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

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A B S T R A C T

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 cellulytic 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.

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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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cellulose polymers, generating new chain ends. Cellulolytic
hydrodrolases remove cellulose from reducing or non-reducing
ends of cellulose polymers. β-Glucosidases hydrolyze cel-
lodextrins and cellobiose to glucose.2 Cellulases have
generated commercial interest in various sectors, including
the food, energy, pulp, and textile industries.3 Among the
two or 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 vari-
ous industrial applications. This enzyme has been industrially
applied in biomass waste management, pulp and paper deink-
ing, 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.6

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

Although the ruminal microbial ecosystem is highly
diverse, the majority of rumen microorganisms are unculturable.10 It is estimated that less than 1% of microbial
species in nature have ever been cultured.10 A culture-
dependent 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 classi-
ﬁed 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 Com-
mittee (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 slaugh-
ter. 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 HindIII to construct a metagenomic fosmid library
by means of the CopyControl™ Fosmid Library Construction
Kit (Epigenes, Madison, WI). The technique was based on
the pCC1FOS vector, according to the manufacturer’s instruc-
tions. Escherichia coli DH5α™ (Life Technologies, Carlsbad,
CA) transformants, carrying various recombinant fosmids,
were transferred to 384 well plates. To determine an aver-
age insert size of the fosmid library, a total of 36 clones
were randomly selected and digested with the restriction
enzyme NotI.

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 (Ad12SD08) 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 (Ad12SD08) 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 Qia-
gen Plasmid Midi Kit (Qiagen, Valencia, CA) and subjected
to shotgun sequencing that comprised two approaches: conven-
tional 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 per-
formed using the HydroShear DNA shearing device (Genomic
Solution, Waltham, MA) under the following processing con-
ditions: 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 Smal site of pUC19. The liga-
tion products were introduced into E. coli DH10B cells by
electroporation. Approximately 192 recombinant plasmids in
the shotgun DNA library were randomly selected for sequenc-
ing. 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 assem-
bled using GS De Novo Assembler, version 2.7.
In silico sequence analyses of the enzyme-positive fosmid clone

All contigs from conventional shotgun sequencing with Sanger methodology and from the pyrosequencing assembly were combined to construct a full metagenomic-insert-DNA sequence using the SeqMan software in Lasergene version 7 (DNASTAR, Madison, WI). Gene prediction in each contig was performed using MetaGeneMark with default parameters. Functional annotation was performed by searching Pfam domains on the Pfam website (http://pfam.sanger.ac.uk). Amino acid sequences were deduced from the nucleotide sequences of the enzyme-positive fosmid clone (Ad12SD08) and were analyzed using BlastP on the National Center for Biotechnology Information (NCBI) database to search for proteins with similarity to the amino acid sequences derived from the identified open reading frames (ORFs). Multiple alignments among amino acid sequences were deduced from the ORFs, and the sequences of other proteins were performed using ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The PROSITE database was used to predict the module structures and signature sequences of the enzyme (http://prosite.expasy.org/). The N-terminal regions were analyzed using Signal IP software for a signal peptide sequence (http://www.cbs.dtu.dk/services/SignalP/) and by HMMTOP 2.0 software for transmembrane regions (http://www.enzim.hu/hmmtop/html/submit.html).

Heterologous expression and purification of the recombinant KG35 endo-β-1,4-glucanase

The KG35 gene (the complete ORF of the putative cellulase gene) was amplified from the enzyme-positive fosmid clone (Ad12SD08) by PCR. The amplicon was ligated into the expression vector pET24 (Novagen, Darmstadt, Germany), and the resulting recombinant vector was introduced into E. coli Rosetta-gami™ (Novagen, Darmstadt, Germany). Additional screening for cellulolytic activity of the enzyme produced by the transformed E. coli cells was performed using the same CMC agar plate method as described previously. The cellulolytic E. coli cells were grown at 37°C and the transgene expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) as described previously. After incubation for 6 h at 26°C, the cells were harvested and disrupted by sonication as described previously. The over-expressed protein was purified from the resulting cell-free extracts using the Chelating Excelsol® Spin Kit (Bioprogen, Daejeon, South Korea). A 2.0-μl aliquot of the purified protein was inoculated into the CMC agar plates to confirm the enzymatic activity. The proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (1970), and the protein concentrations were determined by the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA).

