Carbohydrate Research 352 (2012) 44–50

Contents lists available at SciVerse ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Substrate specificity of the recombinant alginate lyase from the marine bacteria *Pseudomonas alginovora*

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ARTICLE INFO

Article history:
Received 2 December 2011
Received in revised form 7 February 2012
Accepted 10 February 2012
Available online 20 February 2012

Keywords:
Alginate lyase
Alginate
Oligosaccharide
NMR

ABSTRACT

The gene coding for an alginate lyase from the marine bacteria *Pseudomonas alginovora* X017 was cloned and heterologously expressed in *Escherichia coli* strains. The protein was produced in inclusion bodies and the active form was obtained by applying a refolding protocol based upon dilution. The biochemical characterization was performed on the active, refolded form of the alginate lyase. The substrate specificity was monitored by NMR. The degradation products were size-fractioned by size exclusion chromatography. The fractions were subsequently analyzed by ESI-MS to determine the molecular weight of the compounds. The structures of the different oligosaccharides were then elucidated by NMR. The enzyme was shown to be only acting on M–M diads. No enzymatic hydrolysis occurred between M–MG, G–MM or G–MG blocks proving that the sequence accounting for the generated oligomers by enzymatic hydrolysis is M–MM. The unsaturated oligosaccharides produced by the alginate lyase were ΔM, ΔMM, ΔMMM, and ΔMMMM indicating that the minimum structure recognized by the enzyme is the M6 oligosaccharide.

1. Introduction

Alginic acid (alginate) is a linear polysaccharide, composed of (1→4)-β-D-mannuronic acid (M) and (1→4)-α-L-guluronic acid (G) units arranged in homopolymeric regions (MM- or GG-blocks) and alternating heteropolymeric (MG-blocks).[1,2] Alginates are found in great abundance as part of the cell wall and intracellular material in brown seaweeds (Phaeophyceae).[3,4] They are also produced by the two heterotrophic marine bacteria *Azotobacter vinelandii* and *Pseudomonas aeruginosa* (*Pseudomonadaceae*).[5,6] In this respect it is noteworthy that the detailed phylogenetic analysis of the genes of the final steps of alginate synthesis pathway in the brown algae *Ectocarpus siliculosus* indicates that this polysaccharide has been acquired by a lateral gene transfer from an ancestral actinobacteria.[7]

The physical properties of alginates such as gel properties and viscosity are largely correlated to the monomer composition (M/G ratio), their distribution pattern and the size of the polymer.[8,9] The capability to form gel is due to their ability to bind to different divalent or multivalent cations, especially Ca^{2+}. A polymer with a lower M/G-ratio and containing a larger amount of GG-blocks gives a stronger but brittle gel, due to the ability of GG-blocks to form a chelate with calcium. The 'egg-box' model proposes an explanation to this gel formation.[10] Alginates with a larger amount of MM or MG-blocks do not discriminate between calcium and other metal ions and form elastic gels. Owing to their gelling ability, stabilizing properties, and high viscosity, alginates are widely used in the food and pharmaceutical industries.[11,12]

Alginate lyases catalyze the degradation of alginates breaking the (1→4)-O-linkage between monomers by a β-elimination mechanism to yield unsaturated 4-deoxy-L-erythro-hex-4-ene pyranosyluronate (Δ) at the non-reducing end of the resulting oligosaccharide.[13] Although the alginate lyases most currently described are poly (M) lyases, the enzymes display different substrate specificities. Alginate lyases are present in a large diversity of organisms, in majority of marine sources such as marine algae, mollusks, microorganisms, and marine bacteria.[12] The alginate lyases are generally classified according to their dominant cleaving action as poly (M) lyase [(1→4)-β-D-mannuronan lyase; EC 4.2.2.3] and poly (G) lyase [(1→4)-α-L-guluronan lyase; EC 4.2.2.11].[12] The majority of alginate lyases have an *endo*-cleaving activity[12] but some known lyases exhibit an *exo*-cleaving activity,[12] removing alginate monomers or dimers from one end of the alginate polymer. Based on the similarities of their primary structure, alginate lyases are further classified into polysaccharide lyase (PL) families PL5, PL6, PL7, PL14, PL15, PL17 and PL18 (http://www.cazy.org). Moreover, their three dimensional arrangements allow grouping them further into three structural classes, displaying either an α/α_6 helix-barrel fold[14] or a β-sandwich (jelly-roll) fold.[15] Interestingly, although the structural fold or primary sequences have no overall similarity, the spatial arrangement of the catalytic active site residues in all three structures is surprisingly similar. This suggests a convergent evolution event leading to several alginate lyase classes.[15]

