Mannuronate C-5 epimerases and their use in alginate modification

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Abstract

Alginate is a polysaccharide consisting of β-D-mannuronate (M) and α-L-guluronate (G) produced by brown algae and some bacterial species. Alginate has a wide range of industrial and pharmaceutical applications, owing mainly to its gelling and viscosifying properties. Alginates with high G content are considered more valuable since the G residues can form hydrogels with divalent cations. Alginates are modified by lyases, acetylases, and epimerases. Alginate lyases are produced by alginate-producing organisms and by organisms that use alginate as a carbon source. Acetylation protects alginate from lyases and epimerases. Following biosynthesis, alginate C-5 epimerases convert M to G residues at the polymer level. Alginate epimerases have been found in brown algae and alginate producing bacteria, predominantly *Azotobacter* and *Pseudomonas* species. The best characterised epimerases are the extracellular family of AlgE1-7 from *Azotobacter vinelandii*. AlgE1-7 all consist of combinations of one or two catalytic A-modules and one to seven regulatory R-modules, but even though they are sequentially and structurally similar, they create different epimerisation patterns. This makes the AlgE enzymes promising for tailoring of alginates to have the desired properties.

This review describes the current state of knowledge regarding alginate-active enzymes with focus on epimerases, characterisation of the epimerase reaction, and how alginate epimerases can be used in alginate production.

Summary points

- Alginates are widely used industrially due to their viscosifying and hydrogel forming abilities. Alginate-active enzymes, especially alginate epimerases, are important to tailor alginate to the desired properties.
- Alginate epimerases change the C-5 conformation of β-D-mannuronate (M) residues in alginate converting them to α-L-guluronate (G). G residues can chelate divalent cations thereby forming hydrogels.
- Characterisation of the epimerase reaction requires the ability to distinguish between M and G residues. Different methods are used to analyse the alginate G-content, block distribution, chain length, and reaction kinetics.
- The G-content of alginates can be increased using alginate epimerases. Epimerases can be genetically engineered becoming *e.g.* more efficient at creating G-blocks.
- Alginate epimerases have not been fully utilised, and it would be industrially beneficial to develop more robust epimerases that produce well-defined products in a reproducible manner.

1 Introduction

Alginate structure and properties

Alginate is produced by and currently manufactured from harvested brown algae but is also found in *Azotobacter* and some *Pseudomonas* bacteria [1, 2]. Alginates are linear copolymers consisting of (1→4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) [3], which are primarily arranged in blocks of M (polyM), blocks of G (polyG), and blocks of alternating M and G (polyMG) [4] (see Figure 1). Bacterial alginates may be O-acetylated at C-2 and/or C-3 on the M-residues, affecting the polymer's properties [5]. In brown algae, alginates are the most abundant cell wall component [6], whereas in *Azotobacter spp*. alginates are found in the vegetative capsule and is a necessary component of the cyst cell wall [7].

