Mannuronan C-5 epimerases suited for tailoring of specific alginate structures obtained by high throughput screening of an epimerase mutant library

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Mannuronan C-5 epimerases suited for tailoring of specific alginate structures obtained by high throughput screening of an epimerase mutant library

Running title: Alginate epimerases with new properties

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ABSTRACT

The polysaccharide alginate is produced by brown algae and some bacteria and is composed of the two monomers β-D-mannuronic acid (M) and α-L-guluronic acid (G). The distribution and composition of M/G is important for the chemical-physical properties of alginate, and result from the activity of a family of mannuronan C-5 epimerases that converts M to G in the initially synthesised polyM. Traditionally, G-rich alginates are commercially most interesting due to gelling and viscosifying properties. From a library of mutant epimerases we have isolated enzymes that introduce a high level of G-blocks in polyM more efficiently than the wild type enzymes from *Azotobacter vinelandii* when employed for *in vitro* epimerization reactions. This was achieved by developing a high throughput screening method to discriminate between different alginate structures. Furthermore, genetic and biochemical analysis of the mutant enzymes have revealed structural features that are important for the differences in epimerisation pattern found for the various epimerases.

Keywords: alginate, epimerases, high-throughput screening, mutant library

INTRODUCTION

Alginate is a family of linear polysaccharides with numerous present and potential future application areas ranging from food, textile and printing industry to biomedical and biopharmaceutical as well as electrochemical products [1-5]. The polymer is synthesized by brown algae and by *Azotobacter* and *Pseudomonas* species [6, 7] and currently all commercial production is based on extraction from algal resources. The alginate monomers β-D-mannuronic acid (M) and α-L-guluronic acid (G) are arranged in M-, G- and MG-blocks along the polymer chain and the length and distribution of these blocks determine the physicochemical properties of the polysaccharide [8]. Alginates containing G-blocks are dominating in commercial use due to its ability for forming hydrogels. The polymer is first produced as mannuronan (polyM) and subsequently the block structure results from the activity of a family of mannuronan C-5 epimerases catalysing non-random epimerization of β-D-mannuronic acid to α-L-guluronic acid at the polymer level [9]. *Azotobacter vinelandii* encodes a family of seven secreted mannuronan C-5 epimerases, AlgE1-AlgE7, involved in the cellular differentiation of the bacterium [10-13]. These enzymes display a modular structure being composed of one or two catalytic A-modules and from one to seven regulatory R-modules. Although highly homologous, these enzymes each create characteristic M/G patterns producing alginates with different properties. AlgE4 which is the smallest epimerase containing one A-module and one R-module makes predominantly alternating M/G structure acting on polyM by a processive mode of action [14-16]. AlgE1, AlgE2, AlgE3, AlgE5 and AlgE6 make G-blocks of varying lengths, and AlgE6 is the epimerase able to make the longest G-block structures when acting on polyM [17]. AlgE7 encompasses dual functionalities in also having alginate lyase activity [18]. The three dimensional structure of the A- and R-module of AlgE4 has been reported and the catalytic residues in the active site determined [19, 20]. Given the similarity in amino acid sequence of the A-modules of the epimerases, it is clear

that very minor and to a large extent unpredictable changes in the primary structure can lead to alterations in the epimerization pattern.

The microscopic structure i.e. the monomeric sequence distribution of biopolymers like alginate determine the chemical and physical properties of the molecules and thereby the spectrum of possible applications [21, 22]. Being able to structurally design biopolymers is therefore highly desirable for obtaining biomaterials with controllable and targeted properties [2]. For alginates this can be approached by utilizing mannuronan C-5 epimerases with defined properties in *in vitro* epimerization processes of i.e. polyM [23, 24]. As opposed to current manufacturing strategies from algal resources this enzymatic route would reduce batch to batch variations as well as introduce possibilities for obtaining reproducible alginate structures that are not readily obtainable from algae. Additionally, *in vitro* epimerization with specific enzymes could also offer a route for so-called up-grading of algal alginate, i.e. increasing the level of G-blocks [17]. This strategy could represent a valuable supplement to the global alginate market which is facing shortage of G-rich alginates due to lack of algal raw material.

In the present study our main goal has been to develop mannuronan C-5 epimerases that can be used for efficient *in vitro* epimerization of either bacterially produced mannuronan or alginate substrates of algal origin. Based on the *algE1-algE6* genes from *A. vinelandii* a mutant library was constructed by gene shuffling and subsequent error prone PCR. A high throughput screening protocol was developed that enabled discrimination of epimerised alginate based on the M/G content in the samples, i.e. the microscopic structure of the polymers created by the mutant epimerases. To our knowledge high throughput screening studies based on biopolymer structure has not been performed previously. By this approach we have obtained novel mannuronan C-5 epimerases that are more efficient in epimerizing polyM to high levels of G-blocks than any of the wild type enzymes. Time resolved NMR

spectra indicate that at least one of these enzymes has altered enzyme kinetics compared to wild type AlgE6. Furthermore, results obtained in this work indicate that the R-modules of the mannuronan C-5 epimerases play a role also in determining the epimerization pattern, a property which has previously been attributed only to the catalytic A-modules.

MATERIALS AND METHODS

Bacterial strains, growth conditions and DNA manipulations

Escherichia coli strains DH5α (Bethesda Research Laboratories), JM109 (New England BioLabs) and XL1-Blue (Stratagene) were used as general cloning hosts, whereas XL10-Gold® (Stratagene) was used for establishing the mutant library. *E. coli* strains were routinely grown at 37°C in LB medium (yeast extract, 5 g/liter; tryptone, 10 g/liter; and NaCl, 10 g/liter) or on LB agar (LB medium supplemented with 20 g/liter agar). For protein expression, strains were grown in triple strength LB medium (3×LB; yeast extract, 15 g/liter; tryptone, 30 g/liter; and NaCl, 10 g/liter). For growth in 96-well plates a reduced Hi-Ye medium with the following composition was used: $Na_2HPO_4 \cdot 2H_2O$, 12.3 g/liter; KH_2PO_4, 4.29 g/liter; NH_4Cl, 0.43 g/liter; NaCl, 0.71 g/liter; glucose, 2.86 g/liter; yeast extract, 2.86 g/liter; citric acid, 1.43 g/liter; $MgSO_4$, 1.86 mM; Fe(III)-citrate, 118 μM; H_3BO_3, 21.0 μM; $MnCl_2$, 37.6 μM; EDTA, 9.86 μM; $CuCl_2$, 3.86 μM; Na_2MoO_4, 4.29 μM; $CoCl_2$, 4.71 μM and Zn-acetate, 17.3 μM. Cultures were induced for protein expression using an induction solution containing: glycerol (99%), 25.8 g/liter; yeast extract, 24 g/liter and isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.25 mM. When appropriate, ampicillin (200 μg/ml) was added to the growth media. Standard recombinant DNA techniques was performed as described elsewhere [25]. Plasmids were purified by the Wizard®Plus SV Minipreps DNA purification system (Promega) or the QIAGEN Plasmid Plus Midi Kit (QIAGEN). Transformation of

XL10-Gold® ultracompetent cells was performed according to instructions given by the manufacturer, and for other *E. coli* strains according to RbCl transformation protocol (New England BioLabs). DNA sequencing was performed by Eurofins MWG Operon. Construction of vectors expressing epimerases with site-specific mutations and mutant A-modules combined with the R-modules of AlgE6 is described in Table S1 in Supporting Information.

