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Expression and characterization of a bifunctional alginate lyase named Al163 from the Antarctic bacterium *Pseudoalteromonas* sp. NJ-21*

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Abstract In this study, an endolytic alginate lyase, named Al163, was identified, cloned, and characterized from the Antarctic bacterium *Pseudoalteromonas* sp. NJ-21. Comparative sequence analysis showed that the predicted amino acid sequence encoded by *al163* belongs to the polysaccharide lyase 6 (PL-6) family and has a molecular mass of about 80 kDa. Recombinant enzyme was purified by Ni-Sepharose affinity chromatography. Recombinant Al163 exhibited maximum activity (258 U/mg) at pH 7.0 and 40°C, and thermal stability assays showed retention of almost 90% activity after incubation at 30°C for 30 min. Al163 activity was stimulated by Cd^{2+}, Ca^{2+}, Fe^{3+}, and Mn^{2+}, but inhibited by Cu^{2+}, Si^{2+}, Fe^{2+}, and Ni^{2+}. Thin-layer chromatographic analysis indicated that Al163 degraded sodium alginate, polyM, and polyG, generating disaccharides and trisaccharides as the final products. Only a few bacterial strains that produce a bifunctional alginate lyase have been reported. Our results indicate that recombinant Al163 exhibits broad substrate specificity and its products exhibit low degrees of polymerization. Both properties imply high potential for use of the enzyme in several industrial fields, including cosmetics and pharmaceuticals, based on the high demand for biologically active oligosaccharides.

Keyword: *Pseudoalteromonas* sp.; alginate lyase; PL-6 family

1 INTRODUCTION

Alginate is the main component of the cell wall of brown algae and is comprised of α-L-guluronic acid (G) and its C5 epimer β-D-mannuronic acid (M) as the basic framework (Garron and Cygler, 2010). Alginate is also a component of some bacterial biofilms (Haug et al., 1967; Kim et al., 2011). Alginate has many potential applications in the food and pharmaceutical industries because of its high viscosity and gelling properties (Laurienzo, 2010). Alginate oligosaccharides are formed from alginate by alginate lyase through a β-elimination reaction, which produces unsaturated oligosaccharides with double bonds at the non-reducing end. Alginate oligosaccharides exhibit various physiological activities, including promotion of root growth in higher plants (Tomoda et al., 1994; Xu et al., 2003), acceleration of *Bifidobacterium* sp. growth rate (Akiyama et al., 1992), enhancement of penicillin production in *Penicillium chrysogenum* (Ariyo et al., 1998), promotion of macrophage and keratinocyte proliferation and endothelial cell viscosity (Kawada et al., 1999; Iwamoto et al., 2003, 2005; Yamamoto et al., 2007), and lowering of blood pressure in humans. Therefore, the production of value-added oligosaccharides from alginate via alginate lyase is an attractive prospect, especially given the ability of this

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enzyme to directly degrade fronds of brown seaweed to release alginate oligosaccharides and convert elastic seaweed fronds into slurry-like homogenates (Inoue et al., 2014).

Alginate lyases are classified into two groups based on their substrate specificity (polyG lyase, EC4.2.2.11 and polyM lyase, EC4.2.2.3) (Zhu et al., 2015) or mode of action (endolytic or exolytic) (Wong et al., 2000). Because the oligosaccharide products of alginate lyase exhibit many types of bioactivity, a source from which highly-active enzyme can be produced affordably and on a large scale is needed.

Antarctica is too cold and dry for the survival of most organisms; however, there are multiple cold-adapted microorganisms there that make essential contributions to nutrient recycling and organic matter mineralization using a special class of extracellular cold-adapted or cold-active enzymes. Enzymes from Antarctic microorganisms exhibit special properties, including high catalytic efficiency and excellent thermal stability at temperatures below 20°C when compared with those from other microorganisms. These enzymes have potential applications in industrial processes that require reactions at lower temperatures; therefore, the mechanisms associated with cold-adapted or cold-active enzymes have been a focus of study (Carrasco et al., 2012). However, studies of alginate lyases from Antarctic cold-adapted bacteria are scarce, and information on their sequence, structure, and function is required if their industrial potential is to be realized. It is anticipated that such enzymes with high activity and excellent thermal stability could be used to produce alginate-derived oligosaccharides.

In this study, we sequenced, cloned, and characterized an alginate lyase gene, *al163*, and purified and characterized the recombinant protein (Al163) from the Antarctic bacterium *Pseudoalteromonas* sp. NJ21. The biochemical properties of the recombinant Al163, including its specific activity and optimal reaction conditions, were determined, and the enzymatic products were characterized.

2 MATERIAL AND METHOD

2.1 Material

The bacterial strain NJ-21 was isolated from sediment samples from the Antarctic region (75°45.9′E, 68°5.65′S) and belongs to the genus *Pseudoalteromonas*. *Escherichia coli* strains DH5α and BL21 and vectors pUC57 and pET-30a(+) were purchased from GenScript (Nanjing, China). Sodium alginate, agar, agarose, chondroitin B, kanamycin, isopropyl β-D-1-thiogalactopyranoside (IPTG), dinitrosalicylic acid, and other reagents were purchased from Solarbio (Beijing, China). Tryptone and yeast extract were purchased from OXOID (Basingstoke, UK). PolyM and polyG were provided by the Ocean University of China.