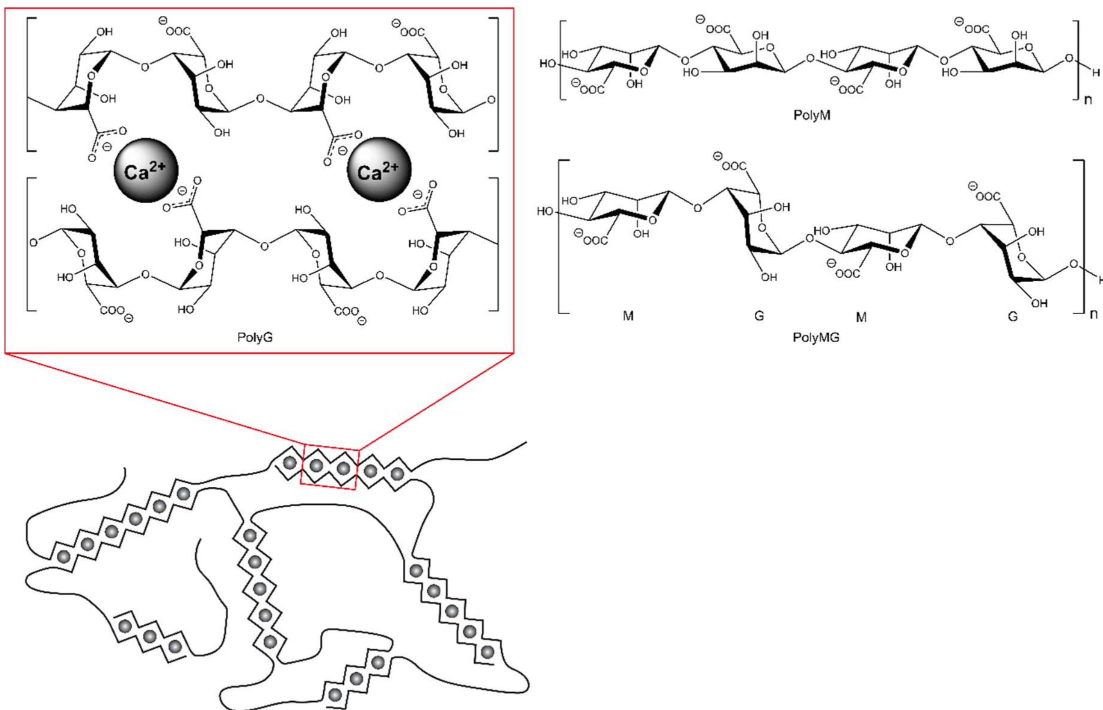

Figure 1: Alginates consist of the monosaccharides (1→4)-linked β-D-mannuronate (M) and α-L-guluronate (G), which can be arranged in three types of block structures; polyG, polyM, and polyMG. The stretches of G-monomers coordinate divalent cations (here Ca^{2+}) resulting in formation hydrogel network, which is also known as the 'egg-box' model [8].

The G-blocks of alginates can bind divalent cations, typically Ca^{2+}-ions, and form hydrogels as described by the so-called egg-box model (Figure 1) [8]. This property along with the ability of alginates to bind water and provide viscosity is the reason for the wide industrial and pharmaceutical use of alginates in for example textile printing, wound dressing, and as a thickening agent in food [9-11]. The M residues do not have the same chelating ability [10].

Biologically, alginates are synthesised as polyM, and then modified post polymerisation by alginate-active enzymes [12, 13].

Alginate-active enzymes

Alginate lyases are enzymes that degrade alginate to oligo- or monosaccharides by breaking the glycosidic bond [14, 15]. Alginate lyases use a β-elimination mechanism, which leads to the formation of 4,5-unsaturated sugars resulting in a 4-deoxy-L-erythro-hex-4-enopyranosyluronate (denoted Δ) at the non-reducing end of the reaction products. The proposed enzymatic mechanism is shown in Figure 2 [16]. In alginate-producing bacteria, a periplasmic alginate lyase is necessary to avoid alginate from accumulating in the periplasm and cause cell lysis [17]. In alginate-degrading bacteria, alginates and alginate oligomers can be degraded to a monosaccharide by alginate lyases [18]. The monosaccharides are linearised by the enzyme KdgF to 4-deoxy-L-erythro-5-hexoseulose uronate (DEH) and is further reduced to 2-keto-3-deoxygluconate (KDG), which can enter the Entner-Doudoroff metabolic pathway of bacteria [19]. A similar pathway has been proposed for the brown algae *Saccharina japonica* [20]. On the contrary, alginate-producing bacteria do not seem able to use alginate as a carbon source.

In alginate-producing bacteria, the nascent polyM is transported through a periplasmic protein channel where the alginate is modified by acetylases and epimerases [21]. O-acetylation protects the M-residues against further modification by epimerases and most lyases. The only known alginate deacetylase is the secreted, bifunctional epimerase and deacetylase PsmE from *P. syringae* where a separate deacetylase module removes acetyl groups and hence allows further epimerisation by the epimerase module [22]. Acetylation also affects viscosity, increase the water binding properties, and reduces gel strength of alginates [5]. After alginate is synthesised as polyM, some M residues are converted to G by alginate epimerases [23].