Construction of an epimerase gene library by staggered extension process (StEP) and error prone PCR

Vectors used and constructed are listed in Table S1 in Supporting Information, whereas sequences of primers utilized are given in Table S2 in Supporting Information. The sequences encoding the A-modules of AlgE1-6 (eight in total) were cloned as NcoI-XmaI fragments into pTrc99A and the resulting vectors used as templates in the StEP reaction. Primers StEP fwd (located 257 bp upstream of NcoI) and StEP rev (loacated 145 bp downstream of XmaI) were used for amplification of PCR fragments and the reaction mixture used were as follows; 12 ng of each template vector, 30 pmol of each primer, 0.2 mM dNTP, 1 × Taq 2000 buffer and 3.75 U Taq 2000 DNA polymerase. PCR conditions used were as follows; 2 min at 96^{o}C, 80 cycles of 30 sec at 95^{o}C and 3-4 sec at 40-45^{o}C. The StEP procedure was repeated several times, and since DNA sequencing of the PCR products revealed a predominance of the gene *AlgE5A* in the recombined sequences for the A-modules, the vector encoding this A-module was omitted from some of the StEP reactions. PCR reactions were treated with DpnI to degrade template DNA, and fragments with correct size (1.1 kb) were isloated from agarose gels. The recombined fragments were digested with NcoI-XmaI and ligated into the same sites of pBL5 creating libraries of hybrid epimerase genes consisting of recombined A-modules (376 amino acid residues) and the R-module (177 amino acid residues) from AlgE4. The ligation mixtures were transformed into XL-10 Gold® cells, the resulting transformants

pooled together, grown for a few generations in LB medium and used for plasmid isolation. Plasmids from each StEP reaction were mixed together and used as template for error prone PCR. Random mutations were introduced into the recombined library by either using the GeneMorph^{TM} PCR mutagenesis Kit from Stratagene (method 1), or by decreasing the fidelity of the Taq polymerase by manipulating the Mn/Mg ratio and the nucleotide concentration in the reaction mixture (method 2). Method 1. Conditions were chosen to give a mutation frequency of 3-7 mutations/kb: 8.5 ng template DNA (corresponding to 1 ng target DNA), 30 pmol each of primers StEP fwd and EU20 (located 52 bp downstream of XmaI), 0.8 mM dNTP, 1× mutazyme buffer and 2.5 U mutazyme in a final volume of 50 µl. Method 2. 78 ng template DNA, 30 pmol each of primers StEP fwd and EU20, 0.2 mM dNTP, 5 µl 5 × PCR-buffer (300 mM Tris-HCl, pH 8.5, 75 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$), 1.25, 2.0, 2.5 or 3.0 µl 10 × mutagenic buffer (8 mM dTTP, 8 mM dCTP, 48 mM $MgCl_2$, 5 mM $MnCl_2$) and 5 U Taq polymerase (Promega) in a final volume of 50 µl. PCR conditions used for both methods were as follows; 1 min at 96^{o}C, 4 cycles of 30 sec at 96^{o}C, 30 sec at 55^{o}C and 2 min at 72^{o}C, and then 27 cycles of 30 sec at 96^{o}C, 30 sec at 60^{o}C and 2 min at 72^{o}C. The five PCR reactions mixtures (one by method 1 and four by method 2) were treated with DpnI, fragments of correct size (1.1 kb) were isolated from agarose gels, digested with NcoI-XmaI and ligated into the corresponding sites of pBL5 to creating recombined, mutated libraries of epimerase genes. The ligations mixtures were transformed into XL10-Gold® cells, the transformants pooled together before addition of glycerol and storage (-80^{o}C) of the library.

Production of alginate substrates and alginate lyases

High molecular weight mannuronan (polyM) was isolated from a mutant strain of *Pseudomonas fluorescens* NCIMB 10525 [26]. ^{13}C-1 labelled mannuronan was produced by growing *P. fluorescens* on a minimal media with 99% D-^{13}C-1 fructose as carbon source. The

obtained mannuronan was selectively enriched to 59% with ^{13}C at carbon position C-1 [15, 27]. Alginate with a strictly alternating structure (polyMG; F_G=0.47 and F_{GG}=0) was prepared by epimerisation of polyM with recombinant mannuronan C-5 epimerase AlgE4 and characterized by NMR as described previously [27]. G-blocks (polyG; F_G=0.94 and DP=18.5) was prepared from *Laminaria hyperborea* stipes as described elsewhere [28, 29]. Production of M-M specific M-lyase (AlxM), G-M and G-G specific G-lyase (AlyA) and G-G specific GG-lyase (AlyA5) by fermentation of recombinant *E. coli* strains was performed as described elsewhere [30].

Robotic screening of the mutant recombined epimerase library

The *E. coli* library was plated on LB-agar in 25 x 25 cm Petri dishes (Corning CLS431301), and incubated overnight at 37 ºC. Colonies were picked using a Genetix Q-Pix2 robotic colony picker and transferred to 96-well microplates (Greiner M3186) containing 80 µl reduced Hi-Ye medium. The microplates were incubated at 30 ºC, 900 rpm (3 mm amplitude) and 80% relative humidity. After 24 h, the microplates were added 40 µl of induction solution using an Asys Hi-Tech Flexispence microplate dispenser. The microplates were incubated at 37 ºC, 900 rpm and 80% relative humidity for 7 h after induction, and were frozen at - 40 ºC prior to analysis.

After thawing, the microplates were added 30 µl B-per II solution (Pierce) (with $CaCl_2$ to a concentration of 25 mM) per well, shaken for 30 s (900 rpm, 3 mm amplitude), and incubated at room temperature for 1 h. After incubation, the microplates were shaken (850 rpm, 3 mm amplitude) for 10 min and then centrifuged for 30 min at 3500 × g. For epimerization of alginate, 10 µl of enzyme extracts was added to 190 µl of assay buffer (40 mM MOPS, 20 mM NaCl, 2 mM $CaCl_2$, pH 6.8) containing polyM alginate (0.1 mg/ml). The plates were sealed after addition of enzyme extract using sterile sealing film (Nunc 236366) and

incubated at 37 ºC for 48 hours. Microplates with epimerized alginate were frozen at - 40 ºC prior to analysis.

For analysis of G-content in epimerized alginate samples, 30 µl samples of alginate in assay buffer were transferred to wells in 384-well microplates (Corning CLS3675). The wells were then added 10 µl of assaybuffer containing the AlyA enzyme (0.14 U/ml on MG alginate) and shaken at 1700 rpm for 1 minute. The microplates were then incubated at 25 ºC for 5 hours. The absorbance at 230 nm (A230) was read in a Beckman Coulter DTX880 microplate reader prior to addition of alginate shortly after mixing and after incubation. The increase in absorption during incubation was calculated, and ΔA230 ($A230_{t=5}$-$A230_{t=0}$) were used for estimation of the total G content of the epimerized alginates.

To be able to discriminate between alginate samples containing MG- and GG-blocks a two-step protocol using an M-lyase and two alginate lyases with different specificity towards G-block and polyMG alginates was developed. 30 µl of epimerized alginate in assay buffer were transferred to wells in 384-well microplates (Corning CLS3675). Each of the wells were added 5 µl of assaybuffer containing M-lyase and shaken at 1700 rpm for 1 minute and the microplates where incubated at 25 ºC for 12 hours. Each of the wells were then added 5 µl of assaybuffer containing either the AlyA enzyme or the AlyA5 enzyme (0.2 U/ml on polyG for both enzymes) and shaken at 1700 rpm for 1 minute. After mixing, the microplates where incubated further at 25 ºC for 12 hours. The absorbance at 230 nm (A230) was read in a Beckman Coulter Paradigm microplate prior to addition of enzymes, shortly after mixing and then each hour after addition enzymes. The difference in absorbance between the two first time points (ΔA230 =$A230_{t=1}$-$A230_{t=0}$) were used for evaluation of the G content and structure of the epimerized alginates. All liquid- and microplate handling was performed by a Beckman Coulter Core system robotic liquid handling workstation.

Protein expression and purification

Epimerase expressing strains were grown in 100 ml of 3×LB medium in 500-ml baffled shake flasks at 37°C for 3 h before induction with 0.5 mM IPTG. Growth was continued for 4 h at the same temperature before harvesting the cells by centrifugation. For preparation of protein extracts, the cells were sonicated in 10 ml of 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 5 mM $CaCl_2$, pH 6.9, and then centrifuged for 30 min at 20 000 ×*g*. The supernatant was filtered (0.2 μm), applied on a 5-ml HiTrap Q HP column, and proteins were eluted using a stepwise NaCl gradient (0 to 1 M) in the same buffer as above. Protein containing fractions were tested for epimerase activity by NMR (see below), and the total protein content was measured by the Bio-Rad micro assay procedure using bovine serum albumin as standard. Purity of protein fractions were determined by SDS-PAGE.

