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BIOENERGY AND BIOFUELS

Depolymerization of alginate into a monomeric sugar acid using Alg17C, an exo-oligoalginate lyase cloned from *Saccharophagus degradans* 2-40

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Abstract Macroalgae are considered to be promising biomass for fuels and chemicals production. To utilize brown macroalgae as biomass, the degradation of alginate, which is the main carbohydrate of brown macroalgae, into monomeric units is a critical prerequisite step. *Saccharophagus degradans* 2-40 is capable of degrading more than ten different polysaccharides including alginate, and its genome sequence demonstrated that this bacterium contains several putative alginate lyase genes including *alg17C*. The gene for Alg17C, which is classified into the PL-17 family, was cloned and overexpressed in *Escherichia coli*. The recombinant Alg17C was found to preferentially act on oligoalginates with degrees of polymerization higher than 2 to produce the alginate monomer, 4-deoxy-L-erythro-5-hexo seulose uronic acid. The optimal pH and temperature for Alg17C were found to be 6 and 40 °C, respectively. The K_M and V_{max} of Alg17C were 35.2 mg/ml and 41.7 U/mg, respectively. Based on the results of this study, Alg17C could be used as the key enzyme to produce alginate monomers in the process of utilizing alginate for biofuels and chemicals production.

Keywords Oligoalginate lyase · Alg17C · Polysaccharide lyase-17 · Exo-type alginate lyase · *Saccharophagus degradans* 2-40

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Introduction

Recently, the use of marine algal biomass for biofuels production has gained increased attention as a potential replacement for terrestrial biomass (Singh et al. 2011). In particular, since macroalgae including red, brown, and green algae are primarily composed of polysaccharides, the enzymatic and fermentative conversion of these polysaccharide into biofuels may be possible (John et al. 2011). Compared to terrestrial biomass, the carbohydrate composition of marine biomass is unique. For example, the carbohydrates of brown macroalgae are mainly composed of alginate, laminaran, fucoidan, and cellulose (Shekharam et al. 1987; Jensen 1993; Jiao et al. 2011). To utilize macroalgae as the biomass for fuel production, the right depolymerizing enzymes for algal biomass and metabolically engineered microorganisms that are capable of converting the algal polysaccharide-derived sugars into fuels would be necessary. In regard to brown macroalgae, there have been several attempts to produce bioethanol using brown algae and their major polysaccharide component, alginate (Aizawa et al. 2007; Adams et al. 2009).

Alginate is a linear polysaccharide composed of α-L-guluronic acid (G) and its C5 epimer, β-D-mannuronic acid (M), as monomeric units (Garron and Cygler 2010). These units are linked in three different configurations: blocks of consecutive Gs; consecutive Ms; and heteropolymeric random Gs and Ms (Gacesa 1988). Alginate is depolymerized by an alginate lyase through a β-elimination reaction to form a double bond between C4 and C5 at the non-

reducing end (Wong et al. 2000; Garron and Cygler 2010). The unsaturated unit with the double bond is then nonenzymatically converted into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) (Miyake et al. 2003; Ochiai et al. 2010).

Alginate lyase was reported to be produced by many bacteria including *Sphingomonas* sp. A1 (Miyake et al. 2003), *Pseudoalteromonas elyakovii* IAM (Sawabe et al. 2001), *Agrobacterium tumefaciens* (Ochiai et al. 2010), and *Pseudomonas* sp. OS-ALG9 (Maki et al. 1993). In the Carbohydrate-Active enZYmes (CAZy) database, alginate lyases belong to the polysaccharide lyase (PL) families (Cantarel et al. 2009). Among a total of 21 PL families, the PL families containing alginate lyases are only PL-5, 6, 7, 14, 15, 17, and 18 (Garron and Cygler 2010). Most alginate lyases that were experimentally characterized were endo-type enzymes producing oligoalginates (Matsubara et al. 1998; Wong et al. 2000; Sawabe et al. 2001; Kobayashi et al. 2009). Meanwhile, different types of alginate lyases that were reported to form monomers from alginate included A1-IV from *Sphingomonas* sp. strain A1 (Miyake et al. 2003) and Atu3025 from *A. tumefaciens* (Ochiai et al. 2010). These monomeric sugar-forming enzymes have been classified into PL-15 (Miyake et al. 2003; Ochiai et al. 2010) whereas the endo-type alginate lyases were classified into PL-5, 6, 7, 14, 17, and 18 (Garron and Cygler 2010).