Zymogram analysis of the recombinant KG35 endo-β-1,4-glucanase was performed using a 10% polyacrylamide gel containing 0.1% CMC under denaturing conditions as described previously.

Enzymatic characterization of the recombinant KG35 endo-β-1,4-glucanase

Specific activities of the purified recombinant KG35 endo-β-1,4-glucanase toward various substrates were evaluated under the following standard conditions. The substrates used in this study were CMC, barley glucan, laminarin, avicel, xylan from birchwood, β-mannan, p-nitropheryl-d-glucopyranoside, and p-nitrophenyl-d-cellobioside. In all the assays, 10 μL of a properly diluted enzyme solution were added to 200 μL of 0.1 M sodium phosphate buffer (pH 7), containing 1% (w/v) of each substrate. The enzymatic reactions were allowed to proceed at 50°C (different reaction duration): 15 min for CMC, glucan, laminarin, and avicel; 10 min for xylan from birchwood and β-mannan; and 20 min for p-nitrophenyl-d-glucopyranoside and p-nitrophenyl-d-cellobioside. Specific activity was defined as the number of activity units per milligram of protein. One unit of enzymatic activity was defined as the amount of enzyme necessary to release 1 μmol of reducing sugar per minute. Additionally, 1 unit of enzymatic activity producing chromogenic sugar derivatives (p-nitrophenyl-d-glucopyranoside and p-nitrophenyl-d-cellobioside) as the substrates was defined as the amount of the enzyme required to release 1 μmol of p-nitrophenol per minute. The amounts of released reducing sugar and p-nitrophenol were determined by measuring the absorbances at 540 or 400 nm, respectively. All assays were performed in triplicate.

Optimal pH was determined by assaying the enzymatic activity of the purified recombinant, KG35 endo-β-1,4-glucanase, at 50°C for 15 min at pH ranging from 4 to 10. To determine the optimal temperature, enzymatic activity was analyzed in 0.1 M sodium phosphate buffer (pH 7) for 15 min at a temperature ranging from 20 to 70°C. All assays were performed in triplicate. The pH stability was assessed after the enzyme was pre-incubated at 4°C for 24 h at pH ranging from 4 to 10. The thermal stability was assessed after the enzyme was pre-incubated for 1 h in 0.1 M sodium phosphate buffer (pH 7) at various temperatures mentioned above. All assays were performed in triplicate.

Nucleotide sequence accession number

The nucleotide sequence of the enzyme-positive fosmid clone (Ad12SD08), including the complete ORF of the KG35 gene (a novel GH5 cellulase gene), was deposited in the GenBank database under the accession number KJ631389.

Results

Construction and functional screening of the metagenomic fosmid library

A metagenomic fosmid library containing 115,200 clones was constructed from the rumen microorganisms of black goats. NotI restriction enzyme analyses revealed that the average insert size was typically 31 kb (data not shown). A total of 155 independent fosmid clones, displaying carboxymethyl cellulase (CMCase) activity, were identified. Among them, a single fosmid clone (Ad12SD08) formed one of the largest clear zones
on the CMC-containing screening plates and was selected for further analysis.

Conventional shotgun sequencing with the Sanger method was performed under 2.7-fold coverage, and ~300 kb of pyrosequencing reads were produced. Consequently, one contig of 42.453 kb was obtained without the pCC1FOS vector sequence. Thirty-four ORFs were predicted in the contig sequence of fosmid clone Ad125D08, and an ORF containing a cellular domain (KG35 gene) was identified and localized within the clone.

**In silico sequence analysis of the putative KG35 endo-β-1,4-glucanase**

Sequence analysis of the putative KG35 gene revealed a single ORF of 963 bp encoding a polypeptide of 320 amino acid residues, with an estimated molecular mass of 35.1 kDa and pl of 5. Signal IP and HMMTOP 2.0 software programs could not detect any known signal peptide sequence or transmembrane region in the KG35 protein. The module structure search in the PROSITE database predicted that the KG35 protein contains a GHS cellulase catalytic domain with a conserved (amino acid) signature sequence VLYIEICNEPN, and its second Glu residue was assumed to play a catalytic role (Fig. 1).22,23

A BlastP search, comparing the amino acid sequence of the KG35 protein with those of other proteins registered in the GenBank database, revealed that this protein has the closest (58%) sequence identity to a hypothetical protein from *Eubacterium cellulosolvens* (WP_051527028.1) and shares even less sequence identity with other GHS family protein homologs from various microbial species (Table 1). The amino acid sequences of these homologous proteins possess characteristic features conserved among the GHS family of enzymes (Fig. 1).

