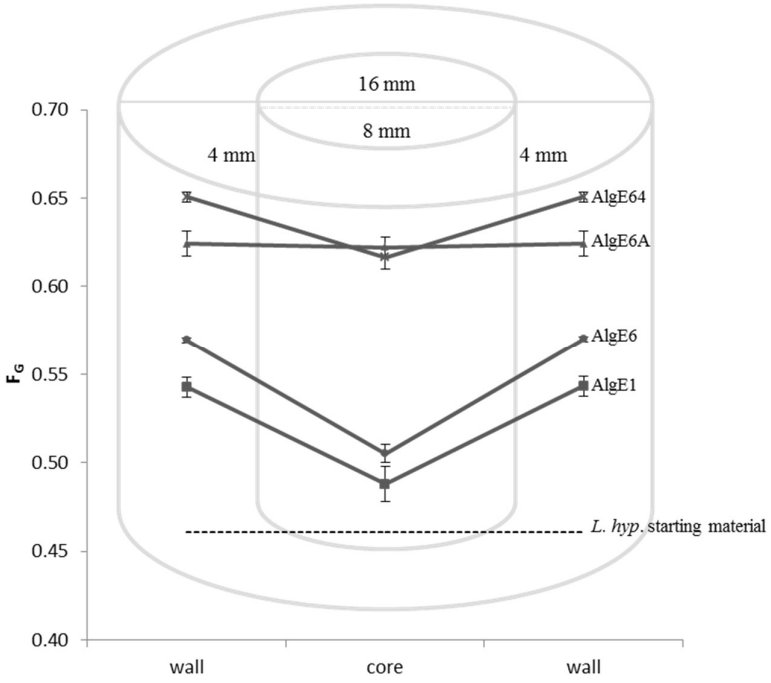


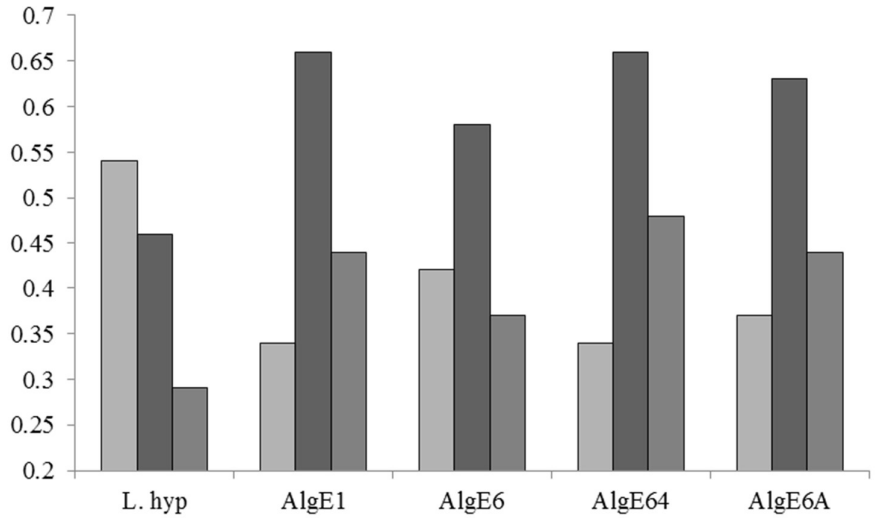
Fig 4. Epimerization of calcium- alginate gel cylinders by AlgE1, AlgE6, AlgE64 and AlgE6A. Gel cylinders [core: diameter 8 mm (light grey dashed line) and external part: diameter outer circle 16 mm] are prepared with 1% (w/v) *Laminaria hyperborea* leaf alginate (Mw = 219,000; F_G = 0.46), as described in the text. Monad fractions F_G are calculated from ^{1}H-NMR spectra. The epimerization gradient is shown from the wall towards the core of the gel cylinder. ■, AlgE1; ●, AlgE6; ▲, AlgE6A; ✕, AlgE64. Values are reported as mean ± s.d. (n=3). Dashed line at the basis of the cylinder indicates G content of the starting material (*L. hyperborea* Mw = 219,000; F_G = 0.46).

