

Enzymatic Alginate Modification

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Abstract Alginate is a linear 1-4-linked copolymer of β -D-mannuronic acid and its C-5-epimer α -L-guluronic acid. The polymer is produced by some algae and bacteria, and is used for numerous purposes in industry. Alginate is initially synthesized as mannuronan, which is then modified at the polymer level by mannuronan C-5-epimerases, alginate lyases, and *O*-acetylases. This generates a variety of heteropolymers where properties such as viscosity, chain stiffness, gel formation, water-binding potential, and immunogenicity are dependent on the action of the

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modifying enzymes. Both alginate lyases and C-5-epimerases can be used in vitro to tailor alginates for specific purposes. The lyases may also be used as tools to better define the sugar monomer sequences of an alginate sample.

1 Introduction

Alginates are structurally heterogenous polysaccharides produced by bacteria belonging to the genera *Pseudomonas* and *Azotobacter*, and by brown seaweeds and some calcareous red algae (Stanford 1883; Gorin and Spencer 1966; Linker and Jones 1966; Govan et al. 1981; Okazaki et al. 1982). In bacteria these polymers probably serve many different functions, as illustrated, for example, by *P. aeruginosa*, where they provide the producing organism with a selective advantage during serious infections of patients suffering from the disease cystic fibrosis (Ramsey and Wozniak 2005). In *A. vinelandii* they play a critical role in the ability of the organism to enter a desiccation-resistant state designated “cyst” (Sadoff 1975). Brown seaweed alginates serve a role in maintaining the integrity of the cell wall, analogous to cellulose and pectins in green land plants, and provide the stiffness or elasticity needed depending on the environment (Haug et al. 1974). The function in red algae is not known although it has been suggested that alginate is involved in the calcification process (Bilan and Usov 2001).

All alginates isolated from natural sources are linear 1-4-linked copolymers of the two sugar monomers β -D-mannuronic acid (M) and its C-5-epimer α -L-guluronic acid (G) (Fig. 1). However, alginate structures can otherwise be quite heterogeneous, even when they are isolated from different parts of the same organism (e.g., seaweed blades and stipes) or from bacteria exposed to different environmental conditions. The structural differences relate to the following: (1) the ratio between M and G residues may vary over a wide range (Ertesvåg et al. 1996); (2) the distribution pattern of the monomers along the polymer chains may vary drastically, even if the fractional composition is similar (Skjåk-Bræk et al. 1986a); (3) bacterial alginates are O-acetylated to varying degrees in the 2 and/or 3 positions, while this is not seen in seaweed alginates (Skjåk-Bræk et al. 1986a); (4) alginate chain lengths vary and are heterogenous even when alginate is extracted from a single source. All these four structural differences have very important consequences for the physicochemical properties of the polymer, and the producing organisms have taken advantage of the structural diversity to serve their functional needs. The diversity also forms the basis for a widespread and varied use of the polymer in industry.

Generally, the presence of G residues in alginates opens the potential for formation of polymer gels in the presence of divalent cations such as Ca^{2+} , but this form of gel formation can only take place if the G residues are found as consecutive stretches (designated “G-blocks”) (Fig. 1). The lengths and numbers of these blocks affect several important physical properties of the gels, such as stiffness, swelling, and porosity. The G residues may also be present as alternating sequences, (MG) n , designated “MG-blocks” (Fig. 1). Finally, alginates with a very low content of G

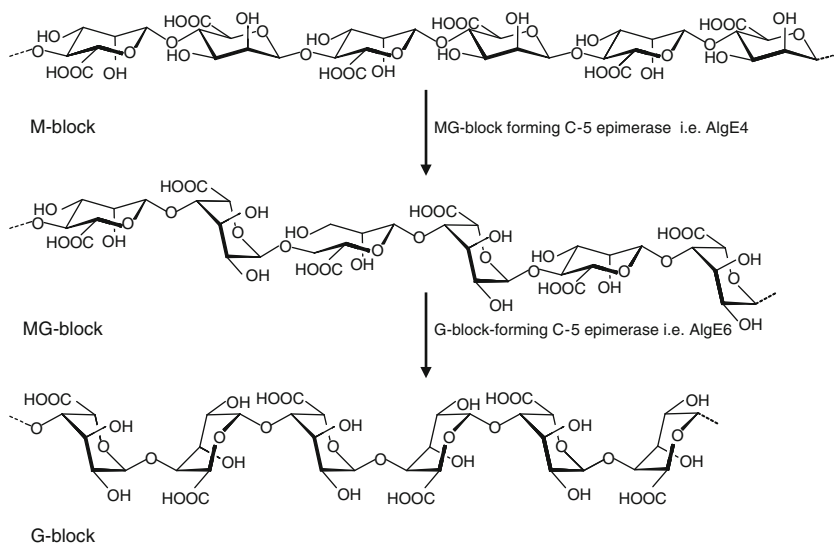


Fig. 1 Alginate structure. Hexamers of the three different structural elements are shown. The mannuronan C-5-epimerases will convert consecutive stretches of β -D-mannuronic acid (M-blocks) to blocks containing single α -L-guluronic acid residues or consecutive stretches of α -L-guluronic acid residues (G-blocks)

residues are referred to as being rich in M-blocks (Fig. 1). Such alginates cannot form cationic gels, but can form acidic gels (Draget et al. 1994). For further details on the relation between monomer composition and physicochemical properties, see Smidsrød and Draget (1996). An additional interesting observation is that some types of alginates exhibit immune-stimulating properties. These activities which include stimulation of cytokine synthesis through the Toll-like receptor system, is correlated with a high content of M interspaced with single G residues, although the sequential structure of the epitope remains to be identified (Flo et al. 2000).