**Purification and SDS-PAGE analyses of the recombinant KG35 endo-β-1,4-glucanase**

The putative endo-β-1,4-glucanase gene (KG35) was cloned and overexpressed in *E. coli* and the resulting recombinant enzyme was purified, as described in the materials and methods section. SDS-PAGE analysis of the purified recombinant KG35 endo-β-1,4-glucanase showed a single band on the gel, with the apparent molecular mass of approximately 35 kDa (Fig. 2). Zymogram analysis revealed a single band corresponding to an active endo-β-1,4-glucanase of the same residue mass as the band (Fig. 2).

![Multiple alignment of the KG35 endo-β-1,4-glucanase GHS domain with other homologous GHS protein domains](attachment:image.png)

**Fig. 1** – Multiple alignment of the KG35 endo-β-1,4-glucanase GHS domain with other homologous GHS protein domains. The deduced amino acid sequence of a GHS family domain in KG35 endo-β-1,4-glucanase was aligned with those of selected homologous enzymes from the following microorganisms: *Eubacterium cellulosolvens* (WP_051527028.1); *Agathobacter rectalis* (WP_055222932.1); *Roseburia inulinivorans* (WP_055169535.1); *A. rectalis* (WP_012741522.1); *Ruminococcus torques* (WP_055163516.1); *Eubacterium rectale* CAG:36 (CDC71888.1); *R. inulinivorans* (WP_055040325.1); and *Lachnospiraceae bacterium* C6A11 (WP_051646390.1). Signature sequences of GHS domains are shaded in gray. The Glu residues under the star symbols indicate the catalytic sites.
Table 1 – Amino acid sequence identity between the novel KG35 endo-β-1,4-glucanase and other (Homologous) proteins.

| Protein name (Organism name) | GeneBank accession number | Query coverage (%) | Identity (%) |
|------------------------------|--------------------------|--------------------|--------------|
| Hypothetical protein (Eubacterium cellulosolvens) | WP_051527028.1 | 98 | 58 |
| Glycosyl hydrolase family 5 protein (Agathobacter rectalis) | WP_055222932.1 | 98 | 52 |
| Glycosyl hydrolase family 5 protein (Roseburia inulinivorans) | WP_055169535.1 | 98 | 51 |
| Endo-1,4-β-glucanase (A. rectalis) | WP_012741522.1 | 98 | 51 |
| Glycosyl hydrolase family 5 protein (Ruminococcus torques) | WP_055163516.1 | 98 | 51 |
| Endoglucanase (Eubacterium rectale CAG:36) | CDC71888.1 | 98 | 51 |
| Glycosyl hydrolase family 5 protein (R. inulinivorans) | WP_055040325.1 | 98 | 50 |
| Hypothetical protein (Lachnospiraceae bacterium C6A11) | WP_051646390.1 | 98 | 50 |

* The novel KG35 endo-β-1,4-glucanase gene was used for BlastP searches using the 98% query coverage cutoff value and 50% identity cutoff value.

Table 2 – Substrate specificity of the novel KG35 endo-β-1,4-glucanase toward various substrates.

| Substrate | Main linkage types | Specific activity (U/mg) |
|-----------|-------------------|-------------------------|
| Carboxymethylcellulose (CMC) | β-1,4-Glucan | 8.40 ± 0.03 |
| Barley glucan | β-1,3/1,4-Glucan | 5.40 ± 0.19 |
| P-nitrophenyl-D-cellobioside | β-1,4-Glucan | 0.51 ± 0.01 |
| Laminarin | β-1,3/1,6-Glucan | 0.48 ± 0.04 |
| Avicel | β-1,4-Glucan | 0.12 ± 0.02 |
| Xylan from birchwood | β-1,4-Xylan | 0.00 ± 0.00 |
| β-Mannan | β-1,4-Mannan | 0.00 ± 0.00 |
| P-nitrophenyl-D-glucopyranoside | β-1,4-Glucan | 0.00 ± 0.00 |

Fig. 2 – SDS-PAGE analyses of recombinant KG35 endo-β-1,4-glucanase produced in E. coli Rosetta-gamiTM. Lane M: protein molecular weight markers; lane 1: total cell-free extracts before induction; lane 2: the soluble fraction after the induction; lane 3: purified KG35 endo-β-1,4-glucanase; lane 4: zymogram analysis of KG35 endo-β-1,4-glucanase.