This reveals the importance of the size of the enzyme for the diffusion into the gel. Indeed, the largest enzymes, AlgE1, AlgE6 and AlgE64, are able to epimerase the external part of the cylinder, but they show reduced activity in the core of the cylinder. In fact, the epimerization of the gel core implies the entrance in the deep gel matrix, which is clearly impeded by the enzyme size. Moreover, the value of F_G and therefore the tendency of epimerization of the different enzymes in the gel matrix could provide an approximate measure of the pores of the gel. For example, AlgE1 which should show a higher F_G value compared with AlgE6 is clearly impeded by its size. Similarly, AlgE64, which benefits from a combination of higher activity, promoted by a stronger binding to the alginate substrate (Buchinger et al., 2014), together with a small size, is still slightly obstructed by its size in the core part. Pore sizes ranging from 50 Å to 1500 Å, have previously been reported in literature (Andresen, Skipnes, Smidsrød, Østgaard & Hemmer, 1977; Turco et al., 2011), however M-rich alginates form a less permeable gel than high-G alginates, which contain a more open gel network caused by the stiffness of G-blocks (Martinsen, Skjåk-Bræk & Smidsrød, 1989). Since the diffusion of molecules into and out of gel systems depends on their molecular weight, shape and charge (Kulseng, Thu, Espevik & Skjåk-Bræk, 1997), it is likely that AlgE64 (100 Å) is more impeded from entering the core gel matrix because the size of the pores is relatively smaller, rather resembling the size of AlgE6A (65 Å), which more easily diffuses into the matrix.

To elucidate how the distance of diffusion influences on the ability for epimerization of the different enzymes, AlgE1, AlgE6, AlgE64 and AlgE6A were incubated with calcium-alginate gel beads ranging

388 in size from 250-1000 µm. The results of the epimerization with all four enzymes are summarized in
389 Fig 5. The epimerases are able to diffuse in the solution and enter the beads.

390



391

392 **Fig 5.** Epimerization of calcium- alginate beads by AlgE1, AlgE6, AlgE64 and AlgE6A. Beads are
393 made with 1% (w/v) *Laminaria hyperborea* leaf alginate (Mw = 219,000; F_G = 0.46), as described in
394 the text. The droplet size was in the range 250-1000 µm. Monads (□ F_M; ■ F_G) and dyads (F_{GG} ■) are
395 calculated from ^{1}H-NMR spectra.
396

397 This demonstrates that the epimerases could convert M-residues present between the junctions of the
398 capsule gel matrix converting this high-M leaf alginate into high-G material. Similar epimerization
399 pattern is displayed by all four enzymes, although AlgE6 stands out as the least effective while AlgE64
400 gives the highest content of G-blocks. As observed for modified alginate substrates the epimerases
401 ability to form G-residues in small beads is probably related to the nature of the enzyme itself, as well
402 as to the size of the enzymes. However, the size of the epimerases has a considerable larger influence
403 when it comes to bigger gel matrices.

404

405 CONCLUSION

406 In this study, the ability of four mannuronan C5-epimerases differing in size to epimerize sterically
407 hindered alginate substrates were evaluated. We found that epimerization of alginates modified by
408 oxidation and acetylation depends on the size of the epimerase used, as well as on other enzyme
409 properties such as interaction with the substrate. Furthermore, for large alginate gel networks, there is
410 a clear correlation between the size of the epimerase and its ability to diffuse into the gel matrix and
411 epimerize available M- and MG-sequences. In particular, the AlgE6A-module seems to be especially
412 suited for performing *in vitro* epimerization of hindered alginate substrates. Important technological
413 applications within biomaterials would involve mannuronan C5-epimerases to construct novel
414 biomaterial by chemo-enzymatic strategies and to introduce additional G-blocks into alginates in the
415 hydrogel state.