2.2 Sequence analysis and gene cloning

Sequencing and annotation of the *Pseudoalteromonas* sp. NJ21 genome were performed by Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) in our preliminary work (data not shown). The *al163* sequence was amplified from the genomic DNA of *Pseudoalteromonas* sp. NJ21 by polymerase chain reaction (PCR) using primers *al163*-F (5′-ACTTGC-CATGCCTATTTGCA-3′) and *al163*-R (5′-TCGTA-GGTCACATTTGAGCTT-3′). The PCR products were purified and ligated into the pUC57 cloning vector. The *al163* sequence was determined by GenScript, and the open reading frame was analyzed using DNAStar Lasergene (v7.1; DNASTAR, Madison, WI, USA). The *al163* nucleotide sequence has been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession number KF700697.

2.3 Construction of expression plasmid and purification of alginate lyase Al163 from *E. coli*

For *al163* ligation into an expression vector, primer pair F 5′-CGC**GGATCC**ACTTGCCATGCCTATTT-GCA-3′ (including a *BamH*I restriction site, in bold letters) and R 5′-CCG**CTCGAG**TCGTAGGTCACA-TTTGA-GCTT-3′ (including an *Xho*I restriction site, in bold) was designed and synthesized with no stop codon and used to amplify the gene by PCR. The amplified fragment was inserted into the pET-30a(+) expression vector, which was used to transform *E. coli* BL21 (DE3) cells. *Escherichia coli* cells harboring the pET30a-Al163 vector were cultured at 37°C for 2–3 h in Lauria-Bertani medium. IPTG was added to a final concentration of 0.5 mmol/L and the cells were cultured at 16°C for an additional 20 h. Cells were pelleted by centrifugation at 12 000×*g* at 4°C for 20 min, washed with 50 mmol/L phosphate buffer (pH 7.5), resuspended in the same buffer, and then disrupted by sonication (Constant Systems Ltd., Daventry, UK). The disrupted cells were centrifuged at 12 000×*g* at 4°C for 15 min and dialyzed against 50 mmol/L phosphate buffer (pH 7.5) for 48 h. The dialyzed

sample was freeze-dried, dissolved in binding buffer [50 mmol/L Tris-HCl (pH 8.0), 300 mmol/L NaCl, and 10 mmol/L imidazole], and loaded onto a Ni-Sepharose column (GE Healthcare, Uppsala, Sweden). The enzyme was eluted with a linear gradient of imidazole (20–500 mmol/L) in 50 mmol/L Tris-HCl (pH 8.0) and 300 mmol/L NaCl. Active fractions were desalted using an ultrafiltration device with a 30-kDa cut-off (Merck Millipore, Darmstadt, Germany) and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the purified enzyme was determined using the Bradford method with bovine serum albumin as a standard (Bradford, 1976).

2.4 Determination of recombinant Al163 enzyme activity

The activity of alginate lyase Al163 was measured using the dinitrosalicylic acid method (Miller, 1959). Briefly, purified Al163 (10 μg) was dissolved in 0.1 mL 20 mmol/L sodium phosphate buffer (pH 7.0) and mixed with 0.9 mL 20 mmol/L sodium phosphate buffer (pH 7.0) containing 0.2% alginate, and the reaction system was incubated at 40°C for 15 min. Al163 activity was analyzed by measuring the mass of reducing sugar released by the reaction system. One unit of Al163 enzyme was defined as the amount of enzyme required to produce 1 μg of reducing sugar per min.

2.5 Characterization of the biochemical properties of recombinant Al163

The optimal temperature for Al163 activity was determined in the reaction system from 0°C to 60°C in 20 mmol/L phosphate buffer (pH 7.0). Al163 thermal stability was investigated by incubating purified enzyme for 30 min at temperatures from 0°C to 60°C, followed by analysis of enzyme activity at 40°C. The optimal pH was evaluated by performing enzyme assays in different buffers (20 mmol/L): acetate buffer (pH 4–5), phosphate buffer (pH 6–8), glycine-NaOH buffer (pH 9–10), and Na_2HPO_4-NaOH buffer (pH 11). The pH stability of Al163 was determined according to the residual activity observed following incubation in the different buffers at different pH values (4.0–11.0) at 4°C for 3 h using the reaction system described above. To investigate the influence of various compounds on enzyme activity, 10 μg purified Al163 was incubated in solutions containing 5 mmol/L of $MnCl_2$, $FeCl_3$, $CaCl_2$, SDS, $MgCl_2$, $ZnCl_2$, KCl, $NiCl_2$, $CuCl_2$, or ethylenediaminetetraacetic acid (EDTA) in 1 mL of 20 mmol/L phosphate buffer (pH 7.0) containing 0.2% alginate at 40°C for 15 min. Enzyme activity was measured as described, with a reaction system lacking the compound used as a control. The influence of NaCl on enzyme activity was measured in buffers containing different concentrations of NaCl (0–60 g/L) in standard enzyme activity assay conditions. An assay using a buffer lacking NaCl was used as a control. The highest activity was defined as 100%.

2.6 Substrate specificity and kinetic parameters of recombinant Al163

To determine the substrate specificity of Al163, 0.2% sodium alginate, polyM, polyG, agar, agarose, and chondroitin B were tested as substrates, and standard enzyme activity assays were performed as described in Section 2.4. The kinetic parameters of the recombinant Al163 with sodium alginate, polyM, and polyG as substrates were evaluated by measuring the initial rate of the reaction at different substrate concentrations (0.05–0.5 mg/mL). K_m and V_{max} values were determined using double-reciprocal (Lineweaver-Burk) plots.

