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A novel oligoalginate lyase from abalone, *Haliotis discus hannai*, that releases disaccharide from alginate polymer in an exolytic manner

Harumasa Suzuki, Ken-ichi Suzuki, Akira Inoue and Takao Ojima*

Laboratory of Marine Biotechnology and Microbiology, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

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Abstract—We previously reported the isolation and cDNA cloning of an endolytic alginate lyase, HdAly, from abalone *Haliotis discus hannai* [*Carbohydr. Res.* **2003**, *338*, 2841–2852]. Although HdAly preferentially degraded mannuronate-rich substrates, it was incapable of degrading unsaturated oligomannuronates smaller than tetrasaccharide. In the present study, we used conventional chromatographic techniques to isolate a novel unsaturated-trisaccharide-degrading enzyme, named HdAlex, from the digestive fluid of the abalone. The HdAlex showed a molecular weight of 32,000 on SDS-PAGE and could degrade not only unsaturated trisaccharide but also alginate and mannuronate-rich polymers at an optimal pH and temperature of 7.1 and 42 °C, respectively. Upon digestion of alginate polymer, HdAlex decreased the viscosity of the alginate at a slower rate than did HdAly, producing only unsaturated disaccharide without any intermediate oligosaccharides. These results indicate that HdAlex degrades the alginate polymer in an exolytic manner. Because HdAlex split saturated trisaccharide producing unsaturated disaccharide, we considered that this enzyme cleaved the alginate at the second glycoside linkage from the reducing terminus. The primary structure of HdAlex was deduced with cDNAs amplified from an abalone hepatopancreas cDNA library by the polymerase chain reaction. The translational region of 822 bp in the total 887-bp sequence of HdAlex cDNA encoded an amino-acid sequence of 273 residues. The N-terminal sequence of 16 residues, excluding the initiation methionine, was regarded as the signal peptide of this enzyme. The amino-acid sequence of the remaining 256 residues shared 62–67% identities with those of the polysaccharide lyase family-14 (PL14) enzymes such as HdAly and turban-shell alginate lyase SP2. To our knowledge, HdAlex is the first exolytic oligoalginate lyase belonging to PL14.

Keywords: Alginate lyase; Oligoalginate lyase; *Haliotis discus hannai*; HdAlex; cDNA cloning; Primary structure

1. Introduction

Alginate is an acidic heteropolysaccharide consisting of β-D-mannuronate (M) and α-L-guluronate (G) arranged as a poly(M)-block, a poly(G)-block, and an alternating or random poly(MG)-block.[1–3] Alginate is produced as a structural polysaccharide by brown algae and as an extracellular polysaccharide by certain bacteria.[1–4] The bacterial alginate is in an acetylated form.[3,4] Alginate lyase is an enzyme that catalyzes the degradation of alginate by a β-elimination mechanism, forming 4-deoxy-L-*erythro*-hex-4-enopyranosyluronate at the new non-reducing terminus. This enzyme is found in herbivorous marine mollusks,[5–11] seaweeds,[12,13] marine and soil bacteria,[3,4,14–18] and in *Chlorella* virus.[19] Alginate lyase is primarily classified into poly(M) lyase (EC 4.2.2.3) or poly(G) lyase (EC 4.2.2.11), acting preferentially on the poly(M)-block or poly(G)-block, respectively. Recently, lyases that can degrade both the

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); PCR, polymerase chain reaction.

* Corresponding author. Tel./fax: +81 138 40 8800; e-mail: ojima@fish.hokudai.ac.jp

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poly(M)-block and poly(G)-block have also been isolated.[14,16] The molluscan enzymes are classified as poly(M) lyase.[3,4,6–11,20,21] On the other hand, Henrissat et al. (http://afmb.cnrs-mrs.fr/CAZY/) classified alginate lyases into seven families, that is, polysaccharide lyase families (PLs) -5, -6, -7, -14, -15, -17, and -18, based on their primary structures. Abalone and *Chlorella* virus alginate lyases belong to PL14.