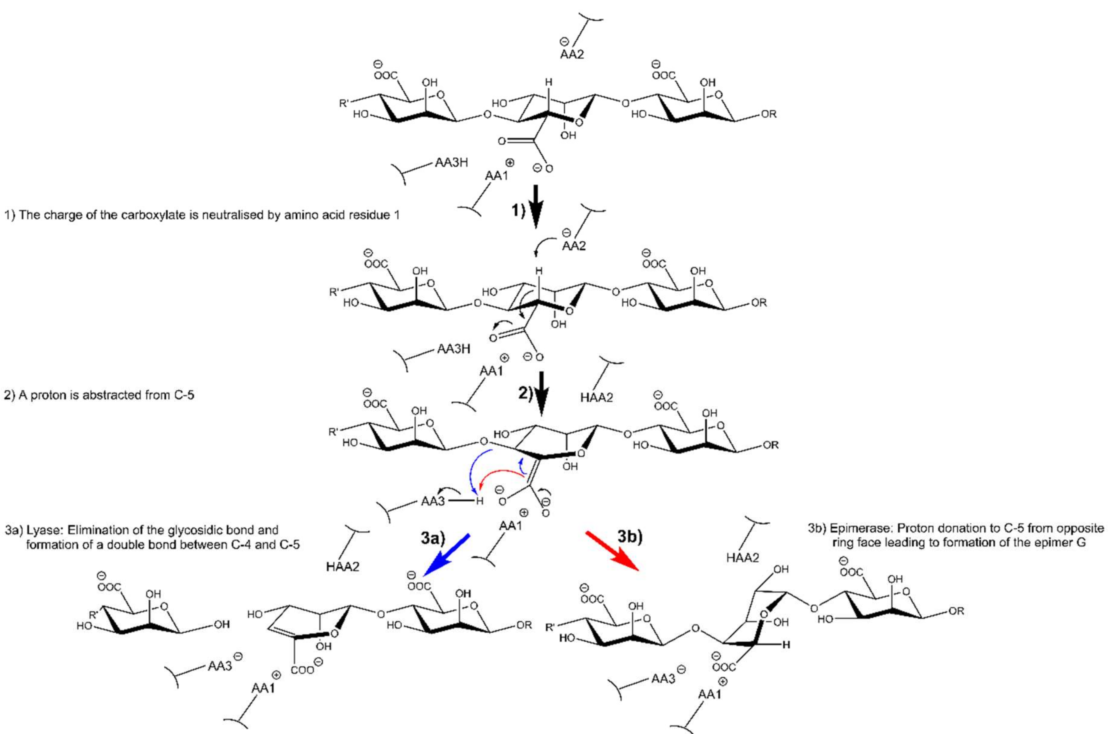

Figure 2: Overall hypothesised reaction mechanism for alginate lyases (blue arrow) and epimerases (red arrow), where AA1, AA2, and AA3 are abbreviations for amino acid residue 1, 2, and 3. The first and second step are identical for the two types of enzymes, whereas the third step differ in how a proton is donated to the intermediate leading either to formation of the lyase or epimerase product [16].

2 Alginate epimerases

Bacterial alginate epimerases (EC 5.1.3.37) can be divided into two categories: AlgG- and AlgE-type epimerases. AlgGs are periplasmic calcium-independent enzymes that epimerise single M residues to G in polyM during translocation of the polysaccharide [24]. AlgE enzymes are calcium-dependent, extracellular, and create MG-blocks and G-blocks [25, 26]. All alginate-producing bacteria encode one AlgG enzyme [23]. *Azotobacter vinelandii* (*Av*) produces seven different AlgE enzymes (AlgE1-7) [25, 27, 28], whereas *Azotobacter chroococcum* (*Ac*) produces three AlgE enzymes [29]. AlgG epimerases have been identified from *Pseudomonas* genera *P. aeruginosa, P. corrugate, P. fluorescens, P. mendocina,* and *P. syringae* [22, 30-33]. Most *Pseudomonas sp.* do not encode AlgE like epimerases, with the exception of some strains of the *P. syringae* [22]. As expected, mannuronan C-5 epimerase genes have been identified in the genomes of brown algae, *e.g*. *Laminaria digitata, Saccharina japonica,* and *Ectocarpus siliculosus* [20, 34, 35]. Each species encodes many different epimerases, which are expressed at different times or in different tissues providing the algae with the ability to tailor its alginate depending on the environmental inputs [36]. The algal epimerases are most similar to the bacterial AlgG type epimerases, although the homology is below 40 % [36]. So far, only two algal epimerases have been successfully expressed and characterised, one of them produced MG blocks while the other increased the content of G blocks [35, 37].

The bacterial AlgEs contain one or two catalytic A modules (see below), and these produce different alginate structures. *Av*AlgE1A2, *Av*AlgE3A2, and *Av*AlgE4A create only MG-blocks, while others also create G-blocks where the length of the blocks differ between the epimerases [38, 39]. Encoding different epimerases thereby allows bacteria to tailor the structure of their alginates to have various functional properties [25].

AlgE enzymes consist of two types of modules

The AlgE epimerases consist of combinations of two types of structural modules, A and R [25, 40]. Only the A-module is catalytically active, whereas the R-module appears to work as a carbohydrate binding domain and is required for full activity as well as reducing the calcium requirement for the reaction [26, 41, 42]. The AlgEs have different combinations of A and R-modules (see Figure 3), where the A-module is always preceding one to four R-modules. The different A-modules result in different epimerisation patterns: *Av*AlgE4 creates MG-blocks, *Av*AlgE1A1, *Av*AlgE2, *Av*AlgE3A1, *Av*AlgE5, *Ac*AlgE1 and PsmE create G-blocks, and *Av*AlgE6 creates long G-blocks [39]. PsmE also contains four other modules, which are responsible for its deacetylase activity [22]. The structures of the A-module of *Av*AlgE4 (PDB ID: 2PYG,2PYH) and *Av*AlgE6 (PDB ID: 5LW3) as well as their R-modules (*Av*AlgE4 PDB ID: 2AGM; *Av*-AlgE6 PDB ID:2ML1, 2ML2, 2ML3) have been determined using X-ray crystallography [43] and NMR [41, 42]. The A-modules form a right-handed 3-4 stranded β-helix, whereas the R-modules form a two-stranded β-roll (see Figure 4). A loop protruding from the 10th turn in the β-helix in the A-modules of *Av*AlgE4 and *Av*AlgE6 is essential for the final alginate structure by affecting substrate binding, and a tyrosine residue in this loop is conserved in all G-block forming AlgEs, whereas in the MG-block forming AlgEs the same position is occupied by a phenylalanine [44].