The biotechnological uses of alginates cover a wide range of applications owing to the consequences of the structural diversity (Smidsrød and Draget 1996). They are, for example, used to affect food texture owing to the ability to form viscous solutions (related to polymer chain lengths), for immobilization of cells owing to the ability to form cationic gels (Draget et al. 2000), and as a feed additive in fish farming owing to their immunogenic properties (<http://www.biomar.no>).

Since all commercial alginates are currently harvested from brown seaweeds, different structural types of alginates must be selected from different types of alga or from specific algal tissues, i.e., alginate enriched in G for making mechanically strong gels is found in the stipes and holdfast of *Laminaria hyperborea*, a plant growing in very exposed coastal areas. Alginates with a high M content (for softer and more elastic gels) can be extracted from the leaves of the same plant or from other species such as *Laminaria digitata*, *Ascophyllum* sp, and *Lessonia* sp. Alginate can also to some extent be fractionated, but the efficiency is low (Haug et al. 1967).

Another advantage of using seaweed alginates compared with using those from bacteria is that they can generally be produced more cheaply from seaweeds than from bacteria. However, bacterial alginates not only present a wider structural variety, but they also offer the potential of designing new alginate structures to meet demands for alginates with improved or new functional properties.

For many types of application, such as for pharmaceuticals, price per unit weight is not necessarily the most critical parameter, but rather the product quality in terms of homogeneity and structural reproducibility and availability. For such purposes there is certainly a potential for using alginates produced by bacteria, as one could envision both stable production and unique quality. To achieve this one must be able to tailor-make the alginate structures *in vitro* or *in vivo*, and in this chapter the methods available for doing this are discussed.

2 Mannuronan C-5-Epimerases

Homopolysaccharides with only one type of bond are synthesized using a nucleotide sugar and one glycosyltransferase. This concept may be extended to linear heteropolysaccharides by suggesting that several glycosyltransferases cooperate, and that the polysaccharide structure will be a product of their relative reaction rates, substrate requirements, and precursor availability. This was also suggested for the biosynthesis for alginate, as Lin and Hassid (1966b) claimed to be able to isolate the potential precursors GDP-D-mannuronic acid and GDP-L-guluronic acid from the brown alga *Fucus gardnerii*. The same authors also showed the presence of enzymes necessary to incorporate mannose 1-phosphate into alginate using ^{14}C -labeled mannose derivatives (Lin and Hassid 1966a). However, Haug and Larsen (1969) were able to show that the bacterium *A. vinelandii* secretes an enzyme that epimerizes M-residues to G-residues on the polymer chain, and this was the first example of an epimerase working on a polymer. This suggested that alginate is synthesized as a homopolymer, as was later confirmed by Chitnis and Ohman (1990). A mannuronan C-5-epimerase was also identified in the algae *Pelvetia canaliculata* (Magdwick et al. 1973). Mammalian cells were found to synthesize heparin in a similar way, where a glucuronan C-5-epimerase converts some of the D-glucuronic acid residues to L-iduronic acid residues (Lindahl et al. 1972).

2.1 Early Studies on the Secreted Mannuronan C-5-Epimerase from *A. vinelandii*

Mannuronan C-5-epimerase could be partly purified from the *A. vinelandii* culture broth using ammonium sulfate precipitation (Larsen and Haug 1971). Further purification was later obtained by using ion-exchange and affinity chromatography (Skjåk-Bræk and Larsen 1982b; Skjåk-Bræk et al. 1985). When the partially

purified enzyme was incubated with alginate in the presence of tritiated water most of the radioactivity was found in the G residues, but a smaller amount was also found in M residues (Larsen and Haug 1971). The authors suggested a model in which the first step in the catalytic reaction is the abstraction of H-5 followed by addition of hydrogen from water to the other side of the uronic acid residue.

The carbazole method can also be used to follow the epimerization reaction (Knutson and Jeanes 1968; Haug and Larsen 1971), since G gives rise to a higher color intensity than M. Infrared light may also give an estimate of the G content (Mackie 1971), and it is possible to measure the increase in G content using G-specific lyases as was first described by Currie and Turvey (1982). However, a more accurate method involves the use ^3H -5-labeled mannuronan as a substrate. During epimerization, this hydrogen atom is exchanged with water and the reaction can be measured by quantifying the radioactivity remaining in the soluble phase after the alginate has been precipitated by ethanol (Skjåk-Bræk and Larsen 1982a).

These methods can quantify the increase in G content as the epimerization reaction proceeds, but cannot provide information regarding the distribution pattern of the G residues (G-blocks or MG-blocks). This can to some extent be achieved by partial acid hydrolysis of the polymer followed by acid precipitation, since the MG-blocks are more soluble in acid than G-blocks and M-blocks (Haug et al. 1967). However, ^1H -NMR and ^{13}C -NMR spectroscopy (Grasdalen et al. 1979, 1981; Grasdalen 1983) is now the preferred method since it can be used to obtain accurate data on the frequencies of the different dimer and trimer distributions in the alginate polymer.