End point and time-resolved NMR analysis of epimerised alginate samples

All experiments were recorded on a BRUKER Avance 600 or DPX 400 spectrometer equipped with a 5 mm cryogenic CP-TCI z-gradient probe and 5 mm z-gradient DUL (C/H) probe, respectively. End-point analysis of epimerised samples were recorded at 90°C, while time-resolved NMR recording of the epimerisation reaction was performed at 40°C. To reduce the viscosity of the alginate samples prior to NMR measurements the samples were depolymerised by mild acid hydrolysis to a final average DP_n ~30 residues [31]. 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (Aldrich, Milwaukee, WI, USA) was used as internal standard for the chemical shift and triethylenetetra-amine hexa-acetate (Sigma-Aldrich) was added to chelate residual calcium ions in end-point epimerised samples. For the time-resolved NMR analysis of epimerisation reactions a stock solution of 22 mg/ml ^{13}C-1-enriched polyM (average DP_n ~70) in 5 mM MOPS, pH 6.9 with 75 mM NaCl in 99.9% D_2O was prepared. Purified enzyme fractions from ion exchange chromatography were

subject to buffer exchange and upconcentrated (final concentration of 1.1-2.3 mg/ml) by spin columns (VivaSpin, Sartorius Stedim Biotech) with molecular cut-off 10kDa. Samples were washed with 5 mM MOPS, pH 6.9 with 75 mM NaCl and 27.5 mM $CaCl_2$ in 99.9% D_2O. Protein concentrations were determined with a Nanodrop ND-1000 to ensure similar enzyme concentration in the epimerisation reaction. 500 µl ^{13}C-1-enriched polyM stock solution was preheated in the NMR instrument and 1D proton and carbon spectra were recorded to ensure that the sample has not undergone any degradation or contamination prior to the time-resolved NMR experiment. 50 µl enzyme solution was added to preheated substrate and mixed by inverting the sample 2-3 times. The sample was then immediately inserted into the preheated NMR instrument and the experiment was started. The recorded spectrum is a pseudo-two-dimensional type experiment recording a 1D carbon NMR spectrum every 15 min. The recorded 1D carbon spectrum (using inverse gated proton decoupling) contains 8K data points, has a spectral width of 80 ppm, 64 scans with a 30 degree flip angle, relaxation delay of 1 s (total recording time of 91s). The NMR data were processed and analysed with Bruker XwinNMR 3.5, TopSpin 2.1 and TopSpin 3.0 software.

Bioinformatics analysis of epimerase mutants

The experimental three-dimensional structure of the A-module from AlgE4 with and without mannuronan trisaccharide bound (Protein Data Bank code 2PYH and 2PYG, respectively) in the substrate binding groove were downloaded from the Rescarch Collaboratory for Structural Bioinformatics Protein Data Bank [32]. The structures were used as template input for the SWISS-MODEL platform [33-35] modelling the mutant A-modules identified from the library. The structures were visualized and analysed with PyMol [36]. Alignment of mutant and wild type protein sequences was done with ClustalX [37] and visualised with TreeView [38].

RESULTS AND DISCUSSION

Construction and characterization of a mannuronan C-5 epimerase mutant library

In order to obtain mannuronan C-5 epimerases with improved properties for *in vitro* epimerization we used the genes encoding the secreted epimerases from *A. vinelandii* as basis for construction of a mutant library. The catalytic site of the epimerases is located in the A-modules of the enzymes, and the gene sequences encoding these modules of AlgE1-AlgE6 (E1A1, E1A2, E2A, E3A1, E3A2, E4A, E5A and E6A) were therefore used as templates in the staggered extension process (StEP). DNA encoding AlgE7 was not included in the recombination reaction due to the combined epimerase and lyase activity of this enzyme [39]. The recombined A-module sequences were ligated into expression vectors containing the R-module sequence of AlgE4 creating complete epimerase genes. The resulting plasmids were transformed into XL10-Gold® cells creating a library of about 120 000 clones. To test the diversity in the library, plasmids from 48 random clones were sequenced to analyse the degree of recombination between the different A-module sequences and the epimerases encoded by the same plasmids were also tested for epimerase activity. Sequence alignment showed that 33 plasmids (69%) encoded shuffled A-modules, and of these 26 (78%) encoded epimerases displaying activity. To further increase the diversity of in the DNA sequences, error prone PCR was performed on the recombined A-module genes from the first library. Conditions were adjusted to achieve a mutation frequency of about 3-8 nucleotide changes per gene. The mutagenized A-modules were ligated into expression vectors containing the R-module sequence from AlgE4, and the resulting plasmids transformed into XL10-Gold® cells creating a final library of about 100 000 clones. To verify the mutation frequency, plasmid DNA from 56 random clones was sequenced and 18 of the mutant enzymes encoded by these plasmids (32%) were found to display epimerase activity. This final library containing

epimerases with recombined and mutated A-modules were used for screening for enzymes with high G-block forming activity.

Screening of the mutant library and isolation of enzymes that are able to epimerise mannuronan to high levels of G-blocks

An initial screening was performed by randomly picking 11 000 colonies from the final library, followed by cultivation and induction for epimerase expression with IPTG in 96-well microtiter plates. Cell-free extract from each culture was prepared and used for epimerisation of polyM. To evaluate the G-content in each epimerised sample, the resulting alginates were degraded using a G-lyase which cleaves G-M and G-G linkages almost equally well (polyMG/polyG activity ratio of 1.2) [40-42]. End point measurements of A_{230} detecting the unsaturated uronic acid residues resulting from lyase degradation made it possible to identify samples containing epimerised alginate among the ones that were not epimerised i.e. containing polyM. However, by this method it turned out to be difficult to distinguish between samples of alginates with medium G-content (~45%) in alternating MG structure, and high G-content (~80%) in block structure. This is due to the similar amounts of linkages available for lyase degradation in the two structurally very different alginates. A two-step degradation protocol utilizing three alginate lyases with different specificities was therefore developed. The strategy was to first use an M-lyase to specifically degrade all the M-M linkages in the alginate samples [43]. Samples containing high or low M would then be subject to extensive or limited degradation by this enzyme, respectively. The next step was to further degrade two parallels of the same samples with G-lyase and GG-lyase, the latter enzyme displaying increased specificity towards G-G linkages (polyMG/polyG activity ratio of 0.1) [42]. The GG-lyase was expected to display lower activity towards a sample containing alginate molecules with alternating MG structure than the G-lyase, whereas the two enzymes were

expected to have similar activity towards samples rich in G-G linkages. To take full advantage of the different specificities of these two enzymes it was necessary to follow the kinetics of alginate degradation. End point measurements would give nearly the same A_{230} for both enzymes, since the GG-lyase has some residual activity towards G-M linkages. The protocol was first tested on alginates with known composition: polyM (F_M=1, F_G=0), polyMG (F_G=0.47, F_{GG}=0) and LF10/60 (F_G=0.66, F_M=0.34, F_{GG}=0.55, F_{MG}/F_{GM}=0.12, F_{MM}=0.22). First, M-lyase was added to the samples and degradation was followed by measuring A_{230} every hour for 12h. Then two parallel samples were added G- or GG-lyase, and incubation continued with A_{230} monitoring for another 12h. As expected, polyM is completely degraded in the M-lyase step i.e. there is no further increase in A_{230} by the addition of G- or GG-lyase (Fig. 1A). Furthermore, the kinetics of degradation of polyMG and LF10/60 was clearly different in the second step (Fig. 1B and C) and the largest difference in activity between the G- and GG-lyase was as expected obtained on the alternating substrate (Fig. 1B). This showed that it was possible to perform a screening of the library based on discrimination between differences in the resulting polymer microstructures created by the mutant enzymes. To our knowledge this kind of high throughput screening studies has not been performed previously. Due to the increasing applications and need for high-G alginates we targeted the current screening approach to isolate mutant enzymes giving high content of G-blocks when epimerising polyM.