ACKNOWLEDGEMENT

This work were financed by the MARPOL project and NNP - Norwegian NMR Platform (grants 221576 and 226244 from the Research Council of Norway, respectively). We thank Wenche Iren Strand and Gerd Inger Sætrom for analyzing the NMR samples. We are also grateful to Marianne Øksnes Dalheim for providing the oxidized/reduced alginate samples.

REFERENCES

Aachmann, F. L., *et al.* (2006). NMR structure of the R-module: a parallel beta-roll subunit from an Azotobacter vinelandii mannuronan C-5 epimerase. *J Biol Chem, 281*(11), 7350-7356.

Aarstad, O., Strand, B. L., Klepp-Andersen, L. M., & Skjåk-Bræk, G. (2013). Analysis of G-block distributions and their impact on gel properties of in vitro epimerized mannuronan. *Biomacromolecules, 14*(10), 3409-3416.

Aarstad, O. A., Tøndervik, A., Sletta, H., & Skjåk-Bræk, G. (2012). Alginate Sequencing: An Analysis of Block Distribution in Alginates Using Specific Alginate Degrading Enzymes. *Biomacromolecules, 13*(1), 106-116.

Andresen, I.-L., Skipnes, O., Smidsrød, O., Østgaard, K., & Hemmer, P. C. (1977). Some Biological Functions of Matrix Components in Benthic Algae in Relation to Their Chemistry and the Composition of Seawater. *Cellulose Chemistry and Technology* (Vol. 48, pp. 361-381): AMERICAN CHEMICAL SOCIETY.

Bakke, I., *et al.* (2009). Random Mutagenesis of the Pm Promoter as a Powerful Strategy for Improvement of Recombinant-Gene Expression. *Applied and Environmental Microbiology, 75*(7), 2002-2011.

Balakrishnan, B., Lesieur, S., Labarre, D., & Jayakrishnan, A. (2005). Periodate oxidation of sodium alginate in water and in ethanol–water mixture: a comparative study. *Carbohydrate Research, 340*(7), 1425-1429.

Bouhadir, K. H., *et al.* (2001). Degradation of Partially Oxidized Alginate and Its Potential Application for Tissue Engineering. *Biotechnology Progress, 17*(5), 945-950.

Buchinger, E., *et al.* (2014). Structural and functional characterization of the R-modules in alginate C-5 epimerases AlgE4 and AlgE6 from Azotobacter vinelandii. *Journal of Biological Chemistry, 289*(45), 31382-31396.

Campa, C., *et al.* (2004). Biochemical analysis of the processive mechanism for epimerization of alginate by mannuronan C-5 epimerase AlgE4. *Biochemical Journal, 381*(Pt 1), 155-164.

Draget, K. I., Østgaard, K., & Smidsrød, O. (1990). Homogeneous alginate gels: A technical approach. Carbohydrate Polymers, 14(2), 159-178.

Ertesvåg, H., Doseth, B., Larsen, B., Skjåk-Bræk, G., & Valla, S. (1994). Cloning and expression of an Azotobacter vinelandii mannuronan C-5-epimerase gene. *J Bacteriol, 176*(10), 2846-2853.

Ertesvåg, H., *et al.* (1995). A family of modular type mannuronan C-5-epimerase genes controls alginate structure in Azotobacter vinelandii. *Mol Microbiol, 16*(4), 719-731.

Ertesvåg, H., Høidal, H. K., Schjerven, H., Svanem, B. I. G., & Valla, S. (1999a). Mannuronan C-5-Epimerases and Their Application for in Vitro and in Vivo Design of New Alginates Useful in Biotechnology. *Metabolic Engineering, 1*(3), 262-269.

Ertesvåg, H., Høidal, H. K., Skjåk-Bræk, G., & Valla, S. (1998). The Azotobacter vinelandii Mannuronan C-5-Epimerase AlgE1 Consists of Two Separate Catalytic Domains. *Journal of Biological Chemistry, 273*(47), 30927-30932.

