

Characterization of alginate lyase gene using a metagenomic library constructed from the gut microflora of abalone

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Abstract A metagenomic fosmid library was constructed using a genomic DNA mixture extracted from the gut microflora of abalone. The library gave an alginate lyase positive clone (AlyDW) harboring a 31.7-kbp insert. The AlyDW insert consisted of 22 open reading frames (ORFs). The deduced amino acid sequences of ORFs 11–13 were similar to those of known alginate lyase genes, which are found adjacent in the genome of *Klebsiella pneumoniae* subsp. *aerogenes*, *Vibrio splendidus*, and *Vibrio* sp. belonging to the phylum *Gammaproteobacteria*. Among the three recombinant proteins expressed from the three ORFs, alginate lyase activity was only observed in the recombinant protein (AlyDW11) coded by ORF 11. The expressed protein (AlyDW11) had the highest alginate lyase activity at pH 7.0 and 45°C in the presence of 1 mM AgNO₃. The alginate lyase activity of ORF 11 was confirmed to be endolytic by thin-layer chromatography. AlyDW11 preferred poly(β-D-mannuronate) as a substrate over poly(α-L-guluronate).

AlyDW11 contained three highly conserved regions, RSEL, QIH, and YFKAGVYNQ, which may act to stabilize the three-dimensional conformation and function of the alginate lyase.

Keywords Abalone · Alginate lyase · Metagenomic library · β-D-Mannuronate · α-L-Guluronate

Introduction

The need for new microbial enzymes is on the rise due to increased industrial demand for novel functional foods and fermentable bio-resources from seaweed. Intestinal microbes from some marine animals that produce a mixture of seaweed-degrading enzymes, including agarase, laminarase, cellulase, and alginate lyase, have been screened [24]. Recently, an enzymatically degraded alginate was found to exhibit certain biological activities, such as promotion of root growth in higher plants, acceleration of the growth rate of *Bifidobacterium* sp., induction of the production of cytotoxic cytokines in human mononuclear cells, suppression of IgE, and antihypertensive effects [1, 6, 11, 12]. Accordingly, both alginate lyase and alginate oligosaccharides have attracted the attention of researchers in the food and pharmaceutical industries [11]. There have been many reports on microbial alginate lyases, which have been classified as polysaccharide lyases, and these lyases can cleave polysaccharides through the β-elimination mechanism, resulting in the formation of a 4-deoxy-L-erythrohex-4-enopyranosyluronate at the new nonreducing terminus [10, 25]. On the basis of the enzymatic cleavage specificity of a nonregular distribution of mannuronic (M) and guluronic (G) acid units, alginate lyase has been primarily classified into poly(M)-lyase (EC 4.2.2.3) and poly(G)-lyase

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(EC 4.2.2.11) preferentially breaking up the poly(M) and poly(G) blocks, respectively [17, 25].

These enzymes were isolated from the gut of abalone and many marine bacteria, including *Pseudoalteromonas* sp. IAM 14594 (AF082561), *Sphingomonas* sp. A1 (AB120939), *Klebsiella pneumoniae* subsp. *aerogenes* (L19657), *Vibrio* sp. QY101 (AY221030), and *Pseudomonas* sp. OS-ALG-9 (AB003330) [3, 9, 19, 21]. To date, approximately 23 open reading frames (ORFs) of alginate lyases have been identified in the GenBank database [31]. Previous studies have revealed the existence of many different bacterial alginate lyases, which have been classified into two types depending on substrate specificity, G-block-specific polyguluronate lyase (EC 4.2.2.11) and M block-specific polymannuronate lyase (EC 4.2.2.3) [26]. Cultivation-independent studies using clone libraries of 16S rRNA genes have been used to assess the total bacterial diversity in the gut of abalone *Haliotis discus hannai*, and *Alpha*-, *Gamma*-, and *Epsilonproteobacteria*, and *Mollicutes* were found to be the major microflora [28]. However, the data contained in the 16S rRNA genes did not provide sufficient information to indicate the biological functions of the microbes [23, 30]. In contrast, a metagenomic library based on a fosmid cloning system has commonly been used to identify novel genes from organisms that are not easily cultured [2]. This screening system, which uses a fosmid vector with an insert size of approximately 40-kb DNA directly isolated from diverse environmental samples, has been used to identify a wide range of genes, such as those encoding chitinase, dehydrogenase, oxidoreductase, amylase, esterase, endoglucanase, and cyclodextrinase [2, 27]. As a result of the limitations of standard culture methods, which allow only less than 1% of microorganisms to be cultured in a laboratory, a metagenomic approach has been used to study the genomes of organisms that are difficult to isolate through a conventional cultivation-based approach [27]. One of the advantages of metagenome screening is that the identification of novel genes from viable, but nonculturable bacteria cells is much more rapid relative to previous approaches, which involved cloning the alginate lyases from isolated alginate-assimilating bacteria. In this study, a metagenomic library from abalone intestine was constructed and screened for alginate lyase genes containing novel amino acid sequences that can produce lower molecular weight alginate than commercial enzymes.

Materials and methods

Metagenomic library construction

Abalone samples were obtained from the South Sea near Yeosu in the Republic of Korea during February 2009.

DNA was prepared by direct DNA extraction and purified as previously described [14]. A metagenomic library was then constructed from these samples using a previously described method, with slight modifications [34]. The DNA was again fractionated by pulsed-field gel electrophoresis (PFGE, BioRad, Hercules, CA, USA) into approximately 36-kb lengths after *HindIII* partial digestion (0.05 U/ml of DNA, 37°C for 3 h). Next, the DNA was purified by PFGE to eliminate humic compounds and small fragments in the soil DNA. The crude DNA samples were fractionated by PFGE in 1% low melting point agarose at 6 V/cm and 12°C for 14 h. A gel slice containing 40–100 kb of DNA was then processed by electro-elution using a dialysis tubing cellulose membrane (Sigma-Aldrich). Finally, the purified DNA was end-repaired and ligated into the pCC1FOS vector for packaging (Epicentre, Madison, WI, USA). EPI300 phage T1-resistant cells (Epicentre) were used as a host for routine library manipulation.