Alginate epimerised by 960 randomly selected mutants were evaluated using the two-step degradation protocol described above (9% of the amount in the initial screen). PolyM was epimerised with protein extracts from the randomly selected mutants, and subjected to lyase degradation as described for the test samples. From data analysis of the degradation kinetics of each sample we identified three for which the degradation with G- and GG-lyase was

almost identical (Fig. 2 A and B) or very similar (Fig. 2C) indicating a high level of G-blocks in the epimerised alginate. Furthermore, around 25 samples displayed degradation kinetics similar to polyMG indicating an F_G of about 0.45 (three examples shown in Fig. 2D-F). To test whether the observed degradation kinetics corresponded to the expected sample properties, a preliminary characterization of crude protein extracts obtained from a total of 11 strains (including those shown in Fig. 2) was performed. Strains were grown in shake flasks, and cell free protein extracts were used to epimerise polyM for NMR-analysis. These analyses showed that alginates with F_G in the range of 0.65-0.80 were obtained for the samples shown in Fig. 2A-C, and 0.40-0.45 for the remaining samples. Taken together, this confirmed the validity of the screening method for identification of samples with a specific composition. Furthermore, it also indicated that about 0.2% of the mutants in the library encoded epimerases that were introducing such a high level of G-blocks into polyM.

It should be pointed out that the kinetic measurements performed to obtain the data underlying the isolation of mutant enzymes are time- and resource demanding. If the aim was to isolate as many mutants as possible an alternative strategy allowing for screening of a larger portion of the library could be considered. As a first step the entire collection of alginate samples would be degraded with M-lyase and end-point A_{230} measurements used to sort out the ones containing polyM (inactive enzymes) and low G (enzymes with low activity). The remaining samples i.e. with A_{230} below 1 like in Fig. 1B and C would then be chosen for further characterization following the kinetics during degradation with G- and GG-lyase. From the original library of about 100 000 clones one would then expect to obtain around 200 mutants resulting in high G when starting with polyM.

Mutant epimerases AlgEM1 and AlgEM2 epimerise polyM to higher G-content than wild type epimerase AlgE6

To characterize enzyme properties in detail, the mutant epimerases corresponding to samples shown in Fig. 2 (designated AlgEM1-AlgEM6), were partially purified by ion exchange chromatography and used for epimerisation of polyM. As a control, polyM was also incubated with AlgE6, which is the wild type epimerase known to give the highest F_G [17]. The monomer composition and the diad and triad frequencies in the different alginate samples are shown in Table S3 in Supporting Information. Two of the mutants, AlgEM1 and AlgEM2 epimerised polyM to F_G=0.85 and 0.83, respectively, which is higher than what was obtained with AlgE6 (F_G=0.77) under the conditions utilized. AlgEM1- and AlgEM2-epimerised alginate also display high F_{GG} (0.78 and 0.74) and low F_{MGM} (0.030 and 0.027) indicating that the majority of guluronic acid residues are present as blocks and that there is very little alternating structure present. Compared to AlgE6, these two enzymes produce alginates in which a larger fraction of the total guluronic acid residues are present as blocks. AlgEM3-5 resulted in alginates with F_G in the range of 0.54-0.67, and where the guluronic acid residues introduced are present both in blocks (F_{GG}=0.22-0.44) and in alternating structures (F_{MGM}=0.15-0.25). AlgEM6 epimerised polyM to F_G=0.4 and most of the guluronic acid residues are present in alternating structures (F_{GG}=0.039 and F_{MGM}=0.33) which is very similar to wild type AlgE4.

The availability of effective enzymes that can epimerise polyM in a single reproducible reaction step is valuable in *in vitro* design of alginates. Another approach for enzymatic preparation of specific alginates is to perform epimerisation of algal alginates with the aim of increasing the G content, and we therefore wanted to test mutants AlgEM1 and AlgEM2 on a predefined complex alginate isolated from leaves of *L. hyperborea* to elucidate the efficacy on this kind of substrate. This natural substrate has F_G=0.50 with G-residues present both as blocks and alternating structures. NMR analysis showed that AlgE6 and AlgEM1 acted very

similar on this substrate, yielding alginates with F_G=0.76 and 0.75 respectively, whereas AlgEM2 reached F_G=0.69. This indicates that the enzymes act quite differentially on various substrates and that in screening for epimerases with targeted properties, the choice of substrate is very important.

AlgEM1 and AlgE6 display different epimerisation kinetics on polyM

The results shown in Table S3 are end point measurements of the final composition of polyM epimerised with the different enzymes. To reveal possible differences in the kinetics of epimerisation for the mutant enzymes AlgEM1 and AlgEM2 compared to AlgE6, continuous NMR-spectra were recorded using ^{13}C-1-enriched polyM. AlgEM1 (Fig. 3B) displayed significantly different epimerisation kinetics than AlgE6 (Fig. 3A), whereas AlgEM2 displayed a spectrum very similar to AlgE6 (spectra not shown). AlgEM1 showed an almost immediate and fast introduction of G-blocks (evident as increase in peak marked G<u>G</u>G) into the substrate. This is accompanied by a simultaneous rapid decline in the content of M-blocks (M<u>M</u>M) as well as a slow accumulation of alternating blocks (G<u>M</u>G and M<u>G</u>M). Moreover the G<u>G</u>M peak that signifies the number of G-blocks remains constant after the initial phase, indicating that the G residues are introduced predominantly as elongation of existing G-blocks. For AlgE6, the formation of G-blocks lagged behind the introduction of G-residues in alternating sequences, indicating that AlgEM1 have higher affinity for the alternating polyMG structure than polyM compared to AlgE6. There are in principle two modes of action that could account for a predominant G-block formation; a processive mode where the enzyme slides along polyM carrying out repetitive epimerisation reactions without dissociating from the substrate, or a preferred attack mode where the enzymes affinity is higher for M-G than M-M. In both cases subsite -1 (by definition epimerization takes place at subsite +1) must preferentially accommodate a G residue. A processive mode where consecutive residues are

epimerized appears unlikely since the uronic acid residues are rotated 180^{o} with respect to each other and the enzyme would then have to rotate while sliding around the polymer chain. We have previously demonstrated processivity for AlgE4 acting on polyM generating long alternating MG stretches, or for AlgE6 when acting on polyMG. In both of these cases the enzymes act in processive modes where every second M is converted while the enzymes slides along the polymer, however this does not require the enzyme to rotate. Whether the properties of AlgEM1 is due to an increase of processivity or a result of an enhanced affinity for pre-existing G residues is not possible to conclude from the present experiments.

Alignment of mutant and wild type A-modules elucidates sequence properties mediating changes in enzymatic activity

To reveal possible structural features in the mutants underlying the enzymatic properties described above, the genes encoding the A-modules of mutants AlgEM1-6 were sequenced and the resulting protein sequences compared to wild type A-modules. Sequence properties i.e. similarity to wild type A-modules and introduction of new amino acid residues due to error prone PCR are shown in Table S4 in Supporting information. Relationship between the A-modules is visualised in a phylogenetic tree (Fig. 4) and alignment of sequences is given as supplementary material (Fig. S1 A-C). The phylogenetic tree displays three groups, which can be characterised as formers of MG-blocks, G-blocks and long G-blocks, the latter group comprising only AlgE6. A general tendency found for AlgE6 is that more amino acid residues promoting hydrogen bonding and hydrophobic interactions are identified along the alginate binding groove in the A-module compared to the other G-block forming enzymes (Fig. S1).