Even in the first papers describing the secreted mannuronan C-5-epimerases it was mentioned that the concentration of divalent calcium ions affected activity (Haug and Larsen 1969, 1971). However, if the reaction was allowed to proceed to the end point, an intermediary concentration of Ca^{2+} (about 1 mM) gave rise to the highest number of G residues. Ca^{2+} also increased the stability of the enzyme. Later, with use of purified epimerase, it was confirmed that at low concentrations of Ca^{2+} G-blocks were preferably made, while at higher concentrations MG-blocks dominated (Larsen et al. 1986; Skjåk-Bræk et al. 1986b). One hypothesis that could explain this observation is that the increase in Ca^{2+} changed the action pattern of the enzyme from a predominantly single-chain mechanism to a multiple-attack mechanism with increasing number of calcium ions (Larsen et al. 1986). However, in a report by Ofstad and Larsen (1981), it was stated that different batches of ammonium sulfate precipitated epimerase varied both regarding their optimal calcium ion concentration for activity and the monomer sequence distribution in the polymer reaction product. These authors suggested that the lack of reproducibility could be explained by assuming the presence of two enzymes with different calcium optimums and preference with respect to the epimerization patterns introduced. The relative amounts of these two enzymes could then vary among different batch cultivations of the bacterium. As discussed later, this prediction later turned out to be correct, except that the complexity is even greater than they anticipated.

2.2 Cloning and Characterization of the Mannuronan C-5-Epimerases from *A. vinelandii*

To resolve the issues described in the previous section and also to obtain larger quantities of the epimerase, the gene or genes encoding them had to be cloned. The purified proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the N-terminal sequence of the protein corresponding to the most intense band (122 kDa) was determined. A degenerate nucleotide probe was constructed on the basis of this information and was used to screen a genomic bacteriophage λ library of *A. vinelandii* (Ertesvåg et al. 1994). Positive clones were identified, and parts of one of these were subcloned into a plasmid vector. Surprisingly, several of the plasmids expressed active mannuronan C-5-epimerases in *Escherichia coli* (Ertesvåg et al. 1994, 1995). The insert in the phage was then sequenced and it was found to contain a gene encoding a protein with the expected N-terminal sequence, but also genes encoding parts of two other homologous proteins. Further analyses of other positive phage clones finally resulted in the identification of five different homologous genes (Ertesvåg et al. 1995). Furthermore, Southern blotting revealed that the *A. vinelandii* chromosome contains even more homologous sequences. The last of these genes were finally cloned by a second screen of the genomic library (Svanem et al. 1999), and it is now clear that the total number of homologous genes is eight, but only seven of them (*algE1*–*algE7*) encode proteins displaying mannuronan C-5-epimerase activity after expression in *E. coli*. The product of the last gene, designated “*algY*,” has no known function (Svanem et al. 1999).

The secreted epimerases are all modular enzymes containing one or two A-modules and one to seven R-modules (Fig. 2). Within each module type the range of homology is between 50 and 100%. The two A-modules from AlgE1 were expressed separately in *E. coli* and both were shown to be active epimerases (Ertesvåg and Valla 1999), proving that this module contained the catalytic site of the enzyme. The presence of the R-modules increased the activity of the enzyme about tenfold. Interestingly, this increase was found when the R-modules were located both N-terminally and C-terminally with respect to the A-module (Ertesvåg and Valla 1999). The R-modules contain four to seven imperfect direct repeats of a nine amino acid long motif found in many proteins exported by ATP binding cassette transporters (Delepelaire 2004). Such type I secreted proteins do not contain an N-terminal secretion signal, but some feature of their C-terminal sequence is necessary for secretion. The genes (*eexDEF*) needed for secretion of the epimerases have been identified (Gimmestad et al. 2006).

The nonameric repeat has been shown to bind calcium (Baumann et al. 1993), and 3D-structure studies of the R-module of AlgE4 support this conclusion (Aachmann et al. 2006). Furthermore, binding and 3D-structure studies of the AlgE4 A-module have demonstrated that this catalytic unit also binds Ca^{2+} (Ertesvåg and Valla 1999; Rozeboom et al. 2008).

The different epimerases produce alginates with distinct epimerization patterns; AlgE2 and AlgE5 seem to predominantly epimerize M residues located next to a





























Enzyme	Modular structure	End products	Other activities
AlgE1	A    A 	G-and MG-blocks	
AlgE2	A    	G-blocks (short)	Weak lyase activity
AlgE3	A    A    	G-and MG-blocks	
AlgE4	A 	MG-blocks	
AlgE5	A   	G-blocks (medium)	Weak lyase activity
AlgE6	A   	G-blocks (long)	
AlgE7	A   	G-and MG-blocks	Lyase activity
PsmE	A   M R N 	G-blocks (long)	Deacetylase

