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ORIGINAL RESEARCH PAPER

Cloning, overexpression and characterization of a new oligoalginate lyase from a marine bacterium, *Shewanella* sp.

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Abstract
Purpose of work Is to report an oligoalginate lyase with high enzymatic activity and high-level expression.Using site-finding PCR and degenerate PCR, a gene (designated *oalS17*) encoding a new oligoalginate lyase was cloned from *Shewanella* sp. Kz7 and expressed in *Escherichia coli.* The gene consisted of 2,292 bp with deduced amino acid size of 763 including a putative signal peptide of 44 amino acid residues belonging to polysaccharide lyase (PL) family 17. The recombinant protein was most active at 50 °C and pH 6.2 in 50 mM phosphate buffer. It degraded alginate more efficiently than polyM and polyG block into a monomeric sugar acid, with a specific activity of 32 U mg^{-1} toward alginate, 24 U mg^{-1} toward polyM and 5 U mg^{-1} toward polyG. With the high-level expression and high enzymatic activity, the recombinant oligoalginate lyase OalS17 could be a potential enzyme for further research on alginate saccharification and biofuels production.

Keywords Biofuel · Cloning · Monomeric sugar acid · Oligoalginate lyase OalS17 · *Shewanella* sp. Kz7

Introduction

Brown algae dominate primary production in temperate and polar rocky shores and represent a huge marine biomass. Indeed, coastal regions are considered as carbon sinks, retaining about 200×10^9 kg C per year. Among these compounds, alginate can account for up to 40 % of the dry weight of the algal biomass (Thomas et al. 2013). Alginate is a linear block polymer of α-L-guluronic acid (G) and β-D-mannuronic acid (M), arranged as a polyM-block, a polyG-block, and an alternating or random polyMG-block. It is a commercially useful polysaccharide that is widely used in food and pharmaceutical industry due to its high viscosity and gelling properties (Draget et al. 2005). When alginate is used as the renewable source for the production of biofuels and chemicals, the

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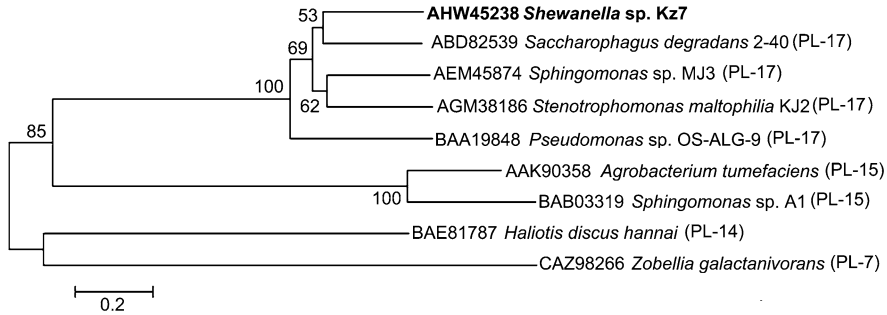

Fig. 1 Phylogenetic analysis of oligoalginate lyase OalS17. The reported alginate lyase (Genbank BAA19848) and oligoalginate lyases protein sequences were aligned using ClustalX, and phylogenetic tree was constructed using MEGA 4.0 via the neighbor-joining method

saccharification of it is prerequisite. Generally, synergistic effects of alginate lyase and oligoalginate lyase are needed for alginate saccharification (Preiss and Ashwell 1962; Hashimoto et al. 2000; Takase et al. 2010) (Supplementary Fig. 1). An engineered microbial platform for direct biofuel production from brown macroalgae has been reported (Wargacki et al. 2012).

Various alginate lyases have been cloned and characterized (Wang et al. 2013), while only six oligoalginate lyases have been reported (Hashimoto et al. 2000; Ochiai et al. 2006; Suzuki et al. 2006; Kim et al. 2012; Park et al. 2012; Thomas et al. 2013). These latter lyases all showed a lower specific activity than alginate lyases. Thus, new oligoalginate lyases with high enzymatic activity and high yield need to be discovered and researched for application in producing monomeric sugar acid from brown macroalgae and developing biofuel further.

