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Purification and characterisation of a bifunctional alginate lyase from novel *Isoptericola halotolerans* CGMCC 5336

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ABSTRACT

A novel halophilic alginate-degrading microorganism was isolated from rotten seaweed and identified as *Isoptericola halotolerans* CGMCC5336. The lyase from the strain was purified to homogeneity by combining of ammonium sulfate fractionation and anion-exchange chromatography with a specific activity of 8409.19 U/ml and a recovery of 25.07%. This enzyme was a monomer with a molecular mass of approximately 28 kDa. The optimal temperature and pH were 50 °C and pH 7.0, respectively. The lyase maintained stability at neutral pH (7.0–8.0) and temperatures below 50 °C. Metal ions including Na^+, Mg^{2+}, Mn^{2+}, and Ca^{2+} notably increased the activity of the enzyme. With sodium alginate as the substrate, the K_m and V_{max} were 0.26 mg/ml and 1.31 mg/ml min, respectively. The alginate lyase had substrate specificity for polyguluronate and polymannuronate units in alginate molecules, indicating its bifunctionality. These excellent characteristics demonstrated the potential applications in alginate oligosaccharides production with low polymerisation degrees.

1. Introduction

Alginate is a linear acidic polysaccharide consisting of 1,4-linked β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G). These uronate residues are randomly arranged as homopolymeric G blocks [poly (G)] and homopolymeric M blocks [poly (M)], and heteropolymeric MG blocks [poly (MG)] (Gacesa, 1992). Alginate oligosaccharides are depolymerization products of alginate by alginate lyases or physicochemical method. They have attracted considerable attention because of their applications in the food industry as growth promoters for plants (Hu, Jiang, Hueymin, Liu, & Guan, 2004) and as therapeutic agents [i.e., anticoagulants, tumour inhibitors and suppression of IgE (An et al., 2009; Iwamoto et al., 2005; Tusi, Khalaj, Ashabi, Kiaei, & Khodagholi, 2011)]. They are also involved in the reduction of blood sugar and lipids (Zhang, Zhou, Jia, Zhang, & Gu, 2004). Therefore, alginate lyases for mild degradation have been the focus of different research groups.

Alginate lyases can catalyse alginate degradation through the β-elimination mechanism, with unsaturated uronic acid products at the non-reducing terminus (Linhardt, Galliher, & Cooney, 1987). Based on the substrate specificities, alginate lyases are classified into mannuronate lyase (EC 4.2.2.3) and guluronate lyase (EC 4.2.2.11) that preferentially break up the M- and G-rich alginates, respectively. Alginate lyases specific to G or M blocks are monofunctional, whereas those specific to MG blocks are bifunctional (Tondervik et al., 2010).

So far, alginate lyases have been widely isolated and studied from various sources including marine molluscs (Sim et al., 2012; Suzuki, Suzuki, Inoue, & Ojima, 2006), seaweeds (An et al., 2008), marine bacteria (Kobayashi, Uchimura, Miyazaki, Nogi, & Horikoshi, 2009; Wakabayashi et al., 2012), marine fungi (Schaumann & Weide, 1990), some bacteriophages (Bartell, Orr, & Lam, 1966) and viruses (Suda, Tanji, Hori, & Unno, 1999). They have been used not only in the production of alginate oligosaccharides, but also in the elucidation of alginate fine structures, cell wall development of *Fucus* (Matsubara, Kawada, Iwasaki, Oda, & Muramatsu) and protoplast production of red and brown algae (Inoue, Mashino, Kodama, & Ojima, 2011). Recent investigations have illustrated the potential application of alginate lyases in cystic fibrosis management in conjugation with other chemotherapeutics (Li, Dong, et al., 2011; Li, Guan, Jiang, & Hao, 2011; Li, Jiang, Guan, Wang, & Guo, 2011) and seaweed waste management (Xiao, Han, Yang, Lu, & Yu, 2006).

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Present studies on alginate lyase are still being developed. Poor sources, narrow substrate specificity and low enzyme activity limit the generation of novel alginate oligosaccharides. Therefore, various isolates with high enzyme activity and wide substrate specificity should be screened to expand the potential applications of alginate lyases.

In this paper, a new alginate lyase-producing strain was isolated and identified as *Isoptericola halotolerans* CGMCC 5336. After the enzyme was purified, its characteristics in terms of enzyme activity were analysed under different temperature and pH conditions. This study is the first to report on a bifunctional alginate lyase derived from the genus *Isoptericola*.

2. Materials and methods

2.1. Microorganism

The alginate lyase-producing bacterial strain used in this study was isolated from decomposed seaweed via an enrichment procedure. Rotting seaweed samples were collected from an industrial production area of sodium alginate in Lianyungang City, China.