Fig. 2 Modular structure and end products of the secreted mannuronan C-5-epimerases. The relationships within the group of A-modules and R-modules (Svanem et al. 1999) are shown by similarities and differences in *cross-hatchings* and *gray tones*. The *Pseudomonas syringae* enzyme also contains two additional calcium-binding modules (*M* and *RTX*) and a module displaying alginate deacetylase activity (*N*). The weak lyase activity of AlgE2 and AlgE5 (0.1–0.3% of the reactions) is probably a result of failed epimerase reactions

preexisting G residue, generating G-blocks (Ertesvåg et al. 1999; Ramstad et al. 1999), while AlgE4 does not do this, and therefore forms alternating sequences, (MG)*n*, in the reaction product (Høidal et al. 1999). As can be seen from Fig. 2, the A-modules of AlgE2 and AlgE5 belong to one homology group of A-modules, while the A-module of AlgE4 belongs to another group. AlgE1 and AlgE3 both contain two A-modules, one from each of these two groups. And when they were expressed separately, the A1-modules make G-blocks, while the A2-modules introduce alternating structures (Ertesvåg et al. 1998b, 1999). However, the A-module of AlgE6 groups with that of AlgE4, but still this enzyme introduces G-blocks into the alginate (Svanem et al. 1999). A study using hybrid A-modules between AlgE2A and AlgE4A might reconcile these findings, since it was shown that only certain parts of the A-module are important for the epimerization pattern (Bjerkkan et al. 2004).

The A-module of AlgE7 is similar to that of AlgE6. However, the end product after incubation of mannuronan with AlgE7 is relatively small oligomers with G residues at the reducing end and an unsaturated residue at the nonreducing end, identifying AlgE7 as being both a mannuronan C-5-epimerase and an alginate lyase (Svanem et al. 2001). The same residues are important for both the epimerase and lyase activities, emphasizing the hypothesis proposed by Gacesa (1987) that alginate epimerases and lyases share the first steps of the catalysis.

The grouping of the epimerases into those generating G-blocks and those preferably introducing alternating structures is fairly crude; there are differences both in the relative numbers of G-blocks and alternating structures made (Fig. 2), the acceptance of alternating structures as a substrate for further epimerization, and the requirements for Ca^{2+} (Ertesvåg et al. 1999; Campa et al. 2004; Holtan et al. 2006).

In the more biological context it has been shown that different epimerases are expressed at different times in the life cycle of *A. vinelandii*, probably reflecting a need for different alginates in the vegetative capsule and in the cyst (Høidal et al. 2000). The crucial role of these enzymes in cyst formation was recently confirmed by studies of the phenotypes of mutants in which all the *algE* genes had been deleted (Steigedal et al. 2008).

A gene encoding a secreted mannuronan C-5-epimerase designated PsmE has also been identified in *P. syringae* (Bjerkkan et al. 2004). This enzyme contains one A-module and three R-modules (Fig. 2). A second module putatively binding calcium is interspersed between the last two R-modules. These modules are followed by a module encoding an alginate deacetylase and lastly by a third type of a calcium-binding module. PsmE was produced recombinantly in *E. coli* and was shown to introduce G-blocks into alginate. Recombinantly produced PsmE is able to remove acetyl groups on acetylated alginate and thus uses acetylated alginate as a substrate for epimerization, in contrast to the AlgE epimerases (Bjerkkan et al. 2004). G-block-containing alginates have not been reported from *Pseudomonas* sp. However, *psmE* was identified as a gene expressed at low (18°C) temperature (Ullrich et al. 2000), and it is possible that alginate has not yet been prepared from the relevant strains growing at the conditions required for production of such an alginate. A-modules are only found in the genomes of *A. vinelandii* and *P. syringae* among all the genomes presently deposited in GenBank.

2.3 Cloning and Characterization of the Bacterial Periplasmic Mannuronan C-5-Epimerases

Chitnis and Ohman (1990) were able to isolate two mutants of *P. aeruginosa* that produced alginate with no G residues, and cloned a DNA fragment that complemented this defect. The DNA fragment was later expressed in *E. coli* and was shown to encode a mannuronan C-5-epimerase that was localized in the periplasm (Franklin et al. 1994). The enzyme was inhibited by acetyl groups on the alginate. Later, similar enzymes were cloned and characterized from *A. vinelandii* (Rehm et al. 1996) and *P. fluorescens* (Morea et al. 2001; Gimmestad et al. 2003). All three characterized AlgG epimerases introduce only single G residues (no G-blocks) into the alginate. However, in a later study, Jerga et al. (2006) claimed that AlgG from *P. aeruginosa* can produce G-blocks in vitro. AlgG does not need calcium for activity (Rehm et al. 1996; Jerga et al. 2006).

AlgG is not only needed for epimerization of the nascent polymer, it also has a structural function in transporting the polymer out of the cell, presumably by being

part of a periplasmic protein complex (Gimmestad et al. 2003; Jain et al. 2003). The *A. vinelandii* AlgG epimerase has fairly low activity in vivo and in vitro (Rehm et al. 1996; Steigedal et al. 2008), indicating that the AlgE epimerases determine the G distribution in this bacterium.