In this study, the gene encoding a new oligoalginate lyase OalS17 was cloned from a recently isolated bacterial strain, *Shewanella* sp. Kz7, and expressed at a high-level in *Escherichia coli*. The encoded enzyme has been characterized.

Materials and methods

Materials

Sodium alginate (from *Macrocystis pyrifera*, M/G ratio: 1.66) was purchased from Bright Moon Seaweed Group (Qingdao, China). PolyM block and polyG block (purity: about 95 %) were kindly provided by Dr. Guangli Yu and Dr. Xia Zhao in Ocean University of China. *Shewanella* sp. Kz7 (CCTCC No. AB2014040) was recently isolated from sea mud collected along the coastal zone of Jiaozhou Bay and now preserved in China Center for Type Culture Collection. *E. coli* strains DH5α and BL21 (DE3) were grown at 37 °C in LB broth supplemented with ampicillin (50 μg ml^{-1}) or kanamycin (30 μg ml^{-1}) when relevant.

Cloning and sequence analysis of the oligoalginate lyase gene

To obtain gene fragments encoding oligoalginate lyase from Kz7, PCR was performed with degenerate primers (PL17-JB-F and PL17-JB-R) designed according to the conserved sequences of PL-17 family oligoalginate lyases. A 494-bp DNA fragment was obtained and sequenced. To obtain the DNA sequence of flanking regions, SiteFinding-PCR (Tan et al. 2005) was performed with six nested gene-specific primers (SFP1&2, Up-oalS17-R1&2&3, Down-oalS17-F1&2&3). Oligonucleotides of degenerate primers and SiteFinding-PCR primers used for the gene cloning of *oalS17* are shown in Supplementary Table 1. The 5′- and 3′-flanking sequences were then assembled with that of the consensus region to form a longer sequence containing the ORF of the oligoalginate lyase gene. Multiple sequence alignments between oligoalginate lyase OalS17 and other known oligoalginate lyases were obtained using the ClustalX program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin et al. 2007). The phylogenetic tree was constructed by MEGA 4.0 (http://www.megasoftware.net/) (Tamura et al. 2007) and the neighbor-joining

Table 1 Effect of metal ions, chelators and detergents on the activity of the purified oligoalginate lyase OalS17

Additives	Concentration (mM)	Relative activity (%)
Control	–	100 ± 3.9[a]
NaCl	1	119 ± 2.3
	10	147 ± 3.5
	100	203 ± 5.2
	300	276 ± 3.4
	500	238 ± 3.0
KCl	1	103 ± 2.5
LiCl	1	96 ± 7.4
NH_4Cl	1	90 ± 9.7
$ZnCl_2$	1	86 ± 4.8
$NiCl_2$	1	40 ± 1.8
$MnCl_2$	1	94 ± 6.9
$CaCl_2$	1	80 ± 6.0
$MgCl_2$	1	94 ± 6.9
$FeCl_3$	1	44 ± 3.3
$AlCl_3$	1	56 ± 2.2
EDTA	1	49 ± 1.8
SDS	1	34 ± 10.6

Data are expressed as mean ± SD, $n = 3$

[a] The activity of control (100 % relative activity) = Table 1 2 U ml^{-1}

method. Protein sequences of enzyme OalS17 and other known oligoalginate lyases were analyzed using Conserved Domain Search Service of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/).

Expression and purification of recombinant OalS17

To facilitate protein expression in the heterologous system, the signal sequence of *oalS17* was removed. The gene coding for OalS17 was PCR amplified and cloned into the expression vector pET-24a using primers OalS17-EF (5′-GGAATTCCATATGTCCTATACCGTAAGCACGCCT-3′) and OalS17-ER (5′-GCCGCTCGAGCTGCGCTTTATTGTTAGTGTGG-3′). The resulting plasmid, pET24-*oalS17*, was then transformed to *E. coli* BL21 (DE3).

