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Identification and characterization of a novel KG42 xylanase (GH10 family) isolated from the black goat rumen-derived metagenomic library

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ABSTRACT

This study was conducted to isolate and functionally characterize a novel xylan-degrading enzyme from the microbial metagenomes of black goat rumens. A novel gene, *KG42*, was isolated from one of the 17 xylan-degrading metagenomic fosmid clones obtained from black goat rumens. The *KG42* gene, comprising a 1107 bp open reading frame, encodes a protein composed of 368 amino acids (41 kDa) with a glycosyl hydrolase family 10 (GH10) domain, consisting of a "salad-bowl" shaped tertiary structure (a typical 8-fold α/β barrel (α/β)8) and two catalytic residues. KG42 xylanase protein has at best 40% sequence identity with other homologous GH10 xylanase proteins. The enzyme displayed its optimum activity at pH 5.0 and 50 °C. The enzyme was thermally stable at pH and temperature ranges of 5.0–10.0 and 20–60 °C, respectively. Substrate specificity and hydrolytic patterns implied that the KG42 xylanase functions as an endo-β-1,4-xylanase (EC 3.2.1.8). The KG42 xylanase was also used for the preparation of bifidogenic xylan hydrolysates, demonstrating its potential applications toward preparing prebiotic xylooligosaccharides.

1. Introduction

Lignocellulosic plants are mainly composed of cellulose, hemicellulose, and lignin. Hemicellulose is the second most common polysaccharide, representing approximately 30% of lignocellulosic materials. A complex structure of hemicellulose contains various monosaccharides, including xylose. Heteropolymeric xylans are the main components of hemicellulose from hardwood, consisting of D-xylosyl units linked by β-1,4-glycosidic bonds as a backbone, usually substituted by D-glucuronic acid, 4-O-methyl-D-glucuronic acid, or L-arabinofuranose [1,2]. Xylans can be hydrolyzed by using enzymes such as endo β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), and α-glucuronidase (EC 3.2.1.139) [3]. Such xylanases are used in food processing industries to accelerate the production rates and improve product quality [4].

Xylooligosaccharides (XOSs) produced using endo β-1,4-xylanase can be used as healthy food ingredients in anti-obesity diets and prebiotics [5]. The prebiotic effects of XOSs are involved in the optimization of colonic function and metabolism. Dietary intake of XOSs improves the intestinal microbiota and exhibits a variety of physiologically beneficial changes, such as increased short chain fatty acids but decreased pH in the colon, increased mineral absorption, and immune stimulation [6]. Therefore, XOSs have currently attracted attention from the global nutraceutical market [7].

The microbial community present in the rumen ecosystem of ruminants is a potentially good source of xylanases. Because ruminants cannot directly digest lignocellulosic biomass, they need lignocellulolytic enzymes produced by the microbial community populating the rumen [8]. Although goats are small ruminants, they display rumination patterns similar to those observed in larger animals. However, they appear to have faster digestion rates and can digest poor roughages [9,10].

Less than 1% of all the microbiota in nature can easily be studied by using conventional culture approaches. However, the rumen microbial community producing lignocellulolytic enzymes shows non-cultivable properties. Therefore, culture-independent metagenomic approaches have been used to study these non-cultivable microbial communities [11]. In our previous study, we constructed a fosmid metagenomic library from rumen microbial community of black goats [12]. In this study, a novel gene (*KG42*), expressing xylanase activity, was selected from the library, and a recombinant KG42 enzyme was functionally characterized and used toward the preparation of bifidogenic xylan hydrolysates.

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2. Materials and methods

2.1. Construction and screening of the metagenomic fosmid library

All animal studies and standard operating procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science, Rural Development Administration, Suwon, Korea (No. 2009-007, D-grade, surgery).

The experimental animals, three 18-month-old Korean black goats, were raised at the National Institute of Animal Science, and had been freely fed with rice-straw and mineral supplements for a month to boost cellulolytic adaptation of microorganisms present within their rumen. The rumen contents of the black goats were gathered promptly after slaughtering.

Metagenomic DNA was extracted according to the method described by Zhou et al. [13]. The DNA fragments (~40 kb) digested by *Hind*III were cloned into a pCC1FOS vector using CopyControl™ fosmid library construction kit (Epicentre, Madison, WI, USA). The metagenomic fosmid library was transformed into *Escherichia coli* DH5α™ cells (Life Technologies, Carlsbad, CA, USA). The transformants containing different recombinant fosmids were transferred to 384-well plates and the fosmid library was screened to select clones displaying high xylan-degrading activity using the method described by Ref. [14]. LB agar plates containing 0.2% (w/v) xylan were used and stained with Congo red in this study. The positive clones with hemicellulolytic activity were identified through the formation of clear zones around the colonies on the red background. A single fosmid clone Ad142N07 was identified as one of the largest clear zone formers, and its sequence has been deposited in the GenBank database under accession numbers KJ631396.1 and MG719279.

2.2. Shotgun sequencing of the enzyme-positive fosmid clone

The bacteria harboring the lignocellulytic fosmid clone Ad142N07 were incubated in 100 mL of LB broth containing 25 μg/mL chloramphenicol, at 37 °C for 20 h, and then harvested by centrifugation at 6000 ×g for 15 min at 4 °C. To utilize for shotgun sequencing, involving two approaches, standard Sanger sequencing and pyrosequencing, DNA was extracted using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, USA).