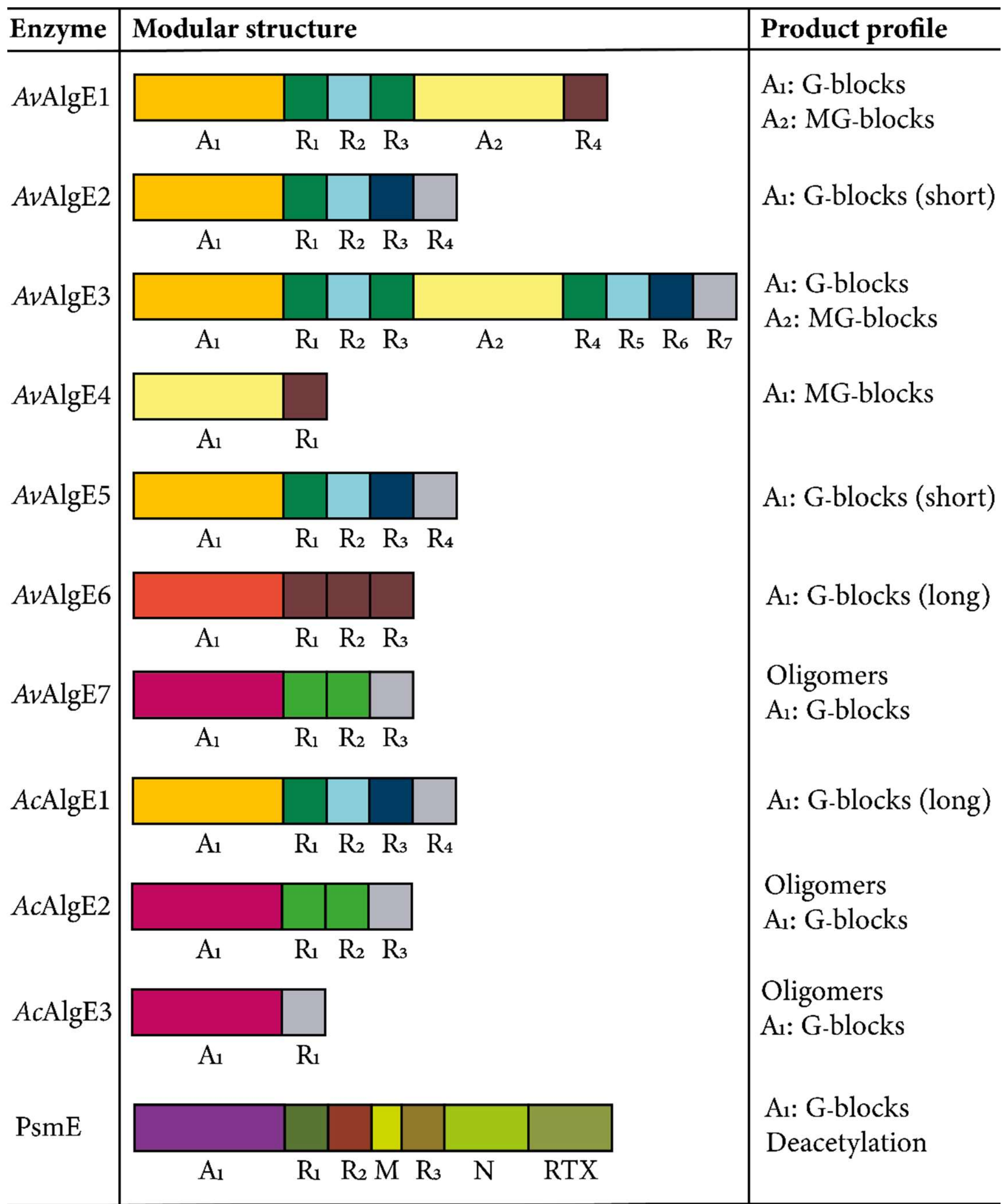

Figure 3: The modular structure of the bacterial AlgE-type epimerases with colours indicating phylogenetic relatedness [25, 39]. $A_{\#}$: Catalytic active module (~385 AA), $R_{\#}$: Repeating module (~150 AA), M: Dystroglycan-type cadherin-like domain (106 AA), N: Mannuronan O-acetyl hydrolase (273 AA), RTX: Repeat in toxic (370 AA) – similar to the R-modules. Furthermore, the profile of the products created by the enzyme activities are shown [26]. The illustration is updated and modified from Ertesvåg (2015) [45].