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doi:10.1016/j.carres.2012.02.014

Since alginate lyases are sequence specific for alginates, they are of great interest in alginate sequencing to evaluate the fine structure of alginate polymers. In this context, an alginate lyase from the marine organism *Pseudomonas alginova* X017 has previously been identified,[16] cloned, and sequenced.[17] After a first characterization as being a guluronate specific enzyme[16] the activity has been corrected to be mannuronate specific,[17] leaving open the question whether *Pseudomonas alginovora* contains several alginate lyases with different substrate specificities. Chavagnat et al.[17] have also performed detailed sequence analyses combined with anti-body cross-reactions highlighting the very low level of sequence similarity between different alginate lyases and classifying the *P. alginovora* enzyme in a third, previously un-described family of alginate lyases. In the present work, we have performed a more detailed NMR study of how the refolded form of an alginate lyase from the marine bacteria *Pseudomonas alginovora* X017 (CIP 102941) degrades alginate polymers and we have analyzed the resulting alginate oligomers by NMR.

2. Results and discussion

2.1. Preparation of enzyme

2.1.1. Cloning and overexpression

The cloning and production of the alginate lyase from *P. alginovora* X017 were reported earlier by Chavagnat et al.[17] The problem of aggregation of the produced protein into inclusion bodies was noticed. To obtain larger quantities for biochemical characterization, the gene was newly submitted to a medium throughput cloning strategy. The gene of the alginate lyase was successfully cloned into the two vectors pFO4 and pGEX-4T-1. The overexpression was tested at several temperatures (12 °C, 20 °C, 30 °C) with the three strains *Escherichia coli* BL21(DE3), C41(DE3) and C43(DE3). In all conditions, the enzyme with the expected size (26.7 kDa), was found only in the insoluble fractions (confirmed by western blotting using an anti-His specific antibody; data not shown). The protein was present in a higher quantity for the strain BL21(DE3) and C41(DE3) with the pFO4 plasmid. The enzymatic assays performed with soluble fractions on the different substrates did not show any activity, but surprisingly, the insoluble fractions in presence of 6 M urea, revealed an alginate lyase activity when the protein was diluted into solutions of native conditions or in presence of a substrate, showing that the protein was able to refold easily.

2.1.2. Purification and refolding

Using the strain BL21(DE3) with pFO4 plasmid, the alginate lyase was highly expressed in 200 mL of ZYP5052 medium at 20 °C. The enzyme was purified from inclusion bodies on Ni(II) HyperCel Column under denaturing conditions. After this affinity chromatography, the pure protein was obtained. The second purification step on size exclusion chromatography (SEC) separated the refolded form from the precipitated protein (ratio 1/12). 5% of glycerol was added to the solution and the protein was concentrated to 10 mg/mL. Thus the overall yield can be estimated to be roughly 100 mg of total protein expression for a 0.5 L culture from which 10 mg of refolded and soluble protein could be recovered. However, as precipitation was observed, the protein sample in presence of urea (after affinity chromatography) was used for the production of oligosaccharides and degradation assays.

2.1.3. Biochemical parameters of alginate lyase

Ion dependency was shown by adding EDTA in the reaction mixture. Concentrations of 5, 10, 50 and 100 μM EDTA did not affect the activity of the enzyme significantly. However, a concentration of 200 μM EDTA inhibited 95% of the activity. Above 300 μM EDTA, no activity was detected. In the presence of substrate and 500 μM EDTA, activity could be recovered by the addition of 20 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $NiSO_4$ and $CoSO_4$, revealing the necessity of a divalent cation for the activity of the enzyme. The enzyme had a higher activity between pH 7 and 9, with a maximum at pH 9.

2.2. Substrate specificity

The substrate specificity was determined by carrying out the enzymatic hydrolysis directly in the NMR tube at 30 °C. The degradation of alginate by alginate lyase resulted in the production of M or G-reducing end residues and of unsaturated 4-deoxy-L-erythro-hex-4-enepyranosyluronate non-reducing end residue (Δ) due to β-elimination.[13] The structure of the reducing end is easily identified by the characteristic chemical shift of its anomeric proton signal.[18,19] Thus a lyase activity on G–M or G–G diads will lead to the appearance of a doublet at 4.88–4.90 ppm with $^3J_{HH}$ = 8.6 Hz corresponding to the β-anomeric proton of a G residue. A lyase activity on M–M or M–G diads will give a signal at 4.90–4.92 ppm corresponding to the β-anomeric proton of a M residue. In contrast, the uronic acid residue initially involved at the other end of the linkage cannot be identified from hydrolysis of the crude