In addition to these microorganisms, *Saccharophagus degradans* 2-40 has also been shown to encode many genes for alginate lyases (Weiner et al. 2008). In fact, several depolymerizing enzymes of *S. degradans* 2-40, including cellulases, xylanases, and agarases, have been evaluated and characterized (Taylor et al. 2006; Ko et al. 2009; Kim et al. 2010; Ha et al. 2011; Hutcheson et al. 2011). However, no study has yet examined alginate degradation by *S. degradans* 2-40. In this study, to develop an enzyme that can be used in producing a monomeric sugar acid from alginate, we cloned, expressed and characterized Alg17C from *S. degradans* 2-40. By the concerted action of an endo-type alginate lyase and an exo-type alginate lyase (e.g., Alg17C), it is possible to efficiently obtain a monomeric sugar acid from alginate. The monomeric sugar acid can be used to elucidate the metabolic pathway of alginate in *S. degradans*, and may be further used to produce biofuels by using a recombinant microorganism that is metabolically engineered to ferment the sugar acid.

Materials and methods

Strains and plasmids

S. degradans 2-40 (ATCC 43961) was cultivated in minimal broth containing 23 g/l of Instant Ocean sea salt (Aquarium Systems, Mentor, OH, USA), 50 mM Tris–HCl, 2 g/l glucose, 1 g/l yeast extract, and 0.5 g/l ammonium chloride at 30 °C for 12 h. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used as the cloning host and the protein expression host, respectively.

Plasmid construction and expression of *alg17C*

The genomic DNA of *S. degradans* 2-40 was obtained using a commercial DNA isolation kit (Qiagen, Valencia, CA, USA). The target gene, *alg17C* (GenBank Accession No. ABD82539), was amplified using pfu DNA polymerase (Fermentas, Glen Burnie, MD, USA). Primers used were 5′-GCGGGATCCCAAGTTTCTGGCAATGGTCATC-3′ (31-mer) and 5′-GCGGCGGCCGCTTTACGTCGAACCACCACGC-3′ (31-mer), and they possessed *Bam*HI and *Not*I sites at their 5′ regions. To facilitate protein expression in the heterologous system, the signal sequence of *alg17C* was removed. The PCR products and pET21a vector were double digested with *Bam*HI and *Not*I, and the resulting DNA fragments were ligated. The plasmid harboring *alg17C* was transformed into *E. coli* BL21(DE3). The cells were grown at 37 °C in Luria–Bertani (LB) broth (BD, Sparks, MD, USA) containing 50 μg/ml of ampicillin until the absorbance at 600 nm reached 0.5. Induction was performed at 16 °C using isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) for 18 h. The cells were harvested by centrifugation, and the cell pellets were stored at −20 °C.

Purification of recombinant Alg17C

The expressed protein was obtained by disrupting cells using a sonicator (Branson, Danbury, CT, USA). The soluble fraction of protein was obtained by centrifugation at 16,000×*g* for 1 h at 4 °C. The target protein was purified from the soluble fraction using a His-trap column (GE Healthcare, Piscataway, NJ, USA). The purified protein was further concentrated using an Amicon tube (Millipore, Billerica, MA, USA). The concentration of the protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The molecular mass of Alg17C was determined by SDS-PAGE on an 8% (w/v) resolving gel.

