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Cloning, Expression, and Characterization of a Cold-Adapted and Surfactant-Stable Alginate Lyase from Marine Bacterium *Agarivorans* sp. L11 S

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The purpose of this study was to find a cold-adapted and surfactant-stable alginate lyase as a candidate for biotechnological and industrial applications. The gene for a new alginate lyase, AlyL1, from *Agarivorans* sp. L11 was cloned and expressed in *Escherichia coli*. The recombinant AlyL1 was most active at 40°C (1,370 U/mg). It was a cold-adapted alginate lyase, which showed 54.5% and 72.1% of maximum activity at 15°C and 20°C, respectively. AlyL1 was an alkaliphilic enzyme and most active at pH 8.6. In addition, it showed high stability in the presence of various surfactants at a high concentration (from 0.1% to 1% (w/v)). AlyL1 was an endo-type alginate lyase that degraded both polyM and polyG blocks, yielding disaccharides and trisaccharides as the main products. This is the first report of the cloning and functional expression of a cold-adapted and surfactant-stable alginate lyase. AlyL1 might be an interesting candidate for biotechnological and industrial applications.

Keywords: Alginate lyase, cold-adapted, surfactant-stable, alkaliphilic, *Agarivorans*

Introduction

Alginate, the most abundant carbohydrate in brown algae, is widely used in food and pharmaceutical industries. It is an acidic heteropolysaccharide consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G), which are arranged as polyM blocks, polyG blocks, and alternating or random polyMG blocks [5]. In recent years, alginate oligosaccharides have captured more and more attention because of their useful and biologically important functions, such as the plant root growth-promoting activities [4, 5], improving the growth of bifidobacteria [15], the induction of cytokine production in mononuclear cells [3], antioxidant activity [1], and protection against endoplasmic reticulum-mediated and mitochondrial-mediated apoptotic cell death and oxidative stress [11].

Alginate lyase catalyzes the depolymerization of alginate by a β-elimination mechanism [7]. Alginate lyases have been obtained from various sources, such as marine algae, marine mollusks, fungi, bacteria, and viruses [5, 16], and are classified into seven polysaccharide lyase (PL) families, namely, PL families 5, 6, 7, 14, 15, 17, and 18 in the CAZy database (http://www.cazy.org/fam/acc_PL.html). To produce oligosaccharides with high value-addition, enabling the biocatalytic processes to run at low temperature often saves energy and production costs, and also reduces the risk of contaminations [13]. In addition, cold-adapted enzymes are heat labile and can be easily and selectively inactivated by moderately elevated temperatures, which have advantages in the industrial processes that do not permit high temperatures for enzyme inactivation [9]. However, a great majority of alginate lyases showed relatively low activity at low temperatures, and none of them was characterized to be a cold-adapted enzyme [2, 9, 14, 16]. Surfactants are common ingredients in detergents, which are often used in the preliminary treatment of brown macroalgae. However, most alginate lyases are relatively unstable in the presence of non-ionic surfactants (Tween-20, Tween-80, and Triton X-100) and anionic surfactants (SDS) [8, 16].

In this study, a cold-adapted alginate lyase (AlyL1)-encoding gene was cloned from a marine bacterium, *Agarivorans* sp. L11, and expressed in *Escherichia coli*. The recombinant AlyL1 was also stable in the presence of various surfactants, making it a good candidate for biotechnological and industrial applications.

Materials and Methods

Materials

Sodium alginate (M/G ratio: 1.66) was purchased from Bright Moon Seaweed Group (Qingdao, China). PolyM and polyG (purity: about 95%) were kindly provided by Prof. Guangli Yu and Prof. Chunxia Li at Ocean University of China. *E. coli* strains DH5α and BL21 (DE3) (Novagen, USA) were grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented with ampicillin (50 μg/ml) or kanamycin (30 μg/ml) when relevant. The pMD18-T (TaKaRa, Dalian, China) and pET-28a (+) (Novagen, USA) plasmids were employed as gene cloning and expression vectors, respectively. Oligonucleotides used for the cloning and expression of AlyL1 are shown in Table S1.

