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High substrate specificity of 3,6-anhydro-L-galactose dehydrogenase indicates its essentiality in the agar catabolism of a marine bacterium

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ABSTRACT

3,6-Anhydro-L-galactose (AHG) is a rare sugar found in red macroalgae. The key metabolic steps in AHG catabolism involve its oxidation into 3,6-anhydrogalactonate (AHGA), followed by cycloisomerization of AHGA into 2-keto-3-deoxy-galactonate. These steps were recently discovered in a marine bacterium *Vibrio* sp. strain EJY3. In this study, we characterized $NAD(P)^+$-dependent AHG dehydrogenase (AHGD) involved in the first step of AHG catabolism. AHGD displayed high substrate specificity for AHG, but showed no catalytic activity toward other aldehyde sugars, including D-form of AHG, glucose, and galactose. This high substrate specificity of AHGD may be associated with the unique chemical structure of its substrate AHG. Unlike other common aldehyde sugars, AHG mainly exists in its hydrated form under aqueous conditions. Growth of EJY3 in presence of AHG, agar, and *Gelidium amansii* but glucose, as the sole carbon source resulted in a significant increase in the AHGD activity of cell-free EJY3 lysates. Amino acid sequence analysis revealed that AHGD is highly homologous to other aldehyde dehydrogenases from agar-degrading bacteria, suggestive of its key role in agar-related metabolism in marine bacteria utilizing red macroalgae. Therefore, AHGD may serve as an important enzyme involved in the bioconversion of red macroalgal biomass to value-added chemicals.

1. Introduction

Marine macroalgae are sustainable sources for the production of bio-based chemicals, owing to their high carbohydrate and very little or no lignin content [1,2]. Agar, the major cell wall component of red macroalgae such as *Agarophytes*, comprises neutral agarose and charged agaropectin [3]. Agarose is a gelling fraction of agar and consists of D-galactose and 3,6-anhydro-L-galactose (AHG), which are alternatively linked by α-1,3- and β-1,4-glycosidic bonds [4]. Agaropectin, a complex polymer of D-galactose and AHG, is a non-gelling fraction of agar. Agaropectin is highly derivatized with ester-sulfates, methyl groups, and pyruvates [5,6]. Therefore, AHG is the most abundant monomeric sugar of red macroalgae along with D-galactose.

The production of monomeric sugars (AHG and D-galactose) from agarose is achieved from its chemical or enzymatic hydrolysis [7]. The enzymatic hydrolysis of agarose may be divided into two steps: liquefaction of agarose using chemicals or enzymes to produce oligomers and the enzymatic saccharification of liquefied agarose to obtain monomers [8–10]. In the chemical liquefaction using acids, α-1,3-glycosidic bonds of agarose are preferentially hydrolyzed, resulting in the formation of agarooligosaccharides (AOSs) [11,12]. During the enzymatic liquefaction of agarose, β-1,4-glycosidic bonds are hydrolyzed by an endo-type β-agarase, resulting in the formation of neoagarooligosaccharides (NAOSs) [9]. AOSs or NAOSs may be further hydrolyzed into neoagarobiose (NAB) by an exo-type β-agarase. Treatment of NAB with an NAB hydrolase (NABH) would finally result in the formation of monomeric sugars, D-galactose and AHG [13]. Despite the advantage of red macroalgae such as the abundant sources of carbohydrates, the fact that AHG is not catabolized by terrestrial organisms such as industrial fermentative strains is the major drawback of red macroalgae as the biomass feedstocks.

A recent study reported a novel AHG catabolic pathway for the first time using a marine bacterium (i.e., *Vibrio* sp. strain EJY3) catabolizing AHG as a sole carbon source [14]. This pathway involved oxidation of AHG into 3,6-anhydrogalactonate (AHGA) by an AHG dehydrogenase (AHGD) and cycloisomerization of AHGA into 2-keto-3-deoxy-galactonate by an AHGA cycloisomerase [14]. An engineered *Escherichia coli* K12 strain harboring the AHG pathway was able to grow in presence of AHG as the sole carbon source [14]. Efficient utilization of AHG by engineered microorganisms demands systematic optimization of each enzyme reaction in the AHG catabolic pathway.