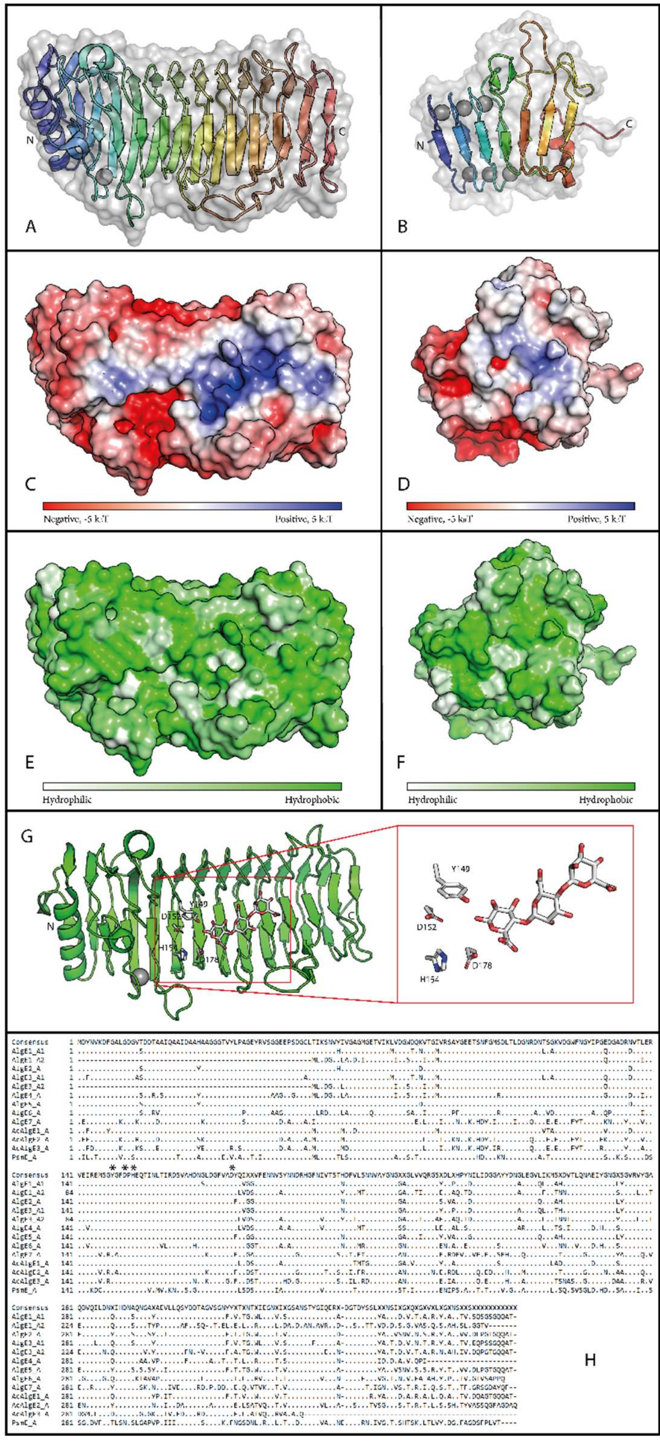

Figure 4: The structure of the A-module (A, C, E, G) from *Av*AlgE4 [43], the R-module (B, D, F) of *Av*AlgE6 [42], and multiple alignment scheme of all A-modules of AlgEs (H). Panel A and B show the secondary structure of the modules with the surface transparently shown. Panel C and D show the electrostatics of the surfaces with red being negatively charged surface area and blue positively charged surface area [46]. Panel E and F show the hydrophobicity of the surface of the modules, where the stronger the colour, the more hydrophobic the surface [47]. In panel G a zoom-inn on the active site of the A-module with catalytically active residues, Ca^{2+}, and a substrate trimer shown. In panel H an alignment of all A modules of the AlgEs are compared to the consensus sequence computed using EMBOSS Cons tool [48].

Reaction mechanism and mode of action

The mechanism of epimerisation is similar to that of the lyase [16], both of which are shown in Figure 2. The epimerisation process occurs in three steps: (1) an amino acid residue neutralises the C-5 acidic group of the substrate lowering the pK_a value of the H-5 proton, (2) the H-5 proton is abstracted, leading to the formation of an enolate intermediate, and (3) a proton is donated from the opposite face of the sugar ring leading to the formation of the C-5 epimer G [16]. In the corresponding third step for a lyase reaction, the hydrogen is added to the glycosidic bond resulting in strand cleavage. Some of the *A. vinelandii* and *A. chroococcum* enzymes, *Ac*AlgE2, *Ac*AlgE3, and *Av*AlgE7, display both lyase and epimerase activity (described below), which supports the theory of similar mechanisms [28, 29, 40].