Library screening and shotgun sequence analysis

Alginate lyase activity was measured using the cetylpyridinium chloride (CPC) assay. The metagenomic DNA ligated into fosmid vectors (pCC1FOS ready vector, Epicentre, USA) was packaged into lambda phages and transfected into *Escherichia coli* EPI300 (Epicentre). The library was replicated into duplicate sets of 96-well microtiter plates. The infected cells were spread on Luria–Bertani (LB, Becton–Dickinson, Sparks, MD, USA) agar containing 0.1% alginate and incubated for 1 day. After 1 h of incubation at 37°C with 10% CPC solution, the samples were examined for the presence of clear zones around the colonies. For the full sequence of the positive clone, a shotgun DNA library was prepared from purified fosmid DNA and a PHRED/PHRAP/CONSED package [4, 5, 7] was used to assemble the shotgun sequencing reads. The DNA sequences were then determined using an ABI 9700 Thermocycler (Applied Biosystems) and an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, version 3.1) in accordance with the manufacturer's instructions. The BLAST program at the National Center for Biotechnology Information (NCBI) was used for database searches and sequence comparisons. The complete fosmid insert was annotated using the Artemis program and functional identification of the protein coding regions was obtained using the BLASTP and PSIBLAST programs. The predicted proteins were classified according to the category of the clusters of orthologous groups (COG) [18, 29, 30]. Nucleotide sequences from ORFs identified in completely sequenced fosmid clones were aligned with homologous genes retrieved from the NCBI nr database using the CLUSTAL_X program [13].

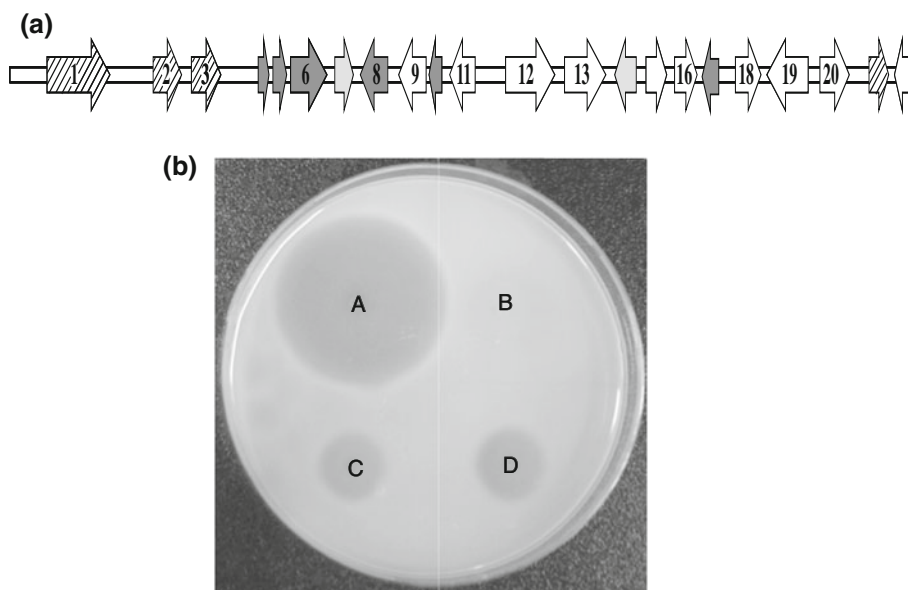


Fig. 1 Schematic representation of the alginolytic metagenome clone (AlyDW) and measurement of alginate lyase activity using a cetylpyridinium chloride (CPC) assay. **a** *Diagonal stripes* indicate ORFs related to cellular processes, *blank arrows* indicate ORFs related to metabolism, and *light gray arrows* indicate ORFs related to information storage. Poorly characterized proteins and hypothetical genes

without homologues are shown in *dark gray*. Different *shadings* indicate the functional categories of the putative genes. **b** CPC assay was performed to measure the degradation of sodium alginate by positive control from *Flavobacterium* sp. (A), negative control from *E. coli* (B), alginolytic metagenome clone (AlyDW, C), and recombinant protein (AlyDW11, D)

Enzyme overexpression and purification

The putative alginate lyase gene was amplified from the metagenomic clone (AlyDW) using the sense primer (5'-TGG GGATCCATGATGAGTTTAAAAACA-3') with a *Bam*HI site (underlined) and antisense primer (5'-GCCAAAGCTTTTT AGTGAGTTACGTTA-3') with a *Hind*III site (underlined). The amplified DNA was then ligated into *Bam*HI and *Hind*III double digested pMal-c2X (New England Bio Labs), after which the construct was transformed into *E. coli* BL21 (DE3) cells. The transformed cells were grown in 500 ml of LB broth at 37°C until the optical density at 600 nm reached 0.5. Isopropyl- β -D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.3 mM, and the flasks were further incubated at 37°C for 4 h to induce expression of the recombinant protein. The cells were lysed by sonication for 2 \times 1 min on ice and the broken cells were centrifuged at 9,000 \times g for 30 min. The crude cell lysate was subsequently loaded onto a 10-ml column containing an amylose resin. The fusion protein was washed with column buffer (200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM β -mercaptoethanol, and 20 mM Tris-HCl, pH 7.4) and eluted with column buffer and 10 mM maltose.