2.7 Analysis of the products of recombinant Al163

The activity of Al163 was measured using alginate, polyM, and polyG. The reaction system (1 mL), including 100 μg purified Al163 and 2 mg substrate, was incubated in phosphate buffer (pH 7.0) for 0–360 min at 40°C. The reaction was stopped by heating to 100°C for 5 min, followed by centrifugation at 12 000×*g* for 5 min to eliminate inactive enzyme. After centrifugation, 2 μL of the supernatant was loaded onto a thin-layer chromatography TLC-60 plate (Merck Millipore) and developed with a solvent comprising 1-butanol, ethanol, and water (2:1:1, v:v:v). After development, the plate was stained by spraying with a color-developing reagent (100 mL acetone, 2 g diphenylamine, 2 mL aniline, 10 mL 85% phosphoric acid, and 1 mL concentrated HCl) and dried at 110°C for 15 min in an oven.

3 RESULT

3.1 Cloning and sequence analysis of alginate lyase Al163

The *Pseudoalteromonas* sp. NJ-21 *al163* gene encoding alginate lyase Al163 was successfully cloned and sequenced using specific primers based on the nucleotide sequence of *al163*. The *al163* sequence

Table 1 Purification of recombinant Al163

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold change)	Yield (%)
Crude enzyme	17.8	1 708.8	96	1	100
Ni-Sepharose	3.5	903	258	5.1	52.9

Table 2 Influence of various compounds on Al163 activity

Reagent	Concentration (mmol/L)	Relative activity (%)
Control	0	100
EDTA	5	89.3±0.05
$MnCl_2$	5	71.3±1.01
$FeCl_3$	5	96.7±6.95
$CaCl_2$	5	116.1±1.65
SDS	5	105.2±0.46
$MgCl_2$	5	102.4±1.73
$ZnCl_2$	5	97.1±1.46
KCl	5	107.1±2.92
$NiCl_2$	5	110.2±2.65
$CuCl_2$	5	106.4±0.93

contained a 2 322-bp open reading frame (GenBank: KU360250) encoding a 773-amino-acid polypeptide that lacked a signal peptide. The translated Al163 sequence had a calculated molecular mass of 84.61 kDa and a pI value of 5.38. A BLAST search of the NCBI database (https://www.ncbi.nlm.nih.gov/) revealed the translated Al163 sequence shares 98% amino acid sequence identity with the putative polyM lyase from *Paraglaciecola mesophila* KMM241 (GenBank: GAC22931) (Fig.1).

Sequence analysis revealed that residues 57 to 426 share high sequence identity with other members of the polysaccharide lyase 6 (PL-6) superfamily, and, interestingly, residues 57 to 458 also share high sequence identity with chondroitin B lyase, whereas residues 524 to 705 match a domain of unknown function (DUF4957). Multiple alignment of the Al163 N-terminal region with AlyGC from *Glaciecola chathamensis* S18K6^T (GenBank: BAEM00000000.1) (Xu et al., 2017), KJ-2 alginate lyase from *Stenotrophomonas maltophilia* (GenBank: AFC88009.1) (Lee et al., 2012), AlyP from *Pseudomonas* sp. OS-ALG-9 (GenBank: BAA01182.1) (Maki et al., 1993), OalS6 from *Shewanella* sp. Kz7 (GenBank: AHC69713.1) (Li et al., 2016), BBFL7 alginate lyase from *Flavobacteria* (GenBank: WP006795620.1), and chondroitin B lyase 1DBG_A from *Pedobacter heparinus* (GenBank: ACU03011.1) revealed a putative calcium-binding site and other residues potentially important for enzyme function (Fig.2).

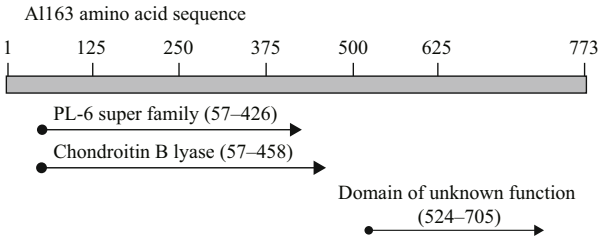

Fig.1 Structure of the translated Al163 amino acid sequence

The sequence contains 773 amino acids. Residues 57–426 share high sequence identity with other members of the PL-6 superfamily, residues 57–458 share similarity with chondroitin B lyase, and residues 524–705 match a domain of unknown function (DUF4957).

3.2 *Al163* expression, and purification of the Al163 protein

The *al163* gene fragment encoding the mature Al163 enzyme was expressed in *E. coli* BL21 (DE3) cells as an intracellular protein in the presence or absence of IPTG induction, and total protein was analyzed by SDS-PAGE. Only one induced protein, which ran at 80 kDa, was observed (Fig.3). Purification details are summarized in Table 1. Overall, about 5.1-fold purification was achieved with 52.9% yield after a single step. The recombinant enzyme had a specific activity of 258 U/mg.

3.3 Biochemical characterization of recombinant Al163

The optimal temperature for recombinant Al163-catalyzed degradation of sodium alginate was 40°C (Fig.4a), and enzyme activity was similar between 30°C and 40°C. Thermal stability analysis showed >90% residual activity following incubation at temperatures ranging from 10°C to 30°C for 30 min, but the residual activity sharply declined at temperatures >40°C (Fig.4b). Enzyme activity was highest in sodium phosphate buffer at pH 7.0 (Fig.4c), and almost 20% of the activity remained after incubation at pH 4.0 for 3 h (Fig.4d).