Sequence alignment analysis shows that the A-module of AlgEM1 is most similar to the G-block forming A1 modules of AlgE1 and AlgE3 before the position of the active site, whereas being most similar to AlgE6 after the active site. Furthermore, in AlgEM1 the alginate

binding subsite at -3, -4 and -6 seems to have more residues supporting alginate binding through hydrogen bonds and hydrophobic interactions (e.g. Arg, Leu, Ser, Asn) than the other G-block formers (Fig. 5, Fig. S1). The AlgEM2 A-module is most similar to AlgE2 and AlgE5 which are also both capable of producing G-blocks. Moreover, AlgEM2 has a substitution in subsite -2 (Ala220Asn) that putatively supports stronger alginate binding through hydrogen bonding. Furthermore, some amino acid residues change the N-terminal α-helix interfacing the rest of the protein and lead to minor rearrangement in packing of the hydrophobic core under subsite +1 (Val136Ala) and -1 (Ile200Val). This might result in a deeper substrate binding grove hereby enlarging the contact surface to the alginate polymer. Since both AlgEM1 and AlgEM2 possibly have improved alginate binding properties compared to AlgE6, this can partially explain their ability to form long G-blocks. The mode of action for the G-block forming A-modules except for AlgE6 acting on polyMG, has been characterised as a preferred attack mechanism [44] and accordingly the N-terminal part before the catalytic site of AlgEM1 and AlgEM2 seem to originate from these A-modules. Combining the preferred attack mode of action with better alginate binding properties i.e. improved processivity may then have resulted in the high G-block forming epimerases AlgEM1 and AlgEM2. The mutants AlgEM3-6 (Table S4) have almost all mutations at the N-terminal part before the catalytic site probably resulting in the ability to form G-blocks. After the active site they are almost identical to the AlgE4 A-module, presumably mediating the ability to create alternating structures (Fig. S1).

Introduction of R-modules from AlgE6 ($R_1R_2R_3$) behind mutant A-modules from AlgEM1 and AlgEM2 modulates the epimerisation pattern of the enzymes

As a consequence of the construction strategy (see materials and methods) all mutants should contain the same C-terminal end i.e. 177 residues constituting the AlgE4 R-module. However,

for AlgEM2 and AlgEM6 a deletion in the XmaI restriction site used for cloning leads to a frame shift and translation into a 52 residue long C-terminal end following the A-module. It has been shown previously that the A-modules alone are sufficient for epimerisation [45], and apparently the addition of 52 random residues C-terminally does not affect the enzymes detrimentally. Although it has been shown that the R-modules are Ca^{2+} binding and stimulate the activity of the A-modules when present [45], the function of the R-modules is not fully understood. AlgE6 is an efficient G-block forming epimerase with three R-modules ($R_1R_2R_3$) and we wanted to explore the effect of combining these R-modules with the mutant A-modules from AlgEM1 and AlgEM2. DNA sequences encoding $R_1R_2R_3$ were synthesised and cloned into the vectors encoding the two mutant A-modules resulting in the expression of hybrid enzymes AlgEM1-$R_1R_2R_3$ and AlgEM2-$R_1R_2R_3$. As described above, polyM was epimerised and the resulting alginate structures analysed by NMR. For both enzymes the F_G obtained was lower for the hybrids containing $R_1R_2R_3$ than for the original mutants isolated from the screen (Fig. 6). It also appeared that the level of alternating MG structures increased with a concomitant decrease in G-blocks. Furthermore, time-resolved NMR spectra recorded from the epimerization of polyM with AlgEM1-$R_1R_2R_3$ (Fig. S2) showed that this enzyme behaved kinetically like AlgE6 and not like AlgEM1. These results show that the R-modules not only modulate the epimerisation rate as previously thought, but also the epimerisation pattern of the individual enzymes. This might be connected to individual differences between the R-modules in binding affinity for the epimerized substrate which will influence on the number of residues that are epimerized before the substrate is released from the enzyme.

Residue Asp119 in the A-module of the epimerases is important for the epimerisation pattern

AlgEM3-5 displayed similar properties in introducing both MG- and G-blocks, although being up to 92% identical to AlgE4 which do not make G-blocks at all. Of these three mutants, AlgEM3 was found to have one substitution which was considered to be of particular interest (Table S4). The tyrosine substituting the aspartic acid residue found at position 119 in all wild type enzymes might influence on the catalytic activity due to being in the vicinity of catalytic site (Fig. 7A). To elucidate whether this residue is somehow determinative for the epimerisation pattern, targeted substitutions were made at position 119 in both AlgE4 and AlgEM3. Site specific mutations were introduced in the corresponding genes, and epimerisation of polyM performed with purified protein samples. Results from NMR analysis of the epimerized polyM are given in Table S5 in Supplementary Information. AlgE4 is forming strictly alternating MG-blocks, but by substitution of Asp119 to Tyr, Phe or Ala the resulting enzymes introduce a low level of two sequential G residues (F_{GG}=0.044 to 0.089). Furthermore, a closer inspection of the endpoint ^{13}C spectrum from the time-resolved NMR experiments shows that AlgEM3 (Fig. 7C) is not able to produce GGG triads. This might indicate that GG-formation takes place because the enzyme only move one residue forward instead of two before making the next epimerisation reaction, and hereafter AlgEM3 dissociate from the alginate polymer. This points to residue 119 as one of probably many that are directly involved in determining epimerisation pattern, and also indicates that a negative charge on the side chain might be essential for obtaining the strictly alternating MG structure as is the case for AlgE4. Effects on epimerisation pattern is also found for AlgEM3 when substituting Tyr119 to Asp or Arg, which in both cases leads to an increase in the frequency of MG. Again this points to charged residues as being determinative for epimerisation pattern.

CONCLUSIONS

In the present study we have constructed a library of mutant mannuronan C-5 epimerases by gene shuffling and error prone PCR. Furthermore, a screening method was developed that enabled the identification of specific alginate sequences created by the mutant enzymes. By screening nearly 1000 mutant strains we were able to isolate two epimerases that are more efficient in introducing G-blocks in polyM than the naturally occurring enzymes, and one of these apparently acts kinetically different than the G-block former AlgE6. Such mutant epimerases with new or improved functionalities can be valuable tools in future *in vitro* design of alginate structures, and especially in manufacturing G-rich alginates of which there is inadequate supplies in the global alginate market. The results obtained also emphasise the need for careful design of the screening protocol, in that the AlgEM1 and AlgEM2 did not display superior properties to AlgE6 in epimerizing an alginate substrate with a complex composition. For isolation of robust enzymes with an industrial potential for up-grading of algal alginates the current method can be expanded to screen for enzymes efficient in epimerizing algal alginates under conditions of e.g. defined pH, temperature, ionic strength and salinity that are relevant for the actual process.

ASSOCIATED CONTENT

Supporting information

Vectors used and constructed and primers utilized are listed in Table S1 and S2 in Supporting Information. Results from NMR analysis of polyM epimerized with AlgE4, AlgE6 and the epimerase mutants are shown in Table S3 and S5. Sequence properties of the A-modules from epimerase mutants AlgEM1-6 are shown in Table S4. Multiple sequence alignment of epimerase A-modules from *A. vinelandii* and the mutant A-modules for AlgEM1-6 is shown in Figure S1. Continuously recorded NMR spectra for epimerization of polyM with AlgEM1-

$R_1R_2R_3$ are shown in Figure S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE LEGENDS

FIGURE 1

Degradation of polyM (A), polyMG (B) and LF10/60 (C) with M-lyase (left) and G- or GG-lyase (right) measured by monitoring A_{230}. Two parallel samples of the alginate substrate (1 mg/ml in MOPS, pH 6.9 with 100 mM NaCl and 1.5 mM $CaCl_2$) was added M-lyase (0.5 U/ml on polyM) and incubated for 12h with A_{230} readings every hour. Then G- and GG-lyase (0.2 U/ml on polyG) were added to parallel samples and incubation and A_{230} reading continued for an additional 12h. Degradation reactions for each sample were performed in 384-well plates in 96 parallels, and the curves shown are representatives of each kind. The standard deviation of the mean for the measurements is below 15 %.