Determination of enzymatic activity

The endoglucanase activity of the enzyme was determined using the Nelson-Somogyi assay, in which a greater

number of reducing sugars leads to increased color intensity in response to enzymatic hydrolysis of the glycosidic bonds of carbohydrates [8]. A standard curve was prepared with glucose at concentrations ranging from 31.5 to 1,000 μ g/ml based on the absorbance at 525 nm using a spectrophotometer (Mecasys Co. Ltd., Korea) [8]. The temperature (30–55°C) and pH (4.0–9.0) were varied to determine the optimal conditions for enzyme activity. In addition, enzyme activity after incubation for 2 h in the following buffers was measured: 0.1 M citrate-phosphate buffer (pH 4.0–7.0), 10 mM phosphate buffer (pH 8.0), and 50 mM glycine-NaOH buffer (pH 9.0). Inhibition and enhancement of the enzyme activity were determined in the presence of metal ions under the optimal conditions (pH 7.0 and 45°C).

Detection of alginate lyase activity

Purified recombinant enzyme AlyDW11 was treated with sample buffer without 2-mercaptoethanol and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to the method described by Laemmli [15]. After SDS-PAGE, the proteins in the gel were renatured by three 30-min incubations in renaturation buffer (50 mM Tris-HCl buffer, pH 7.0, 10 mg/ml of casein, 2 mM EDTA, 0.01% NaN_3) containing 25% methanol. The polyacrylamide gel was then washed with 0.1 M

Table 1 Predicted protein-encoding genes in the metagenomic fragment AlyDW

ORF no.	Homologous to BLASTP	Organism/phylum or domain	Position	BLAST e-value	No. of TMHs ^a	COG no.	COG category
1	(NiFe) hydrogenase maturation protein hypF	<i>Ferrimonas balearical</i> Gammaproteobacteria	1,280...3,652	0.00E+00	0	O	COG0068
2	Hydrogenase accessory protein HypB	<i>Ferrimonas balearical</i> Gammaproteobacteria	5,025...6,128	5.00E−105	0	OK	COG0378
3	Hydrogenase expression/formation protein HypD	<i>Ferrimonas balearical</i> Gammaproteobacteria	6,365...7,492	2.00E−137	0	O	COG0409
4	Hypothetical protein	<i>Vibrio</i> sp./Gammaproteobacteria	8,687...9,202	1.00E−47	1		NA ^b
5	Hypothetical protein	<i>Vibrio</i> sp./Gammaproteobacteria	9,206...9,823	3.00E−258	1		NA
6	Hypothetical protein	<i>Vibrio coralliilyticus</i> / Gammaproteobacteria	9,823...11,250	1.00E−73	1		NA
7	Transcriptional regulator	<i>Vibrio parahaemolyticus</i> / Gammaproteobacteria	11,377...12,189	2.00E−79	0	K	COG0583
8	Hypothetical protein	<i>Photobacterium</i> sp./ Gammaproteobacteria	12,290...13,306	2.00E−61	1		NA
9	Multidrug resistance efflux pump	<i>Vibrio vulnificus</i> / Gammaproteobacteria	13,611...14,642	2.00E−166	2	Q	COG1566
10	Hypothetical protein	<i>Vibrio vulnificus</i> / Gammaproteobacteria	14,646...15,212	3.00E−55	3		NA
11	Alginate lyase = poly (β-D-mannuronate) lyase	<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i> /Gammaproteobacteria	15,417...16,388	6.00E−117	0		NA
12	Lyase, putative	<i>Vibrio splendidus</i> / Gammaproteobacteria	17,370...19,223	0.00E+00	0		NA
13	Alginate lyase	<i>Vibrio</i> sp./Gammaproteobacteria	19,452...21,017	0.00E+00	0		NA
14	Transcriptional regulator, LysR family	<i>Vibrio splendidus</i> / Gammaproteobacter	21,253...22,143	9.00E−122	0	K	COG0583
15	Probable oxidoreductase	<i>Vibrio</i> sp./Gammaproteobacteria	22,309...23,121	1.00E−95	0	QR	COG1028
16	Oxygen-independent coproporphyrinogen III oxidase, putative	<i>Vibrio splendidus</i> / Gammaproteobacter	23,333...24,208	6.00E−138	0	C	COG1032
17	Flavodoxin reductase family I protein	<i>Vibrio parahaemolyticus</i> / Gammaproteobacteria	24,294...24,989	4.00E−72	0	R	COG3217
18	Dihydroorotase	<i>Vibrio coralliilyticus</i> / Gammaproteobacteria	25,446...26,477	2.00E−171	0	F	COG0418
19	Glycerol kinase	<i>Vibrio cholera</i> / Gammaproteobacteria	26,549...28,063	0.00E+00	0	C	COG0554
20	Glucose-1-phosphate adenylyltransferase	<i>Vibrio shilonii</i> / Gammaproteobacteria	28,362...29,579	0.00E+00	0	G	COG0448
21	ABC transporter, periplasmic substrate-binding protein	Vibrionales bacterium/ Gammaproteobacteria	30,103...30,930	2.00E−88	0	P	COG0226
22	NodN-related protein	<i>Vibrio orientalis</i> / Gammaproteobacteria	31,010...31,696	6.00E−80	0	I	COG32030

TMH transmembrane helix

^a Based on TMHMM search

^b Not assigned to COG group

citrate–phosphate buffer (pH 7) and overlaid onto a 1.5% agarose gel containing 1 mg/ml sodium alginate, 10 mg/ml NaCl, and 0.1 M citrate–phosphate buffer (pH 7). The gel was incubated at 45°C for 20 h, flooded with an excess of 10% CPC solution for 1 h, and then washed with excess distilled water.