In the marine bacterium, the entry of AHG in the catabolism pathway is mediated by AHGD, an aldehyde dehydrogenase [14]. In biological systems, aldehyde dehydrogenases play a key role in

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mitigating oxidative/electrophilic stress through the oxidation of endogenous and exogenous aldehydes into carboxylic acids [15]. Most aldehyde dehydrogenases have broad substrate specificities toward various aldehyde substrates such as acetaldehyde, glyceraldehyde, and glycolaldehyde [16,17]. However, a specific aldehyde dehydrogenase (i.e., AHGD) is required for AHG, which may be due to the unique chemical structure of AHG comprising a bridged bicyclic structure with fused five- and six-membered rings [18].

In this study, we have characterized AHGD, the unique enzyme involved in the first enzymatic step of AHG catabolism, which was discovered in the marine bacterium *Vibrio* sp. strain EJY3 growing on AHG as the sole carbon source. The importance of AHGD in the metabolism of red macroalgae by the marine bacteria was studied using AHGD from EJY3. AHGD, a keystone enzyme for AHG catabolism, may have potential applications in the production of high-value chemicals with red macroalgal biomass.

2. Materials and methods

2.1. Overexpression and purification of recombinant AHGD

The gene VEJY3_09240 encoding AHGD was cloned into pBAD/myc-His vector and the recombinant plasmid was transformed into *E. coli* BL21(DE3) in the previous study [14]. To produce recombinant AHGD protein, the recombinant *E. coli* cells were grown at 37 °C in Luria-Bertani broth (BD, San Jose, USA) containing 100 μg/mL of ampicillin (Sigma-Aldrich, St. Louis, USA) until the mid-exponential phase. The protein expression was induced with 0.2% L-(+)-arabinose (Sigma-Aldrich) at 16 °C for 16 h. Following induction, cells were harvested by centrifugation at 5000*g* for 15 min at 4 °C and resuspended in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4). The cell suspension was disrupted by a sonicator (Branson, Danbury, USA) and the supernatant containing soluble recombinant protein was collected by centrifugation at 16,000*g* for 1 h at 4 °C. The recombinant protein was purified using a His-Trap column (GE Healthcare Life Sciences, Pittsburgh, USA). The purified protein was desalted using a HiTrap desalting column (GE Healthcare Life Sciences), followed by protein quantification with bicinchoninic acid protein assay kit (Pierce, Dallas, USA).

2.2. Production and purification of AHG as the substrate for AHGD reaction

We carried out the enzymatic saccharification of agarose, followed by purification to produce high purity AHG. For the enzymatic saccharification, three in-house recombinant enzymes; an endo-type β-agarase, an exo-type β-agarase, and an NABH were used. Those recombinant proteins were overexpressed in *E. coli* BL21(DE3) and purified using a His-Trap column. Aga16B, an endo-type β-agarase, was added into 2% (w/v) agarose in 200 mL of 20 mM Tris-HCl buffer (pH 7.4) and the reaction mixture was incubated at 55 °C for 12 h to produce NAOSs [9]. Aga50D, an exo-type β-agarase, was then added to the reaction product of Aga16B to hydrolyze NAOSs into NAB, followed by the incubation of the reaction mixture at 30 °C for 24 h [19]. Finally, NABH was added to the reaction product of Aga50D and the reaction mixture was incubated at 30 °C for 2 h to produce AHG and D-galactose [20]. The final reaction product from the three consecutive enzymatic saccharification steps of agarose was lyophilized until purification.

To purify AHG from the reaction product, the lyophilized reaction product was subjected to silica gel chromatography. A column (I.D 4 × 100 cm) was packed with silica gel 60 (70–230 mesh ASTM) and equilibrated with dichloromethane. The reaction product containing AHG was loaded onto the top of the silica gel column and eluted with a mobile phase comprising dichloromethane/methanol/water (78:20:2, v/v/v). Fractions containing only AHG were collected and dried in a rotary vacuum evaporator at 40 °C to obtain purified AHG.