Escherichia coli BL21 (DE3) cells harboring pET24-*oalS17* were grown at 37 °C in LB medium containing 30 μg kanamycin ml^{-1} until the OD_{600} reached 0.8, and IPTG was then added to give 0.5 mM. Cultivation was continued further for 12 h at 25 °C and 100 rpm. Cells were harvested by centrifugation (6,000×*g*, 10 min), resuspended in phosphate buffer (20 mM, pH 7.0; 500 mM NaCl), and disrupted by ultrasonication. The supernatant was obtained by centrifugation at 10,000×*g* for 30 min at 4 °C, and then the target protein was purified from the supernatant using a HiTrap HP column (1 ml, GE Healthcare, Piscataway, USA). The purity and molecular weight of OalS17 were determined using SDS-PAGE. Protein was measured using the Bradford method with BSA as standard.

Assay of oligoalginate lyase activity

Oligoalginate lyase OalS17, 100 μl, was incubated with 900 μl sodium alginate solution (3 g l^{-1} in 20 mM phosphate buffer, pH 7.0) at 50 °C for 10 min. Relative enzyme activity was measured by using the dinitrosalicylic acid (DNS) method with glucuronic acid as the standard. One unit was defined as the amount of enzyme causing release of 1 μmol reducing sugar at the above conditions.

Characterization of the purified OalS17

For the studies of substrate specificities, alginate, polyM and polyG were used as substrates. The optimal pH of the enzyme was determined by measuring its activity in different buffers at 50 °C for 10 min (Li et al. 2013). Its optimal temperature was determined by monitoring enzymatic activity at various temperatures in 20 mM phosphate buffer (pH 7.0) for 10 min. To determine acidity stability of the purified OalS17, the residual activity was measured after enzyme was incubated in different buffers at 4 °C for 6 h. Its thermostability was determined after it was incubated in 20 mM phosphate buffer (pH 7.0) at various temperatures for 1 h. The effects of metal ions and chelators on OalS17 activity were examined by monitoring enzymatic activity in the presence of various cation ions or chelators.

Analysis of degradation products

Purified enzyme, 0.5 ml (2 U ml^{-1}) was incubated with 2 ml sodium alginate, polyM or polyG solution (3 g l^{-1} in 20 mM phosphate buffer, pH 7.0) for 3 h at 50 °C then analysed by TLC (Yao et al. 2013).

Nucleotide sequence accession numbers

The 16S rRNA and oligoalginate lyase gene *oalS17* of strain Kz7 have been deposited in GenBank under accession numbers KF673475 and KJ094505, respectively.

Results and discussion

Sequence analysis of the gene *oalS17*

The coding gene of protein OalS17 was amplified by degenerate PCR and SiteFinding-PCR. The ORF of the gene *oalS17* consisted of 2,292 bp with deduced amino acid size of 763 (85.6 kDa) including a putative signal peptide of 44 amino acid residues. The recombinant protein with 729 amino acids had a calculated molecular weight of 82,334 Da. In the Carbohydrate-Active enZYmes (CAZy) database, alginate lyases belong to the polysaccharide lyase (PL) families (Cantarel et al. 2009). The protein OalS17 showed the highest sequence identity of 65 % with PL-17 protein sequence Sfri_3104 from *Shewanella frigidimarina* NCIMB 400 and 49 % with the oligoalginate lyase OAL (PL-17) from *Stenotrophomonas maltophilia* KJ-2. When the protein sequence of OalS17 was submitted to NCBI for searching conserved domains, OalS17 together with other PL-17 oligoalginate lyases all showed an alginate lyase superfamily domain in the *N*-terminal region and a heparinase II/III family domain in the *C*-terminal region (Supplementary Fig. 2). Multiple sequence alignments analysis showed that the catalytic active sites and substrate interacting sites of OalS17 and other PL-17 family members were conserved (Supplementary Fig. 3). A phylogenetic tree was constructed for all oligoalginate lyases and one alginate lyase AlyII (GenBank BAA19848). The oligoalginate lyase OalS17, along with PL-17 family oligoalginate lyases Alg17C (GenBank ABD82539), AlgL (GenBank AEM45874), OAL (GenBank AGM38186) and alginate lyase AlyII formed a deeply branched cluster in the phylogenetic tree and was thus a new member of PL-17 family clearly distinct from other oligoalginate lyases (Fig. 1).