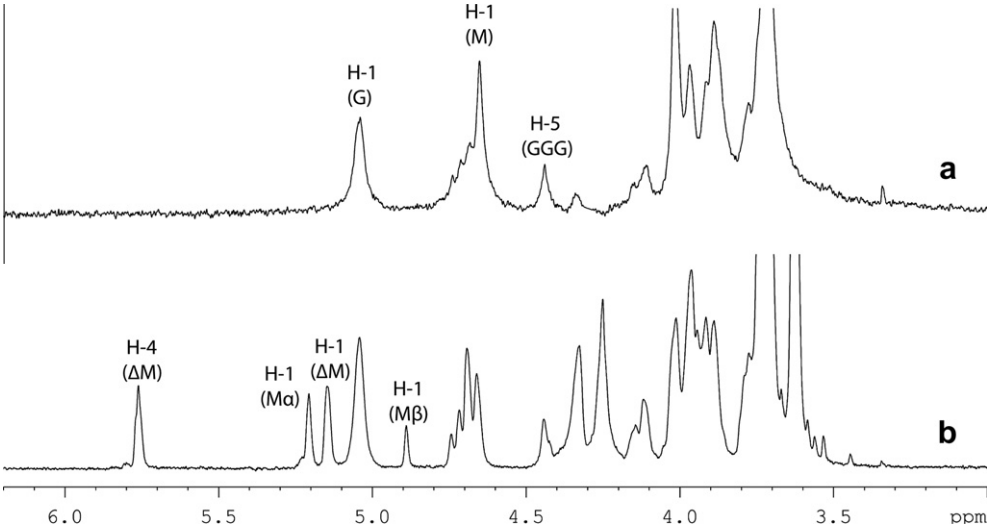

Figure 1. ^{1}H NMR (400 MHz) spectra at 70 °C of 0.5 mL solutions (5 mg/mL alginate in 100 mM deuterated phosphate buffer, pH 7.6, 100 mM NaCl and 5 mM $CaCl_2$) of sodium alginate with M/G ratio 0.9, (a) alone and (b) in the presence of alginate lyase from the marine bacteria *Pseudomonas alginovora*.

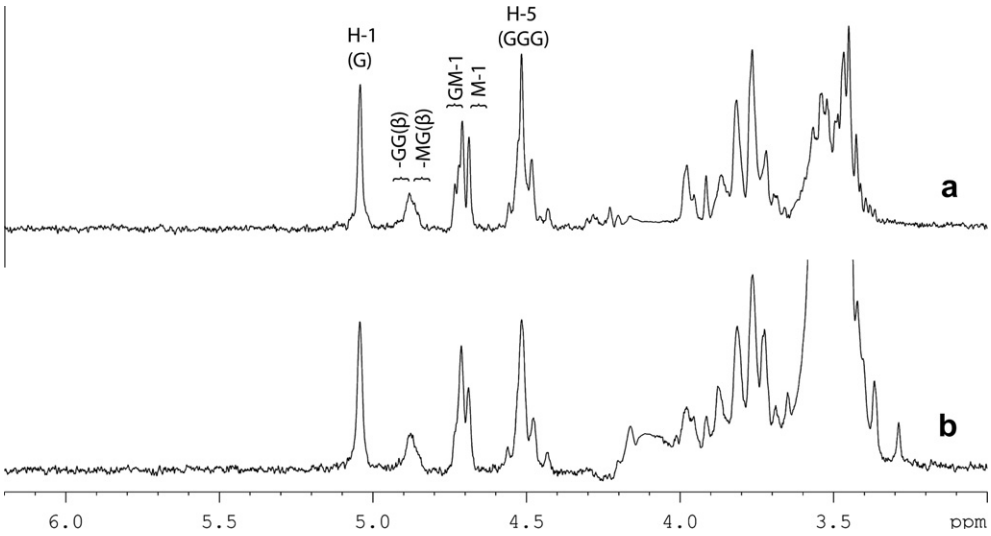

Figure 2. ^{1}H NMR (400 MHz) spectra at 70 °C of 0.5 mL solution (5 mg/mL poly-MG in 100 mM deuterated phosphate buffer, pH 7.6, 100 mM NaCl and 5 mM $CaCl_2$) (a) alone and (b) after addition of alginate lyase from the marine bacteria *Pseudomonas alginovora*.