2.4 Mannuronan C-5-Epimerases from Algae

As already mentioned, mannuronan C-5-epimerase activity has been described from several brown algae. However, genes encoding putative algal epimerases seem to be difficult to express recombinantly. Nyvall et al. (2003) were able to show that *L. digitata* expresses several genes sharing significant sequence similarities with the bacterial epimerases, especially the AlgG group. The expression pattern of these genes coincided with the level of mannuronan C-5-epimerase, strengthening the hypothesis that these genes encode such enzymes. The data indicated that *L. digitata* encodes a family of at least 21 mannuronan C-5-epimerases. It seems probable that the high number of different epimerases enables the algae to tailor alginates according to the needs of the season and tissue concerned, an explanation analogous to that for the existence of the AlgE epimerase family in *A. vinelandii*. Alginate-related genes from red algae have not yet been reported.

2.5 Structure of the Mannuronan C-5-Epimerases

The 3D structures of the A- and R-modules of AlgE4 were recently published (Aachmann et al. 2006; Rozeboom et al. 2008), and were both found to be right-handed parallel β -rolls (Fig. 3a). The A-module consists of one β -helix with positively charged residues facing a shallow groove. One calcium ion was identified in the structure (Fig. 3b) (Rozeboom et al. 2008). When the crystal was soaked in a solution containing a tetramer of M residues, it was found that the oligomer bound with the reducing end closest to the N-terminal end of the A-module. The R-module forms a narrower β -roll that also binds an oligomer of M residues. On the basis of this, it was proposed that the whole enzyme probably forms one elongated protein with the positively charged alginate binding site spanning both modules (Aachmann et al. 2006).

The catalytic site of AlgE4 seems to consist of Asp152, His154, and Tyr149 (Fig. 3b). This assumption is consistent with the structure data, and even conservative substitutions of one of these amino acids decreased the enzyme activity to less than 1% (Rozeboom et al. 2008). The studies also showed that this catalytic site is very similar to the corresponding site reported for five alginate lyases. The similarity between the lyases was first observed by Osawa et al. (2005). AlgG from *P. aeruginosa* is predicted to be structurally similar to that of the A-module (Douthit et al. 2005), and also shares some sequence similarity to AlgE4 around the catalytic site.

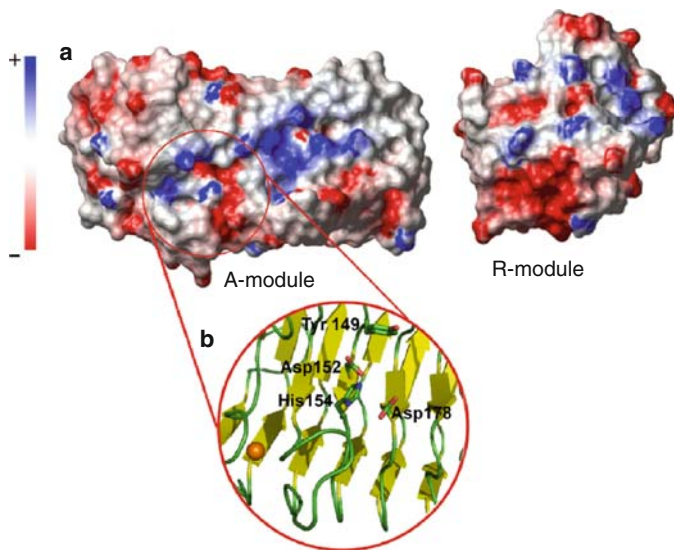


Fig. 3a Electrostatic surface model of the A- and R-modules of AlgE4. **b** The catalytic site of AlgE4 is enlarged and shown in stick representation. The bound Ca^{2+} ion is shown as an *orange sphere*. The models were constructed using MOLMOL (Koradi et al. 1996) and PyMOL (DeLano 2002). (a Reproduced with permission from Achmann et al. 2006)

Rozeboom et al. (2008) also proposed a catalytic mechanism for the epimerases based on the structure and activities of mutant proteins (Fig. 4). In this model Asp152 (or possibly Asp178) protonates the +1 M residue. Tyr149 abstracts the C-5 proton from the protonated sugar, while His154 subsequently donates a proton to C-5 from the other side of the sugar.

2.6 Mode of Action of the Secreted Mannuronan C-5-Epimerases

Each of the AlgE1–AlgE7 epimerases generates specific nonrandom epimerization patterns when acting upon mannuronan or any other alginate as a substrate. Such nonrandom block structures are typical in all alginates and have traditionally been attributed to the processive mode of action of polymer-modifying enzymes. “Processivity” refers to the average number of times a reaction is repeated between association and dissociation of the enzyme-substrate complex. High processivity is common for enzymes taking part in the replication and modification of RNA and DNA (Kornberg and Baker 1992). However, any enzyme that has more than one substrate-binding subsite and performs multiple modifications on a substrate may display processivity (Hartmann et al. 2002). Several examples of this type of action have been confirmed for polysaccharides, mainly for exolytic enzymes (Ernst et al. 1998;