Analysis of reaction products by thin-layer chromatography

A cellooligosaccharides standard mixture (cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, celloheptaose, and cellooctaose) was purchased from

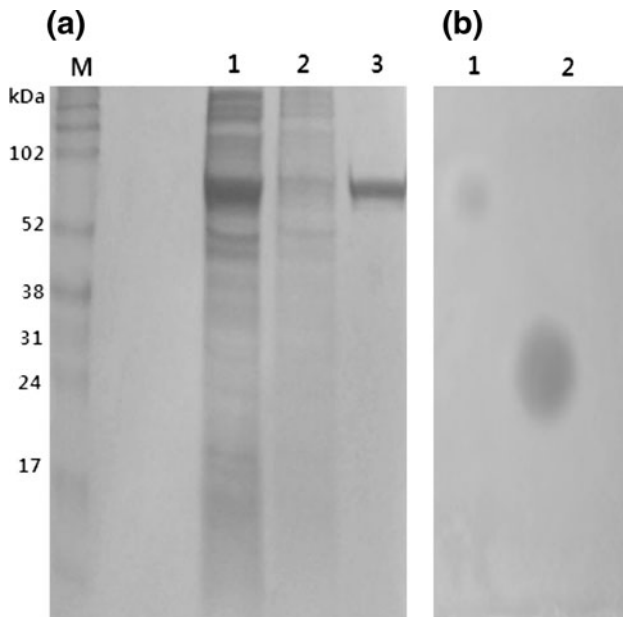


Fig. 2 SDS-PAGE analysis and zymogram activity staining of the metagenomic clone (AlyDW) and recombinant protein (AlyDW11). **a** 10% SDS-PAGE showing overexpression of recombinant pMAL-c2X-AlyDW11. *M* molecular weight markers, *1* total cell lysate from induced cells, *2* proteins obtained after washing step, *3* purified recombinant protein. **b** Results of zymogram activity staining of AlyDW11 (lane *1*) and alginate lyase obtained from *Flavobacterium* sp. (lane *2*) in agarose gel containing 0.1% alginate

Sigma-Aldrich. To determine if the test clone had alginate lyase activity, 0.1% alginate was digested with 50 μ l of recombinant alginate lyase (AlyDW11, 1.79 mg/ml) in 0.1 M citrate–phosphate buffer (pH 7) at 45°C. Subsequently, the reaction products were developed in a mixture of 1-propanol, nitromethane, and water (5:3:2, v/v/v) for 2 h. The hydrolysis products were then separated by thin-layer chromatography (TLC) on a silica gel plate (Merck KGaA, 64271 Darmstadt, Germany). Following separation, the sugars were visualized by spraying the plates with a mixture of 1 ml sulfuric acid and 10 ml stock solution (1 g diphenylamine, 1 ml aniline, 100 ml acetone) [32].

Nucleotide sequence accession number

The accession number of the AlyDW11 DNA sequence in GenBank is JN392921.

Results and discussion

Screening of alginate lyase gene (AlyDW11)

One bacterial metagenomic clone (AlyDW) displaying alginate lyase activity on alginate was selected from 3,840 clones after screening a total of 90,000 clones constructed

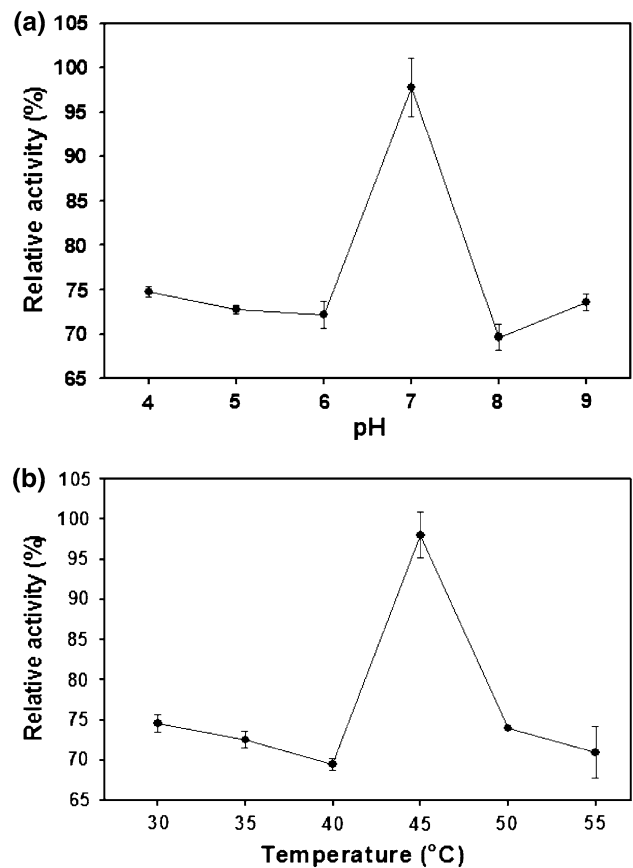


Fig. 3 Effect of pH **a** and temperature **b** of the recombinant protein (AlyDW11) on the alginate lyase activity in a reducing sugar assay. The pH (4.0–9.0) and temperature (30–55°C) were varied to determine the optimal conditions for enzyme activity. In addition, enzyme activity after incubation for 2 h in the following buffers was measured: 0.1 M citrate–phosphate buffer (pH 4.0–7.0), 10 mM phosphate buffer (pH 8.0), and 50 mM glycine–NaOH buffer (pH 9.0)

from the gut microbial flora of abalone. Shotgun sequencing data analysis of the metagenomic clone (AlyDW) revealed the presence of a 31.7-kbp insert, which had an average G+C content of 43.3% and contained 22 predicted ORFs (Fig. 1a; Table 1). Of these ORFs, 17 showed significant similarities to genes of known function in the NCBI nr database, whereas five were homologous to hypothetical proteins in the NCBI microbial genome database. There were six ORFs related to cellular processing and information storage and ten ORFs related to metabolism. Three ORFs (11–13) showed similarity to known protein sequences of the alginate lyase gene, which is found adjacent in the genome of *Klebsiella pneumoniae* subsp. *aerogenes*, *Vibrio splendidus*, and *Vibrio* sp. belonging to the phylum *Gammaproteobacteria*. Following zymogram active staining in 10% SDS-PAGE under renaturation conditions, the cell-free extracts of the metagenomic clone (AlyDW) only were shown to display alginate lyase activity in a clear band with a calculated molecular mass of