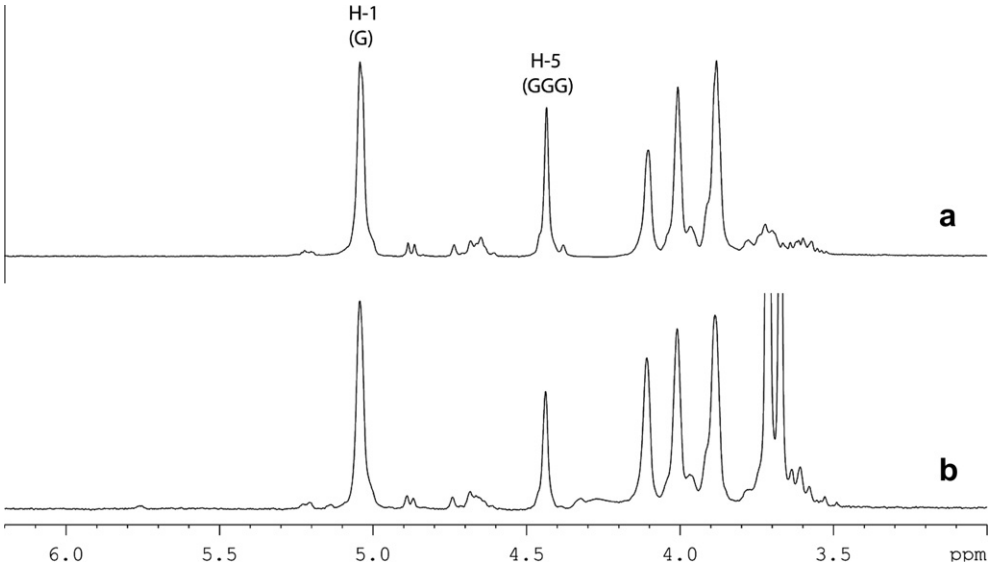

Figure 3. ^{1}H NMR (400 MHz) spectra at 70 °C of 0.5 mL solution (5 mg/mL poly-G in 100 mM deuterated phosphate buffer, pH 7.6, 100 mM NaCl and 5 mM $CaCl_2$) (a) alone and (b) after addition of alginate lyase from the marine bacteria *Pseudomonas alginovora*.

alginate since β-elimination on β-D-mannuronosyluronate or on α-L-gulupyranosyluronate will produce, in both cases, the nonreducing end 4-deoxy-L-erythro-hex-4-enepyranosyluronate.

At 30 °C, the temperature at which the progress of the enzymatic reaction was followed by NMR, the signals from NH_2 of urea overlap with the signal from H4(Δ). Thus, after the reaction was completed, the NMR spectra were also obtained at 70 °C since at this temperature the NH_2 of urea was not visible due to rapid rate of exchange with water.

Figure 1 shows that when the alginate was submitted to the action of the lyase, the characteristic H-1 signal at 4.91 ppm appeared in the NMR spectrum demonstrating that the enzyme cleaves the alginate substrate to give an M-reducing end. The shifts of the protons from the unsaturated non-reducing end Δ are dependant on the nature of the nearest residue and the sugar residue neighbor to Δ can therefore be identified from the chemical shift of the H-4 (Δ) signal. Thus, a signal at 5.85 ppm indicates that the neighbor is a G residue while a signal at 5.75 ppm indicates that the neighbor to the unsaturated non-reducing end is an M residue.[18] The signal at 5.75 ppm demonstrates that an M residue is neighboring the non-reducing Δ residue.

These data indicate the predominant formation of $\Delta M(X)_nM$ oligosaccharides as the end products, with X being a G or M. This is in agreement with the previously observed mannuronate-specific activity of both a recombinant form and the wildtype enzyme as described by Chavagnat et al.[17] To determine if the lyase performs β-elimination on both M–G and M–M diads or only on M–M diads, the cleavage of poly-G, poly-M and poly-MG[†] blocks was monitored by ^{1}H NMR spectroscopy. Figure 2 shows that the NMR spectrum of the poly-MG block is unchanged upon incubation with the lyase indicating that the enzyme is not active on this substrate. No cleavage occurred on the GG-blocks (Fig. 3). The

[†] Poly-G stands for alginate polymer with predominantly G–G diads; Poly-M stands for alginate polymer with predominantly M–M diads; Poly-MG stands for alginate polymer with predominantly M–G diads.