Isolation and Identification of Bacteria

Decaying brown seaweed samples were collected from the coastal zone of Jiaozhou Bay, Qingdao, China. They were immersed, diluted, and spread on selective medium plates (3 g sodium alginate, 3 g KH_2PO_4, 7 g $K_2HPO_4{\cdot}3H_2O$, 2 g $(NH_4)_2SO_4$, 30 g NaCl, 0.05 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MgSO_4{\cdot}7H_2O$, and 15 g agar in 1 L of distilled water, pH 7.0), and then the detectable colonies were purified and inoculated into fermentation medium (3 g sodium alginate, 3 g KH_2PO_4, 7 g $K_2HPO_4{\cdot}3H_2O$, 2 g $(NH_4)_2SO_4$, 30 g NaCl, 0.05 g $FeSO_4{\cdot}7H_2O$, and 0.01 g $MgSO_4{\cdot}7H_2O$ in 1 L of distilled water, pH 7.0) and assayed for alginate lyase activity [12]. The 16S rRNA gene of strain L11 was amplified by PCR from the genomic DNA and aligned with its closely related sequences retrieved from GenBank using the BLASTn and ClustalX programs. The phylogenetic tree was constructed by MEGA 4.0.

Cloning and Sequence Analysis of the Alginate-Lyase-Encoding Gene

Degenerate primers (PalyL1-F1 and PalyL1-R1) were designed according to the conserved sequences of alginate lyases in PL-7. The partial AlyL1-encoding gene (390 bp in length) was amplified from the genomic DNA of *Agarivorans* sp. L11. Inverse PCR was used to amplify the flanking sequences with two pairs of nested primers (PalyL1-F2, PalyL1-R2, PalyL1-F3, and PalyL1-R3). The PCR products were purified, sequenced, and assembled with the initial fragment to obtain the full-length alginate lyase gene, *alyL1*. The signal peptide was predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP). Multiple sequence alignments between AlyL1 and other alginate lyases were obtained using the ClustalX program. The theoretical molecular weight (Mw) and isoelectric point (pI) were calculated using the Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html).

Expression and Purification of Recombinant AlyL1

For expression of AlyL1, the DNA fragment containing the *alyL1* gene without signal sequence and stop codon was amplified using the primers PalyL1-EF and PalyL1-ER to introduce *Nco*I and *Xho*I sites into *alyL1*, and then ligated into the *Nco* I and *Xho* I sites of plasmid pET28a. The recombinant plasmid pET28a-alyL1 was transferred into *E. coli* BL21 (DE3). *E. coli* BL21-pET28-alyL1 cells were cultured in LB medium containing 30 μg/ml kanamycin at 37°C until the OD_{600} reached 0.6. Afterwards, the expression of the target gene was induced by 0.1 mM isopropyl-β-thiogalactoside at 25°C and 100 rpm for 36 h. Precipitated cells were resuspended in Tris-HCl buffer (20 mM, pH 8.6; 500 mM NaCl) and ultrasonically disrupted. The recombinant AlyL1 was purified from the soluble fraction using a Ni-Sepharose column. The protein concentration

Table 1. Effects of various surfactant agents on the activity of AlyL1.

Reagent added	Concentration (%)	Relative activity (%)	Reagent added	Concentration (%)	Relative activity (%)
None	-	100 ± 1.8			
Triton X-100	0.1	100.8 ± 3.1	Tween-20	0.1	105.3 ± 1.3
	0.2	106.3 ± 4.2		0.2	110.5 ± 3.1
	0.5	122.7 ± 2.4		0.5	113.4 ± 3.9
	1	109 ± 2.5		1	108 ± 2.9
	2	52.1 ± 3.5		2	47.1 ± 3.4
SDS	0.1	107.7 ± 3.2	Tween-80	0.1	108.8 ± 2.7
	0.2	99.3 ± 2.4		0.2	104.3 ± 1.9
	0.5	101 ± 2.9		0.5	111.6 ± 2.6
	1	64.9 ± 2.3		1	100.5 ± 3.4
	2	38.7 ± 1.9		2	31.6 ± 1.4

The data were expressed as the mean ± SD, $n = 4$. The activity of the control (100% relative activity) was 13.5 U/ml.