2.3. Activity assay of AHGD and the effect of pH and temperature on AHGD activity

The activity of AHGD was determined by measuring the reduction of NAD^+ to NADH coupled with the oxidation of AHG to AHGA at 340 nm, as described in a previous study [14]. The reaction mixture containing 1 mM AHG, 1.5 mM NAD^+ (as the cofactor; Sigma-Aldrich), and 0.05 mg/mL (equivalent to 0.94 μM) of AHGD in 50 mM sodium phosphate buffer (pH 7) was incubated at 30 °C for 5 min. The increase in the absorbance at 340 nm was measured with a spectrophotometer (xMark Microplate Spectrophotometer, Bio-Rad, Hercules, USA). An extinction coefficient of 6220 M^{-1} cm^{-1} was used to determine the amount of NADH formed during the enzymatic reaction. One unit of AHGD was defined as the amount of enzyme required to reduce 1 μmol of NAD^+ to NADH in 1 min under above reaction conditions.

To study the effect of pH on AHGD activity, the enzymatic reaction was performed from pH 4–10 at 30 °C using 50 mM citrate buffer (pH 4–5), sodium phosphate buffer (pH 6–7), Tris-HCl buffer (pH 8–9), and glycine-NaOH buffer (pH 10). The effect of temperature on AHGD activity was measured from 10 °C to 60 °C at pH 7 in 50 mM sodium phosphate buffer.

2.4. Analysis of the enzymatic reaction product by liquid chromatography-mass spectrometry and gas chromatography/time-of-flight mass spectrometry

To analyze the AHGD reaction product, liquid chromatography-mass spectrometry (LC/MS) analysis was performed using an LC/MS ion-trap (IT) and time-of-flight (TOF) system (Shimadzu, Kyoto, Japan) equipped with a Thermo Hypercarb porous graphitic carbon LC column (100 mm × 2.1 mm packed with a 3 μm particle size; Thermo Fisher Scientific, Waltham, USA). Electrospray ionization was operated in the positive ion mode. The mobile phase comprised solution A (25 μM lithium chloride [LiCl]) and B (acetonitrile). The gradient elution was from 0% to 80% in 41 min at a flow rate of 0.2 mL/min, while the injection volume was 20 μL. The temperature of the LC column and autosampler was maintained at 70 °C and 10 °C, respectively. Source-dependent parameters were set as follows: nebulizing gas flow, 1.5 L/min; interface voltage, 4.5 kV; detector voltage, 1.65 kV; a curved desolvation line (CDL) temperature, 200 °C; and heat block temperature, 200 °C. The mass range was set from 100 to 700 *m/z*. LabSolutions LCMS software (version 3.8, Shimadzu) was used for the analysis of LC/MS data.

For gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS), the enzymatic reaction mixture of AHGD was centrifuged at 16,000*g* for 5 min at 4 °C. The supernatant (20 μL) was vacuum-dried for 1 h using a speed vacuum evaporator. Methoxyamination and trimethylsilylation were performed for derivatization of the dried sample, as previously described [14]. Briefly, 10 μL of 40 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich) was added to the dried sample and incubated for 90 min at 30 °C. Following incubation, the sample was treated with 45 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Fluka, Buchs, Switzerland) for 30 min at 37 °C to complete the derivatization process. Agilent 7890A GC (Agilent Technologies, Wilmington, USA) coupled to a Pegasus HT TOF MS (LECO, St Joseph, USA) was used to analyze the derivatized sample. An RTX-5Sil MS column (30 m × 0.25 mm, 0.25-μm film thickness; Restek, Bellefonte, USA) coupled to a 10-m guard column was used. The sample (1 μL) was injected into GC in splitless mode. The oven temperature was programmed as follows: the initial temperature of 50 °C for 1 min was ramped to 330 °C at 20 °C/min and the temperature was then held for 5 min. Electron ionization was performed at 70 eV; the temperature of the ion source and transfer line was 250 °C and 280 °C, respectively.