Table 2 Effect of metal cations on alginate lyase activity

Chemicals	Fold activity ^a
None	1.00 ± 0.05
EDTA (1 mM)	0.75 ± 0.05
CaCl ₂ (1 mM)	0.95 ± 0.03
MgCl ₂ (1 mM)	0.91 ± 0.03
KCl (1 mM)	0.92 ± 0.01
MgSO ₄ (1 mM)	0.93 ± 0.15
FeSO ₄ (1 mM)	0.90 ± 0.14
CoCl ₂ (1 mM)	1.08 ± 0.05
MnCl ₂ (1 mM)	0.85 ± 0.01
CuCl ₂ (1 mM)	1.01 ± 0.08
NaCl (1 mM)	0.96 ± 0.06
AgNO ₃ (1 mM)	1.31 ± 0.03

^a Fold activity was measured relative to the control reading as 1. These data are presented as the mean ± standard deviation of three experiments

35 kDa, which corresponded to alginate lyase coded by ORF 11 (data not shown). The CPC plate was loaded with 10 µl (2.0 mg/ml) of protein samples. The CPC plate assay in the cell-free extract of *E. coli* (negative control, B) did not show alginate lyase activity, whereas enzyme activity was observed in the cell-free extracts of *Flavobacterium* sp. (positive control, A, Sigma-Aldrich, MO, USA), metagenome clone (AlyDW, C), and recombinant protein (AlyDW11, D) coded by ORF 11 (Fig. 1b).

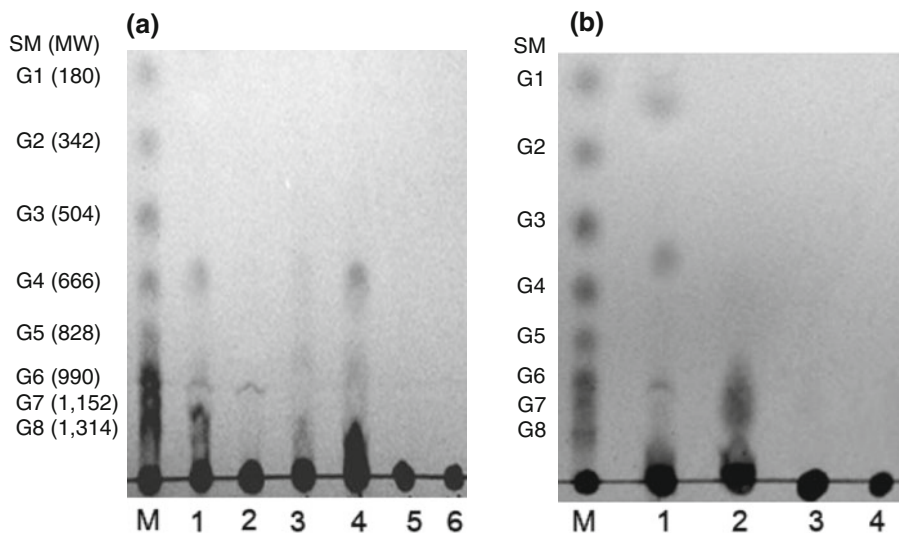


Fig. 4 TLC analysis of depolymerization of alginate by recombinant protein (AlyDW11). **a** Hydrolysis profiles of poly(β -D-mannuronate) (lane 1) and poly(α -L-guluronate) (lane 2) after 3 h of incubation at 45°C. Positive controls from *Flavobacterium* sp. [poly(β -D-mannuronate) (lane 3) and poly(α -L-guluronate) (lane 4)]; negative controls [poly(β -D-mannuronate) (lane 5) and poly(α -L-guluronate) (lane 6)]; SM (MW) standard mixture (molecular weight) of G1 glucose,

Fig. 5 Multiple amino acid sequence alignment of the AlyDW11 with putative alginate lyases of other bacterial strains. Amino acid sequences were aligned using the CLUSTAL_X program. The GenBank accession numbers of *Klebsiella pneumoniae* subsp. *aerogenes*, *Saccharophagus degradans* 2-40, *Microbulbifer* sp. 6532A, *Microbulbifer* sp. 6532A, *Vibrio harveyi* 1DA3, *Cellulophaga lytica* DSM 7489, *Vibrio* sp. A9m, *Vibrio alginolyticus* 40B, *Pseudoalteromonas* sp. CY2, *Vibrio splendidus* 12B01, *Polaribacter* sp. MED152, and *Agarivorans* sp. JAM-A1m are Q59478, ABD81738, BAJ62034, ZP_0617515, YP_004263738, BAH79133, ZP_06182095, ACM89454, ZP_00990010, ZP_01052859, and BAG70358, respectively

Characterization of AlyDW11

After subcloning ORF 11 into the pMAL-2cX expression vector and purifying the protein over an amylose resin, 30-µl aliquots (1.8 µg/µl) of the protein from the original total cell lysates, wash, and elution fraction were loaded onto a 10% SDS-PAGE and subjected to electrophoresis. The purified mannose binding protein fused alginate lyase (AlyDW11) was about 77.5 kDa (Fig. 2a). The alginate lyase activity of recombinant proteins was analyzed by SDS-PAGE under renaturation conditions and then subjected to activity staining. The alginate lyase activity of AlyDW11 was detected in a clear band with a molecular weight of approximately 77.5 kDa (Fig. 2b). The maximum alginolytic activity in the reducing sugar assay was observed at 45°C and pH 7 (Fig. 3). Approximately 70% residual activity was detected over all temperature (30–55°C) and