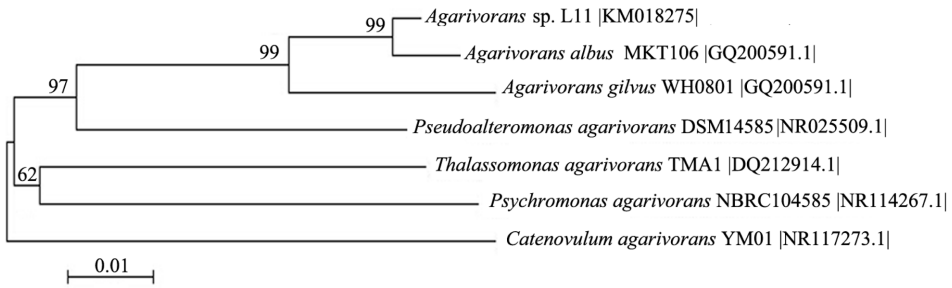

Fig. 1. Neighbor-joining phylogenetic tree of strain L11 based on 16S rRNA gene sequences.
Numbers at nodes are levels of bootstrap support (%). The scale bar represents two nucleotides substitution per 100 nucleotides.

was measured with the bicinchoninic acid method with bovine serum albumin as the standard. The molecule weight of the purified AlyL1 was determined by SDS-PAGE on a 10% (w/v) resolving gel.

Enzymatic Activity Assay

The enzymatic hydrolysis reaction was conducted in 20 mM Tris-HCl buffer (pH 8.6) containing 0.3% (w/v) sodium alginate at 40°C for 10 min. Then, alginate lyase activities were measured as the increase in absorbance at 235 nm. One unit (U) was defined as the amount of enzyme required to increase the absorbance at 235 nm by 0.1 per minute. For the studies of substrate specificities, polyM and polyG were used as the substrate in the same condition.

Determination of Kinetic Parameters

Initial velocities were determined in the standard assay mixture with 20 mM Tris-HCl buffer (pH 8.6). The kinetic parameters of AlyL1 were measured by using ten different concentrations of alginate (ranging from 0.1 to 8 mg/ml). The K_m and V_{max} were then analyzed by using Lineweaver-Burk's methods.

Analysis of Reaction Product and Reaction Pattern

Fifteen units of the purified enzyme (0.5 ml) was incubated with 2 ml of alginate (2 g/l in 20 mM Tris-HCl buffer, pH 8.6) for various times (1, 5, 15, 30, and 60 min) at 40°C. The reaction products were boiled for 10 min, centrifuged, and analyzed using an ÄKTA FPLC system (GE Health, USA) with a Superdex peptide 10/300 column (GE Health, USA) using 0.2 M ammonium bicarbonate (0.3 ml/min) as the mobile phase [7]. To identify the structure of hydrolysis products, the eluted fractions were analyzed using negative-ion electrospray ionization mass spectrometry (ESI-MS), and ^{1}H- and ^{13}C-NMR spectrometries as described previously [18].

Nucleotide Sequence Accession Numbers

The 16S rRNA gene and alginate lyase gene (*alyL1*) of strain L11 have been deposited in GenBank under the accession numbers KM018275 and KM018274, respectively.

Results and Discussion

Isolation and Identification of Strain L11

Through sole carbon source culturing, an alginate-lyase-producing bacterium, L11, was isolated from Jiaozhou Bay, Qingdao, China. The 16S rRNA gene of strain L11 was amplified and sequenced. A 1,497 bp fragment of the gene was compared with those of other strains.

The highest nucleotide sequence match was that of *Agarivorans albus* MKT106 (NR_024788.1) with 99.6% identity. The phylogenetic tree (Fig. 1) also showed that the strain L11 belongs to genus *Agarivorans* and was therefore named as *Agarivorans* sp. L11.

