BRAZILIAN JOURNAL OF MICROBIOLOGY XXX (2017) XXX–XXX

BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Biotechnology and Industrial Microbiology

Isolation and characterization of a novel endo-β-1,4-glucanase from a metagenomic library of the black-goat rumen

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ARTICLE INFO

Article history:
Received 10 August 2016
Accepted 1 March 2017
Available online xxx
Associate Editor: Solange I. Mussatto

Keywords:
Black goats
Endo-β-1,4-glucanase
Glycosyl hydrolase family 5 (GH5)
Metagenomic library
Rumen

ABSTRACT

The various types of lignocellulosic biomass found in plants comprise the most abundant renewable bioresources on Earth. In this study, the ruminal microbial ecosystem of black goats was explored because of their strong ability to digest lignocellulosic forage. A metagenomic fosmid library containing 115,200 clones was prepared from the black-goat rumen and screened for a novel cellulolytic enzyme. The *KG35* gene, containing a novel glycosyl hydrolase family 5 cellulase domain, was isolated and functionally characterized. The novel glycosyl hydrolase family 5 cellulase gene is composed of a 963-bp open reading frame encoding a protein of 320 amino acid residues (35.1 kDa). The deduced amino acid sequence showed the highest sequence identity (58%) for sequences from the glycosyl hydrolase family 5 cellulases. The novel glycosyl hydrolase family 5 cellulase gene was overexpressed in *Escherichia coli*. Substrate specificity analysis revealed that this recombinant glycosyl hydrolase family 5 cellulase functions as an endo-β-1,4-glucanase. The recombinant KG35 endo-β-1,4-glucanase showed optimal activity within the range of 30–50 °C at a pH of 6–7. The thermostability was retained and the pH was stable in the range of 30–50 °C at a pH of 5–7.

Introduction

Cellulose is a major polysaccharide compound in the plant cell wall and is composed of repeating cellobiose units linked *via* β-1,4-glycosidic bonds. It is one of the most abundant renewable bioresources in nature.[1] The enzyme cellulase can be categorized into three major classes based on its catalytic action: endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), or β-glucosidases (EC 3.2.1.21). Endo-β-1,4-glucanases randomly attack internal amorphous sites in

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http://dx.doi.org/10.1016/j.bjm.2017.03.006

Please cite this article in press as: Song Y, et al. Isolation and characterization of a novel endo-β-1,4-glucanase from a metagenomic library of the black-goat rumen. *Braz J Microbiol.* (2017), http://dx.doi.org/10.1016/j.bjm.2017.03.006

cellulose polymers, generating new chain ends. Cellobiohydrolases remove cellobiose from reducing or non-reducing ends of cellulose polymers. β-Glucosidases hydrolyze cellodextrins and cellobioses to glucoses.[2] Cellulases have generated commercial interest in various sectors, including the food, energy, pulp, and textile industries.[3] Among the three types of cellulases, endo-β-1,4-glucanase can release smaller cellulose fragments of random length.[4] Consequently, endo-β-1,4-glucanase has a biotechnological potential in various industrial applications. This enzyme has been industrially applied in biomass waste management, pulp and paper deinking, and textile biopolishing.[5] In addition, this enzyme can be practically used to produce better animal feeds, improve beer brewing, decrease the viscosity of β-glucan solutions, and improve biofinishing in the textile industry.[5]

Cellulases can be produced using a wide range of microorganisms, plants, and animals. Symbiotic microorganisms in the rumen of herbivores can hydrolyze and ferment cellulosic polymers, thereby allowing the host to obtain energy from the indigestible polymers.[6] Therefore, rumen microbial communities in ruminants are attractive sources of cellulases because these communities have adapted to utilization of lignocellulosic plant biomass.[6–9]

Although the ruminal microbial ecosystem is highly diverse, the majority of rumen microorganisms are unculturable.[6] It is estimated that less than 1% of microbial species in nature have ever been cultured.[10] A culture-independent method has been developed to overcome this problem. This approach involves isolating metagenomic DNA from the environment and cloning this DNA into a vector to generate a metagenomic library. The library is subsequently screened for genes of interest *via* the detection of genotypic or phenotypic biomarkers using DNA–DNA hybridization, polymerase chain reaction (PCR), or enzymatic assay techniques.[11]