Enzyme assay of Alg17C

To measure the enzyme activity of Alg17C, the reaction was conducted with 50.6 nmol of Alg 17 C in 100 μl of 20 mM Tris–HCl buffer (pH 6.0) containing 2% (w/v) sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) at 40 °C for 30 min. The reaction was quenched by immersing the reaction mixture in boiling water for 5 min. Relative enzyme activity was measured by using the dinitrosalicylic acid (DNS) method (Miller 1959) with D-glucose as the standard. One unit was defined as the amount of enzyme required to

release 1 μmol of the reducing sugars (based on glucose) per minute at the above conditions.

Enzyme characterization of Alg17C

The changes in relative activities at different pHs were measured using 2% of sodium alginate in 50 mM citrate buffer (pH 4.0–5.0) and 20 mM Tris–HCl (pH 6.0–8.0) at 40 °C for 30 min. The effect of temperature was also examined at 20–70 °C using 2% sodium alginate in 20 mM Tris–HCl (pH 6.0) for 30 min. Kinetic parameters of Alg17C, such as the maximal reaction velocity (V_{max}) and the Michaelis constant (K_M) were determined using a Lineweaver–Burk plot. In these experiments, the substrate concentrations were varied from 6.8 to 47.9 mg/ml in Tris–HCl (pH 6.0) at 40 °C for 30 min.

Mode of action of Alg17C

To determine the properties of Alg17C, four different types of substrates, such as alginate, oligoalginates released by Alg7D, polymanuronic acid (poly M), and polyguluronic acid (poly G), were used. Sodium mannuronate, the M-rich fraction as poly M and sodium guluronate, the G-rich fraction as poly G, were prepared from sodium alginate after partial acid hydrolysis, and poly M and poly G were fractionated at different pHs (Haug et al. 1966; Gacesa and Wusteman 1990; Sim et al. in press).

TLC and HPLC analyses of reaction products

The changes in the profile after digestion with Alg17C were examined using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) at different reaction times (0, 5, and 30 min). Reaction products were developed with the solvent mixture system of *n*-butanol/acetic acid/water (3:2:2 by volume) and visualized using 10% (v/v) sulfuric acid solution in ethanol followed by heating the TLC plate at 130 °C for 5 min.

The reaction products were also analyzed using an Agilent 1100 HPLC (Agilent, Waldbronn, Germany) with a cation exclusion column (TSK-Gel SCH (H^+), Tosoh Bioscience, Japan) at 235 nm, in which the column oven temperature was 35 °C, and the mobile phase was 0.1 % (w/v) phosphoric acid at 0.8 ml/min.

LC-MS analysis of reaction products

The molecular mass of the products obtained from the reaction of alginate by Alg17C was determined using an ultra performance liquid chromatography/quadruple time of flight tandem mass spectrometry (UPLC/Q-TOF tandem MS) using ACQUITY UPLCTM (Waters, Milford, MA, USA) equipped with an ACQUITY BEH C18 column (100×2.1 mm, 1.7 μm; Waters). The two mobile phases included Solution A, which consisted of 0.1 % (w/v) formic acid in distilled water, and Solution B, which consisted of 0.1% (w/v) formic acid in acetonitrile. The flow rate was set at 0.3 ml/min with a linear gradient, and the mass spectrum was obtained using a Q-TOF Micro mass detector (Waters).

Results

Amino acid sequence analysis of Alg17C

The amino acid sequence of the protein encoded by *alg17C* was deduced from the nucleotide sequence available in CAZy database (Cantarel et al. 2009). The *alg17C* gene encodes a protein that contains 733 amino acids with a calculated molecular mass of 81.6 kDa protein. This enzyme belongs to the PL-17 family and has a high similarity with other bacterial PL-17 enzymes such as 744 amino acids (82.6 kDa) of Smal_2067 from *Strenotrophomonas maltophilia* R551-3, 742 amino acids (82.4 kDa) of Smlt2602 from *Stenotrophomonas maltophilia* K279a, 738 amino acids (82.4 kDa) of AlylL from *Pseudoalteomonas haloplanktis* TAC125, and 742 amino acids (81.3 kDa) of Avin_46500 from *Azobacter vinelandii* (strain DJ/ATCC BAA-1303). The amino acid sequence of Alg17C of *S. degradans* 2-40 was found to be highly homologous to the PL-17 enzymes described above with more than 50% DNA homology and 40% protein identity.