The effects of various compounds on enzyme activity were tested (Table 2). Ca^{2+}, SDS, Mg^{2+}, K^+, Ni^{2+}, Cu^{2+}, and Ca^{2+} enhanced Al163 activity by up to 16.1% in the best case, while EDTA, Mn^{2+}, Fe^{2+}, and

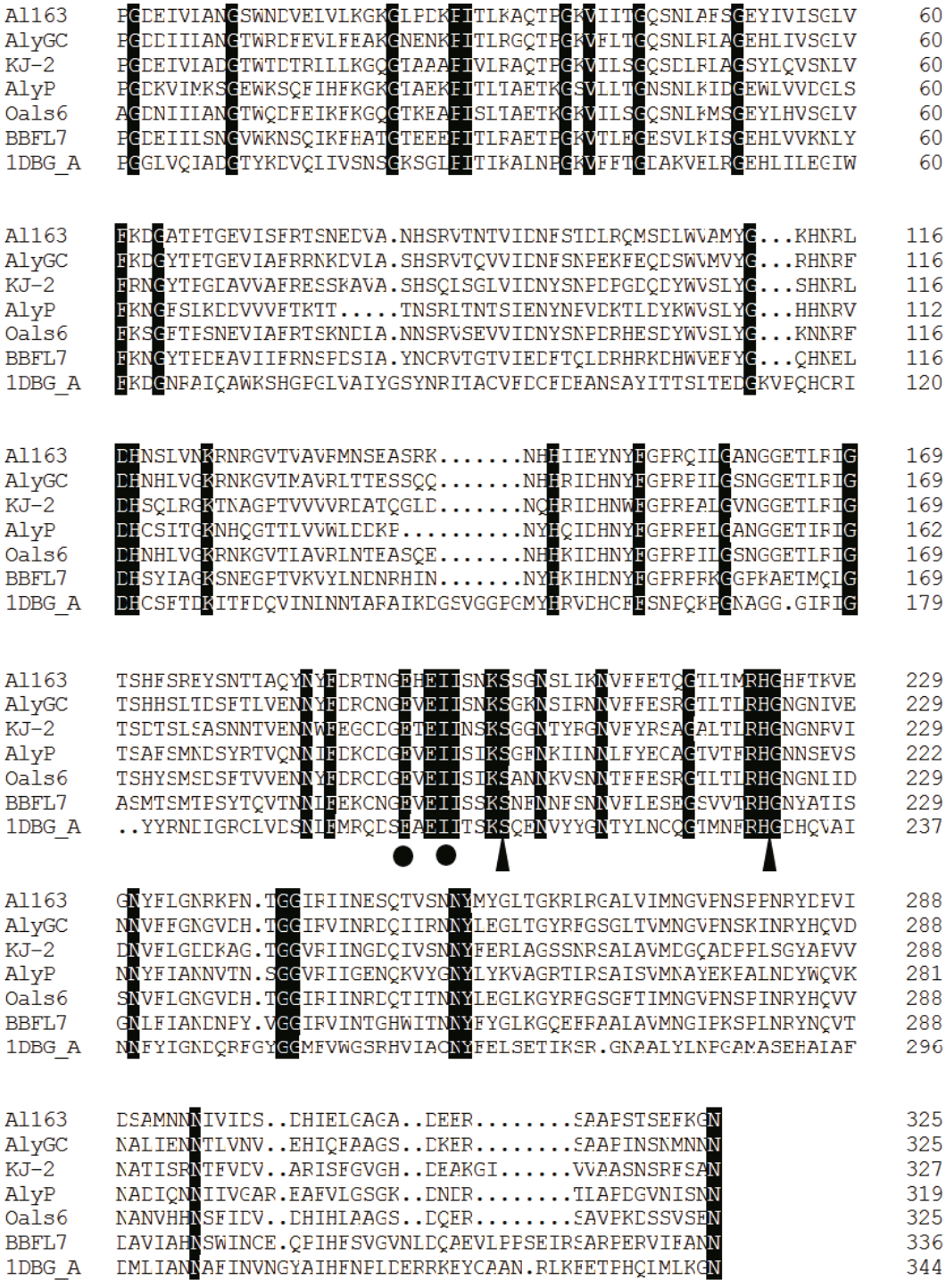

Fig.2 Multiple sequence alignment of the N-terminal region of Al163 with other alginate lyases

Filled circles indicate putative calcium-binding site residues, and filled triangles indicate probable functionally important residues. Sequence alignment was performed using DNAMAN software for homology modeling (Lynnon Corporation, San Ramon, CA, USA).