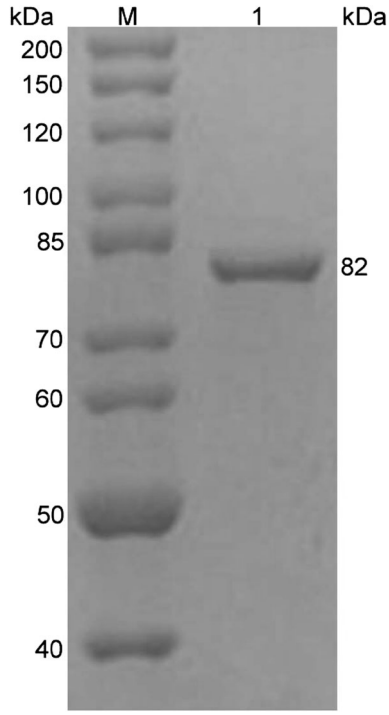

Fig. 2 SDS-PAGE of the recombinant OalS17. *Lane M*, protein markers; *lane 1*, purified enzymes by His-tag affinity chromatography

Purification and biochemical characterization of the recombinant OalS17

The recombinant OalS17 was highly expressed as soluble body forms in *E. coli*. 150 mg recombinant OalS17 was purified to homogeneity from 1 l LB culture. The enzyme showed a single band with an apparent molecular weight of 82 kDa on SDS-PAGE (Fig. 2). The recombinant OalS17 was most active at 50 °C and pH 6.2 in 50 M phosphate buffer (Fig. 3a, b). The enzyme retained 88 % of the original activity after incubation at the temperatures below 40 °C for 1 h (Fig. 3c), and it was stable between pH 6–9 (Fig. 3d). The effects of metal ions, detergents and chelating agents on the activity of OalS17 are shown in Table 1. Although its activity was enhanced by NaCl, the enzyme was active in the absence of NaCl. The enzyme was sensitive to surface active agent SDS and trivalent metal ions including Al^{3+} and Fe^{3+}. The chelating agent EDTA inhibited the activity of OalS17, suggesting that it was a metalloenzyme. However, the required divalent metal ion was not