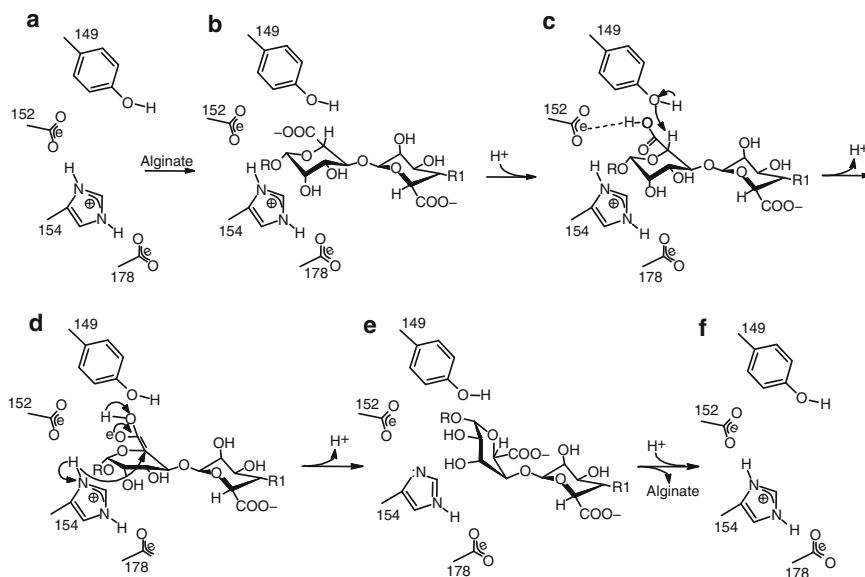


Fig. 4 Proposed catalytic mechanism of AlgE4. The alginate enters the catalytic site (a, b). The carboxylate group is protonated, allowing it to form a hydrogen bond with Asp152 (or Asp178) (c). Tyr149 is deprotonated (via Arg195), and the alkoxide group extracts H-5 from the mannuronic acid residue in the catalytic site (c). A double bond is formed, resulting in a nearly planar hex-4-enepryanosyluronate residue (d). The protonated His145 performs a nucleophilic attack on the C-5 atom of this sugar from the *si* face, flipping the sugar ring into the ¹C₄ chair conformation of guluronic acid (d, e). The carboxylic acid moiety is deprotonated and leaves the active site (e, f). His154 is protonated again and the epimerase is ready to perform a new reaction (f). (Modified with permission from Rozeboom et al. 2008)

Breyer and Matthews 2001). In alginates, enzyme processivity influences whether G units are introduced at random or successively as blocks. The formation of long blocks could thus be explained by a high degree of processivity. However, these nonrandom patterns can also arise from a preferred attack mechanism by which the affinity for the substrates depends on preexisting G residues. To distinguish between these two mode of action experimentally is, however, difficult.

Results from kinetic experiments using time resolved NMR spectroscopy and modeling based on Monte Carlo simulation suggest that AlgE4 acts processively (Høidal et al. 1999; Hartmann et al. 2002). Evidence for processivity also comes from single molecular pair unbinding studies of mannanuronan and AlgE4 using atomic force microscopy (Sletmoen et al. 2004). The degree of processivity was estimated, using specific degrading enzymes, NMR, electrospray ionization mass spectrometry, and capillary electrophoresis. On average, AlgE4 epimerizes ten M residues before the enzyme-substrate complex dissociates, implying that the enzyme, converting every second residue, propagates a distance corresponding to 20 M residues (Campa et al. 2004).

AlgE1 and AlgE6 introduce G-blocks in a processive manner when acting on polyMG as a substrate. (Holtan et al. 2006). The remaining epimerases act according to either a preferred attack mechanism (AlgE2 and AlgE5) or a combination of the two mechanisms. A polymer-modifying enzyme, independent of whether it acts processively or in a preferred mode, would have to interact with more than one residue in the polymer substrate. AlgE4 requires a hexameric mannuronan oligomer as the minimum size of the substrate to support epimerase activity, while seven to eight residues and eight to ten residues appear to be the minimum size for AlgE6 and AlgE1, respectively (Holtan et al. 2006).

The recently elucidated crystal structure (Rozeboom et al. 2008) of the A-module reveals that AlgE4A has the structural basis for processivity, since the substrate is partially enclosed in a groove with a relatively large interaction surface, and with a clamp at each side of the active site that would enclose the polymer and help it to slide rather than dissociate.

2.7 Enzyme Specificity; Epimerization of Nonnatural Uronans and Poly(guluronic acid)

The AlgE epimerases are specific for polymeric β -1•4-linked mannuronic acid found naturally in alginates or in C-6-oxidized mannose-containing polysaccharides. AlgE1, AlgE4, and AlgE6 are able to epimerize M-residues in various nonnatural uronans obtained by a selective 2,2,6,6-tetramethylpiperidine-1-oxyl-mediated hypohalite oxidation of mannose containing polysaccharides, leading to new polyelectrolytes with enhanced gelling properties in aqueous systems containing Ca^{2+} ions (Crescenzi et al. 2000, 2002).

The epimerization reaction of D-ManA to L-GulA is analogous to the conversion of D-GlcA into L-IdoA (Lindahl et al. 1972; Hagner-McWhirter et al. 2000) in the biosynthesis of heparin, heparan sulfate, and dermatan sulfate, and glucuronan C-5-epimerase is the only other known polymer-level C-5-epimerase. It has therefore been of considerable interest to establish if mannuronan C-5-epimerases also could epimerize polymer-linked GlcA. In 2000 an astonishing paper by Chang et al. (2000) was published in which they claimed that epimerase isolated from *A. vinelandii* was able to epimerize polymer-linked uronic acid in a range of C-6-oxidized polymers (cellulose, amylose, pullulan, and chitosan) and in native pectin. Over the years, we and other groups have tried to reproduce their results but without success (Holtan 2006) (A. Hotchkiss, USDA ARS, USA, and V. Crescenzi and M. Dentini, University La Sapienza, Rome, Italy, personal communications). Both wild-type and recombinant epimerases have been tested alone or in combinations on the following substrates: C-6-oxidized cellulose and amylose, microbially produced glucuronan from *Rhizobium meliloti*, pectate, and heparin precursors such as *E. coli* K5 capsular polysaccharide and sulfaminoheparosan. No epimerization was detected.

