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Cyclodextrin glycosyltransferase encoded by a gene of *Paenibacillus azotofixans* YUPP-5 exhibited a new function to hydrolyze polysaccharides with β-1,4 linkage

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ABSTRACT

The bacteria with hydrolysis activity to glucomannan were isolated from the rhizosphere of *Amorphophallus konjac* through enrichment cultivation. One strain with strong activity in degrading glucomannan was identified preliminarily as *Paenibacillus azotofixans* YUPP-5 according to the sequence analysis of 16S rDNA. This strain is able to hydrolyze many polysaccharide with β-1,4 linkage, including glucomannan, galactomannan, xylan, carboxymethyl cellulose, and chitin. One hydrolytic enzyme band of approximately 70 kDa was examined from the supernatants of YUPP-5 by using zymogram with mixture polysaccharides as substrate. The encoding gene had an open reading frame of 2157 bp, which deduced cyclodextrin glycosyltransferase (CGTase), including 718 amino acids with a signal peptide in the N-terminal region. When the gene was expressed in *Escherichia coli* BL21, the recombinant CGTase exhibited strong activity in degrading polysaccharides with β-1,4 linkage, and in forming cyclodextrin by using carboxymethyl cellulose as substrate. This CGTase exhibited some new functions. Finally, the hydrolytic oligosaccharides from galactomannan or glucomannan were detected by thin layer chromatography. Pentasaccharide, tetrasaccharide, trisaccharide, and disaccharide could be examined as reaction time went on.

1. Introduction

Polysaccharides from plants, mushrooms, shrimps, and crab shells are commercially available and important natural resources widely used in food processing, industrial applications, and pharmaceuticals. Starch serves as a major energy source in the diet of humans, whereas cellulose is the main food source of herbivorous animals and insects. Other polysaccharides, such as chitin, chitosan, agar, alginate, arabic gum, carrageenan, glucomannan, guar gum, locust bean gum, pectin, dextrin, pullulan, xanthan, and xylan, are important materials with desirable properties.

Polysaccharides with complex structure can be divided into certain groups according to different glycosidic bond linkage. In contrast to starch with α-1,4 linkage, cellulose, chitin, glucomannan, guar gum, locust bean gum, xylan, and other polysaccharides have β-1,4 linkage. These polysaccharides cannot be digested by people. However, their derivative oligosaccharides have special functions. Many oligosaccharides are available commercially as prebiotic agents for humans and animals that stimulate bifidobacteria [1,2]. Chitin oligosaccharide was reported to induce defense responses in mammalian and insect cells [3], as well as in a wide range of plant cells [4,5]. In addition to chitin oligosaccharide, galactoglucomannan-derived oligosaccharides were also demonstrated to induce resistance for cucumber against tobacco necrosis virus [6]. Chito-oligosaccharides are able to directly inhibit the growth of *Botrytis cinerea* [7] and show a potential for antitumor growth and antimetastasis in the Lewis lung carcinoma cells of mice [8].

An increasing number of oligosaccharides and their derivatives are prepared through polysaccharide-degrading enzymes. There is a kind of special hydrolytic enzymes, cyclodextrin glycosyltransferase (CGTase). That enzyme is able to cleave starch into linear malto-oligosaccharide and change them into cyclodextrins (CDs) through an intramolecular transglycosylation reaction. Up to now cyclodextrins produced by enzymes are usually composed of glucose units with α-1,4 linkage. Except for that function, CGTase is

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also able to catalyze coupling and disproportionation reactions [9]. In a coupling reaction, CGTase can open the CD ring and transfer it into a second linear acceptor. In a disproportionation reaction, two linear oligosaccharides are converted into a longer linear structure by CGTase.

CGTase has been reported in many bacteria, including *Anaerobranca gottschalkii* [10], alkalophilic *Bacillus* sp. [11], *Bacillus circulans* [12], *Bacillus firmus* [13], *Bacillus licheniformis* [14], *Bacillus macerans* [15], *Bacillus megaterium* [16], *Bacillus obhensis* [17], *Bacillus stearothermophillus* [18], *Brevibacillus brevis* [19], *Geobacillus stearothermophilus* [20], *Klebsiella pneumoniae* [21], *Paenibacillus* sp. [22], and *Thermoanaerobacter* sp. [23]. The CGTases in the above-mentioned bacteria only act on starch and polysaccharides with α-1,4 linkage [24]. However, we cloned a new gene encoding CGTase from *Paenibacillus azotofixans* YUPP-5, isolated from the rhizosphere of *Amorphophallus konjac* field. This CGTase is able to make use of glucomannan, a polysaccharide with β-1,4 linkage, to form CD. This enzyme is also able to degrade galactomannan (locust bean gum) and xylan, as well as carboxymethyl cellulose (CMC) and chitin. These functions of CGTase are different from those of other CGTases reported by previous references.