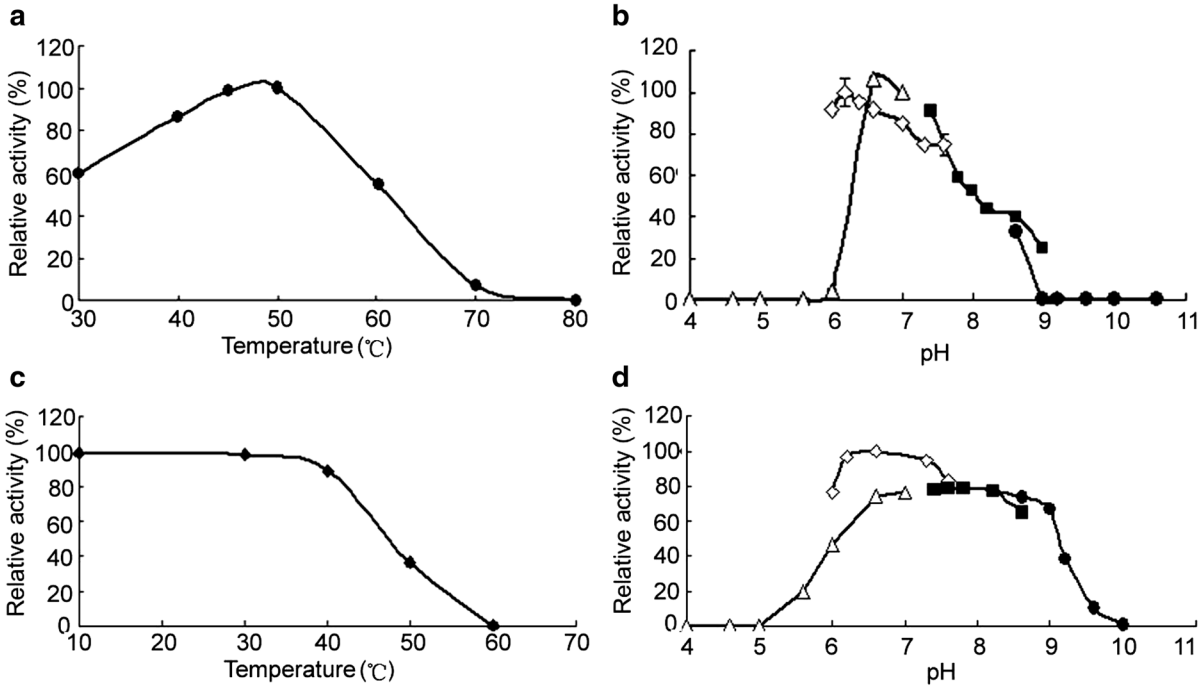

Fig. 3 Effects of pH and temperature on activity and stability of the purified oligoalginate lyase OalS17. Buffers used were 50 mM Na_2HPO_4-citric acid (*open traingle*), 50 mM Na_2HPO_4-NaH_2PO_4 (*open diamond*), 50 mM Tris/HCl (*filled square*) and 50 mM glycine/NaOH (*filled circle*). **a, b** Temperature and pH dependence, respectively. **c, d** Thermostability and pH stability, respectively. The relative activity of 100 % was 2 U ml^{-1}. Experiments were conducted three times

detected. The analysis of the substrate specificity of the recombinant OalS17 showed that it degraded alginate more efficiently than polyM and polyG block into a monomeric sugar acid, with a specific activity of 32 U mg^{-1} toward alginate, 24 U mg^{-1} toward polyM and 5 U mg^{-1} toward polyG (Fig. 4).

Analysis of reaction product

The reaction mixture was analyzed using TLC when different substrates were completely degraded by the recombinant OalS17 (Fig. 4). The major spots of the same mobility from alginate, polyG, and polyM that have greater mobility than monosaccharide have been identified as DEH (4-deoxy-L-*erythro*-5-hexoseulose uronic acid) through chemical analysis (Preiss and Ashwell 1962). In addition, they showed a major molecular ion peak at 175.02 *m/z* $[M\text{-}H]^-$ in ESI-MS analysis, which was corresponded to the monomeric sugar acid (Supplementary Fig. 4). Thus, the recombinant OalS17 is an oligoalgiante lyase that can mainly degrade the glycosidic bond of different substrates into a monomeric sugar acid which spontaneously converts into DEH.

The degradation of alginate into monomeric units by oligoalginate lyase is a critical prerequisite step for alginate metabolism and biofuels production (Wargacki et al. 2012). However, in contrast to various reported alginate lyases, only 6 oligoalginate lyases have been reported, and they all showed low specific activities. Although oligoalginate lyases Alg17C and AlyA5 showed higher activity than the recombinant OalS17, Alg17C preferred to form a substantial amount of inclusion bodies, while AlyA5 displayed a narrow range of pH tolerance (Kim et al. 2012; Thomas et al. 2013). Thus, the recombinant OalS17 would be a more proper enzyme for further research on producing biofuels from alginate.

