International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Characterization of a new endo-type alginate lyase from *Vibrio* sp. W13

Benwei Zhu[a,b], Haidong Tan[a], Yuqi Qin[c], Qingsong Xu[a], Yuguang Du[a,d,*], Heng Yin[a,*]

[a] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] National Glycoengineering Research Center, Shandong University, Jinan 250100, PR China
[d] Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, PR China

ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form 14 January 2015
Accepted 16 January 2015
Available online xxx

Keywords:
Alginate lyase
Polysaccharide lyase family 7
Vibrio sp.

ABSTRACT

A gene, encoding a new alginate lyase Algb, was identified and cloned from marine bacterium *Vibrio* sp. W13. The recombinant alginate lyase was characterized followed by being purified on Ni-NTA Sepharose. It exhibited the highest activity (457 U/mg) at pH 8.0 and 30 °C. Interestingly, Algb possessed broader substrate specificity. It showed activities toward both polyM (poly β-D-mannuronate) and polyG (poly α-L-guluronate). Furthermore, K_m values of Algb toward alginate (0.67 mg/ml) and polyMG (0.50 mg/ml) are lower than those toward polyG (1.04 mg/ml) and polyM (6.90 mg/ml). The TLC and ESI-MS analysis suggested that Algb mainly released oligosaccharides with DP of 2–5 from the four kinds of substrates in an endolytic manner. Therefore, it may be a potent tool to produce alginate oligosaccharides with low DP.

1. Introduction

Alginate is the most abundant polysaccharide of brown algae, which consists of β-D-mannuronate (M) and α-L-guluronate (G) as monomeric units [1]. These units are linked in three kinds of different blocks, poly β-D-mannuronate (polyM), poly α-L-guluronate (polyG) and the heteropolymer (polyMG) [1]. Alginate oligosaccharides are depolymerization products of alginate by alginate lyase or physicochemical method. They have attracted increasing attention due to their wide applications in food and pharmaceutical industry. They can be used as growth promoters for plants and therapeutic agents such as anticoagulants and tumor inhibitors [2–4]. They can also induce the cytokine production and regulate the blood sugar as well as lipid [5]. Therefore, alginate lyases for mild degradation have been the focus for various fields.

Alginate lyase can degrade alginate by β-elimination of glycosidic bonds and produce unsaturated oligosaccharides with double bonds at the non-reducing end [6]. A number of alginate lyases have been identified, cloned their genes, purified and characterized from various sources [7–10], including marine and terrestrial bacteria, marine mollusks and algae. They can be classed into two groups due to their substrate specificities [6], one is G block-specific lyase (polyG lyase, EC4.2.2.11), and the other is M block-specific lyase (polyM lyase, EC4.2.2.3). This classification has been widely accepted, but some enzymes show activities toward both polyM and polyG [11–15], which may degrade alginate more effectively. In terms of the mode of action, alginate lyase can be grouped into endolytic and exolytic alginate lyase [6]. Endolytic alginate lyase cleaves glycosidic bonds inside alginate polymer and releases unsaturated oligosaccharides (di-, tri-, and tetra-saccharides) as main products [16], while exolytic alginate lyase can further degrade oligosaccharides into monomers [17–19]. Now alginate lyases, especially endolytic alginates, have been widely used in the production of alginate oligosaccharides [20], the elucidation of the fine structures of alginate [21] and the preparation of protoplast of red and brown algae [22,23]. Furthermore, alginate lyase also shows great potential application in treatment of cystic fibrosis by degrading the polysaccharide biofilm of bacterium [24–26]. Therefore, considering the poor sources, narrow substrate specificity and low activity of alginate lyase, it is in great urgency to obtain alginate lyase with high-activity and broad substrate specificity.

In this work, a new alginate lyase with broad substrate specificity has been identified and characterized from marine bacteria *Vibrio* sp. W13 by using PCR with homologous nucleotide sequences-based primers. The kinetics and analysis of degrading products were also characterized, suggesting that it would be potential candidate for expanding application of alginate lyases.

* Corresponding authors at: Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, PR China. Tel.: +86 411 84379061; fax: +86 411 84379061.
E-mail addresses: duyg@dicp.ac.cn (Y. Du), yinheng@dicp.ac.cn (H. Yin).

http://dx.doi.org/10.1016/j.ijbiomac.2015.01.053
0141-8130/

2. Materials and methods

2.1. Materials

The strain *Vibrio* sp.W13 was isolated and conserved in our laboratory [27]. Sodium alginate (*Macrosystis pyrifera* origin, M/G ratio 77/23) was purchased from Sigma–Aldrich (St. Louis, MO, USA). PolyM (M/G ratio 97.3/2.7, purity: about 99%) and polyG (M/G ratio 1.8/98.2, purity: about 99%) were purchased from Qingdao BZ Oligo Biotech Co., Ltd (Qingdao, China). PolyMG (M/G ratio 37/63) was donated by Biomedical engineering group of our institute. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were used as the cloning host and the protein expression host, respectively.

2.2. Cloning of alginate lyase gene algb

The genomic DNA of *Vibrio* sp. W13 was obtained by using a commercial DNA purification kit (Thermo Scientific, Beijing, China).