2. Methods

2.1. Materials and medium

Galactomannan (Locust bean gum, LBG), birchwood xylan, CMC, and chitin were purchased from Sigma (St. Louis, MO, USA). Glucomannan was obtained from Nongtai Co. Ltd. (Hubei province, China). Konjac curd was bought from the local market.

The basic medium consists of 0.5% (w/v) polypepton, 0.2% (w/v) yeast extract, 0.2–2% (w/v) konjac glucomannan, 0.1% (w/v) KH_2PO_4, and 0.02% (w/v) $MgSO_4$. Different sources of carbon were added to replace konjac glucomannan for different experiments.

2.2. Isolation bacteria with the activity in degrading konjac curd

Five kilograms of soil samples from *A. konjac* were placed into a glass pot. Then, 500 g of konjac curd was buried into soil samples and incubated at 25 °C. After 7 d, the konjac curd disintegrated and liquefied. One milliliter liquid from the glass pot was transferred into a flask with 500 g sterilized konjac curd and incubated overnight at 25 °C. When the konjac curd liquefied again, 50 μL of the liquid was sprayed into the agar plate with the basic medium and incubated at 25 °C for 20 h. Colonies were inoculated into the same position in two Petri dishes with the basic medium: one plate for bacterial growth and the other for dyeing using a 0.2%:2% (w/v) iodine-KI solution. The colony with hydrolytic zone was the target bacteria.

2.3. Identification of konjac curd-degrading bacteria

The 16S rDNA gene of the YUPP-5 strain was amplified by using primer 1 (5′-GAGTTTGATCCTGGCTCAG-3′, positions 9–27 relative to *Escherichia coli* 16S rDNA) and primer 2 (5′-AGAAAGGAGGTGATCCAGCC-3′, positions 1525–1542 relative to *E. coli* 16S rDNA). Polymerase chain reaction (PCR) products were inserted into the pGEM T-easy vector (Promega Co., USA) for sequencing. Phylogenetic analysis was performed using the software ClustalX and TreeView.

2.4. Hydrolysis activity of the YUPP-5 strain for different polysaccharides

To investigate the hydrolytic activity of the YUPP-5 strain for different polysaccharides, we supplemented 0.2% of galactomannan, CMC, xylan, chitin, or starch in the basic medium instead of konjac glucomannan, respectively. The YUPP-5 strain was streaked on the solid medium and incubated at 25 °C for 20 h. The hydrolytic zone was detected by dyeing using a 0.2%:2% iodine-KI solution.

2.5. Zymograms

Zymograms were performed according to the method of Jiang et al. [25], with slight modification. The crude enzyme was concentrated from the culture supernatant by vacuum freeze-drying. To investigate the hydrolytic enzyme species of different substrates, we performed a zymogram by co-polymerizing 12.5% (wt/vol) of polyacrylamide with 0.15% (wt/vol) of konjac glucomannan, 0.15% (wt/vol) of CMC, or 0.15% (wt/vol) of chitin, respectively.

For the analysis of multifunctional hydrolytic enzymes in degrading various polysaccharide substrates, zymogram was prepared by co-polymerizing 12.5% (wt/vol) polyacrylamide with mixture substrates, including 0.05% (wt/vol) of konjac glucomannan, 0.05% (wt/vol) of CMC, and 0.05% (wt/vol) of chitin.

After electrophoresis, the gel was soaked in 25% (vol/vol) of isopropanol with gentle shaking to remove the sodium dodecyl sulfate. The gel was then washed three times in 50 mM of citrate buffer (pH 6.5) at 4 °C for 30 min. After further incubation at 37 °C for 12 h, the gel was stained with Congo red solution (0.1%, wt/vol) and decolorized with 1 M of NaCl. The enzyme band appeared as a clear colorless zone.