As shown in Fig. S1, Alg17C was found to be only 20% similar to PL-15 oligoalginate lyases such as A1-IV and Atu3025 from *Spingomonas sp.* strain A1 and *Agrobacterium tumefaciens*, respectively (Hashimoto et al. 2000; Ochiai et al. 2010). These two oligoalginate lyase shows more than 50% protein identity and DNA homology each other. In addition, Alg17C did not possess the amino acid residues that are conserved in the interacting site for

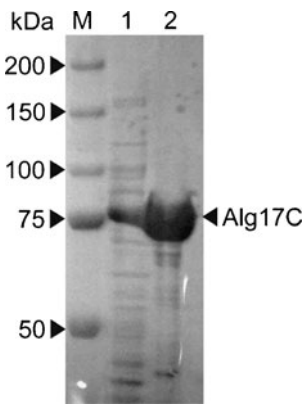

Fig. 1 SDS–PAGE of the recombinant Alg17C. Lanes: *M* protein markers, *1* crude enzyme produced by the recombinant *E. coli* harboring *alg17C*, *2* purified Alg17C by His-tag affinity chromatography

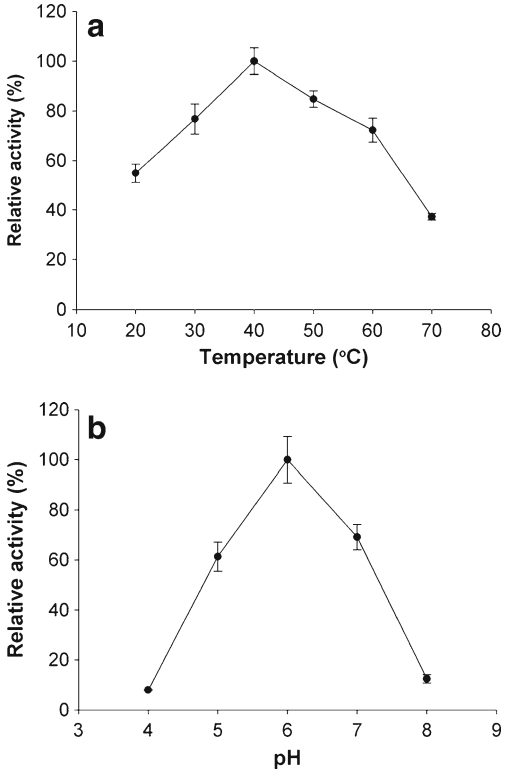

Fig. 2 Relative activity of Alg17C at different **a** temperatures and **b** pHs. In **a**, the reaction mixture was incubated in 20 mM Tris–HCl buffer at pH 6.0 for 30 min at temperatures ranging from 20 °C to 70 °C. In **b**, the enzyme reaction was performed at 40 °C for 30 min at pH 4.0–5.0 using 50 mM citrate buffer and at pH 6.0–8.0 using 20 mM Tris–HCl buffer

alginate-derived substrates as was found in Atu3025 and A1-IV (Arg^{199}, His^{311}, Tyr^{365}, Trp^{467}, and His^{531}) (Ochiai et al. 2010).

Overexpression and purification of recombinant Alg17C

To facilitate the expression of the recombinant Alg17C in a heterologous system, a signal peptide gene from *alg17C* was removed. When the recombinant protein was induced using 0.1 mM IPTG, a substantial amount of inclusion bodies were formed at 37 °C. However, when the induction temperature was lowered to 16 °C, the production of inclusion bodies was reduced. The molecular mass of the expressed recombinant Alg17C was estimated to be approximately 79.1 kDa by SDS-PAGE (Fig. 1), which was in good agreement with its theoretical molecular mass.