Zn^{2+} repressed Al163 activity, with the largest decrease caused by Mn^{2+} (71.3% residual activity compared with controls containing no additives). NaCl also increased Al163 activity (Fig.5), with the highest activity measured in the presence of 40 g/L NaCl, which was nearly 3-fold higher than that measured in the absence of NaCl. Furthermore, Al163 activity steadily increased from 0 to 40 g/L NaCl, suggesting that Al163 was activated by NaCl over a broad concentration range and can be considered a salt-activated alginate lyase. Many NaCl-activated enzymes have been isolated from marine organisms,

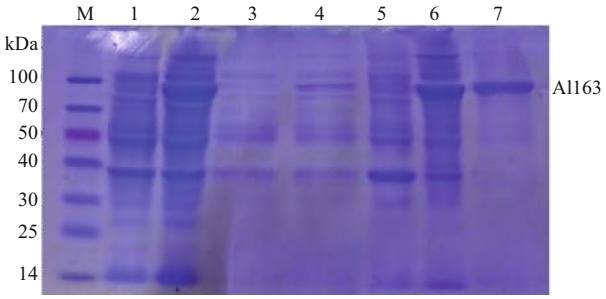

Fig.3 Purification of recombinant Al163

Lane M: prestained standard protein markers; lane 1: proteins from uninduced whole cells; lane 2: proteins from whole cells induced with 0.5 mmol/L IPTG; lane 3: uninduced-cell supernatant after disruption; lane 4: induced-cell supernatant after disruption; lane 5: insoluble material (pellet) from uninduced cells; lane 6: insoluble material (pellet) from cells induced with 0.5 mmol/L IPTG; lane 7: recombinant Al163 purified from induced-cell soluble extract by Ni-Sepharose affinity chromatography.

in which the presence of NaCl in varying amounts is indispensable for survival.

3.4 Substrate specificity and kinetic parameters of recombinant Al163

Alginate, polyM, polyG, agar, agarose, and chondroitin B were tested as potential substrates of the recombinant Al163 (Fig.6). The enzyme degraded alginate, polyM, and polyG, but showed no activity toward agar, agarose, or chondroitin B. The highest Al163 activity was observed toward polyG, almost 2-fold higher than that observed toward polyM or alginate.

Kinetic parameters of the recombinant Al163 with alginate, polyM, and polyG were calculated from a series of initial rates (V, U/mg) measured at various