Cloning and Sequence Analysis of the Alginate Lyase Gene

The alginate lyase gene, *alyL1*, consisted of an open reading frame of 1,050 bp, encoding 349 amino acid residues, including a signal peptide of 27 residues. The theoretical Mw and pI of the mature enzyme were 36,358 Da and 5.41, respectively. AlyL1 had the highest identity of 65.6% with putative alginate lyase (WP_026025726) from *Vibrio rumoiensis*. Recently, there are only two alginate lyases (A1m and AlmU) that have been isolated from the genus *Agarivorans* [6, 12]. In this study, AlyL1 showed the highest homology to Alm and AlmU with only 28.1% and 16.5% identity, respectively. These results showed that alginate lyase, AlyL1, was a new member from the genus *Agarivorans*. AlyL1 contained three conserved regions, RSELRAM, QIH, and YFKAGXYXQ, which may act to stabilize the three-dimensional conformation and function of the PL-7 family alginate lyases [10].

Expression and Purification of AlyL1

The *alyL1* gene was expressed in the pET28a (+)/*E. coli*

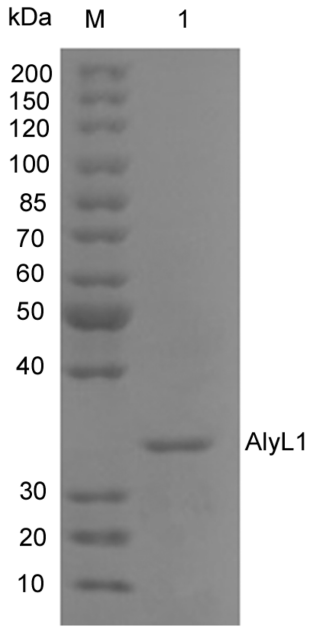

Fig. 2. SDS-PAGE of AlyL1.
Lane M, molecular weight markers; Lane 1, purified AlyL1.

BL21 (DE3) system. The recombinant AlyL1 was purified 45.3-fold to homogeneity with a specific activity of 1,370 U/mg by the Ni-Sepharose column chromatography (yield: 69%). The Mw of recombinant AlyL1 was estimated to be approximately 35 kDa by SDS-PAGE (Fig. 2), which was in a good agreement with the theoretical Mw.

Biochemical Characterization of AlyL1

The optimal temperature of AlyL1 was 40°C, and it showed 54.5% and 72.1% of the maximal activity at 15°C and 20°C (Fig. 3A), respectively, indicating that AlyL1 was a cold-adapted alginate lyase. Alginate lyases from *Vibrio*, *Agarivorans*, *Bacillus*, *Aplysia kurodai*, and *Haliotis discus* showed less than 40% of the maximal activity at 20°C [2, 5, 9, 14, 16, 18]. Although the alginate lyase from *Pseudomonas* sp. QD03 showed approximately 50% of the maximal activity at both 15°C and 20°C, the application of this enzyme was limited by the low specific activity (188.5 U/mg) [17]. To the best of our knowledge, AlyL1 was the first cold-adapted alginate lyase, which retained high activity at low temperature. Most of the activity of AlyL1 was lost