FIGURE 2

Degradation of polyM epimerised with mutant epimerases AlgEM1-6. Two parallel samples of each were first degraded with M-lyase for 12h (not shown in the figure), and then samples were added G- (circles) and GG-lyase (squares) and incubation and A_{230} reading continued for additional 12h. The figures show only the A_{230} measurements up to 8 hour since after that there was no change in absorbance. The reaction conditions and enzyme concentrations used and the experimental uncertainty for the measurements were as described in the legend to Fig. 1.

FIGURE 3

Continuously recorded NMR spectra showing epimerisation of ^{13}C-labelled polyM with AlgE6 (A) and mutant enzyme AlgEM1 (B). Substrate (20 mg/ml) and enzyme were mixed and immediately inserted into the NMR instrument before recording of spectra every 15 min. Reactions were performed in MOPS, pH 6.9 with 75 mM NaCl and 2 mM $CaCl_2$. The

position of each of the eight possible triads in the spectra is indicated, and the M or G moiety giving rise to the signal is underlined. Arrows indicate increasing reaction times. It should be noted that the enzyme reactions were not run to complete epimerisation of the substrate, so the end composition of the resulting alginates in this experiment is not directly comparable to results given in Table S3.

FIGURE 4

Phylogenetic tree displaying the relationship between the wild-type and mutate A-modules in relation to their function on alginates. Alignment of mutant and wild type protein sequences was done with ClustalX [37] and visualised with TreeView [38].

FIGURE 5

Ribbon structure of the A-module from AlgE4 with subsites for the substrate indicated. The division of the subsites is based on prolongation of the mannuronan trisaccharide bound to A-module from AlgE4 (Protein Data Bank code 2PYH) in the substrate binding groove. Subsite +4: Met1-Glu51, +3:Pro52-Ser91, +2: Ala92-Asn123, +1:Gly124-156+Asp178, -1:Thr157-Asp178+Leu228, -2:Tyr179-Leu228, -3:Glu229-Lys255, -4: Met256-Tyr278, -5:Gly279-Tyr318, -6:Thr319-Arg343, -7:Asn344. The structures were visualized and analyzed with PyMol [36].

FIGURE 6

Relative frequencies of F_G, F_{GGG} and F_{MGM} obtained by epimerisation of polyM with mutant enzyme AlgEM1 and AlgEM1-$R_1R_2R_3$ i.e. A-module from AlgEM1 and R-modules from AlgE6 (A). Corresponding results for AlgEM2 and AlgEM2-$R_1R_2R_3$ (B). Epimerisation was

performed with polyM (10 mg) and enzyme (0.2 mg) in MOPS, pH 6.9 with 100 mM NaCl and 1.5 mM $CaCl_2$.

FIGURE 7

View of the active site with substrate bound (PDB code: 2PYH) and ^{13}C NMR spectra for end-point products from the epimerization reaction. (A) The bound mannuronan trisaccharide (only two (M-1 and M-2) of the sugar units are shown on the figure), the catalytic residues (Tyr149, Asp152, His154, and, Asp 178) and ionic pair (Lys117, Asp119) are shown in stick representation. The ionic pair is involved in the coordinate of the carboxyl group on the mannuronan at subsite +1. The structure was visualized with PyMol [36]. (B) End-point products as a result of epimerization with the GG-block forming epimerase AlgE6. (C) End-point products as a result of epimerization use mutant epimerase AlgEM3. This clearly shows that the mutant epimerase AlgEM3 is not able to form more then only GG-block and not polyG and fill in a G in a GMG sequence.

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FIGURE 1

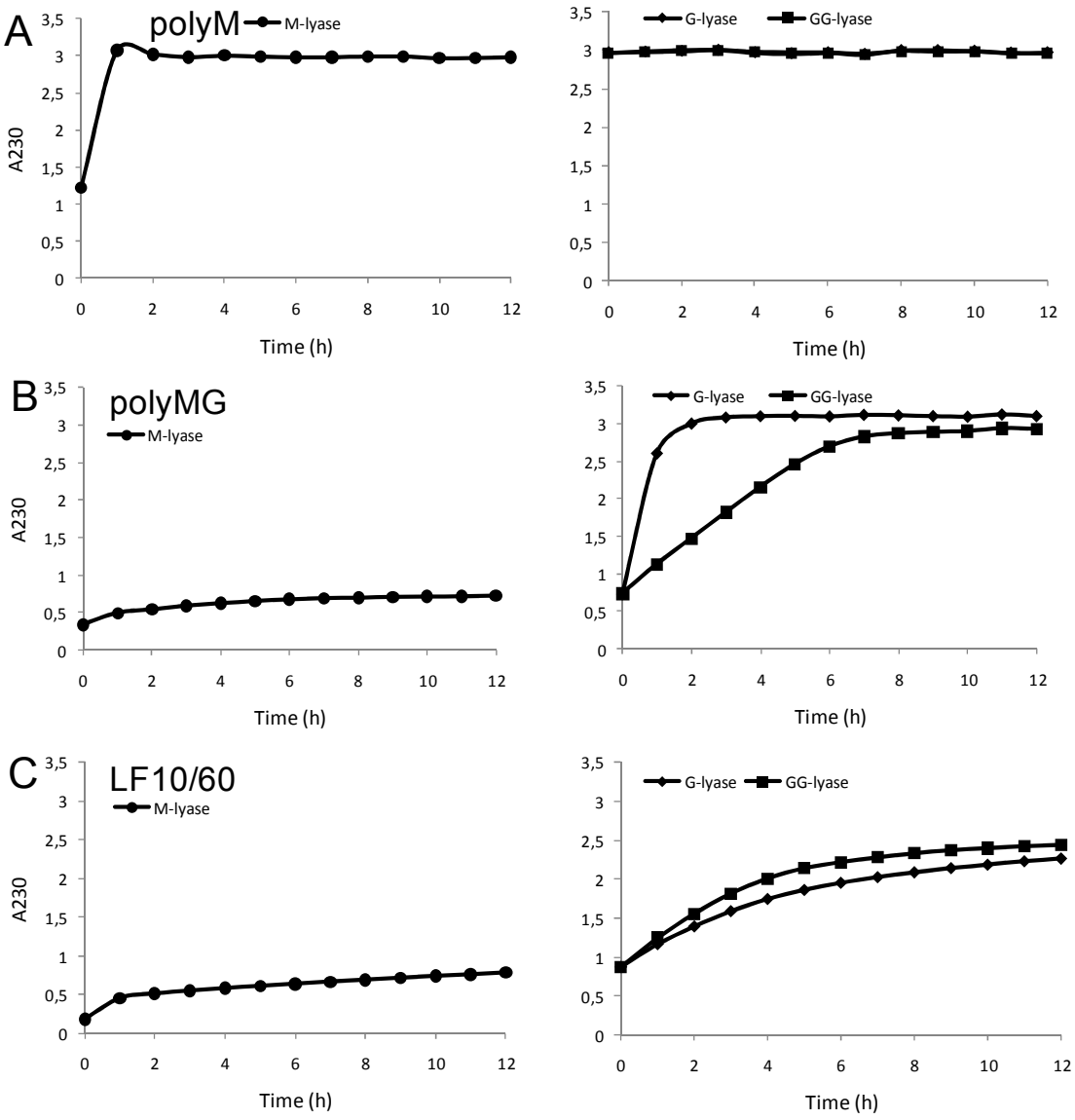

A
polyM
M-lyase
G-lyase
GG-lyase
A230
Time (h)
B
polyMG
M-lyase
G-lyase
GG-lyase
A230
Time (h)
C
LF10/60
M-lyase
G-lyase
GG-lyase
A230
Time (h)