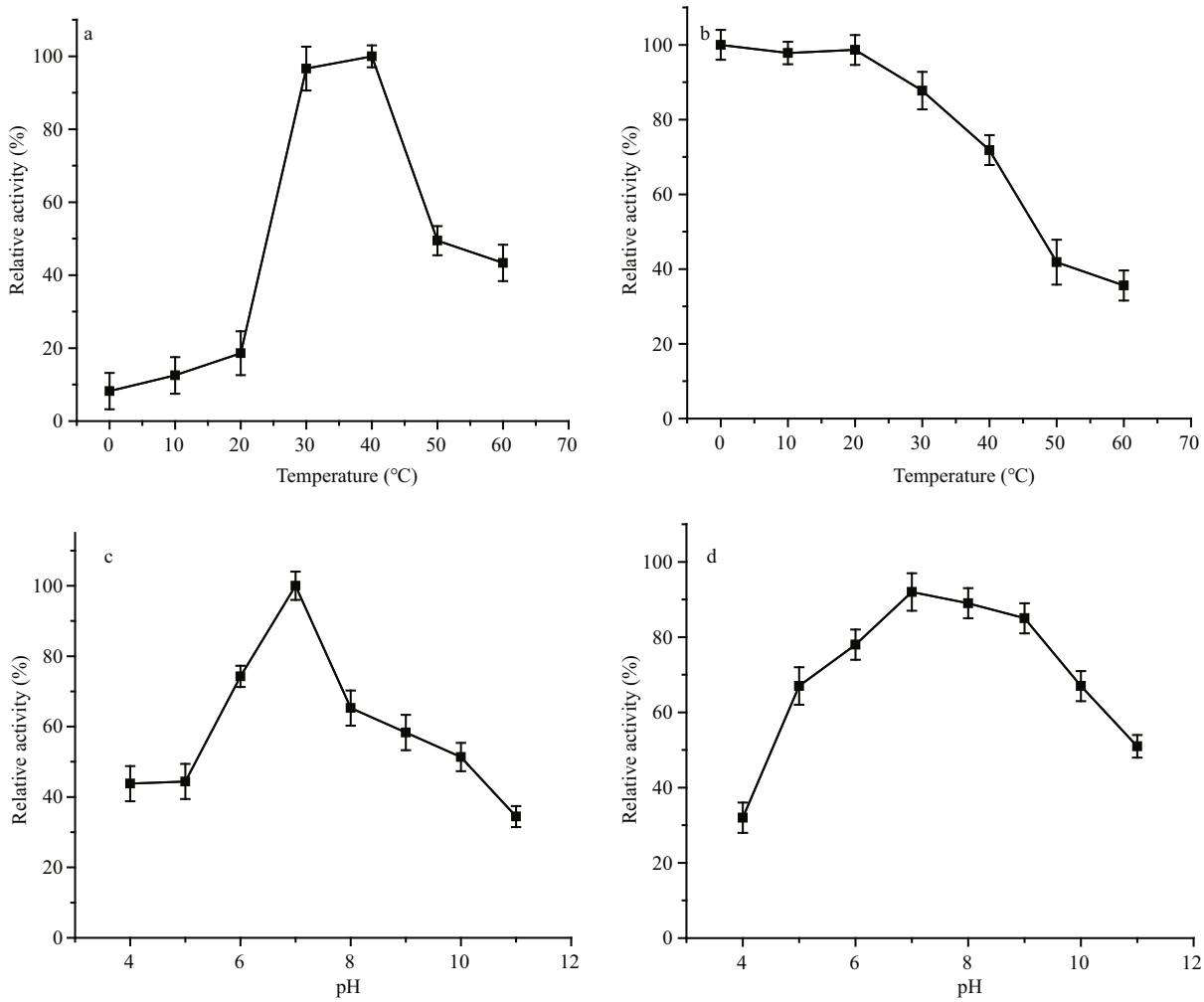

Fig.4 Biochemical characterization of recombinant Al163

a. influence of temperature on Al163 activity in 20 mmol/L phosphate buffer (pH 7.0); reaction at 0°C to 60°C for 15 min; b. Al163 thermal stability was determined by preincubation at different temperatures (0–60°C) for 30 min, followed by measurement of enzyme activity in 20 mmol/L phosphate buffer (pH 7.0) at 40°C; c. effect of pH on Al163 activity was determined at 40°C for 15 min in 20 mmol/L buffer; d. Al163 pH stability was estimated by preincubation at a pH from 4.0 to 11.0 at 4°C for 3 h, followed by measurement of enzyme activity in 20 mmol/L sodium phosphate buffer (pH 7.0) at 40°C. The highest activity in each set of experiments was defined as 100%.

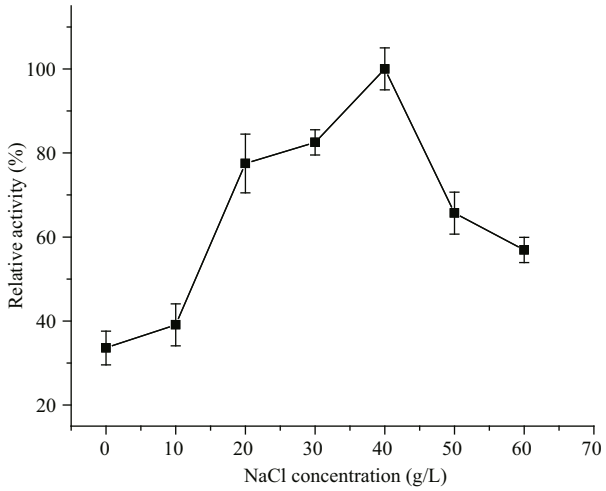

Fig.5 Influence of NaCl concentration on Al163 activity

The highest observed activity was defined as 100%.

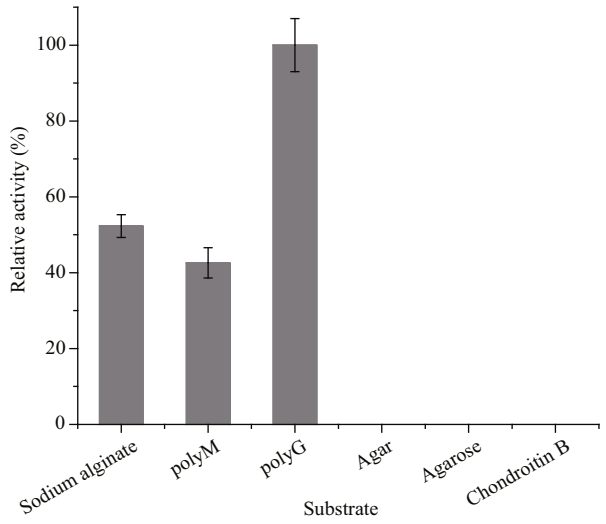

Fig.6 Substrate specificity of recombinant Al163

Phosphate buffer (1 mL, pH 7.0) containing the tested substrate (0.2%, w/v) was mixed with 100 μg purified Al163 and incubated for 15 min at 40°C. The highest observed activity was defined as 100%.

substrate concentrations and estimated using Lineweaver-Burk plots (Fig.7). The resulting V_{max} values for alginate, polyM, and polyG were 303 U/mg, 256 U/mg, and 625 U/mg, respectively, and the corresponding K_m values were 1.787 mg/mL, 1.301 mg/mL, and 0.875 mg/mL. The K_m value for polyG was clearly lower than those for alginate and polyM, which was consistent with the results of substrate specificity analysis.

3.5 Analysis of the degradation products of substrates of recombinant Al163

Degradation products of alginate, polyM, and polyG were investigated after incubation with recombinant Al163 for 0–360 min by thin-layer chromatography. Purified unsaturated dimeric, tetrameric, and heptameric agar oligosaccharides were used as standard markers (Fig.8). For all three