The strain *Vibrio* sp. W13 (accession number: KC777293) was identified to be close to *Vibrio alginolyticus* based on 16S rDNA analysis with identity of 99% [27]. And the genome of *V. alginolyticus* NBRC15630 could be found in NCBI Genome Project (http://www.ncbi.nlm.nih.gov/genome/). Five putative alginate lyase genes were found in a cluster for alginate degradation. Therefore, we could design PCR primers based on these genes to clone the alginate genes. The primers for cloning algb were designed based on the homolog sequence of putative alginate lyase gene sequence (hypothetical protein N646_4461) from *V. alginolyticus* NBRC15630 (NC_022359.1). The forward primer was designed as 5′-ATGCGCTCAGAAGTTCGTGA-3′ and the reverse primer was designed as 5′-CTATTGATGAAGAGTGCTCA-3′. The PCR products were purified and ligated to pEASY-blunt cloning vector (TransGen Biotech, Beijing, China). The nucleotide sequence was sequenced by BGI (Beijing, China) and analyzed with Vector-NTI program, and the open reading frame was searched by using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/). The signal peptide cleavage site was predicted using SignalP 4.1 server.

2.3. Expression and purification of the recombinant alginate lyase Algb

In order to subclone the alginate lyase gene into expression vector, the forward primer (5′-CGCGGATCCATGCGCTCAGAAGTTCGTGA-3′) containing restriction site *BamH* I and the reverse primer (5′-CCGCTCGAGTTGATGAAGAGTGCTCAAAG-3′) containing restriction site *Xho* I were designed without stop codon. The alginate lyase gene was subcloned and ligated into pET-21a (+) expression vector. The recombinant *E. coli* BL21 (DE3) harboring the pET-21a (+)/*algb* was cultured in an LB medium supplemented with 100 μg ampicillin/ml for 2–3 h with shaking at 200 rpm and 37 °C up to an OD_{600} of 0.4–0.6. The cells were induced by addition of 0.3 mM IPTG at 20 °C for 20 h. The cells were then harvested and sonicated in lysis buffer (50 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 10 mM imidazole). The cell homogenate containing the recombinant protein was loaded on Ni-NTA Sepharose column (GE Healthcare, Uppsala, Sweden) equilibrated with lysis buffer. The column was washed with wash buffer (50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole), and the recombinant alginate lyase was eluted with elution buffer (50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 300 mM imidazole). The active fraction was desalted using HiTrap™ desalting column (Amersham Biosciences, Buckinghamshire, UK) and analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Enzymatic activity assay of the recombinant enzyme

Purified enzyme (0.1 ml) was mixed with 0.9 ml Tris–HCl (20 mM, pH 8.0, 1% sodium alginate) and incubated at 30 °C for 10 min. The reaction was stopped by heating in boiling water for 10 min. The enzyme activity was then assayed by measuring the increased absorbance at 235 nm due to the formation of double bond between C4 and C5 at the non-reducing terminus by β-elimination. One unit was defined as the amount of enzyme required to increase the absorbance at 235 nm by 0.1 per min. The protein concentrations were determined with a protein quantitative analysis kit (Beyotime Institute of Biotechnology, Nantong, China).

2.5. Substrate specificity and kinetic measurement of alginate lyase

The purified enzyme was reacted with 1% of sodium alginate, polyMG, polyM and polyG, respectively. The assay of enzyme activity was defined as described previously.

The kinetic parameters of the purified enzyme toward these four kinds of substrates were determined by measuring the enzyme activity with substrates at different concentrations (0.2–6.0 mg/ml). The K_m and V_{max} values were calculated by double-reciprocal plots of Lineweaver and Burk.

2.6. Effects of temperature and pH on alginate lyase activity and stability

The effects of pH on the enzyme activity were evaluated by incubating the purified enzyme in buffers with different pH (4.0–11.0) under the assay conditions described previously. The pH stability depended on the residual activity after the enzyme was incubated in buffers with different pH (2.0–11.0) for 24 h. Meanwhile, the effects of temperature (20–80 °C) on purified enzyme were investigated at pH 7.0. The thermal stability of the enzyme was determined under the assay conditions described previously after incubating the purified enzyme at 20–80 °C for 30 min.

2.7. Effects of NaCl and metal ions

The effects of NaCl on enzyme activity were measured in buffers with different concentrations of NaCl (100–900 mM) under standard test conditions. The enzyme activity without NaCl served as control. The influence of metal ions on the activity of the enzyme were performed by incubating the purified enzyme at 4 °C for 24 h in the presence of various metal compounds at a concentration of 1 mM. Then the activity was measured under standard test conditions. The reaction mixture without any metal ion was taken as control.

2.8. TLC and ESI-MS analysis of the degradation products of the alginate lyase

The reaction mixtures (800 μl) containing 1 μg purified enzyme and 2 mg substrates (sodium alginate, polyMG, polyM or polyG) were incubated at 30 °C for 0–72 h. After incubation, the mixture solutions were boiled for 10 min and then centrifuged at 12,000 rpm for 10 min to remove the unsolved materials. The hydrolysates were loaded onto a carbograph column (Alltech, Grace Davison Discovery Sciences, United Kingdom) to remove salts after removing proteins, and then concentrated, dried and re-dissolved in 1 ml methanol.

The degradation products were firstly analyzed by TLC plate (TLC silica gel 60 F_{254}, Merck KGaA, Darmstadt, Germany) with the solvent system (1-butanol/acetic acid/water 3:2:3) and visualized by

Please cite this article in press as: B. Zhu, et al., Int. J. Biol. Macromol. (2015), http://dx.doi.org/10.1016/j.ijbiomac.2015.01.053