FIGURE 2

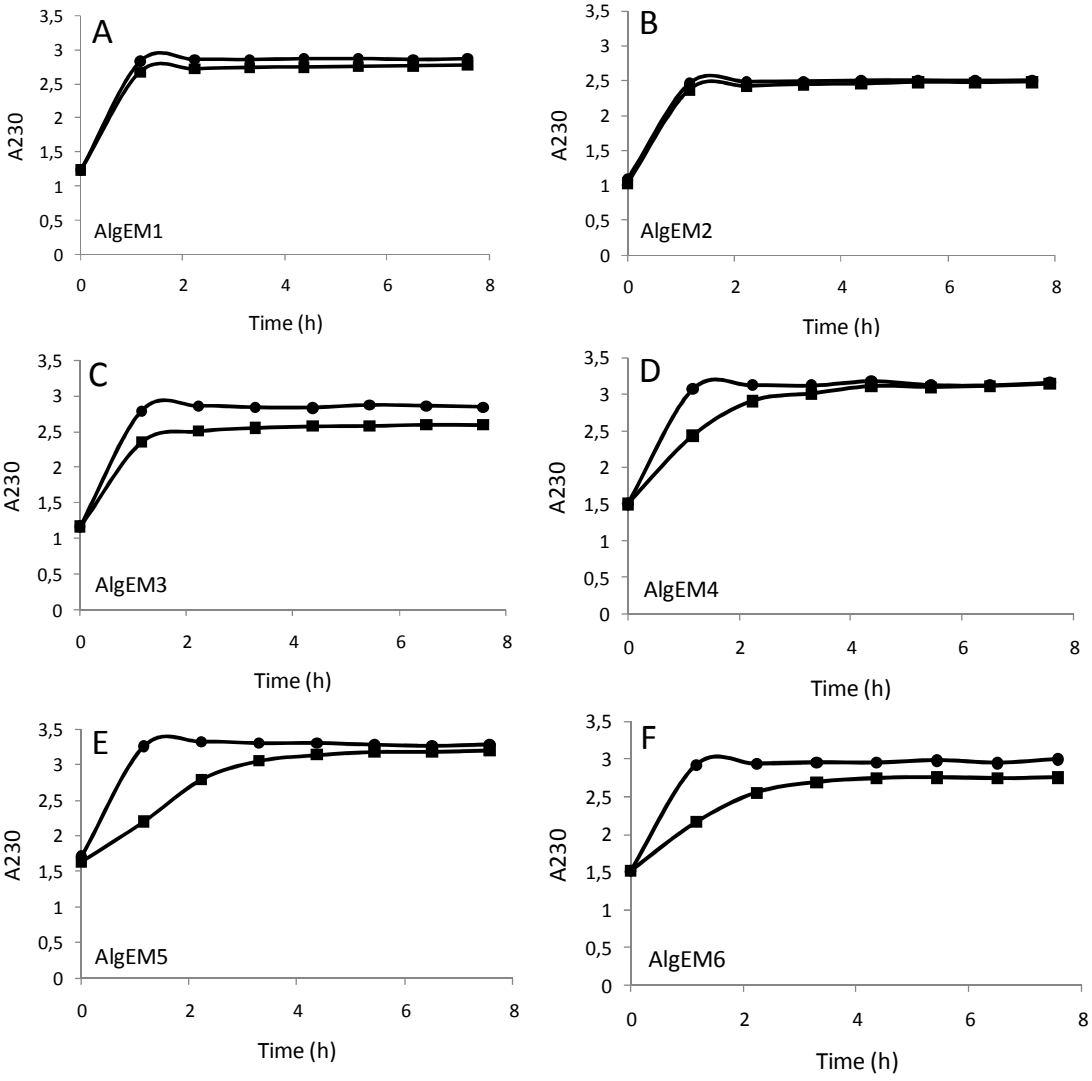
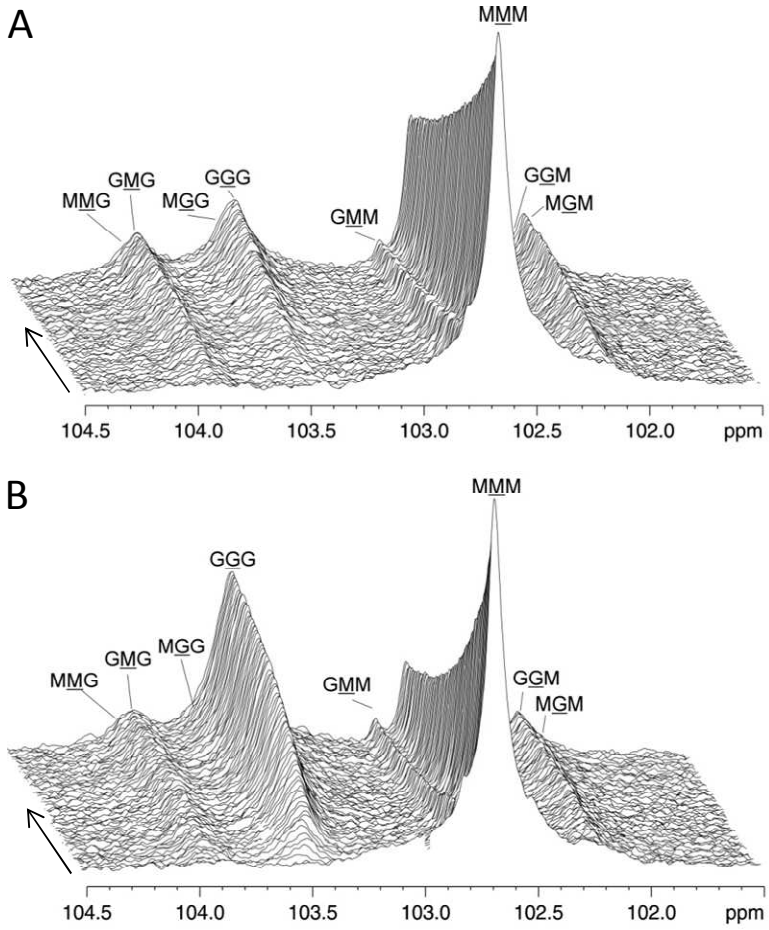

A
AlgEM1
B
AlgEM2
C
AlgEM3
D
AlgEM4
E
AlgEM5
F
AlgEM6
A230
Time (h)

FIGURE 3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MMM
GMG
GGG
MMG
MGG
GMM
GGM
MGM
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104.0
103.5
103.0
102.5
102.0
ppm
B
MMM
GGG
GMG
MGG
MMG
GMM
GGM
MGM
104.5
104.0
103.5
103.0
102.5
102.0
ppm

FIGURE 4

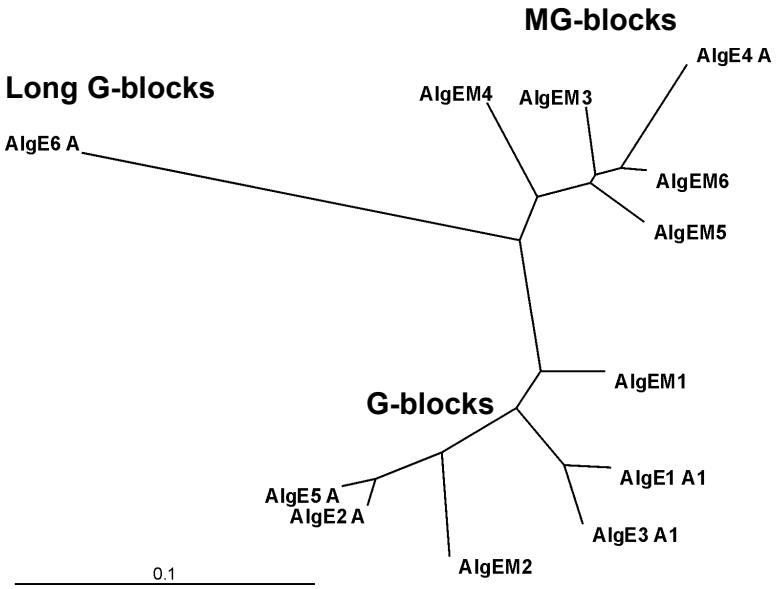

MG-blocks
AlgE4 A
Long G-blocks
AlgEM4
AlgEM3
AlgE6 A
AlgEM6
AlgEM5
AlgEM1
G-blocks
AlgE1 A1
AlgE5 A
AlgE2 A
AlgE3 A1
AlgEM2
0.1