There is, however, indicative evidence that the epimerase binds to GlcA-containing polymers. In a study on the action of the mannuronan C-5-epimerases on C-6-oxidized

konjac glucomannan (Crescenzi et al. 2002) a GlcA- α -L-(1 \rightarrow 4)GulA- β -D-(1 \rightarrow 4)ManA sequence could be identified after incubation with AlgE4 and AlgE6. This indicates that the AlgE4 subsite (-1) is able to bind to β -D-glucuronic acid. Increasing the AlgE4 and AlgE6 incubation time led to depolymerization of the polymer by a β -elimination mechanism. Similar observations have been reported for C-6-oxidized cellulose (β -(1 \rightarrow 4)-D-glucuronic acid) after incubation with AlgE4 (Crescenzi et al. 2000; Crescenzi et al. 2002). This indicates that GlcA, which is a C-2-epimer of ManA, can to some extent be accepted in the active site of AlgE4 mannuronan C-5-epimerase such that the proton on C-5 is abstracted although epimerization to L-iduronic acid is not taking place. Because GlcA is able to recognize β -(1,4)-D-glucuronic acid and act as a lyase, only subtle conformational modifications of AlgE4 or another epimerase might turn it into an active glucuronan C-5-epimerase.

2.8 In Vitro Epimerization with AlgE Epimerases

Alginate gels represent very challenging semisolid structures with applications in biotechnology and industrial fields. The ability to control and tune the mechanical properties represents a key feature for improving the applications of such biomaterial. The availability of AlgE epimerases provides a powerful tool to design the sequential structures and allows monitoring of the effect of compositional modifications on the functional properties of alginate gels. The AlgE enzymes, which in their natural state act in the exocellular environment, are soluble, stable, and require no cofactor except calcium ions. They have been proven to be very effective in modifying all types of alginate in vitro (Mørch et al. 2007). In particular AlgE1, AlgE4, and AlgE6 are technically useful owing to their lack of lyase activity. AlgE4 is highly effective in converting M-blocks into polyMG sequences in any block-structured alginate. When acting on pure mannuronan, this enzyme is able to convert the entire polymer into polyMG (Fig. 1) without breaking the polymer backbone (Hartmann et al. 2002). As described earlier, AlgE1 and AlgE6 generate long G-blocks either by elongating preexisting G-blocks or by condensing them by epimerizing single M residues flanked by Gs (Fig. 1). PolyG is still difficult to make since the calcium ions, required by the enzyme, are gradually depleted owing to their cooperative binding to the G-blocks generated. Moreover, binding of calcium ions could lead to gel formation, rendering the molecules less accessible as a substrate for the enzyme. With use of suboptimal concentrations of calcium ions in the presence of high concentrations of antigelling ions such as sodium, high molecular weight alginates with 97% G were obtained (Holtan et al. 2006). Since three to four M residues at the reducing and nonreducing ends cannot be attacked by the enzyme, this probably represents the end point of the epimerization.

The availability of pure alginates resembling separately the three extreme block sequences present in the natural polysaccharide, namely, G-blocks, M-blocks, and MG-blocks, was found to be a fundamental tool to point out their role in the final hydrogel (Donati et al. 2005).

3 Alginate Lyases

The viscosity of alginates is mostly dependent on molecular weight, although the G distribution affects the stiffness of the chain and thereby also the viscosity. Alginate lyases split the polymer by β -elimination, leaving an unchanged saturated uronate on the reducing end and an unsaturated (4-deoxy-L-erythro-hex-4-enepyranosyluronate) residue, symbolized by Δ , at the nonreducing end. Alginate lyases are widely distributed in nature, in organisms growing on alginate as a carbon source, in alginate-producing organisms, and in some bacteriophages (Hashimoto et al. 2009). In this review we concentrate on lyases produced by alginate-producing bacteria and those used to characterize alginate structure.

3.1 Alginate Lyases as Tools To Determine Alginate Structure

Specific degrading enzymes are invaluable tools for structural analysis of macromolecules. This is also the case with alginate-specific degrading enzymes, of which all known can be classified as lyases. The specificity of these enzymes is commonly directed towards one of the residues in the glycosidic linkage, i.e., the lyase from *Klebsiella aerogenes* cleaves G-G and G-M specifically for the guluronate in the glycon position, leaving a reducing guluronate end and an unsaturated end originating from either M or G (Boyd and Turvey 1978). The lyase from *A vinelandii* is specific for M in the glycon position, cleaving M-M and M-G bonds (Ertesvåg et al. 1998a). Other enzymes are specific for the sugars on both sides of the bond, such as the M-M-specific lyase from *P. alginovora* (Chavagnat et al. 1996). Although the chemical identity of the aglycon is lost by the lyase-catalyzed reaction, the unsaturated end functions as a chromophore (UV 230 nm) and is also easily identified by NMR. Combined with mass spectrometry and NMR, specific lyases have been used successfully in analyses of subsite specificity for the epimerases (Campa et al. 2004) as well as in analysis of block length and block length distribution (Holtan et al. 2006). We are currently using enzyme engineering in our search for more specific lyases to be used in alginate sequencing.