Ertesvåg, H., & Skjåk-Bræk, G. (1999). Modification of Alginate Using Mannuronan C-5 Epimerases. In C. Bucke (Ed.), *Carbohydrate Biotechnology Protocols* (pp. 71–78). Totowa, NJ: Humana Press.

Ertesvåg, H., & Valla, S. (1999b). The A Modules of the Azotobacter vinelandii Mannuronan-C-5-Epimerase AlgE1 Are Sufficient for both Epimerization and Binding of Ca(2+). *Journal of Bacteriology, 181*(10), 3033-3038.

Gimmestad, M., *et al.* (2003). The Pseudomonas fluorescens AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. *J Bacteriol, 185*(12), 3515-3523.

Gorin, P. A. J., & Spencer, J. F. T. (1966). Exocellular alginic acid from Azotobacter vinelandii. Canadian Journal of Chemistry, 44(9), 993–998.

Govan, J. R., Fyfe, J. A., & Jarman, T. R. (1981). Isolation of alginate-producing mutants of Pseudomonas fluorescens, Pseudomonas putida and Pseudomonas mendocina. J Gen Microbiol, 125(1), 217–20.

Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., & Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: The egg-box model. *FEBS Letters, 32*(1), 195-198.

Hartmann, M., Holm, O. B., Johansen, G. A., Skjåk-Bræk, G., & Stokke, B. T. (2002). Mode of action of recombinant Azotobacter vinelandii mannuronan C-5 epimerases AlgE2 and AlgE4. *Biopolymers, 63*(2), 77-88.

Haug, A., & Larsen, B. (1971). Biosynthesis of alginate. II. Polymannuronic acid C-5-epimerase from Azotobacter vinelandii (Lipman). Carbohydrate Research, 17(2), 297–308.

Holtan, S., Bruheim, P., & Skjåk-Bræk, G. (2006). Mode of action and subsite studies of the guluronan block-forming mannuronan C-5 epimerases AlgE1 and AlgE6. *Biochemical Journal, 395*(Pt 2), 319-329.

Høidal, H. K., Glærum Svanem, B. I., Gimmestad, M., & Valla, S. (2000). Mannuronan C-5 epimerases and cellular differentiation of Azotobacter vinelandii. *Environ Microbiol, 2*(1), 27-38.

Kristiansen, K. A., Potthast, A., & Christensen, B. E. (2010). Periodate oxidation of polysaccharides for modification of chemical and physical properties. *Carbohydr Res, 345*(10), 1264-1271.

Kristiansen, K. A., *et al.* (2009). Novel alginates prepared by independent control of chain stiffness and distribution of G-residues: Structure and gelling properties. *Carbohydrate Polymers, 77*(4), 725-735.

Kulseng, B., Thu, B., Espevik, T., & Skjåk-Bræk, G. (1997). Alginate polylysine microcapsules as immune barrier: permeability of cytokines and immunoglobulins over the capsule membrane. *Cell Transplant, 6*(4), 387-394.

494 Larsen, B., & Haug, A. (1971a). Biosynthesis of alginate. 1. Composition and structure of alginate
495 produced by Azotobacter vinelandii (Lipman). Carbohydrate Research, 17(2), 287–296.

496 Larsen, B., & Haug, A. (1971b). Biosynthesis of alginate. 3. Tritium incorporation with
497 polymannuronic acid. Carbohydrate Research, 20(2), 225–232.

498 Linker, A., & Jones, R. S. (1966). A New Polysaccharide Resembling Alginic Acid Isolated from
499 Pseudomonas. Journal of Biological Chemistry, 241(16), 3845–3851.

500 Martinsen, A., Skjåk-Bræk, G., & Smidsrød, O. (1989). Alginate as immobilization material: I.
501 Correlation between chemical and physical properties of alginate gel beads. *Biotechnol Bioeng, 33*(1),
502 79-89.