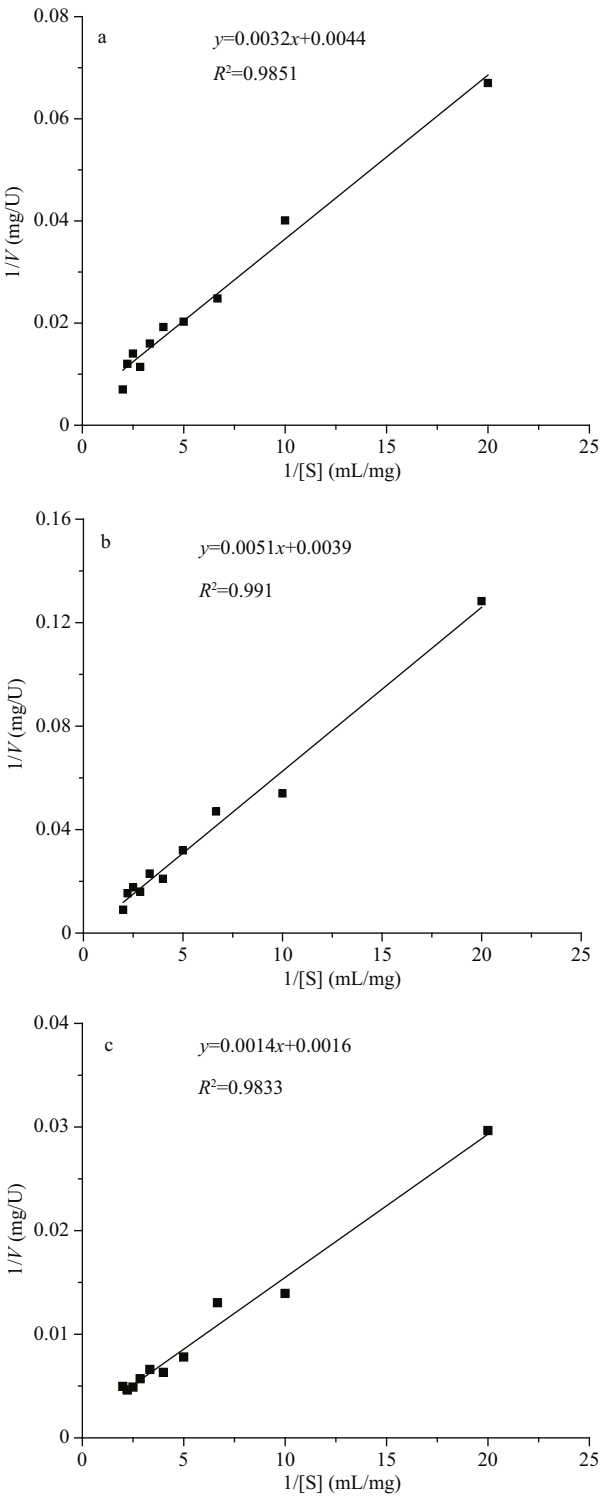

Fig.7 Kinetic parameters of recombinant Al163

Kinetic parameters were measured in the presence of (a) alginate, (b) polyM, and (c) polyG as substrates. The initial reaction rates were measured at concentrations of 0.05–0.5 mg/mL substrate. V indicates the initial reaction rate, and [S] indicates the substrate concentration.

Table 3 Comparison of alginate lyases belonging to the PL-6 superfamily

Enzyme	Source	Signal peptide	Amino acid	pH	Temperature (°C)	Prefer substrate	Product
Al163	*Pseudoalteromonas* sp.	No	773	7	40	PolyG	DimerTrimer
AlyGC	*Glaciecola chathamensis S18K6*^{T}	Yes	754	7	30	PolyG	Monomer
KJ-2	*Stenotrophomas maltophilia*	Yes	454	8	40	PolyMG	DimerTrimer
Oals6	*Shewanella* sp.	Yes	770	7.2	40	PolyG	Monomer

substrates, the main degradation products were dimers and trimers. Small quantities of pentamers and heptamers were visible after only 15 min of incubation, while dimers began to appear after 2 h, suggesting Al163 initially hydrolyzed substrates into trimers, pentamers, and heptamers that were subsequently degraded into dimers and trimers. These results indicate that Al163 may hydrolyze substrates in an endolytic manner.

4 DISCUSSION

Hundreds of alginate lyases have been obtained from multiple sources, including marine algae, marine mollusks, and a wide range of marine and terrestrial bacteria (Zhu and Yin, 2015). According to their amino acid sequence similarities, alginate lyases are classified into seven PL families (PL-5, -6, -7, -14, -15, -17, and -18) in the Carbohydrate-Active enZYmes (CAZy) database (Cantarel et al., 2009). In this study, alginate lyase Al163 from the Antarctic bacterium *Pseudoalteromonas* sp. NJ-21 was determined to belong to the PL-6 family, and was successfully expressed in *E. coli* BL21 (DE3) cells. Al163 shared >90% sequence identity with a putative polyM lyase from *P. mesophila* KMM241 (GenBank: GAC22931) and enzymes of unknown function from *Pseudoalteromonas atlantica* T6c (GenBank: ABG42142), and *Paraglaciecola agarilytica* (GenBank: WP008301990). Most endolytic bacterial alginate lyases have been assigned to the PL-5 and -7 families (Hashimoto et al., 2005; Yamasaki et al., 2005; Ogura et al., 2008), and there have been few reports of enzymes from the PL-6 family; only AlyGC, KJ-2, AlyP, and OalS6 have been characterized as alginate lyases belonging to the PL-6 superfamily (Table 3). Most PL-6 alginate lyases prefer polyG as the substrate and share similar optimal conditions for enzymatic degradation of polysaccharides.

Sequence alignment of Al163 with other alginate lyases revealed a putative calcium-binding site, and enzyme assays showed that Ca^{2+} had the greatest effect on stimulating enzyme activity, supporting an important role for the putative calcium-binding site. The role of Ca^{2+} in AlyGC-degradation of alginate was investigated recently, revealing a Ca^{2+} ion is required for catalysis (Xu et al., 2017). Interestingly, based on amino acid sequence alignment and modeling analysis, Al163 shares high sequence similarity with chondroitin B lyase; however, Al163 did not exhibit chondroitin B lyase activity. Structure-function relationship comparisons between Al163