The Korean black goat is a grazing herbivore with a dietary preference for herbaceous and woody dicots, such as forbs, shrub leaves, and stems.[12] Black goats are classified as small ruminants, and their foregut digestion allows feed particles to move more rapidly through the alimentary tract, promoting higher herb intake in comparison with other larger herbivores.[13] Therefore, the objectives of this study were to isolate and functionally characterize a novel cellulase metagenomically derived from the rumen contents of black goats.

Materials and methods

Construction and functional screening of a metagenomic fosmid library

All animal studies and standard operating procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Animal Science, Rural Development Administration, Suwon, Korea (No. 2009-007, D-grade, surgery). Three 18-month-old Korean black goats were raised at the National Institute of Animal Science and were freely fed rice straw and mineral supplements 30 d prior to the experiment to maximize cellulolytic adaptation of microorganisms in their rumens. Rumen contents of the three black goats were collected immediately after slaughter. Metagenomic DNA was extracted from the contents using hexadecyl trimethyl ammonium bromide and sodium dodecyl sulfate (CTAB-SDS).[14] DNA fragments ranging from 10 to 50 kb were obtained by partial digestion with the restriction enzyme *Hind*III to construct a metagenomic fosmid library by means of the CopyControl™ Fosmid Library Construction Kit (Epicentre, Madison, WI). The technique was based on the pCC1FOS vector, according to the manufacturer's instructions. *Escherichia coli* DH5α™ (Life Technologies, Carlsbad, CA) transformants, carrying various recombinant fosmids, were transferred to 384 well plates. To determine an average insert size of the fosmid library, a total of 36 clones were randomly selected and digested with the restriction enzyme *Not*I.

A fraction of the library was spread on Luria–Bertani (LB) agar plates supplemented with chloramphenicol (15 μg/mL) and 0.2% carboxymethyl cellulose (CMC; Sigma–Aldrich, St. Louis, MO), which is a substrate for screening for endo-β-1,4-glucanase activity. After incubated and stained as described previously,[15] fosmid clones with cellulolytic activity were detected upon the formation of clear zones around the colonies. Activity-based metagenomic screening yielded a single fosmid clone (Ad125D08) that formed one of the largest clear zones.

Shotgun sequencing of the enzyme-positive fosmid clone

Shotgun sequencing and molecular analyses were performed to localize the cellulase gene within the enzyme-positive fosmid clone and to subclone the gene for effective biochemical characterization. Bacteria carrying the cellulolytic fosmid clone (Ad125D08) were cultured in 100 mL of the LB medium containing 25 μg/mL of chloramphenicol at 37 °C for 20 h. After the cells were harvested, the DNA was extracted using a Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA) and subjected to shotgun sequencing that comprised two approaches: conventional Sanger sequencing and pyrosequencing. For shotgun sequencing with Sanger methodology, 15 μg of DNA from the cellulolytic fosmid clone was randomly fragmented into sections measuring 2–3 kb in size. Fragmentation was performed using the HydroShear DNA shearing device (Genomic Solution, Waltham, MA) under the following processing conditions: sample volume 200 μL, speed code 11, and 20 shearing cycles. Small fragments were removed and were subsequently repaired with DNA polymerase and the polynucleotide kinase method (BKL Kit; TaKaRa, Shiga, Japan). The prepared DNA fragments were ligated into the *Sma*I site of pUC19. The ligation products were introduced into *E. coli* DH10B cells by electroporation. Approximately 192 recombinant plasmids in the shotgun DNA library were randomly selected for sequencing. Each plasmid DNA was bidirectionally sequenced as described previously.[16] The sequencing data was assembled using Phred and Phrap software (University of Washington, Seattle, WA). One pyrosequencing run was conducted on GS Junior sequencing system (Roche Diagnostics, Oakland, CA) as described previously.[17] The pyrosequencing data were assembled using GS De Novo Assembler, version 2.7.

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