3.2 An Alginate Lyase Is Needed To Remove Aberrantly Localized Alginate

In all alginate biosynthetic gene clusters sequenced so far a gene, *algL*, encoding an alginate lyase has been found. Initially this was surprising since degradation of a newly synthesized polymer seems a futile reaction. It was suggested that AlgL could be involved in determination of the polymer length, making primers for alginate synthesis or releasing the cells from alginate (Boyd et al. 1993). Later it

was shown that AlgG, AlgK, and probably AlgX form a protein complex guiding the nascent alginate chain from the polymerase in the inner membrane and through the secretion pore AlgE (AlgJ in *A. vinelandii*) in the outer membrane (Rehm et al. 1994; Rehm 1996; Jain and Ohman 1998; Gimmetstad et al. 2003; Jain et al. 2003; Robles-Price et al. 2004). If any of these three proteins are missing, the product is unsaturated oligomers, suggesting that the protein complex protects the alginate against AlgL. It was then shown that alginate production in the absence of AlgL is lethal to the cells (Bakkevig et al. 2005; Jain and Ohman 2005). The explanation seems to be that the polyanion alginate in the periplasm will attract cations and thus create an osmotic stress that leads to cell lysis. If one of the alginate-producing complexes does not function properly, this will happen, and AlgL is present in the periplasm as a repair system to remove these lethal molecules.

P. aeruginosa has been found to encode a second alginate lyase (Yamasaki et al. 2004) that prefers MG-rich alginate. The biological function of this lyase is not known.

4 Alginate Acetylation

Bacterial, but not algal, alginates are acetylated at O2, O3, or both (Skjåk-Bræk et al. 1986a). Acetylation increases the water-binding capacity of alginate and its viscosity (Skjåk-Bræk et al. 1989). It has also been shown that acetylation increases intermolecular association of alginate molecules (Windhues and Borchard 2003).

The genes, *algI*, *algJ* (*algV* in *A. vinelandii*), and *algF* encoding the proteins needed for acetylation are encoded in the alginate biosynthetic gene clusters (Franklin and Ohman 1996; Vazquez et al. 1999). However, the gene products are not necessary for alginate production (Shinabarger 1993). Still, acetylation takes place in the periplasm (Franklin and Ohman 2002), indicating that although the nascent alginate is not accessible to AlgL it is accessible to AlgF.

For *P. aeruginosa*, acetylation is necessary for the formation of microcolonies during the early stages of biofilm development (Tielen et al. 2005). This suggests that acetylation of alginate is important for cell–cell attachment. Acetylation will also protect the alginate against degradation by many alginate lyases. For *A. vinelandii*, the G distribution of the alginate is determined by the secreted mannuronan C-5-epimerases. Since these enzymes will not epimerize acetylated residues, acetylation may be a way for the bacterium to determine the final degree of epimerization.

5 Use of Alginate-Modifying Enzymes for Biotechnological Purposes

The studies on alginate biosynthesis have established that alginate is synthesized as the homopolymer mannuronan, and that the diversity of structural and functional properties found in different alginates results from the action of C-5-epimerases,

lyases, and acetylases. So far the secreted mannuronan C-5-epimerases have the greatest potential from a biotechnological point of view. Pure mannuronan can now be produced by mutants lacking epimerases (Gimmestad et al. 2003). When this polymer is used as a substrate for the AlgE epimerases, alone or in combination, it is thus for the first time feasible to produce alginates with predetermined structures. In addition to compositionally homogeneous alginates (mannuronan, guluronan, and polymers with strictly alternating M–G) (see Fig. 1), this could also include other alginates with extreme composition and sequential structure not found in nature. Of particular interest is a type of alginate comprising only G-blocks interspaced with polyMG sequences. This material, which was made by a two-step conversion of polyM with AlgE4 and AlgE1, forms very strong, elastic, nonswelling gels, particularly suitable for making immune-protective microcapsules for cell transplantations (Mørch et al. 2007, 2008).

Specific alginate lyases have also been used for preparation of pure uronate blocks (Heyraud et al. 1998). Studies on oligomeric G-blocks have shown that they can affect many properties of an alginate gelling system (Jørgensen et al. 2007). Alginate lyases have also been suggested as mucolytic agents in the treatment of cystic fibrosis (Mrsny et al. 1994)

A further challenge would be to produce tailored alginates *in vivo*. This might be done by first making an epimerase-negative strain (including an epimerization-defective mutation in *algG*) of *A. vinelandii* and then introducing specific epimerase genes controlled by a heterologous promoter. *A. vinelandii* has a high alginate lyase activity which needs to be controlled to obtain alginate of a sufficiently high molecular weight, but it has the ability to secrete the AlgE epimerases and has been shown to make alginates with a high fractional content of G residues (Skjåk-Bræk et al. 1986a).

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