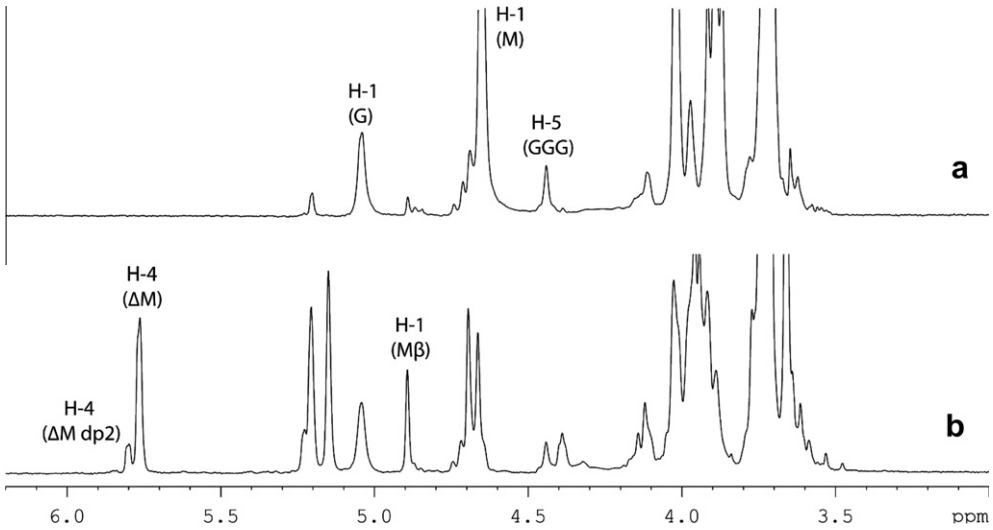

Figure 4. ^{1}H NMR (400 MHz) spectra at 70 °C of 0.5 mL solution (5 mg/mL poly-M in 100 mM deuterated phosphate buffer, pH 7.6, 100 mM NaCl and 5 mM $CaCl_2$) (a) alone and (b) after addition of alginate lyase from the marine bacteria *Pseudomonas alginovora*.

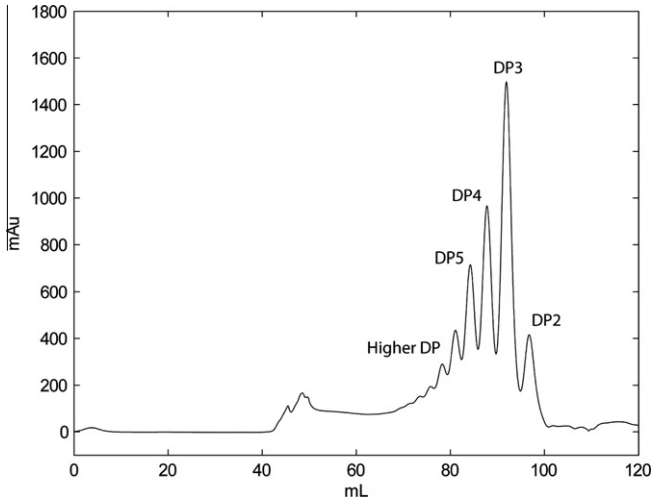

Figure 5. Size-exclusion chromatography of unsaturated oligosaccharides obtained after degradation by *Pseudomonas alginovora*. Eluted with 0.1 ammonium acetate at a flow rate of 0.8 ml/min with detection a 235 nm.

products observed in the NMR spectrum arise from the cleavage of MM diads present in the polymer, as demonstrated by the chemical shift of the H4Δ signal at 5.75 ppm. A clear cleavage on MM-block was observed (Fig. 4) confirming that the enzyme cleaves between two M residues.

2.3. Characterization of end products

The oligosaccharides produced by degradation of the alginate, with M/G ratio 0.9, by the lyase were separated according to size using size exclusion chromatography. The separation profile of the oligosaccharides showed four major peaks (Fig. 5) and the molar fractions of these different products were estimated from the SEC chromatogram (Table 1). The ESI-MS spectrogram showed *m/z* 351.0, 526.8, 703.0, 879.0 [M−H] corresponding to di-, tri-, tetra-, and pentasaccharide fragments, respectively. Due to the low amount of higher molecular weight oligosaccharides (Table 1), these fractions were not further analyzed by ESI-MS or NMR. Trisaccharides and tetrasaccharides were the most abundant oligomers

Table 1
Amount of different oligosaccharides produced by *Pseudomonas alginovora* and assessed by SEC.

Oligomer	Molar fraction %
Disaccharide M	12
Trisaccharide MM	49
Tetrasaccharide MMM	21
Pentasaccharide MMMM	12
Higher MW oligosaccharide	4

produced by the alginate lyase with a total content of 49% and 21%, respectively. Only 12% of di- and pentasaccharide oligomers were isolated. The uniform sized fractions were not separated further and the structure of the oligosaccharides, present in each fraction, was determined by NMR. The NMR spectra of the disaccharide showed that only ΔM was produced, as evidenced by the characteristic signal for ΔM from H-1 reducing end at 4.91 ppm, and the H-4 (Δ) signal at 5.80 ppm. Distinct signals are observed for the protons on the reducing and non-reducing residues due to the two anomeric forms α- and β- of the M sugar. Depending on the form of the reducing-end, either furanose or pyranose, a further contribution to the H-4 signal can be distinguished.[20] The fractions corresponding to the trisaccharide, tetrasaccharide, and pentasaccharide showed that only the ΔMM, ΔMMM, and ΔMMMM oligosaccharides were produced (Fig. 6).