G2 cellubiose, G3 cellotriose, G4 cellotetraose, G5 cellopentaose, G6 cellohexaose, G7 celloheptaose, and G8 cellooctaose. **b** Hydrolysis profiles of alginate (lane 1) after 3 h of incubation at 45°C. Alginate lyase from *Flavobacterium* sp. was used as a positive control (lane 2); buffer and 0.1% sodium alginate (lane 3) or buffer and AlyDW11 (lane 4) were used as a negative controls

Metagenomic clone (AlyDW11)	1	10	20	30	40	50
<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i>	F D L L G W T V S V P	-- I D S D G D	-- G K S D Q I K E K E L A A G Y S D A N Y F Y L A E D	-- G G M		
<i>Saccharophagus degradans</i> 2-40	F E L S G W S L S V P	-- V D S D N D	-- G K A D Q I K E K T L A A G Y R N S D F F T L S D A	-- G G M		
<i>Microbulbifer</i> sp. 6532A	F D L W P W Y L S V P	-- T D T D G S	-- G T A D S I K E S D L N A G Y E S S E F F Y T A A D	-- G G M		
<i>Vibrio harveyi</i> 1DA3	F D L A A W Y L S V P	-- T D D D G N	-- G R A D S I Y E A E L N S G Y G N S N Y F Y T G E D	-- G G M		
<i>Cellulophaga lytica</i> DSM 7489	F D L L D W K L D L P	-- V D D N G N A S G D A Q E V K E D E L S S G F E N S E F F Y T G D D	-- G G L			
<i>Vibrio</i> sp. A9m	F N L A T W N I S I P	-- I D E D N N	-- N K A D T V K E N E L N N G F T N D N Y F Y T A A D	-- G G M		
<i>Vibrio alginolyticus</i> 40B	F D L T H W Y L S Q P	-- F D H D K N	-- G K P D D V S E W N L A N G Y Q H P E I F Y T A D D	-- G G L		
<i>Pseudoalteromonas</i> sp. CY2	F D L T R W K L T T P	-- F D H D K D	-- G R A D D I D E W D L A N G F Q H S D I F Y T A D D	-- G G M		
<i>Vibrio splendidus</i> 12B01	F D L S Y W Y L S Q P	-- F D H D K N	-- E R P D D V S E W N L A N G Y Q H P E I F Y T A D D	-- G G L		
<i>Polaribacter</i> sp. MED152	F D M T H W Y L S Q P	-- F D H D K N	-- G K P D D V S E W N L A N G Y Q H P E I F Y T A D D	-- G G L		
<i>Agarivorans</i> sp. JAM-A1m	F D L S T W K L N T P	-- E N N G S	-- G F S K T I S V S E I N G G Y E D D N Y F Y T G T D	-- G G L		
	L D L S C W K V T L P	V S L D N G	-- D R P T E F S E K E I A K G A M H E D Y F Y V N E A K D G V			
Metagenomic clone (AlyDW11)	51	60	70	80	90	100
<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i>	V F K A P V K G A K T S K N T T Y T R S	E L R E M I R R G N T Q Y K T	K G V G G N N W F S T A P Q			
<i>Saccharophagus degradans</i> 2-40	V F K A P I S G A K T S K N T T Y T R S	E L R E M L R K G D T S I A T	Q G V S R N N W L S S A P L			
<i>Microbulbifer</i> sp. 6532A	V F K C P V A G F K T S T N T S Y T R V L	R E M L R R G N S I S T	Q G V N G N N W F G S A P Q			
<i>Vibrio harveyi</i> 1DA3	V F R C P I A G F K T S T N T S Y T R E L	R G M L R R G D T S I S T	Q G V N K N N W F S S A P I			
<i>Cellulophaga lytica</i> DSM 7489	V F I S P V E G A T T S A N T K Y T R S E M	R E M L R R G D T S I S T	T G I T K N N W F F A S A P S			
<i>Vibrio</i> sp. A9m	V F K C P I D G F K T S E N T K Y T R T E L	R E M L R A G N T N I D T	Q G V T K N N W F G S A P Q			
<i>Vibrio alginolyticus</i> 40B	V F K S Y V K G V R T S K N T K Y A R T E L	R E M M R R G D Q S I S T	K G V N K N N W F S S A P E			
<i>Pseudoalteromonas</i> sp. CY2	V F K S Y V K G A R T S E N T K Y A R T E L	R T M L R A G D K S Y S T	K G V N P N N W F S S A P V			
<i>Vibrio splendidus</i> 12B01	V F K S Y V K G V R T S K N T K Y A R T E L	R E M L R R G N M S H K T	K G V N K N N W F S S A P E			
<i>Polaribacter</i> sp. MED152	V F K A Y V K G V R T S K N T K Y A R T E L	R E M M R R G D Q S I S T	K G V N K N N W F S S A P E			
<i>Agarivorans</i> sp. JAM-A1m	V F K C P V E G F K T S A N T S Y T R V L	R E M L R G T D T S I P T	Q G V N K N N W F G S S P T			
	V F R S P I Q G I K T S K N T K Y V R S E L	R E M M R C G D T S H K T	K G V N G N N W F S H N E S			
Metagenomic clone (AlyDW11)	110	120	130	140	150	
<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i>	A D -- Q K V A G G V D G T L E A T L A V N H V	T T T G K N W --	Q V G R V I I G Q I H S N N N E P			
<i>Saccharophagus degradans</i> 2-40	S E -- Q K K A G G V D G T L E A T L S V D H V	T T T G V N W --	Q V G R V I I G Q I H A N N D E P			
<i>Microbulbifer</i> sp. 6532A	S D -- L N A A G G I D G L H A T L A V N K V	T T T H G D G F E Y Q V G R V I I G Q I H A N D D E P				
<i>Vibrio harveyi</i> 1DA3	A A -- R E A A G G V D G V L R A T L A V N H V	T T T G D S G --	Q T G R V I V G Q I H A N D D E P			
<i>Cellulophaga lytica</i> DSM 7489	D D -- Q N N S G G V D G V L E A T L A V N A V	T T T G D S S --	Q V G R V I V G Q I H A N N D E P			
<i>Vibrio</i> sp. A9m	Q D -- I T N A G G Y D G L H A T L A V N H V	T T T G D D D --	Q V G R V I I G Q I H A N D D E P			
<i>Vibrio alginolyticus</i> 40B	A D -- L K A A A G I D G V L E A T L K I D H A	T T T G N A N --	E V G R F I I G Q I H D Q N D E P			
<i>Pseudoalteromonas</i> sp. CY2	E D -- Q K E A G G V D G T L E A T L K I D H A	T T T G Q S H --	E V G R F I I G Q I H D K D N D E P			
<i>Vibrio splendidus</i> 12B01	A D -- L K A A G G I D G V L E A T L K I D H A	T T T G A A N --	E V G R F I I G Q I H D Q N D E P			