Another mechanism has been proposed, in which a lyase reaction occurs first, and then the product of the first reaction is epimerised and glycosidic is formed again [49]. However, this mechanism seems unlikely as release of lyase product is not observed for most epimerases.

For all AlgEs the motif YG(F/I)DPH(D/E) is conserved. In this motif three amino acids essential for activity have been identified: Y149, D152, and H154, but the exact role of each residue is yet unclear [28, 34, 43]. The structure of the active site of AlgE4 is shown in Figure 4G.

The mechanism of AlgGs has been investigated along with its structure, and the catalytically active residues have been identified. AlgG from *P. aeruginosa* adopts the same fold as *Av*AlgE4 with a similar active site architecture, but *Av*AlgE4 contains a Ca^{2+} binding site that is not present in AlgG [50].

The AlgEs are processive enzymes that bind alginate, epimerise several residues, and then disassociate again, and each epimerase shows different degrees of processivity [51-54]. Only every other residue is epimerised by the AlgEs [55], and there is a lower limit to the degree of polymerisation of the substrates the different AlgEs can interact with [54, 55]. There are indications that the AlgEs move along the alginate chain from the non-reducing end to the reducing end [54, 55]. The binding groove of *Av*AlgE4 is lined with positive charges (Figure 4C), which seem to control the initial binding of alginate. It has been proposed that after an epimerisation reaction, negatively charged residues facilitate substrate dissociation in order to allow for translocation of the substrate inside the binding groove, which can explain the proposed processive action of the alginate epimerases [56].

The kinetic properties of the alginate epimerase reaction have not been characterised in dept due to the complexity of the reaction. Some studies have described the epimerisation by measuring the number of G-residues formed over time [51, 57], but these studies do not describe the kinetics of the enzymes in full.

Bifunctional enzymes can perform both lyase and epimerase reactions

The alginate epimerases *Av*AlgE7, *Ac*AlgE2, and *Ac*AlgE3 have a dual activity as both epimerases and lyases [28, 29, 58]. Calcium promotes the lyase activity of *Av*AlgE7, whereas sodium decreases lyase activity [58]. A mutation study of *Av*AlgE7 revealed that the amino acid residue R148 is the main contributor to the enzyme's lyase activity [58]. Assumingly, the bifunctional enzymes use the same active site for both lyase and epimerase activity [28, 58]. *Ac*AlgE2 is only active when there are M residues

in position -1 and +2 relative to the cleavage site, whereas *Ac*AlgE3 can accept M and G in all positions, creating a wider range of products [29].

An AlgG-type epimerase *Pm*C5A from *P. mendocina* has been shown to have lyase activity as well as epimerase activity [33], showing that bifunctionality might be more widespread than previously assumed.

3 Characterisation of epimerase reactions

The fundament of alginate characterisation is the ability to distinguish between M and G residues in the polymeric chains, which allows for analysis of the reaction products of epimerase reactions. Following the product formation of the epimerisation reaction is important for optimal application of epimerases and requires more refined characterisation techniques. An overview of the most commonly used characterisation techniques for alginate epimerisation is given in the following.

Circular dichroism

The relative amounts of M and G in alginates can be estimated using circular dichroism (CD) [59, 60], thereby the amount of epimerisation can be determined. M-blocks, MG-blocks, and G-blocks give different CD spectra when absorbing circular polarised light because of the local ring geometry around the carboxyl and the effect of the neighbouring residue [60]. CD is used to determine if there is an increase of G after enzymatic treatment of a sample [61].

Dische carbazole reaction

The Dische reaction is a chromogenic reaction with hexuronic acids using carbazole and sulfuric acid [62]. The colour intensity is higher for G than M, which is used to quantify the degree of epimerisation [63, 64]. The drawbacks of the carbazole reaction are that the reproducibility and sensitivity are low, making it disadvantageous for measuring kinetics [65].

Tritium release

By using tritium labelled substrates, i.e. ^{3}H-5 alginate, an epimerisation reaction can be followed by measuring the release of tritium into water [51, 57, 65, 66]. This method is suited for measuring V_{max} and K_M, but the method requires preparation of the ^{3}H-5 substrate in advance [65]. The reaction mechanism of the lyase and epimerase reaction both release a proton from C-5 of alginate, so measuring the release of tritium into water will not be able to distinguish between these two activities [65].

Absorption-based assays

The lyase reaction products contain an unsaturated moiety, Δ, as described in the introduction. This moiety absorbs light at 230 nm, thus the formation of Δ can be followed by measuring the absorbance [14]. This can be utilised to make a coupled assay, where epimerised alginate is treated with an

alginate lyase specific for G-M and G-G bonds, *e.g*. AlyA from *Klebsiella pneumoniae*, so that the measured absorbance is directly proportional with the amount of G produced by epimerisation [39, 67].