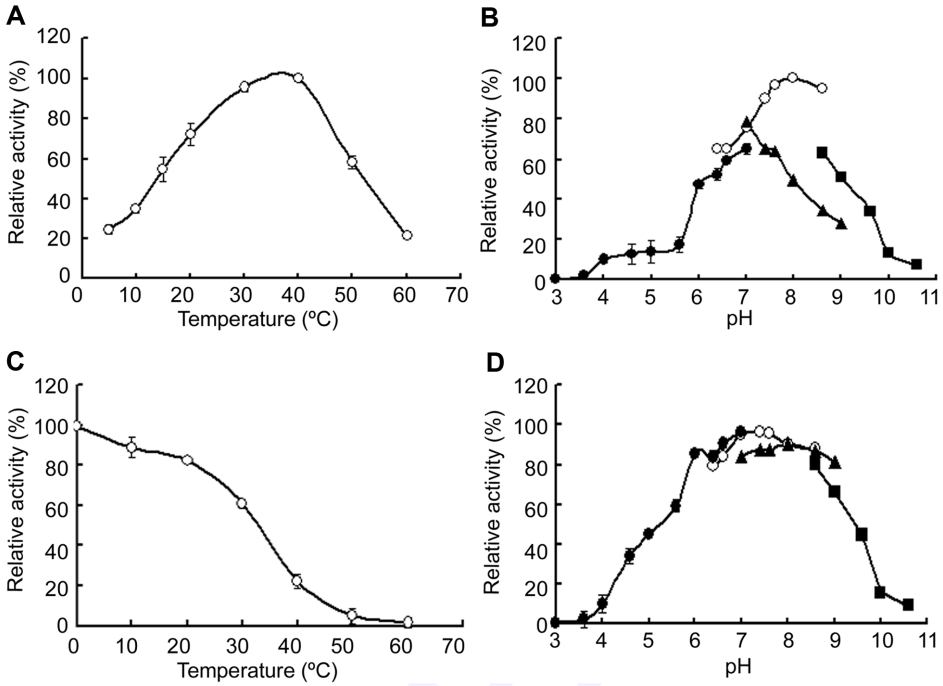

Fig. 3. Effects of pH and temperature on the activity and stability of AlyL1.
(**A**) The optimal temperature of AlyL1 was determined by measuring the activities at various temperatures (5–60°C). (**B**) The optimal pH of AlyL1 was determined by measuring the activities at 40°C in 50 mM Na_2HPO_4-citric acid buffer (*open rhombus*), 50 mM Na_2HPO_4-NaH_2PO_4 buffer (*filled circle*), 50 mM Tris-HCl buffer (*open triangle*), and 50 mM Gly-NaOH buffer (*filled rhombus*), respectively. (**C**) The thermostability of AlyL1 was studied by measuring the residual activities after the enzymes were incubated at different temperatures (0–60°C) for 1 h in 20 mM Tris-HCl buffer (pH 8.6). (**D**) pH stability of AlyL1. The residual activities were measured at 40°C in 20 mM Tris-HCl buffer (pH 8.6) after incubation in the above buffers at 4°C for 6 h. The activity of the control (100% relative activity) was 22.6 U/ml.

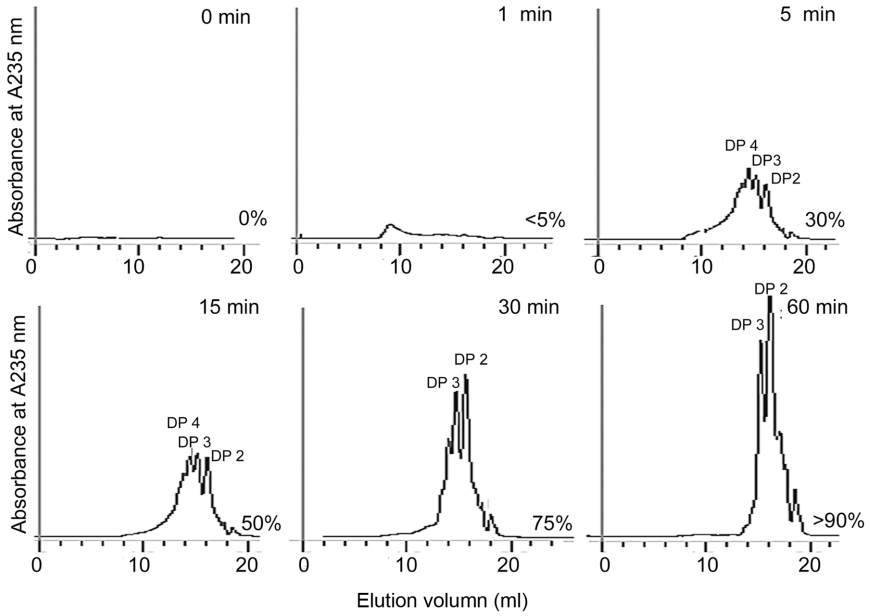

Fig. 4. Size-exclusion chromatography of the action products of the alginate by AlyL1.
The profiles were recorded at several intervals of incubation that were correlated with percentages of degradation determined by the reducing-sugar assay. DP2 to DP4 correspond to the peaks of the oligoalginate.

after being incubated at over 30°C for 1 h (Fig. 3C), indicating that it was heat labile and could easily be regulated in the alginate oligomeric degradation processes.