<i>Polaribacter</i> sp. MED152	S D -- L E S A A G I D G V L E A T L K I D H A	T T T G N A N --	E V G R F I I G Q I H D Q N D E P			
<i>Agarivorans</i> sp. JAM-A1m	S D -- K N A A A G F D G E M N V T L A V N H V	T T T G S S S --	H V G R V I I G Q I H A N D D E P			
	A E S L K N A A G V D G N L K A T L S V D H V	T S T G E I W --	Q G R V I I G Q I H A P D D E P			
Metagenomic clone (AlyDW11)	160	170	180	190	200	
<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i>	I R L Y Y R K L P Q N E T G S I Y F A H E P R K	-- A F G K E T W H N I I G N S L P D Y W H Q D A T				
<i>Saccharophagus degradans</i> 2-40	I R L Y Y R K L P H H Q K G S V Y F A H E P R K	-- G F G D E Q W Y E M I G T L Q P S H G N Q T A A				
<i>Microbulbifer</i> sp. 6532A	I R L Y Y R K L P S N S K G S I Y F A H E L	-- -- L D G D D T W H E M I G S R G -- -- -- D N A S				
<i>Vibrio harveyi</i> 1DA3	L R L Y Y R K L P D N S K G S I Y I A H E I	-- -- K G G D D T W Y E M I G S R S -- -- -- S S A S				
<i>Cellulophaga lytica</i> DSM 7489	I R L Y Y R L L P G H T K G S L Y F A H E P N E D A S S D P E Q F I N L I G S S A	-- -- -- S N A S				
<i>Vibrio</i> sp. A9m	L R I Y Y R K L P N N T L G S I Y I A H E	-- -- N A A A E T Y Y D L I G S R S -- -- -- K S A A				
<i>Vibrio alginolyticus</i> 40B	I R L Y Y R K L P N Q P T G A V Y F A H E S Q D	-- -- A T K E D F Y P L V G D L T -- -- -- A E V G				
<i>Pseudoalteromonas</i> sp. CY2	I R L Y Y R K L P D Q P T G T V Y F A H E K T K	-- -- T G T E D Y Y S L V G D M T -- -- -- G E I G				
<i>Vibrio splendidus</i> 12B01	I R L Y Y R K L P N Q A T G A V Y F A H E S Q D	-- -- A T K E D F Y P L V G D L T -- -- -- A E V G				
<i>Polaribacter</i> sp. MED152	I R L Y Y R K L P N Q E T G A V Y F A H E S Q D	-- -- A T K E D F Y P L V G D M T -- -- -- A E V G				
<i>Agarivorans</i> sp. JAM-A1m	V R L Y Y R K L P N N T L G A I Y F A H E P R D	-- -- G F G S E Q W Y D M I G S R S -- -- -- N S A A				
	V K I Y Y R K L P N H E T G S V W I S H E P N	-- -- G G D D I F P M I G P T K P N Y W K Q K D K				
Metagenomic clone (AlyDW11)	210	220	230	240	250	
<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i>	P T -- E P T D G I K L N E K F S Y R I Q V K G N E L S V T	I M R D G K K D L V K T V D M S E S G F				
<i>Saccharophagus degradans</i> 2-40	P T -- E P E A G I A L G E T F S Y R I D A T G N K L T V T L M R E G R P D V V K T V D M S K S G Y					
<i>Microbulbifer</i> sp. 6532A	--- P A D G I A L D E T F S Y E I D V R G N T L T V T I M R E G K P D V T K V L D M S A S G Y					
<i>Vibrio harveyi</i> 1DA3	--- N P E D G I A L N E I F S Y E I R V E G N T L T V T I F R E G K D D V I Q M V D M S E S G Y					
<i>Cellulophaga lytica</i> DSM 7489	--- E P E D G I A L N E L F F Y R I E V Q G N Q L I V T I K R D D H E D V T E V D M T T S G Y					
<i>Vibrio</i> sp. A9m	--- P E D G I A L D E K F S Y S V K V T G N N M W V T I N R D G K D A I E Q F V D M T N S G Y					
<i>Vibrio alginolyticus</i> 40B	--- E D G I A L G E K F S Y R I E V K G N T M T V S L M R E G H D V V Q V V D M S D S G Y					
<i>Pseudoalteromonas</i> sp. CY2	--- D D G I A L G E K F S Y I I D V K G N T M T V T V K R D G K D V V V Q V V D M S D S G Y					
<i>Vibrio splendidus</i> 12B01	--- E D G I A L G E V F S Y R I E V V G N T M T V T V S R E G K E D A V Q V V D M S D S G Y					
<i>Polaribacter</i> sp. MED152	--- D D G I A L G E V F S Y R I D V K G N T M T V T L I R E G K D D V V Q V V D M S N S G Y					
<i>Agarivorans</i> sp. JAM-A1m	--- N P T D G I P L D E K F S Y Q I K T V N N T L T V T I S R E G K P D V V Q V V D M S N S G F					
	D I P K S Y N D G I A L G E K F S Y E I D I N G S D M T V T I S R E G K A D V V Q K V D M Q N S G Y					
Metagenomic clone (AlyDW11)	260	270	280	275		
<i>Klebsiella pneumoniae</i> subsp. <i>aerogenes</i>	G E G G Q Y M Y F K A G V Y N Q N K T G	D A D D Y A Q A T F Y K L N V T H	275			
<i>Saccharophagus degradans</i> 2-40	S E A G Q Y L Y F K A G V Y N Q N K T G	K P D D Y V Q A T F Y R L K A T H	275			
<i>Microbulbifer</i> sp. 6532A	D E G G Q Y M Y F K A G V Y N Q N N S G	P D D D Y V Q A T F Y A L E A T H	269			
<i>Vibrio harveyi</i> 1DA3	D T E D Q Y M Y F K A G V Y N Q N N S G	D S D Y V Q A T F Y A L E N S H	266			
<i>Cellulophaga lytica</i> DSM 7489	D V G G Q Y M Y F K A G V Y N Q N S G	D P T D Y V Q A T F Y L T N S H	272			
<i>Vibrio</i> sp. A9m	D V G G Q Y M Y F K A G V Y N Q N T G	T A T D Y V Q A T F Y S L E S G H	266			
<i>Vibrio alginolyticus</i> 40B	D V G G K Y M Y F K A G V Y N Q N I S G	D L D D Y S Q A T F Y Q L D V S H	265			
<i>Pseudoalteromonas</i> sp. CY2	D E G G R Y M Y F K A G V Y N Q M Y G	N P D D Y A Q A T F Y K L K --	262			
<i>Vibrio splendidus</i> 12B01	D V G G K Y M Y F K A G V Y N Q N I S G	D L D D Y S Q A T F Y K L K A T H	265			
<i>Polaribacter</i> sp. MED152	D A G G K Y M Y F K A G V Y N Q N I S G	D L D D Y S Q A T F Y Q L D V S H	265			
<i>Agarivorans</i> sp. JAM-A1m	D Q T G Q Y Q Y F K A G L Y H V N N S G	A A T D Y A Q A T F Y Q I E K S H	267			
	G L K D Q W M Y F K A G V Y N Q N R S	G N P E D Y V Q A T F Y S L D V N H	277			