Optimal temperature and pH of Alg17C

To investigate the optimal temperature and pH of the recombinant Alg17C, its relative enzyme activity toward alginate was measured at different temperatures and pHs. When the enzyme reaction was performed at temperatures ranging from 20 °C to 70 °C, as shown in Fig. 2a, the enzyme activity was lower at temperatures higher or lower than 40 °C, and the highest relative activity was detected at 40 °C. In regard to the effect of pH on Alg17C activity (Fig. 2b), the enzyme showed the highest activity at pH 6.0, which sharply decreased when the pH was higher or lower than pH 6.0.

Kinetic parameters of Alg17C

To determine the K_M and V_{max} of Alg17C, enzyme reactions with alginate by Alg17C were carried out at pH 6.0 and 40 °C. By using a Lineweaver–Burk plot at different substrate concentrations, the values of K_M and V_{max} were determined to be 35.2 mg/ml and 41.7 U/mg, respectively (Fig. S2).

Mode of action of Alg17C

The mode of action of Alg17C was examined using the following three different types of substrates: sodium alginate, oilgoalginates produced from alginate by an endo-type alginate lyase (Alg7D), poly M, and poly G (Sim et al. in press). When alginate was used as a substrate of Alg17C, a single spot was detected by the TLC after 5 and 30 min of reaction times (Fig. 3a). In the HPLC analysis, as reaction time

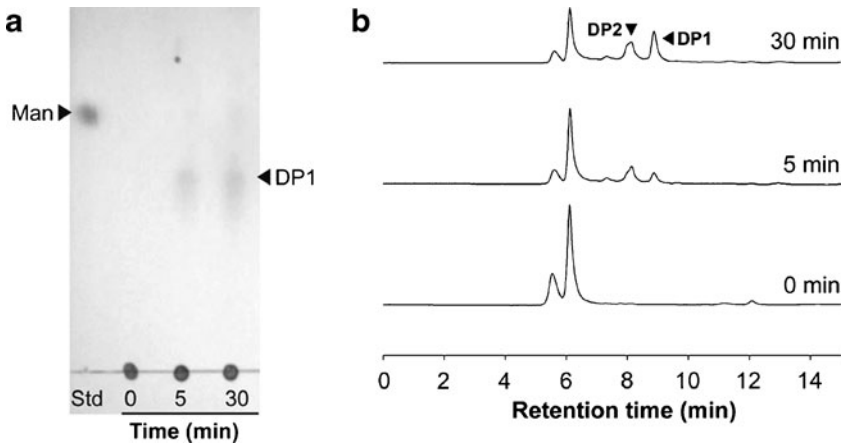

Fig. 3 **a** TLC and **b** HPLC analyses of reaction products from alginate as the substrate. The reactions were performed by Alg17C at 40 °C in 20 mM Tris–HCl (pH 6.0). *Man* mannose as the monomeric sugar standard