AlyL1 showed the highest activity in Tris-HCl buffer at pH 8.6 (Fig. 3B) and it was stable within a range of pH 6.0–9.6 (Fig. 3D). So far, only three bacterial alkaliphilic alginate lyases have been identified; namely, alginate lyase from *Bacillus* sp. M-2, A1m from *Agarivorans* sp. JAM-A1m, and MytC from *Saccharophagus* sp. Myt-1 [6, 8]. From its sequence analysis, AlyL1 shared low identity (<30%) in amino acids with the reported alkaliphilic alginate lyases, indicating that it is a new alkaline alginate lyase.

The effects of metal ions on the activity of AlyL1 are shown in Table S2. The effects of surfactants on the activity of AlyL1 were also examined (Table 1). The activity of AlyL1 was enhanced in the presence of a relatively high concentration of surfactant agents such as Tween-20, Tween-80, and Triton X-100 (from 0.1% to 1% (v/v)). Similarly, in the presence of 0.1% to 0.5% (w/v) SDS, a strong anionic surfactant, the enzyme retained 100% of its activity. In fact, AlyL1 retained 64.9% and 38.7% of its activity upon treatment with 1% and 2% SDS, respectively. The stability of purified enzymes towards surfactant agents is an important characteristic for their eventual use in the industrial applications. To the best of our knowledge, only one alginate lyase, MytC, from *Saccharophagus* sp. Myt-1 has been identified to be highly stable in the presence of SDS and oxidizing agents at a really low concentration (from 0.001% to 0.01%) [12].

The catalytic efficiency (k_{cat}/K_m) of AlyL1 against alginate was 9,952.8 ± 33.1 mg ml^{-1} s^{-1}. AlyL1 showed an apparent K_m of 0.19 ± 0.04 mg/ml and a V_{max} of 907.8 ± 72.5 U/mg protein. The V_{max} of AlyL1 was much higher than Atu 3025 (20.5 U/mg) from *Agrobacterium tumefaciens* C58, A1-IV (17.2 U/mg) from *Sphingomonas* sp. A1, and alginate lyase (173.4 U/mg) from *Pseudomonas* sp. QD3 [7, 8]. These results indicated that AlyL1 showed a high affinity towards alginate and efficiently depolymerized alginate polymers into oligosaccharides.

Action Mode and Reaction Product of AlyL1

The action mode of AlyL1 was monitored by size-exclusion chromatography (Fig. 4). The rapid depolymerization, the increase of polydispersity, and the production of all possible oligosaccharides showed that AlyL1 acted in an endolytic mode. After completion of alginate degradation by AlyL1, the main product was purified and analyzed by negative-ion ESI-MS, and ^{1}H- and ^{13}C-NMR spectrometries. The spectra (data not shown) showed good agreement with those of trisaccharides and disaccharides reported previously [18], indicating that the main products of AlyL1 were trisaccharides and disaccharides (more than 90% of the

total product).

In this study, we cloned, expressed, and characterized a new alginate lyase, AlyL1, from *Agarivorans* sp. L11. The enzyme is a cold-adapted alginate lyase and is stable in the presence of various surfactant agents (Tween-20, Tween-80, Triton X-100, and SDS) at a high concentration. Cold-adapted alginate lyases can offer advantages over the currently used alginate lyases in the industrial processes where heating is economically counterproductive or where low temperatures are required. This surfactant-stable alginate lyase holds high activity even with the presence of detergent residue after the preliminary treatment of brown macroalgae. These results indicate that AlyL1 might be an interesting candidate for industrial applications in alginate oligomeric production.

Acknowledgments

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