2.4. Minimal recognition oligosaccharide

The high content of di-, tri-, tetra-, and pentasaccharide and a small amount of hexasaccharide indicates that the smallest substrate of the alginate lyase from *Pseudomonas alginovora* is the hexasaccharide MMMMMM. This was confirmed by the observation that the pentasaccharide is not cleaved by the alginate lyase.

2.5. Evolution

The sequence of the *P. alginovora* alginate lyase was used with the BLAST algorithm to find out new information about the enzyme class. No significant similarities were found between this alginate lyase and any known alginate lyase (or even a characterized enzyme) that would have helped to shed light on the evolutionary

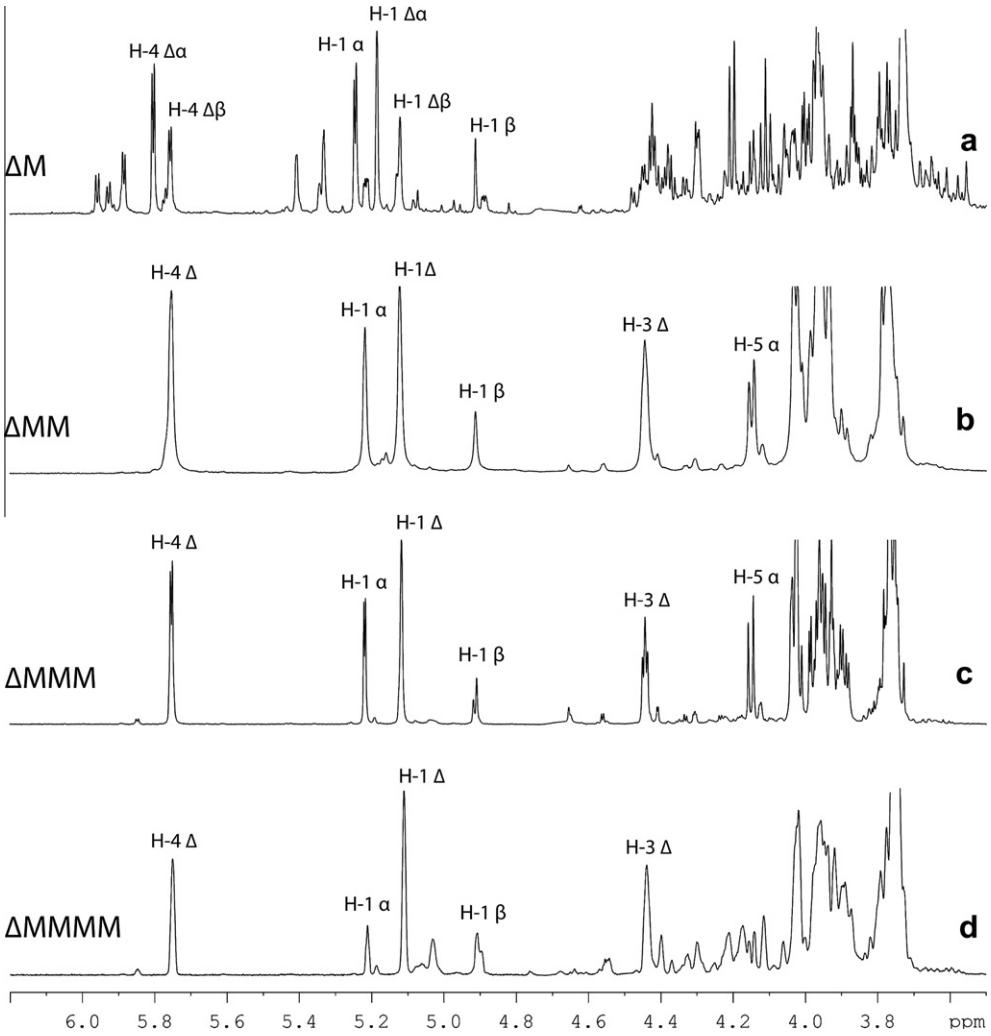

Figure 6. ^{1}H NMR (600 MHz) spectra of products (after enzymatic hydrolysis) in D_2O at 30 °C of (a) the disaccharide ΔM, (b) the trisaccharide ΔMM, (c) the tetrasaccharide ΔMMM and (d) the pentasaccharide ΔMMMM. The most relevant signals are pointed out as following: the (Δ) refers to the unsaturated non-reducing end.