To carry out the Sanger sequencing, a total of 15 μg DNA from the enzyme-positive fosmid clone Ad142N07 was fragmented into 2–3 kb fragments by using the HydroShear DNA Shearing Device (Genomic Solution, Waltham, MA, USA) with the following parameters: 200 μL sample volume, speed code 11, and 20 shearing cycles. Removal and repair of the small size fragments was done using the CHROMA SPIN +TE1000 column (BD Biosciences Clontech, Heidelberg, Germany) and the polynucleotide kinase method (BKL Kit, TaKaRa, Shiga, Japan) with DNA polymerase, respectively. The resulting DNA fragments were ligated into the dephosphorylated *SmaI* site of pUC19 (Qbiogene, Carlsbad, CA, USA) and transformed into *E. coli* DH10B cells by electroporation (Bio-Rad, Hercules, CA, USA). Approximately 192 recombinant plasmids were randomly selected from the shotgun DNA library for sequencing. Then, plasmid DNAs were bidirectionally sequenced for each recombinant plasmid with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) and the ABI 3730 automatic sequencer (Applied Biosystems). The sequencing data were compiled by using Phred and Phrap (University of Washington, Seattle, WA, USA). For pyrosequencing, one run was conducted on a GS junior system (454 Life Sciences, Branford, CT, USA). The results obtained from pyrosequencing were assembled using the GS De Novo Assembler version 2.7 (http://www.454.com).

2.3. Sequence and domain analyses of the enzyme-positive fosmid clone and gene

A full metagenomic-insert-DNA sequence was established by assembling all the DNA fragments obtained from the two-way shotgun sequencing by using the Seqman software from Lasergene version 7 (DNASTAR, Madison, WI, USA). Prediction of potential open reading frames (ORFs) in assembled contigs was performed using the MetaGeneMark program [15]. Functional protein domains of the ORFs were identified on the Pfam website (http://pfam.xfam.org) [16].

The nucleotide sequence of the *KG42* gene of the fosmid clone (Ad142N07) was translated into an amino acid sequence using an *in silico* program (http://insilico.ehu.es/translate/). The module structures and signature sequences of the KG42 protein sequence were identified using the PROSITE database (http://prosite.expasy.org/). Regarding the N terminal region, a signal peptide sequence and a transmembrane region were identified by the Signal P program (http://www.cbs.dtu.dk/services/SignalP) and the HMMTOP 2.0 program (http://www.enzim.hu/hmmtop/html/submit.html), respectively.

A search for similarities between sequences was conducted by using the online BlastP program provided by the National Center for Biotechnological Information (NCBI) website (http://blast.ncbi.nih.gov/Blast/). Multi-alignments of the assumed protein sequences from the ORF (*KG42* gene) against other proteins were performed with the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.4. Tertiary structural analysis of KG42 xylanase

The tertiary structure of KG42 xylanase was predicted using the I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) online platform, based on homology modeling, the LOMETS program, and the iterative TASSER assembly simulation program [17]. The predicted tertiary model was further visualized and analyzed with the SPDBV 4.0.4 program, using the Verify3D (http://services.mbi.ucla.edu/Verify_3D) online platform, to obtain information about the fitness and validate the predicted KG42 protein model. Additionally, hydrogen bonds and salt bridges (distances ≤ 3.2 Å) were predicted using the UCSF Chimera 1.11.2 and VMD 1.9.3 platforms, respectively.

2.5. Expression and purification of the recombinant KG42 xylanase

The *KG42* gene encoding a putative xylanase was amplified from the enzyme-positive fosmid clone Ad142N07 by polymerase chain reaction (PCR) with a pair of primers 5′- cg ggatcc ATG AAG AAA GGA ATC GTG TGG CTG-3' (BamHI site underlined) and 5′- ccc aagctt TCA ATT CAC GGA TTC CGG ATC G-3' (HindIII site underlined). The PCR amplicon was cloned into the expression plasmid pQE80L (Qiagen, Valencia, CA, USA) and overexpressed in *E. coli* BL21 (New England biolabs Inc., Ipswich, MS, USA). The resulting *E. coli* strain harboring the *KG42* gene on pQE80L was cultivated at 37 °C in Terrific Broth containing ampicillin (100 μg/mL final concentration) until an optical density of 0.6 at 600 nm was reached. The expression of the recombinant protein was then induced by adding IPTG to 0.2 mM, followed by further incubation for 4 h at 37 °C. Cells were harvested by centrifugation and washed with 50 mM Tris-HCl buffer (pH 7.0) and disrupted by sonication at 4 °C. The overexpressed recombinant KG42 enzyme was purified from the clarified supernatants using the Chelating Excellose® Spin kit (Bioprogen, Daedeok, Daejeon, South Korea), as recommended by the manufacturer.

The purity and molecular mass of the purified enzyme were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [18]. The protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Activity staining of the purified enzyme was performed by zymography. Zymography of recombinant KG42 xylanase activity was done using 15% SDS-PAGE gel containing 0.6% xylan, under renaturing conditions.