pH ranges (pH 4–6 and pH 8–9) tested. The effects of metal ions on the activity of alginate lyase are summarized in Table 2. These experiments revealed that silver ions increase the alginate lysase activity by 1.3-fold relative to the control without metal cation, whereas EDTA inhibited alginate lyase activity by about 25%. The reaction rate of this enzyme was $2.35 \mu\text{M min}^{-1} \text{mg}^{-1}$ protein under the optimum conditions. The alginate lyases from marine mollusks including *Haliotis* spp., *Littorin* spp., and *Turbo cornutus* are endo-poly(M) and exo-poly(G) lyases, which were shown to have optimal pH values ranging from 4.0 to 9.6 and temperatures ranging from 25 to 50°C in the presence of divalent cations such as Ca^{2+} or Mg^{2+} [16, 22, 32, 33].

Different profiles of alginate oligomers were obtained by digestion of alginate with an alginate lyase (AlyDW11). The enzymatic degradation products of alginate by AlyDW11 were determined by TLC after 3 h of incubation (Fig. 4). Like most of the alginate lyases from marine mollusks [33], this enzyme had endolytic activity and preferred poly(β -D-mannuronate) over poly(α -L-guluronate) (Fig. 4a). This enzyme degraded alginate into a mixture of products with low molecular weights ($\leq 1,000$ Da), whereas the commercial enzyme originated from *Flavobacterium* sp. preferred poly(α -L-guluronate) over poly(β -D-mannuronate) and degraded alginate into higher molecular weight products (1,152–1,314 Da) (Fig. 4b). This indicates that AlyDW may be a useful enzyme for producing lower molecular weight alginate products than the commercial enzyme.

The homologies of the deduced amino acid sequence of the metagenomic enzyme (AlyDW11) with the Sde_2478 (*Saccharophagus degradans* 2-40) and alyA (*Klebsiella pneumoniae* subsp. *aerogenes*) genes were 63 and 65%, respectively, whereas those of the algMsp (*Microbulbifer* sp. 6532A), VME_15370 (*Vibrio harveyi* 1DA3), Celly_3050 (*Cellulophaga lytica* DSM 7489), alg (*Vibrio* sp. A9 m), VMC_35250 (*Vibrio alginolyticus* 40B), alyPI (*Pseudoalteromonas* sp. CY2), V12B01_24259 (*Vibrio splendidus* 12B01), MED152_06195 (*Polaribacter* sp. MED152), and AlgL (*Agarivorans* sp. JAM-A1) genes were between 54 and 60% (Fig. 5). Polysaccharide lyase (PL) family 7 alginate lyases contained three highly conserved amino acid sequences, (R/E)(S/T/N)EL, Q(I/V)H, and YFKAG(V/I)YNQ, which were believed to act as substrate binding and catalytic sites [23]. The enzyme (AlyDW11) possessed RSEL, QIH, and YFKAGVYNQ, which have also been identified in the G- and M-specific lyases (AlyA) of *K. pneumoniae*, M-specific lyase (AlxM) of marine bacterium ATCC 433367, and G-specific lyase (ALY-1) of *Corynebacterium* sp. These amino acid sequences are considered essential to maintaining the stable

three-dimensional conformation and function of alginate lyases [19, 20].

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