aspects of the enzyme mechanism. The same sequence was also submitted to the server Phyre.[21] A faint similarity (<12% identity and an *E*-value of 2.6) can be detected to an alginate lyase from *Alteromonas* sp. strain no. 272 (pdb idcode 1J1T) that is not classified in any PL family, but has the β-sandwich/jelly roll fold, similar to family PL7 enzymes. The sequence similarity however is too low and *P. alginovora* alginate lyase could therefore still not be assigned to any PL family. Consequently, even if this alginate lyase has an *endo*-cleaving activity with preferences for poly(M) substrate, as most of the alginate lyases studied so far, it appears that it has evolved through a different evolutionary pathway, leading to a alginate lyase activity by convergent evolution.

3. Experimental

3.1. Material

Sodium alginate samples with M/G ratio of 0.9 and 1.5 were provided by Danisco AS, Denmark. The alginate with M/G ratio of 0.9 was used to study the mode of action of the alginate lyase and to obtain the G-blocks by action of the enzyme. The alginate with M/G ratio of 1.5 was used to obtain M-blocks, G-blocks, and heteropolymeric blocks by acid hydrolysis according to Haug et al.[22] The degree of polymerization (DP) of the oligooalginates prepared by this method is dependent on the alginate structure.

The pentasaccharide of homopolymeric mannuronic acid was purchased from Shanghai Biochemical Co., Ltd, China.

3.2. Preparation of enzyme

3.2.1. Cloning

The alginate lyase (Mw 25.87 kDa) was included in a medium throughput cloning strategy as described by Groisillier et al.[23] The gene of alginate lyase (702 bp) from *Pseudomonas alginovora* X017 was amplified by PCR and cloned in two expression vectors, pFO4 for expression of recombinant proteins with N-terminal His-tag and pGEX-4T1 for N-terminal GST-tag. *Bam*HI and *Eco*RI were used as restrictions sites. The *Escherichia coli* strains BL21(DE3), C41(DE3) and C43(DE3) were transformed with the two plasmids.

3.2.2. Overexpression

Overexpression assays were prepared by growing strains until saturation in 2 mL of auto-induction ZYP5052 medium at 20 °C.[24] The cells were pelleted then lysed with the lyse buffer BugBuster (Novagen). After centrifugation, the soluble fraction was separated and the pellet was resuspended in 6 M urea ('insoluble fraction').

Soluble and insoluble fractions were analyzed for presence of the target protein on SDS–PAGE and tested for activity. The described conditions did not reveal any expression for the GST-tagged construction and only the insoluble fraction of the His-tagged construction showed significant over expression of the alginate lyase. A scale up production of the His-tagged construction was therefore performed in 200 mL of ZYP5052 medium at 20 °C.

3.2.3. Purification and refolding

The cells were pelleted and resuspended in 10 mL buffer (Tris 50 mM, pH 7.5, NaCl 100 mM). Lyse was performed with a One-shot cell Disruptor (Constant system). The supernatant was discarded and the pellet was resuspended in 5 mL of 8 M guanidine using a potter. After centrifugation (30 min, 40,000*g*), the supernatant was loaded on Ni HyperCel (PALL, 10 mL), pre-equilibrated in 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 10 mM imidazole, 6 M urea (buffer A). The column was washed with buffer A and the protein eluted by a gradient of 60 mL (1 mL/min) to 100% of 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 300 mM imidazole, 6 M urea (buffer B). The purity of fractions was analyzed on SDS–PAGE. The SDS–PAGE of the purified and refolded enzyme is shown in Figure 7. The refolding of the alginate lyase was performed by size exclusion chromatography injecting 5 mL of sample solution on a Superdex 75 HL (GE Healthcare) at a flow rate of 1 mL/min. The protein eluted from the column with an elution time compatible with the theoretical molecular weight of 26 kDa.

3.2.4. Enzyme activity

The enzymatic degradation of alginate was followed by measuring the release of the unsaturated nonreducing end (Δ) with C=C double bounds at 235 nm with a Shimadzu UV-2401PC UV–VIS spectrophotometer. The substrate, alginate (alginic acid, sodium salt 180947, Sigma), was dissolved at a concentration of 0.25% in 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$. The assay was performed using 1 mL of substrate solution and 10 μL of protein sample (concentration 50 μg/mL) at 30 °C for 600 s. The ion dependency of the enzyme was tested by adding different concentrations of EDTA to the reaction. Different solutions of ions (final concentration 10 mM) were also tested. The optimum pH was determined using sodium citrate (0.05 M) for pH 4–5, sodium phosphate (0.1 M) for pH 6–8, and glycine (0.1 M) for pH 9–10.