FIGURE 5

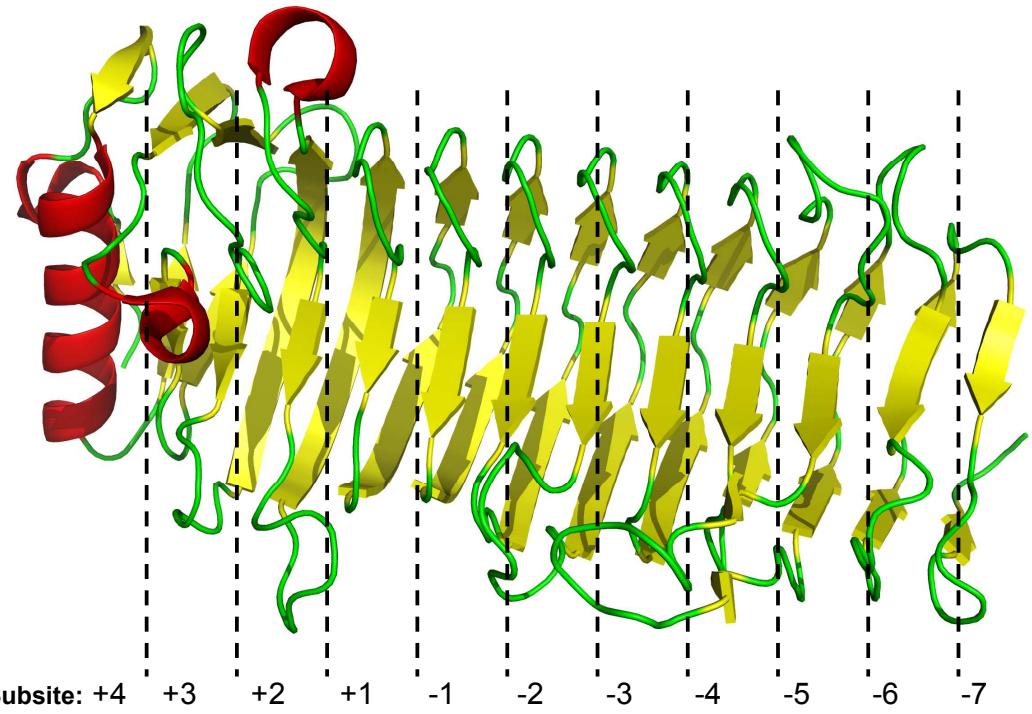


FIGURE 6

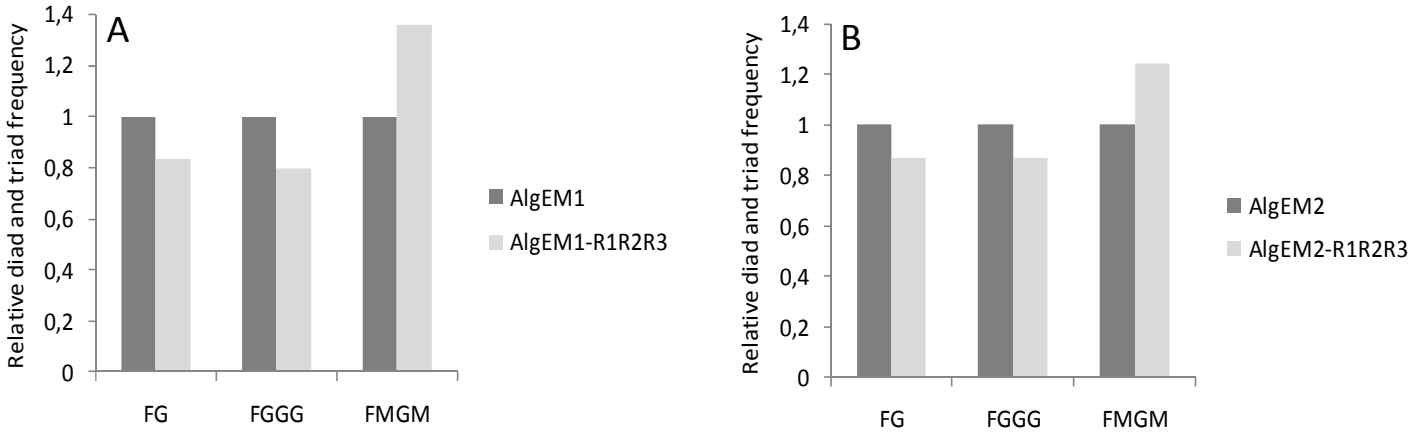
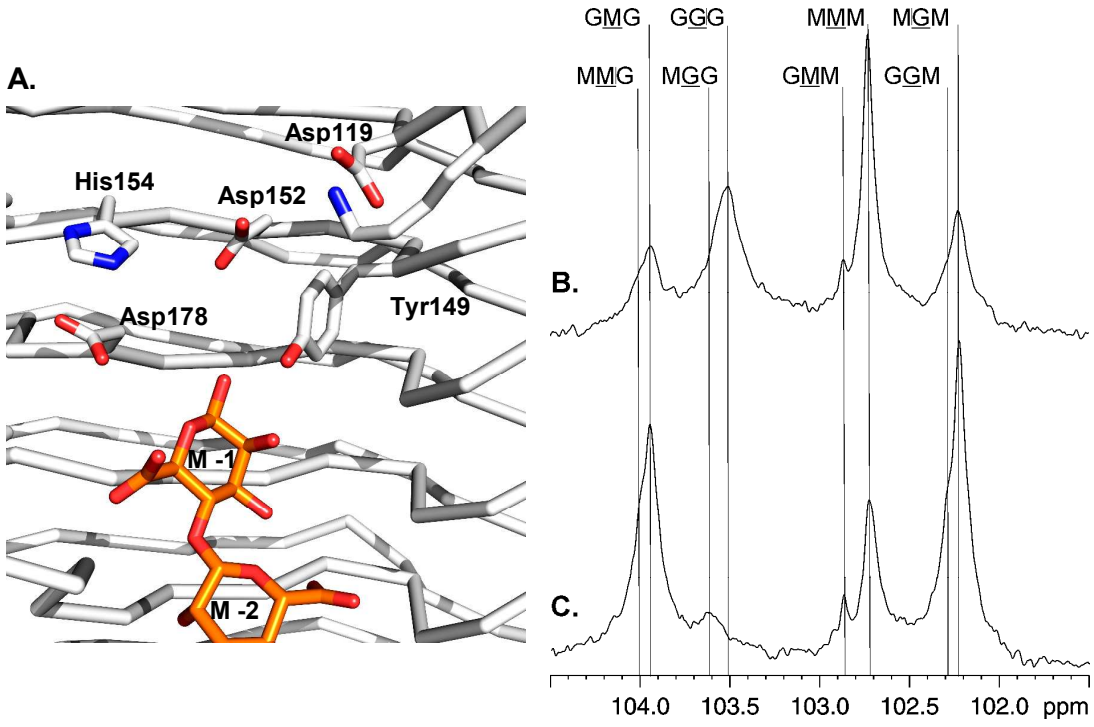

A
Relative diad and triad frequency
1,4
1,2
1
0,8
0,6
0,4
0,2
0
FG
FGGG
FMGM
AlgEM1
AlgEM1-R1R2R3
B
Relative diad and triad frequency
1,4
1,2
1
0,8
0,6
0,4
0,2
0
FG
FGGG
FMGM
AlgEM2
AlgEM2-R1R2R3

FIGURE 7


A.
Asp119
His154
Asp152
Asp178
Tyr149
M -1
M -2
B.
C.
GMG
GGG
MMM
MGM
MMG
MGG
GMM
GGM
104.0
103.5
103.0
102.5
102.0
ppm

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Mannuronan C-5 epimerases suited for tailoring of specific alginate structures obtained by high throughput screening of an epimerase mutant library

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