Alginate lyases and fragment analysis

By combining alginate lyases with different substrate specificities, the block distribution in alginates can be assessed [68]. The alginate lyases are chosen to degrade specific bonds in the polymer and leave the blocks of interest intact. For instance, M-specific lyases can be used to analyse G-block formation. The size and composition of the resulting fragments can then be determined using *e.g*. SEC, NMR, HPAEC-PAD, and SEC-MALS [68].

Acid hydrolysis and NMR spectroscopy

The block composition of alginate can be assessed by controlled partial acid hydrolysis. Alternating MG-blocks are most easily hydrolysed and thereby released into solution first. By dissolving the insoluble part and using acid precipitation to quantify G-blocks, the relative amount of the three block structures can be estimated [69]. A more precise method uses either ^{13}C NMR or ^{1}H NMR spectroscopy to measure the relative amounts of monomer dyads and triads. Here, mild acid hydrolysis to slightly depolymerise the alginate and reduce viscosity is necessary prior to analysis [70-73].

Time-resolved ^{13}C and ^{1}H NMR spectroscopy can be used to follow the epimerisation reaction, allowing for kinetic measurement and characterising the mode of action of the enzyme [28, 39, 42, 74]. In time-resolved NMR spectroscopy a 1D spectrum is recorded at specific time intervals, allowing to follow the disappearance of substrate and appearance of intermediates and products. It is also possible to run time-resolved NMR spectroscopy with ^{13}C-1-labelled substrates which helps in the product identification as it alleviates the number of significant signals in the spectrum and increases signal intensity making it easier to distinguish between M and G. Time-resolved NMR is especially useful to characterise enzymes that have both lyase and epimerase activity, as the relative reaction rates and products formed by either activity can be analysed [28, 29, 58]. An example of a time-resolved NMR proton spectrum can be seen in Figure 5.

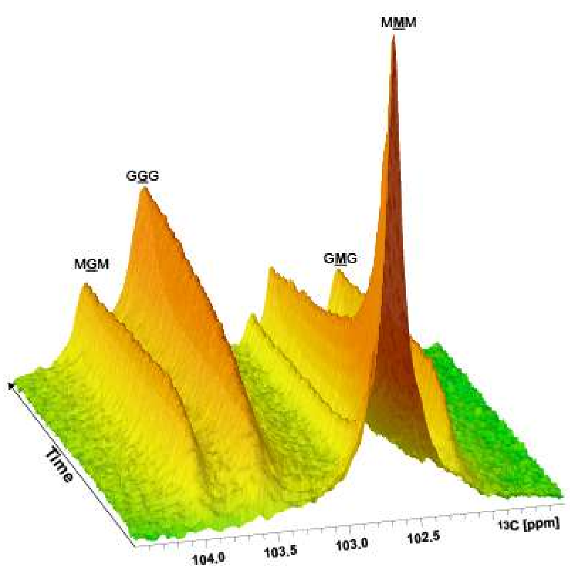

Figure 5: Time-resolved analysis of the reaction between *Av*AlgE1 (8 µM) and ^{13}C-1-labelled polyM (10 mg/mL) (similar to [74]). This pseudo-2D experiment consist of a 1D ^{13}C spectrum recorded every 10 min for a total of 16 h reaction time. The spectrum was recorded at 25 °C on a Bruker Avance III HD 800 MHz spectrometer using a 5 mm Z-gradient CP-TCI (H/C/N) cryogenic probe at the NV-NMR-Center/Norwegian NMR Platform (NNP) at the Norwegian University of Science and Technology (NTNU).

Several of the methods are limited by not being able to follow the epimerisation reaction in real time and therefore do not contribute to understand the reaction kinetics. A way to circumvent this limitation is to stop the reaction after certain reaction times and analyse the products formed. However, most useful for following the epimerisation reaction is time-resolved NMR spectroscopy, although this approach requires the availability of defined polysaccharides as substrates.

By using combinations of the different methods, it has been possible to generate information about G-content, block distribution, chain length, substrate preference, product formation etc. and thereby gain a thorough understanding of the epimerases and their overall reactions.

4 Tailoring of alginates and alginate epimerases

Alginates extracted from most brown algal species contain less than 50 % G residues [11, 75]. By using epimerases, it is possible to increase the G content of alginates [75], thereby opening for a new way of commercially producing G-rich alginates. In one study, *Av*AlgE4 and *Av*AlgE1 were used in combination to increase the G-content of alginate isolated from harvested seaweed from 32 % to 81 % [75]. *Av*AlgE4 creates MG-blocks, and *Av*AlgE1 creates long G-blocks on MG-alternating substrates [54, 76].