Most alginate lyases are endolytic enzymes that degrade internal glycoside linkages in the alginate chain.[3,4] However, there are also a few alginate lyases possessing exolytic activity. Recently, the exolytic alginate lyase A1-IV that belongs to PL15 was isolated from *Sphingomonas* sp. strain A1 and sequenced.[22,23] A1-IV was originally isolated as the oligoalginate lyase responsible for the complete decomposition of oligoalginates produced by endolytic lyases A1-I, -II, and -III from the same bacterium. In addition, A1-IV exhibited exolytic lyase activity toward alginate polymer, that is, it cleaved the glycoside linkage located in the non-reducing terminus of the alginate chain, producing unsaturated monosaccharide. *Sphingomonas* sp. strain A1 can utilize this monosaccharide as a carbon source.[22–25] The exolytic alginate lyases reported so far all belong to PL15.[22,23,26]

On the other hand, the alginate lyases from mollusks such as abalone,[9–11] turban-shell,[8,21] and *Littorina*[7] are endolytic enzymes that degrade poly(M)-rich substrate producing unsaturated oligosaccharides possessing the unsaturated sugar at the non-reducing terminus, such as unsaturated tri- and disaccharide. The molluscan enzymes are usually incapable of degrading unsaturated oligosaccharides smaller than tetrasaccharides, although the *Haliotis tuberculata* enzyme has been reported to degrade saturated trisaccharide to unsaturated disaccharide and monosaccharide with a low reaction rate.[10] These indicate that mollusks require some other unsaturated-oligoalginate-degrading enzymes for complete decomposition of alginate polymer. At present, there are no reports on the isolation of enzymes that can degrade unsaturated trisaccharide from mollusks, and, of course, no sequence data for this type of enzyme are available.

Previously, we isolated an endolytic alginate lyase, HdAly, from the pacific abalone *H. discus hannai* and cloned its cDNA.[11] HdAly showed high sequence similarity to turban-shell enzyme,[27] and like other molluscan enzymes, produced unsaturated tri- and disaccharides as major end products. On the other hand, we have recently become aware of the presence of an enzyme that can degrade the unsaturated trisaccharide in the digestive fluid of abalone. Here, we report the isolation and characterization of this enzyme. This enzyme could also degrade polymer alginate with an exolytic manner. Further, we cloned cDNA encoding this enzyme and deduced its amino-acid sequence. Accordingly, we found that the sequence showed appreciably high sequence identity relative to the PL14 endolytic alginate lyases. To our knowledge, this is the first report on the isolation and cDNA cloning of the molluscan exolytic alginate lyase that belongs to PL14.

2. Materials and methods

2.1. Materials

The pacific abalone, *H. discus hannai*, (shell size: 6×7 cm) was obtained from the Hokkaido Taisei Aquaculture Center (Taisei, Hokkaido Prefecture, Japan). The endolytic alginate lyase, HdAly, was prepared from the abalone digestive fluid as described previously.[11] TOYOPEARL CM-650M was purchased from Toyo Soda (Tokyo, Japan). Sephacryl S-200 HR, Hydroxyapatite Fast Flow, and Bio-Gel P2 were purchased from Amersham Biosciences AB (Uppsala, Sweden), Wako Pure Chemical Industries (Osaka, Japan), and Bio-Rad Laboratories (Hercules, CA, USA), respectively. The other chemicals used were of reagent grade from Wako Pure Chemical Industries.

2.2. Substrates for alginate lyases

Sodium alginate (*Macrocystis pyrifera* origin; Sigma–Aldrich (St. Louis, MO, USA)) was dissolved in distilled water to make 1% (w/v) and dispersed by heating at 90 °C for 1 h before use. Poly(M)-rich, poly(G)-rich, and random(MG) substrates were prepared from the alginate by the method of Gacesa and Wusteman.[28] The mannuronate content in the original alginate was approximately 60%, whereas the mannuronate contents of the poly(M)-rich and the random(MG) substrates were 86% and 64%, respectively, according to circular dichroism analysis with a J-600 spectropolarimeter (Jasco, Tokyo, Japan). The guluronate content in the poly(G)-rich substrate was 99%. Unsaturated tetra-, tri-, and dimannuronate were prepared from poly(M)-rich substrate by HdAly digestion as follows: poly(M)-rich substrate (0.2 g) was dissolved in 5 mL of 10 mM sodium phosphate (pH 7.0) and digested with 100 U of HdAly at 30 °C for 15 h. The digested material was heated at 95 °C for 5 min and centrifuged at 10,000*g* for 15 min to remove insoluble materials. The supernatant, containing unsaturated oligosaccharides, was lyophilized, dissolved in 1 mL of distilled water, applied to a Bio-Gel P2 column (2.4×95.8 cm) pre-equilibrated with 50 mM sodium phosphate (pH 7.0), and eluted with the same buffer. The oligosaccharides eluted from the column were detected by measuring absorbance at 235 nm, followed by TLC. The unsaturated tetra-, tri-, and disaccharide fractions were pooled, lyophilized, and desalted by gel-filtration through a Bio-Gel P2 column (2.0×47.5 cm) pre-equilibrated with distilled water. Trisaccharide possessing no unsaturated sugar