Isolation of a Novel Freshwater Agarolytic Cellvibrio sp. KY-YJ-3 and Characterization of Its Extracellular β-Agarase

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A novel agarolytic bacterium, KY-YJ-3, producing extracellular agarase, was isolated from the freshwater sediment of the Sincheon River in Daegu, Korea. On the basis of Gram-staining data, morphology, and phylogenetic analysis of the 16S rDNA sequence, the isolate was identified as Cellvibrio sp. By ammonium sulfate precipitation followed by Toyopearl QAE-550C, Toyopearl HW-55F, and Mono-Q column chromatographies, the extracellular agarase in the culture fluid could be purified 120.2-fold with a yield of 8.1%. The specific activity of the purified agarase was 84.2 U/mg. The molecular mass of the purified agarase was 70 kDa as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The optimal temperature and pH of the purified agarase were 35°C and pH 7.0, respectively. The purified agarase failed to hydrolyze the other polysaccharide substrates, including carboxymethyl-cellulose, dextran, soluble starch, pectin, and polygalacturonic acid. Kinetic analysis of the agarase hydrolysis catalyzed by the purified agarase using thin-layer chromatography showed that the main products were neoagarobiose, neoagarotetraose, and neoagarohexaose. These results demonstrated that the newly isolated freshwater agarolytic bacterium KY-YJ-3 was a Cellvibrio sp., and could produce an extracellular β-agarase, which hydrolyzed agarase to yield neoagarobiose, neoagarotetraose, and neoagarohexaose as the main products.

Keywords: Freshwater environment, agarolytic Cellvibrio sp., β-agarase, purification, neoagarooligosaccharide

Agar, which is composed of agarose and agaropentin, is usually found in the cell wall of marine red algae. Agarose consists of alternating residues of α-1,3-linked 3,6-anhydro-β-D-galactopyranose and β-1,4-linked D-galactopyranose. Agaropentin has the same repeating units, although some of the 3,6-anhydro-β-D-galactose residues are replaced with α-D-galactose sulfate, and there can also be partial replacement of D-galactose residues with the pyruvic acid acetal 4,6-O-(1-carboxyethylidene)-D-galactose [7]. Agarase divides into two groups, α-agarase and β-agarase, depending on their mode of action [40]. These enzymes catalyze the hydrolysis of agar to produce agarooligosaccharides and neoagarooligosaccharides, respectively. Agarooligosaccharides can be generated from agarose by treatment with either α-agarase or acid, whereas neoagarooligosaccharides are generated from agarose only by treatment with β-agarase. The neoagarooligosaccharides are known to possess various chemical and biological activities. These include antitumor activity [9], macrophage-stimulating activity to increase immune functions [46], antioxidizing activity [6, 43], and prebiotic activity [14]. The low polymerization degree product, such as neoagarobiose (NA2), has moisturizing effect on skins and whitening effect on melanoma cells [15, 19, 24].

Agarolytic bacteria were first isolated from seawater by Gran in 1902 [38]. Since then, many genera of agarolytic bacteria have been identified, mainly from marine environments, seawater, and marine sediment, including Agarivorans [10, 20, 23, 24], Alterococcus [35, 36], Alteromonas [16, 18, 25, 33, 45], Cytophaga [12, 41], Microbulbifer [28, 31], Microscilla [47], Pseudoalteromonas [19], Pseudomonas [22, 27, 42], Saccharophagus [8], Thalassomonas [32], Thermoanaerobacter [3], Vibrio [2, 11, 39], and Zobellia [1]. On the other hand, the identification of agarolytic bacteria from nonmarine environments has been poorly reported, because only a few bacterial genera such as Bacillus, Cytophaga, Paenibacillus, and Streptomyces...
have been isolated as agarolytic bacteria in freshwater environments [17] or terrestrial soil environments [5, 13, 40].

In the present study, we describe the identification of a novel freshwater agarolytic bacterium, Cellvibrio sp. KY-YJ-3, isolated from the Sincheon River in Daegu, Korea, and purification and characterization of its extracellular β-agarase.

**Materials and Methods**

**Agarolytic Bacterial Strain and Culture Condition**

The bacterial strain Cellvibrio sp. KY-YJ-3 used in this study was one of the freshwater agarolytic bacteria isolated from the sediment of the Sincheon River in Daegu, Korea. Twenty ml of the basal medium containing (0.2% NaNO₃, 0.05% polypeptone, 0.05% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.01% CaCl₂·2H₂O, 0.01% NaCl, 0.002% FeSO₄·7H₂O, 0.02% MnSO₄·4H₂O) [17], containing 0.5% agar as sole carbon source, was inoculated with 0.2 ml of the sample and incubated at 30°C for 5 days. Subsequently, 0.2 ml of the cultures liquefied by agarolytic bacteria were then inoculated to 20 ml of the basal medium containing 0.5% agar and cultured for another 5 days. Then 0.1 ml of the culture was properly diluted and plated on the basal medium containing 1.8% agar. Plates were incubated at 30°C and examined daily for agarolytic activity, assessed by liquefaction or shallow depressions appearing around the colonies. The purity of the isolate was checked by further streaking and by microscopic examination.