Characterization of the purified recombinant KG35 endo-β-1,4-glucanase

Substrate specificity toward various substrates was determined (Table 2). The endo-β-1,4-glucanase showed strong specific activities toward CMC (8.40 U/mg) and barley glucan (5.40 U/mg). In contrast, this enzyme showed only trace enzymatic activity toward laminarin (0.51 U/mg), P-nitrophenyl-D-cellobioside (0.12 U/mg), and avicel (0.00 U/mg). In addition, the endo-β-1,4-glucanase could not hydrolyze P-nitrophenyl-D-glucopyranoside, xylan from birchwood, or β-mannan.

The optimal pH and temperature of the recombinant enzyme were determined under various pH and temperature conditions (Fig. 3). The optimal pH and temperature of the recombinant KG35 endo-β-1,4-glucanase toward CMC was approximately 6–7 and 30–50°C, respectively.

In addition, the enzymatic stability was tested under various pH and temperature conditions (Fig. 4). The endo-β-1,4-glucanase retained more than 70% of activity after 24 h of incubation at pH 5–7. The same enzyme showed thermostability after a 1-h incubation at 30–50°C; however, it declined in activity by greater than 60% after a 1-h incubation above 50°C. Our empirical data collectively indicates that the recombinant enzyme retained its pH and temperature stability approximately within its optimal pH and temperature ranges, respectively.

Discussion

It has been reported that the GH5 family of cellulases contain the conserved amino acid signature sequence pattern:
Therefore, the recombinant KG35 cellulase was maximally active and enzymatically stable at a pH range of 6–7 and at a temperature range of 30–50 °C. The optimum and stability characteristics of the recombinant enzyme were similar to those of other cellulase enzymes from rumen microorganisms of other
ruminants. Other cellulases from rumen microorganisms of the cow were reported to have the optimal pH range of 5–7 and an optimal temperature of approximately 50 °C. The cellulase from rumen-resident bacteria was reported to retain 70% of its activity in the pH range from 5 to 7 and in a temperature range from 30 to 50 °C.

The ruminal pH of small ruminants ranges from 5 to 7, under normal dietary conditions, and the ruminal temperature can vary from 38 to 41 °C. The cellulyotic reactions of ruminal-bacteria cellulase have been reported to be relatively more resistant at the pH of 6–5–7 in culture, but the reactions were weakened as the culture pH decreased. KG35 endo-β-1,4-glucanase may be well-adapted to the ruminal environments of black goats because the pH and temperature conditions under which the recombinant KG35 endo-β-1,4-glucanase showed relatively higher activity and stability were in agreement with the ruminal pH and temperature conditions that occurs naturally in ruminants, including goats. The above observation can explain why the recombinant KG35 endo-β-1,4-glucanase functions better at mildly acidic/neutral pH and a moderate temperature than at an acidic/alkaline pH or strongly unphysiological temperature.

Cellulase can effectively convert cellulose, an anti-nutritional component, in animal feedstocks into its hydrolysates, nutritive ingredients, yielding improved performance of animals. Therefore, applications of different cellulases from various sources have attracted considerable attentions in the feed industry. KG35 endo-β-1,4-glucanase may be exploited as a feed additive to improve feed utilization of ruminants, because the enzyme may be well-adapted to the ruminal environments. Moreover, the enzyme can be potentially used to make cellulose ethanol, second generation biofuel, from cellulosic feedstocks such as grass, wood, and crop residues.

This study presents novel results pertaining to the construction and screening of a metagenomic library from the rumen microorganisms of black goats for identification and characterization of a novel cellulytic enzyme. A total of 155 cellulytic fosmid clones were selected from the metagenomic library. Among them, the KG35 gene was successfully isolated as a clone with the strongest cellulose-degrading activity. The KG35 gene was found to encode a novel member of the GHS family of cellulases, and this novel gene was efficiently translated and overexpressed in E. coli Rosetta-gami™. Substrate specificity analysis revealed that the recombinant KG35 cellulase functions as an endo-β-1,4-glucanase. The recombinant KG35 endo-β-1,4-glucanase optimally functions in the pH range of 6–7 and temperature range of 30–50 °C. The endo-β-1,4-glucanase retained enzymatic stability in the pH range of 5–7 and the temperature range of 30–50 °C.

**Conflicts of interest**

The authors declare no conflicts of interest.

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