Conclusion

A recombinant OalS17 was functionally expressed as soluble protein in *E. coli*. The high-level expression of

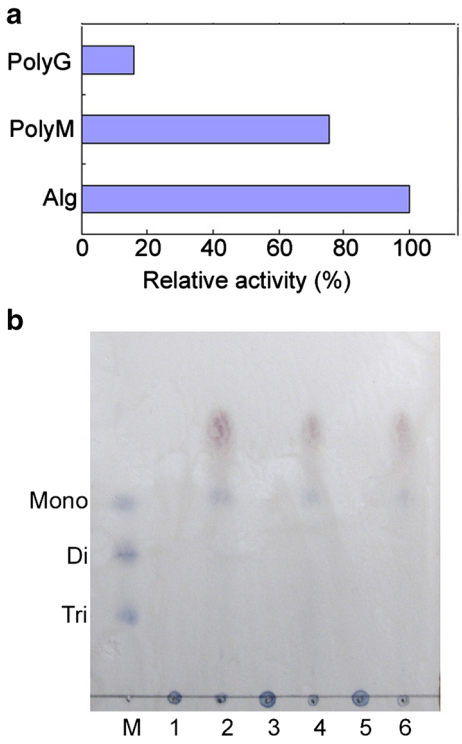

Fig. 4 Substrate specificity of reaction products from different substrates. **a** Three g different substrates l^{-1} in 20 mM phosphate buffer (pH 7.0) was used to perform the reactions. **b** The reaction products were separated on a HPTLC plate developed with n-butanol/formic acid/water (2:1:1, by vol.) and they were visualized with a diphenylamine/aniline/phosphate reagent. *Lane M*, The purified monomeric sugar, dimer and trimer standards; *lane 1*, control, alginate with inactivated OalS17; *lane 2*, reaction products of alginate degraded by OalS17; *lane 3*, control, polyG with inactivated OalS17; *lane 4*, reaction products of polyG degraded by OalS17; *lane 5*, control, polyM with inactivated OalS17; *lane 6*, reaction products of polyM degraded by OalS17

oligoalginate lyase OalS17, together with its high enzymatic activity, made it a potential enzyme for further research for producing biofuels from alginate.

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Supporting information Supplementary Table 1. Primers used for cloning of the gene *oalS17*.

Supplementary Fig. 1 The metabolic process of alginate. Alginate lyase depolymerizes alginate into oligosaccharides by cleaving the glycosidic bonds through a β-elimination reaction. Then, the oligosaccharides are degraded into monomeric unit by oligoalginate lyase, which spontaneously convert into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH). Subsequently, DEH reductase (DehR) reduces DEH into 2-keto-3-deoxygluconate (KDG), a common metabolite that is fed into the Entner–Doudoroff (ED) pathway to produce ethanol. *Thick* and *dotted arrows* indicate the cleavage sites for oligoalginate lyases and alginate lyases, respectively. This scheme was based on the modification of the figure made by Miyake et al. (2003).

Supplementary Fig. 2 Conserved domains in the protein sequences of oligoalginate lyase OalS17 and other oligoalginate lyases. Protein sequences were analyzed using Conserved Domain Search Service of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/). GenBank accession numbers of exotype oligoalginate lyases atu3025 from *Agrobacterium tumefaciens* strain C58, A1-IV from *Sphingomonas* sp. A1, OAL from *Stenotrophomonas maltophilia* KJ-2, AlgL from *Sphingomonas* sp. MJ3, Alg17C from *Saccharophagus degradans* 2-40 and AlyA5 from *Zobellia galactanivorans* DsiJT are AAK90358, BAB03319, AGM38186, AEM45874, ABD82539 and CAZ98266, respectively.

Supplementary Fig. 3 Protein sequence alignment of oligoalginate lyase OalS17, alginate lyase AlyII from *Pseudomonas* sp. OS-ALG-9, oligoalginate lyase AlgL from *Sphingomonas* sp. MJ3, oligoalginate lyase Alg17C from *Saccharophagus degradans* 2-40 and putative alginate lyase Sfri-3104 from *Shewanella frigidimarina* NCIMB 400 using ClustalX. Identical amino acid residues are *boxed* in *dark shade*, and amino acid residues above 70 % consensus are *boxed* in *pale shade*. The *filled triangle* and *filled circle* indicate the proposed catalytic sites, and substrate interacting sites for Alg17C, respectively.

Supplementary Fig. 4. ESI−MS analysis of the major end products from the reaction with alginate by oligoalginate lyase OalS17.

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