The properties of gels of alginate epimerised with these two enzymes have been investigated, showing that the use of epimerases to engineer alginate can greatly improve the properties of alginate gels [77]. The enzymatically engineered gels were found to be more elastic, more compact, less permeable, and very stable compared to native alginate gels, all which greatly increases the possibilities of using alginate microbeads for medicinal purposes [77]. Another possibility is sequential epimerisation of polyM with *Av*AlgE4 and *Av*AlgE6 to create alginate of up to 76 % G residues. In one study this strategy was used to create alginate for cell encapsulation [78].

Alginate can also be functionalised chemically, mainly using three different methods. It is possible to attach cell adhesion ligands containing the RDG amino acid motif to alginate using carbodiimide chemistry, which improves the cell encapsulation abilities of alginate [79]. Alginate can be oxidised using periodate oxidation enabling further functionalisation of alginate [80]. The reducing end of alginate oligomers can be coupled to another polysaccharide using a click-like reaction [81, 82].

Lastly, a way of producing alginates with specific properties is the use of bacteria for biosynthesis of alginates, which for example has been explored using *A. vinelandii* [76, 83, 84] and *P. fluorescens* [85].

Production of epimerases

To tailor alginates by *in vitro* epimerisation, effective recombinant production of epimerases is necessary. The most common method for production of alginate epimerases is to express them in *Escherichia coli* [25, 40, 51, 86]. However, alternative hosts like *Lactococcus lactis* has also been investigated as a possible host [87]. A recent study explored the methylotropic yeast *Hansenula polymorpha* for production and secretion of *Av*AlgE1, *Av*AlgE4, and *Av*AlgE6 [88]. Compared to expression in *E. coli*, using *H. polymorpha* could simplify the downstream processing of the epimerases due to the secretory properties of the yeast [88].

Tailoring of epimerases

Due to the structural similarity of the AlgEs, it is possible to interchange and combine the A and R-modules of different AlgEs and retain and modify activity. One study screened nearly 1000 different mutants and identified two epimerases that were more efficient in creating G-blocks in polyM than the naturally occurring epimerases [39]. The exchange of the R-modules of *Av*AlgE4 and *Av*AlgE6 results in a new enzyme, AlgE64, which is more efficient than *Av*AlgE6 in creating G-blocks [42, 44]. AlgE64 has been used to create high G-content alginate for studies of the impact of G-blocks on gel properties [89].

During epimerisation the substrate can form a gel, which greatly hampers epimerisation. Engineering AlgEs to reduce enzyme size enables epimerisation in regions inaccessible for native epimerases,

which was shown by comparing the large *Av*AlgE1 and *Av*AlgE6 with engineered AlgE64 (containing only one A and one R-module) and the A-module of *Av*AlgE6 alone [90]. Gel formation is mediated by G-blocks binding calcium ions, so if the epimerisation could be run without calcium present the gel formation would be avoided and leave the substrate available for epimerisation. However, calcium ions are necessary for the function of native AlgEs and engineering of a calcium independent epimerase will be needed to alleviate the problem of gel formation during reaction.

5 Conclusion and prospects for alginate epimerase research

Alginate epimerases have been studied since 1969 when Haug and Larsen [12] identified an *A. vinelandii* enzyme that epimerised M residues to G residues. Since then, the knowledge of epimerases has greatly advanced through molecular techniques and characterisation methods, so that it now is possible to use alginate epimerases to tailor alginate within certain limitations [39]. Alginates optimised by engineered epimerases are already promising materials for *e.g*. tissue engineering [11, 78, 91].

Several aspects of the alginate epimerases are not completely understood, most notably the structure-function relationship. Further research into *e.g*. substrate interactions and the reaction mechanism of epimerases could allow for modulation of the epimerase and lyase activity. Shedding light on the unanswered questions of the epimerases will require cutting-edge characterisation techniques, possibly even the development of new methodologies.

Accordingly, the alginate epimerases have not been fully exploited yet. Alginate epimerases that create very high G-content alginate have been explored [76], but the product formation is difficult to control. Especially, for the medical industry it would be relevant to engineer epimerases that create well-defined products with high reproducibility. It would also be relevant to engineer epimerases that are more robust for industrial use. Further development of feasible industrial production of epimerases is also necessary to make alginate production and tailoring via epimerases applicable on an industrial relevant scale. Lastly, the majority of research has focused on bacterial AlgEs from *A. vinelandii* and *Pseudomonas* spp., while epimerases from other bacteria and especially brown algae are not as well characterised. Many epimerases from other organisms, particularly brown seaweed, may yet be discovered, with potentially useful properties. An increased knowledge about alginate epimerases and alginate-active enzymes in general will broaden the possibilities for the rational design of enzymes capable of tailoring the properties of alginates.

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