503 Matricardi, P., Alhaique, F., & Coviello, T. (2015). Polysaccharide Hydrogels: Characterization and
504 Biomedical Applications (Pan Stanford).

505 Ofstad, R., & Larsen, B. (1981). The effect of calcium-ion concentration on poly-D-mannuronate C-5-
506 epimerase. In T. Levring (Ed.). *Proceedings of the 10th International Seaweed Symposium*. Berlin,
507 Germany.

508 Onsøyen, E. (1996). Commercial applications of alginates. Carbohydr Eur, pp. 26–31.

509 Painter, T. J. (1983). 4 - Algal Polysaccharides A2 - Aspinall, G. O. In The Polysaccharides (pp. 195–
510 285). Academic Press.

511 Pindar, D. F., & Bucke, C. (1975). The biosynthesis of alginic acid by Azotobacter vinelandii. Biochem
512 J, 152(3), 617–22.

513 Rozeboom, H. J., *et al.* (2008). Structural and mutational characterization of the catalytic A-module of
514 the mannuronan C-5-epimerase AlgE4 from Azotobacter vinelandii. *Journal of Biological Chemistry,*
515 *283*(35), 23819-23828.

516 Sadoff, H. L. (1975). Encystment and germination in Azotobacter vinelandii. *Bacteriological Reviews,*
517 *39*(4), 516-539.

518 Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor
519 Laboratory Press.

520 Sandvig, I., *et al.* (2015). RGD-peptide modified alginate by a chemoenzymatic strategy for tissue
521 engineering applications. *J Biomed Mater Res A, 103*(3), 896-906.

522 Skjåk Bræk, G., & Draget, K. I. (2012). Alginates: Properties and Applications. In I: Polymer Science:
523 A Comprehensive Reference (213-220). Elsevier.

524 Skjåk-Bræk, G., Grasdalen, H., & Larsen, B. (1986). Monomer sequence and acetylation pattern in
525 some bacterial alginates. *Carbohydr Res, 154*, 239-250.

526 Skjåk-Bræk, G., & Larsen, B. (1985). Biosynthesis of alginate: Purification and characterisation of
527 mannuronan C-5-epimerase from azotobacter vinelandii. Carbohydrate Research, 139, 273–283.

528 Skjåk-Bræk, G., Larsen, B., Grasdalen, H. (1985). The role of *O*-acetyl groups in the biosynthesis of
529 alginate by *Azotobacter vinelandii*. *Carbohydrate Research, 145*, 169-174.

530 Sletta, H., *et al.* (2007). The Presence of N-Terminal Secretion Signal Sequences Leads to Strong
531 Stimulation of the Total Expression Levels of Three Tested Medically Important Proteins during High-

Cell-Density Cultivations of Escherichia coli. *Applied and Environmental Microbiology, 73*(3), 906-912.

Strand, B. L., Gåserød, O., Kulseng, B., Espevik, T., & Skjåk-Bræk, G. (2002). Alginate-polylysine-alginate microcapsules: effect of size reduction on capsule properties. *J Microencapsul, 19*(5), 615-630.

Svanem, B. I., Skjåk-Bræk, G., Ertesvåg, H., & Valla, S. (1999). Cloning and expression of three new Azotobacter vinelandii genes closely related to a previously described gene family encoding mannuronan C-5-epimerases. *J Bacteriol, 181*(1), 68-77.

Turco, G.*, et al.* (2011). Mechanical spectroscopy and relaxometry on alginate hydrogels: a comparative analysis for structural characterization and network mesh size determination. *Biomacromolecules, 12*(4), 1272-1282.

Tøndervik, A.*, et al.* (2013). Mannuronan C-5 Epimerases Suited for Tailoring of Specific Alginate Structures Obtained by High-Throughput Screening of an Epimerase Mutant Library. *Biomacromolecules, 14*(8), 2657-2666.