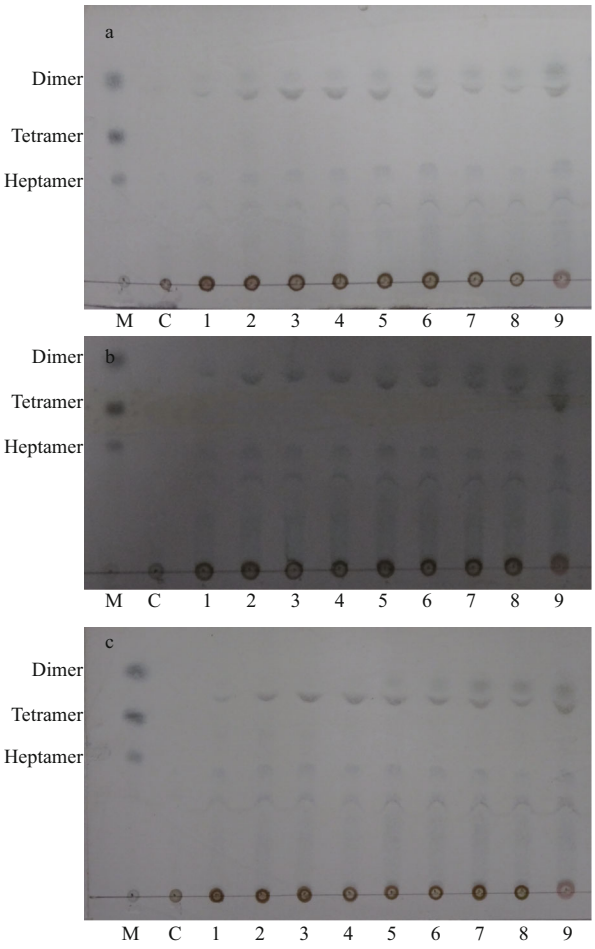

Fig.8 Analysis of Al163 degradation products

Products from the substrates (a) alginate, (b) polyM, and (c) polyG following incubation for 0–360 min. Lane M, purified agar oligosaccharides (dimer, tetramer, and heptamer); lane C, control with inactive Al163; lanes 1–9, hydrolysates after reaction for 15, 30, 45, 60, 120, 180, 240, 300, and 360 min, respectively.

and chondroitin B lyase will require analysis of enzyme structures.

Hundreds of alginate lyase sequences have been deposited in the NCBI database; however, few originate from organisms that inhabit extreme environments. The putative polyM lyase from the cold-adapted bacterium *P. mesophila* KMM241 (GenBank: GAC22931) (Qin et al., 2014) was predicted to be an alginate lyase, but the protein has not been purified and characterized. Studies of enzymes from extreme environments are important for developing more environmentally-friendly industrial catalysts.

Depolymerized alginates with low degrees of polymerization derived from enzymatic hydrolysis possess various biological activities (Wong et al., 2000; Hu et al., 2004; Zhang et al., 2004; An et al., 2009; Kobayashi et al., 2009; Zong et al., 2014), and numerous attempts have been made to produce value-added materials, such as functional oligosaccharides and bioethanol, from alginate (Takeda et al., 2011; Wargacki et al., 2012; Enquist-Newman et al., 2014). There are many advantages in producing functional oligosaccharides using enzymatic methods rather than traditional approaches such as acid hydrolysis of alginate or supersonic schizolysis. The primary goal for developing enzymatic methods is the discovery of efficient and stable alginate lyases. Those described to date, including flalya (Inoue et al., 2014), A9m (Uchimura et al., 2010), and Algb (Wong et al., 2000), are gradually inactivated at temperatures >40°C. We found that the recombinant Al163 exhibited good stability at low temperatures (10–40°C) and maintained almost 20% residual activity even in extreme pH conditions (4.0 and 11.0). Additionally, recombinant Al163 was capable of degrading both polyM and polyG simultaneously, indicating bifunctionality. Previous studies reported that only a few bacterial strains produce bifunctional alginate lyases (Iwamoto et al., 2001). Bifunctional alginate lyases were found to assist in the recovery of pathogenic bacteria sensitive to antibiotics by degrading the alginate polysaccharides in biomembranes (Chung et al., 2007). Additionally, the recombinant Al163 exhibited broad substrate specificity, and its catalytic products exhibited low degrees of polymerization. Both properties suggest high potential for industrial use. Because Al163 was easily expressed in an *E. coli*-expression system, recombinant Al163 could provide a firm basis for future biotechnological studies of alginate lyases. Structural and functional studies of recombinant Al163, including those involving mutagenesis, are currently underway in our laboratory.

5 CONCLUSION

We cloned and characterized a novel PL-6 family endolytic alginate lyase (Al163) derived from the Antarctic bacterium *Pseudoalteromonas* sp. NJ-21. Recombinant Al163, which was purified by Ni-Sepharose affinity chromatography, had a molecular mass of 84.61 kDa. The purified enzyme showed maximum activity (258 U/mg) at pH 7.0 and 40°C. Substrate specificity experiments revealed its ability to degrade alginate, polyM, and polyG, with the highest affinity for polyG, indicating Al163 might be a polyG lyase (EC4.2.2.11). Al163 degraded alginate to oligosaccharide products with low degrees of polymerization (dimers and trimers), suggesting its potential for producing oligosaccharides with interesting, high-demand bioactivities.

6 DATA AVAILABILITY STATEMENT

Sequence data supporting the findings of this study have been deposited in GenBank under accession numbers KF700697 (16S rDNA sequence of *Pseudoalteromonas* sp. NJ21) and KU360250 (*Pseudoalteromonas* sp. NJ21 *al163* gene sequence).

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