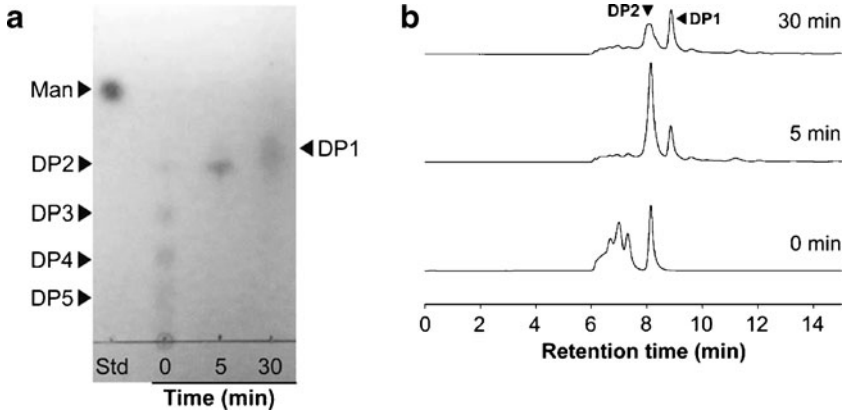

Fig. 4 **a** TLC and **b** HPLC analyses of reaction products from oligoalginates with DP2, 3, 4, and 5. The reactions were performed by Alg17C at 40 °C in 20 mM Tris–HCl (pH 6.0). *Man* mannose as the monomeric sugar standard

increased from 5 to 30 min, the abundance of the monomer (degree of polymerization 1 [DP1]) significantly increased after 30 min compared to that after 5 min (Fig. 3b).

To identify the substrate specificity of Alg17C depending on the DP of alginate, oligoalginates with DP2 to DP5, which were produced from alginate by Alg7D, were incubated with Alg17C for enzymatic reaction. As shown in Fig. 4a and b, four spots and four peaks were detected by the TLC and HPLC analyses at time 0, respectively. After 5 min of reaction, the spots for DP3, 4, and 5 disappeared, and the spot of DP2 became stronger. After 30 min, the spot of DP2 became lighter but that of DP1 appeared (Fig. 4a). These results were confirmed by the HPLC analysis that the reaction with oligoalginates by Alg17C progressed with converting DP3-5 to DP2 after 5 min and then to DP1 after 30 min (Fig. 4b). When poly M or poly G was incubated as a substrate of Alg17C (Fig. S3), single spots were detected in the TLC analyses of the reaction products of both poly M and poly G with Alg17C. Based on the TLC and HPLC analyses, Alg17C was found to be capable of forming a monomeric sugar acid from alginate, oligoalginates, poly M, or poly G.

Identification of reaction products by LC-MS

The reaction products of alginate by Alg17C were analyzed by LC-MS (Fig. 5). The major peak detected at 175 *m/z* $[M-H]^-$ was shown to correspond to the monomeric sugar acid from the reaction by Alg17C. The mass of the reaction product was also as same as the mass of DEH, which is the monomeric end product formed by depolymerization of alginate (Takase et al. 2010).

Discussion

Although marine biomass is thought to be the most abundant and sustainable resource for fermentative biofuel production (Jensen 1993; John et al. 2011; Singh et al. 2011), fermentable sugar availability or yield from marine biomass, such as macroalgae, remains a challenge due to the unique carbohydrate composition of macroalgae (Jensen 1993; Takase et al. 2010; Yun et al. 2011). The microbial fuel synthesis using macroalgal polysaccharides requires the degradation of polymers into oligomeric or monomeric sugars and the capability of metabolizing monomeric sugars. Alginate is the main component of brown macroalgae, and it is known to be metabolized through two different mechanisms: (1) a polymeric form of alginate is uptaken by bacteria and intracellularly degraded into smaller unit (Hashimoto et al. 2004); or (2) a polymeric form of alginate is degraded into oligomeric or monomeric unit by extracellular enzymes and oligomers or monomers are then transported inside bacterial cells (Preiss and Ashwell 1962). Alginate-derived monomeric sugar acids are known to be metabolized by the modified Entner–Doudoroff (ED) pathway (Preiss and Ashwell 1962), and many bacteria possess the ED pathway or modified ED pathways.