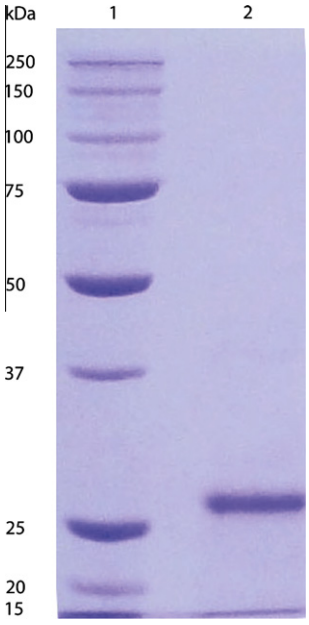

Figure 7. SDS–PAGE of the refolded alginate lyase from *Pseudomonas alginovora*. Lane 1, 3 size standards. Lane 2, purified alginate lyase having a theoretical Mw 25,87 kDa.

3.3. Determination of enzyme specificity

Substrate specificity of enzyme was performed by degradation of sodium alginate with *P. alginovora* alginate lyase[17] directly in the NMR tube in presence of crude alginate, G-, M- and alternating MG-blocks. The substrates at a concentration of 5 mg/mL were dissolved in a 100 mM deuterated phosphate buffer, pH 7.6, with 100 mM NaCl, and 5 mM $CaCl_2$. The optimum temperature for the enzymatic reaction was 30 °C.

3.4. Characterizations of end products

3.4.1. Preparation of end products

The enzymatic degradation was performed in the presence of 10 mL of alginate samples at 5 mg/mL and 2.5 mL of alginate lyase (0.14 mg/mL) at 30 °C for 18 h. The sample was then centrifuged, the supernatant lyophilized, and dissolved in 0.1 M ammonium acetate.

3.4.2. Size exclusion chromatography

The oligosaccharides were fractionated according to their size by preparative gel filtration chromatography on ÄKTA system with a HiLoad 16/60 Superdex 30 column from Amersham Biosciences (Uppsala, Sweden). The elution was done with 0.1 M ammonium acetate at a flow rate of 0.8 mL/min at room temperature. The relative amounts of the different end products were determined as the peak area. The alginates were detected at 235 nm. The fractions belonging to the same peak were pooled, lyophilized, and purified further by repeating SEC on the Superdex 30 column. The pure fractions were lyophilized for molecular weight analysis.

3.4.3. Electrospray ionization mass spectrometry

Mass-spectra of the fraction products from the SEC separation were analyzed by ESI-MS using a Bruker Esquire-LC mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). The samples were dissolved in 1:1 MeOH–H_2O and were directly injected into the electrospray with a flow of 2 μL/min. The mass range that was scanned was from 100 to 1100 atomic mass units (amu). All acquisitions were recorded in both negative and positive mode and the data treated by Bruker Daltonics esquire LC 4.5, data analysis version 3.0.

3.4.4. NMR spectroscopy

The NMR experiments were performed on a Bruker DRX-400 MHz spectrometer using a 5.0 mm $^{1}H/^{13}C/^{15}N/^{31}P$ QNP probe or on a Bruker Avance III 600 MHz spectrometer using a 5.0 mm $^{1}H/^{13}C/^{15}N/^{31}P$ inverse detection QXI probe, both equipped with z-gradients and controlled by Topspin 1.3 and 2.1 software, respectively. The ^{1}H NMR experiments were recorded at 30 and 70 °C using a spectral width of 3000 Hz, an acquisition time of 5.62 s, a pulse width of 8.5 μs, a relaxation time of 4 s, and 128 scans. The residual HOD signal was suppressed using the NOESY presaturation pulse sequence in which saturation was applied during the relaxation delay of 4 s and the mixing time of 50 ms.

3.4.4.1. Monitoring of degradation reaction. Prior to an enzymatic reaction, a reference spectrum of the substrate was acquired. The sample was then removed from the probe and the enzyme was added at the proper concentration and the tube was then put back into the probe. The degradation reaction was monitored as a function of time. To monitor the progress of degradation of the sample, a series of ^{1}H NMR spectra were recorded at 30 °C using the multizg or multi-zgvd programs from the Bruker library.

3.4.4.2. Identification of end products. The oligosaccharide signals were assigned from one and two dimensional TOCSY NMR experiments using a mixing time of 90 ms (data not shown). The NMR spectra were referenced at 30 °C using acetone (δ_H 2.225) as internal reference and the chemical shift of the most relevant signals are reported in Supplementary data.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement No. 222628 (www.polymode.eu). We thank G. Michel and A. Groisillier for the setup and testing of the heterologous protein expression strategy used in this report.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2012.02.014.

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