**Purification of Chromosomal DNA and 16S rRNA Gene Amplification**

Chromosomal DNA of the agarolytic bacterial cells was purified as previously described [17], and dissolved in dH₂O. The 16S rRNA gene (~1.5 kb) was selectively amplified from the chromosomal DNA by polymerase chain reaction (PCR) with oligonucleotide primers designed to anneal to conserved positions in the 5′ and 3′ regions of the 16S rRNA genes. The forward primer 27f (5′-GAGTTTGATCCAGGCAGCAG-3′) and the reverse primer 1525r (5′-AGGAATTGAGGGTGAAAGGAGG-3′) were used. The PCR product was ligated with the TA cloning vector and then the ligation mixture was used for transformation of Escherichia coli DH5α. Subsequently, the recombinant plasmid was purified from the transformant for analysis of the 16S rDNA sequence.

**DNA Sequence Analysis**

The recombinant plasmid DNA construct containing the PCR-amplified 16S rDNA gene was purified from the transformant of E. coli and was subjected to DNA sequencing. The DNA sequence analysis was performed by the cycle sequencing method with the ABI BigDye terminator, with an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The 16S rDNA gene sequence was compared with sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) to obtain closely matched species. The phylogenetic tree of the strain KY-YJ-3 was constructed using the biological software MEGA4.

**Assay of Agarase Activity**

Quantitative assay for agarase activity was performed by the method of Somogyi [37], which detects reducing sugars released from agarose. The enzyme solution (500 µl) was mixed with an equal volume of 0.2% agarose dissolved in 20 mM McIlvaine buffer (pH 7.0). After incubation for 30 min at 30°C, reducing sugars formed in the reaction mixture were measured colorimetrically using the Somogyi-Nelson reagent. One unit of the enzyme activity was defined as the amount of the enzyme that produced reducing power equivalent to 1 µM of d-galactose per minute.

**Purification of Agarase and Protein Quantitation**

Unless otherwise indicated, all purification procedures were performed at 4°C. The basal medium containing 0.5% agar as sole carbon source was inoculated with KY-YJ-3, and cultivated at 30°C for 3 days with shaking. After centrifugation of the culture fluid at 10,000 rpm for 20 min, the supernatant was recovered. Solid ammonium sulfate was added to the culture supernatant to 20% saturation. After standing overnight, the precipitate was removed by centrifugation at 20,000 rpm for 20 min. Solid ammonium sulfate was added to 70% saturation. The precipitated agarase was collected by centrifugation at 20,000 rpm for 20 min, dissolved in 50 ml of 20 mM phosphate buffer (pH 8.0) and then dialyzed in the same buffer overnight. The dialyzed samples (70 ml) were applied to an anion-exchange column (Toyopearl QAE-550C; Tosho, Tokyo, Japan) equilibrated with 20 mM phosphate buffer (pH 8.0). The column was washed with 300 ml of the same buffer. The column was eluted sequentially with a linear gradient of 0 M to 1 M NaCl in 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (35 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions of agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.). The concentrated samples were loaded onto a gel filtration chromatography column (Toyopearl HW-55F; Tosho, Tokyo, Japan) and eluted with 20 mM phosphate buffer (pH 8.0). Fractions with agarase activity were collected (5 ml) and concentrated by ultrafiltration using a YM-10 filter membrane (Amicon, Beverly, MA, U.S.A.).

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)**

SDS–PAGE was carried out on 10% polyacrylamide gel by the method of Laemmli [21]. Protein standards (PageRuler Prestained Protein Ladder; Fermentas Life Science, Ontario, Canada) were used as molecular marker standards. The gel was stained with Coomassie brilliant blue (CBB) R-250 to detect the protein band.

**Thin-Layer Chromatography (TLC)**

TLC of the hydrolysates of agarose by the purified agarase was performed on a Silica Gel 60 glass plate (F254 Merck, Darmstadt, Germany) and developed with 1-butanol–acetic acid–H₂O [2:1:1 (v/v)]. The oligosaccharides were detected by spraying with 10% H₂SO₄, followed by heating (80°C). D-Galactose (Sigma Chemical, St. Louis, MO, U.S.A.), neogarobiase, neoagarotetraeose, and neoagarohexaose were used as standards. The standard mixture of neoagaroligosaccharides containing neoagarobiase, neoagarotetraeose, and neoagarohexaose was provided by Dr. Sang-Hyeon Lee after being prepared as described elsewhere [23].