2.2. Media and culture conditions

All chemicals were of reagent grade. Sodium alginate minimal medium (Li, Dong, et al., 2011; Li, Guan, et al., 2011; Li, Jiang, et al., 2011) containing 5.0 g/l sodium alginate, 5.0 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 30.0 g/l NaCl, 1.0 g/l $MgSO_4{\cdot}7H_2O$ and 0.01 g/l $FeSO_4{\cdot}7H_2O$ at pH 7.0 was used for the bacterium isolation. An optimised fermentation medium for growth enrichment was composed of 6.0 g/l sodium alginate, 5.0 g/l tryptone, 2.5 g/l yeast extract, 25.0 g/l NaCl, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM KH_2PO_4, 0.2 mM $FeSO_4$, and 0.3 mM $MnSO_4$ at pH 8.0. Solid medium was prepared by adding 20 g/l of agar to the above medium. Bacteria were aerobically cultured in the 250 ml shake flasks containing 20 ml liquid medium at 25 °C and 200 rpm, which was a result of optimisation.

2.3. Strain isolation and identification

The preliminary screening for the alginate-degrading microorganism was conducted as follows: A 1 g sample of decomposed seaweed sample was suspended in 10 ml minimal medium and 1 ml suspension samples were transferred in a test tube with 10 ml minimal medium for enrichment. The inoculated test tubes were cultivated at 25 °C for 48 h with continuous shaking at 200 rpm. Microorganisms that could grow on a minimal medium using sodium alginate as the sole carbon source were accumulated by subculturing, which was repeated more than thrice every two days. The accumulated cultures were subsequently diluted and spread on minimal medium plates containing 2% agar for isolation until pure cultures were obtained.

The re-screening process was conducted as follows. Strains with clear hydrolytic zones were selected and incubated aerobically in a fermentation medium under the same conditions as above. Furthermore, the activity of alginate lyase was determined by 3,5-dinitrosalicylic acid (DNS) colorimetry (Preiss & Ashwell, 1962). Among the isolates, the most active strain WX was selected for further studies.

Bacterial identification was performed based on 16S rDNA gene sequence, which was amplified using polymerase chain reaction (PCR) with the following universal primers: GAGAGTTTGATCCTGGCT CAG (P_0) and CTACGGCTACCTTGTTACGA (P_6) (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR conditions were as following: initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation (45 s, 95 °C), annealing (45 s, 56 °C), extension (60 s, 72 °C) and final extension of 72 °C for 10 min. Moreover, 16S rDNA was sequenced by Sangon Biotech (Shanghai) and compared using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information databases (NCBI) and registered at the GeneBank database.

2.4. Enzyme purification

All the purification procedures were carried out at 4 °C.

Step 1: Ammonium sulfate precipitation

A 1 L aliquot of the supernatant separated from bacteria cells were obtained via centrifugation at 6000 rpm for 30 min. Solid ammonium sulfate was added to the crude enzyme solution to obtain 50% to 70% saturation. The resultant precipitate was centrifuged at 6000 rpm for 30 min, dissolved in 50 ml sodium phosphate buffer (PB, pH 7.0) and then dialyzed overnight against a large volume of the same buffer.

Step 2: Anion-exchange chromatography

The crude enzyme solution from step 1 was filtered through a 0.45 μm filter membrane to remove any undissolved substances. The concentrated crude enzyme solution was then applied to an anion-exchange column of Q-Sepharose Fast Flow (2.6 cm × 30 cm, Pharmacia), which had been equilibrated with 50 mM PB (pH 7.0) in advance. The absorbed proteins were eluted with a gradient elution at 0.3 M NaCl followed by a linear gradient of 0.3 M to 1 M NaCl at a low flow rate of 1 ml/min. The eluted fractions were collected every 5 ml and assayed for the enzyme activity. Main active fractions were pooled and lyophilised. The dried powder dissolved in a certain PB (pH 7.0) was used as a purified enzyme for further experiments.

2.5. Assay for alginate lyase activity and protein concentration

Alginate lyase activity was determined through 3,5-dinitrosalicylic acid (DNS) colorimetry. Briefly, 1 ml of the reaction mixture containing 900 μl of 1% sodium alginate (dissolved in 50 mM PB at pH 7.0) and 100 μl of the prepared enzyme was incubated at 40 °C for 15 min. The activity was measured by monitoring the increased absorbance of the reaction products (oligosaccharides) at 520 nm. One unit of alginate lyase activity (U) was defined as the amount of enzyme required to generate 1 μg glucuronic acid per min. Protein concentration was determined through Bradford's method using bovine serum albumin as the standard (Marion, 1976).

2.6. Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 10% poly-acrylamide gel with 0.1% SDS according to method described by Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R-250 followed by destaining with methanol–acetic acid (2:1, v/v). The unstained protein molecular weight marker (Fermentas, China) was served as the molecular mass marker.

2.7. Characterisation of alginate lyase

2.7.1. 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 PB at pH 5.5 to 9.5 under the standard assay conditions. The pH stability depended on the residual enzyme activity when the enzyme was conserved in different pH buffers at 4 °C for 12 h in advance. Meanwhile, the effects of temperature (25–65 °C) on the purified alginate lyase were investigated at pH