2.6. Fosmid library construction

P. azotofixans YUPP-5 was cultured in the liquid basic medium at 25 °C for 16 h. Genomic DNA was isolated according to the protocol for Gram-positive bacteria with the Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA). The fosmid library was constructed using EpiFOS™ Production Kit (Epicentre Technologies, USA) according to the manufacturer's protocol. Briefly, the purified genomic DNA was randomly sheared into approximately 40 kb fragments, and the ends were blunted and ligated into pEpiFOS™-5 Fosmid Vector. The ligated DNA was packaged with MaxPlax™ Lambda Packaging Extracts to form the fosmid library. The library was transduced into EPI100™-T1^R Plating strain *E. coli* and spread on Luria–Bertani (LB) agar plates containing 12.5 μg/mL of chloramphenicol.

2.7. Gene cloning

To prepare the library, transformants were inoculated into the same position in two Petri dishes. The medium consists of 0.5% (w/v) polypepton, 0.2% (w/v) yeast extract, 0.2% (w/v) konjac glucomannan, 0.1% (w/v) KH_2PO_4, and 0.02% (w/v) $MgSO_4$. One dish was used for dyeing with a 0.2%:2% iodine-KI solution. The colony with the clear colorless zone was chosen. Plasmid holding approximately 40 kb genomic fragment of the YUPP-5 strain was extracted and partially digested with *Sau*3AI. The 5 kb fragments were collected and linked with pUC18 plasmid to transform *E. coli* DH5α. Transformant with hydrolytic activity was used for sequencing.

2.8. cgt gene expression and purification

The *cgt* gene was amplified from the YUPP-5 strain using the upstream-primer 5′-AGGGTGTCGACTCGATGCGGATACGGCTGTC-3′ and the downstream-primer 5′-AATTATGTCGACTTATTGCCAGTTTACCGT-3′ (underlined section shows restriction enzyme sites of *Sal*I). PCR products (without signal peptide enclosed) and pGEX-6p-1 expression vector were digested with the restriction enzyme *Sal*I and linked with the T4 DNA polymerase. The recombinant plasmid was transformed into *E. coli* BL21 for gene expression. CGTase protein was purified using GST Purification Kit (Thermo Scientific, USA).

2.9. Hydrolysis activity of recombinant CGTase

To detect the hydrolytic activity of recombinant CGTase for different polysaccharides, we supplemented 0.2% of CMC, galactomannan, xylan, chitin, or starch in the basic medium instead of konjac glucomannan, respectively. The recombinant CGTase was dissolved with 50 mM of acetate buffer (pH 5.5) and transferred 10 μL in the center of solid medium and incubated at 25 °C for 20 h. The hydrolytic zone was detected by dyeing using a 0.2%:2% iodine-KI solution.

2.10. CGTase cyclization activity assay

The cyclization activity of CGTase was determined by the phenolphthalein method [26], with some modification. The gel with a groove in the center was prepared by mixing 1.0% of agar with 0.3% of CMC in 50 mM of acetate buffer (pH 5.5). Recombinant CGTase was dissolved with 50 mM of acetate buffer (pH 5.5) and transferred into the groove for incubating overnight at 40 °C. A 50 mM acetate buffer (pH 5.5) should be added around the gel to avoid dehydration. CGTase from *B. circulans* 251 and 50 mM of acetate buffer (pH 5.5) acted as the control.

The incubated gel was dyed with phenolphthalein solution, which was prepared by mixing 3 mL of phenolphthalein solution (1 g phenolphthalein dissolved in 100 mL of 50% [v/v] ethanol) with 24 mL of sodium hydroxide solution (0.03 M). Usually, the color reaction of phenolphthalein in basic environment is red. When cyclodextrin exists, it can form complex with phenolphthalein. Therefore, the lighter color around groove indicated that CD was produced by CGTase.

2.11. Thin layer chromatography

Galactomannan (0.2%) or glucomannan (0.2%) was dissolved in 50 mM of acetate buffer (pH 5.5) to prepare the substrate solution. First, 10 μL of recombinant CGTase was added into 100 μL of the substrate solution. To investigate the composition of oligosaccharides, the hydrolytic sample was taken out every 40 min and heated at 100 °C for 2 min to stop the reaction. A manno-oligosaccharide mixture from mannobiose to mannohexaose was used as the standard. Hydrolytic products were analyzed by thin layer chromatography (TLC) using TLC-60 plates (MERCK MGaA, Germany) and a developing solvent composed of 1-propanol, nitromethane, and water (7:1:2, v/v/v). The sugar levels on the plate were detected by spraying 5% of sulfuric acid in ethanol and heating at 110 °C for 5 min.