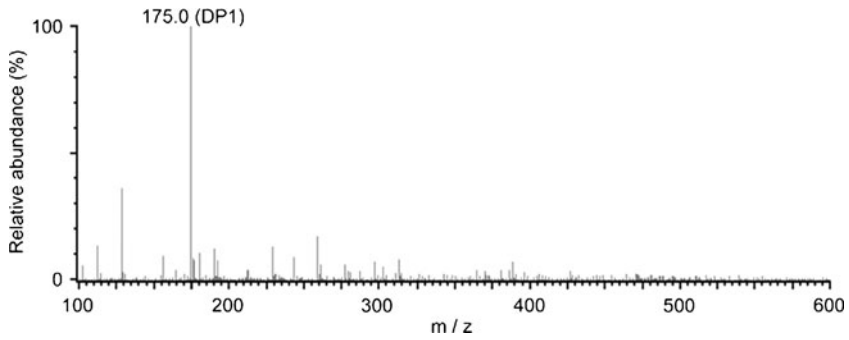

Fig. 5 LC/Q-TOF tandem mass chromatogram of the reaction product from the reaction with alginate by Alg17C. The reaction was performed in 20 mM Tris–HCI buffer (pH 6.0) at 40 °C for 30 min

Based on the CAZy database (Cantarel et al. 2009), three oligoalginate lyases were reported to form monomeric units. They include A1-IV from *Sphingomonas* sp. strain A1 (Miyake et al. 2003) and Atu3025 from *A. tumefaciens* (Ochiai et al. 2010), which are both classified into PL-15. Although *S. degradans* is known to possess a wide variety of carbohydrate degradation enzymes, it does not possess family PL-15 oligoalginate lyases but rather possesses a PL-17 oligoalginate lyase, Alg17C (Cantarel et al. 2009). The sequence analysis revealed that both DNA and amino acid sequences of Alg17C were highly homologous to those of other PL-17 enzymes but not to PL-15 enzymes (Fig. S1) (Miyake et al. 2003; Ochiai et al. 2010). In this study, we demonstrated that Alg17C possessed a similar exolytic reaction mode and substrate affinity as PL-15 alginate lyases. However, the catalytic residues for alginate that are present in the PL-15 enzymes (Arg^{199}, His^{311}, Tyr^{365}, Trp^{467} and His^{531}) (Ochiai et al. 2010) were not found in Alg17C. Due to the difference in the catalytic residues of Alg17C and possibly differences in their three-dimensional structures, Alg17C could function through a mechanism that is completely different from that of PL-15 enzymes.

All the reactions using alginate, oligoalginates (DP2 to DP5), poly M, and poly G with Alg17C resulted in the formation of a monomeric sugar acid (Figs. 3, 4, and S3). Meanwhile, Alg17C seemed to have different affinities depending on the DP of oligoalginates. As shown in Figs. 3 and 4, the alginate dimer (DP2) was formed and decreased as the reactions progressed when sodium alginate and oligoalginates containing DP2, 3, 4, and 5 were used as the substrates. The remaining alginate dimer and no other alginate oligomers after reaction in Figs. 3 and 4 implies that the alginate dimer is the least favorable substrate among the alginate oligomers with DP2 to DP5. This lower affinity of ALg17C to the alginate dimer is similar to that observed for a PL-15 oligoalginate lyase, A1-IV (Miyake et al. 2003), which has a higher affinity towards the alginate trimer than dimer (Miyake et al. 2003). Therefore, it is likely that the reaction mode of Alg17C is similar to that of the previously reported exolytic alginate lyases from PL-15 (Miyake et al. 2003) despite the differences in amino acid residues at the catalytic sites (Ochiai et al. 2010). Based on these findings, we propose the following hypothetic reaction mechanism of Alg17C: (1) access to the end of alginate; (2) recognition of residue units more than two oligoalginate units; then (3) cleavage of alginate into monomeric units by sliding the substrate through the active site. The crystal structure analysis of Alg17C is currently being conducted and will provide a better understanding of the reaction mechanism and properties of Alg17C (Miyake et al. 2003).

In conclusion, Alg17C was an exo-type oligoalginate lyase that decomposes alginate oligomers into the monomeric sugar acid (i.e., DEH). Considering the utilization of alginate as the biomass for fuels or chemicals production, Alg17C seems to be a suitable enzyme for the production of a monomeric sugar acid prior to the main fermentation process for ethanol production.

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