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A novel multifunctional cellulolytic enzyme screened from metagenomic resources representing ruminal bacteria

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ABSTRACT

Metagenomic resources representing ruminal bacteria were screened for novel exocellulases using a robotic, high-throughput screening system, the novel *CelEx-BR12* gene was identified and the predicted CelEx-BR12 protein was characterized. The *CelEx-BR12* gene had an open reading frame (ORF) of 1140 base pairs that encoded a 380-amino-acid-protein with a predicted molecular mass of 41.8 kDa. The amino acid sequence was 83% identical to that of a family 5 glycosyl hydrolase from *Prevotella ruminicola* 23. Codon-optimized CelEx-BR12 was overexpressed in *Escherichia coli* and purified using Ni–NTA affinity chromatography. The Michaelis–Menten constant (K_m value) and maximal reaction velocity (V_{max} values) for exocellulase activity were 12.92 μM and 1.55×10^{-4} μmol min^{-1}, respectively, and the enzyme was optimally active at pH 5.0 and 37 °C. Multifunctional activities were observed against fluorogenic and natural glycosides, such as 4-methylumbelliferyl-β-D-cellobioside (0.3 U mg^{-1}), CMC (105.9 U mg^{-1}), birch wood xylan (132.3 U mg^{-1}), oat spelt xylan (67.9 U mg^{-1}), and 2-hydroxyethyl-cellulose (26.3 U mg^{-1}). Based on these findings, we believe that CelEx-BR12 is an efficient multifunctional enzyme as endocellulase/exocellulase/xylanase activities that may prove useful for biotechnological applications.

1. Introduction

Materials containing cellulose, hemicellulose, and lignin (called lignocellulosic materials) are the most abundant type of natural biomass. Cellulose, which is a linear polymer of glucose residues linked by 1,4-β-D-glucosidic bonds, is the main constitute of plants and is the most abundant renewable organic resource in nature [1]. Cellulose can be degraded to glucose through the synergistic action of three classes of glycoside hydrolases (GHs): (1) endo-β-1,4-glucanase (EG; EC 3.2.1.4), which randomly attacks the cellulose polymer by endo action; (2) the exo-β-1,4-cellobiohydrolases, CBH I and CBH II (EC 3.2.1.91), which remove cellobiose from the non-reducing and reducing ends, respectively, of the cellulose chain; and (3) β-glucosidase (BGL; EC 3.2.1.21), which hydrolyzes cello-oligosaccharides and cellobiose to glucose [2,3]. Exocellulase, which is the only enzyme known to degrade highly ordered crystalline regions, is very important for the degradation of crystalline cellulose [4]. Finally, the released cellobiose is converted to glucose by β-glucosidase.

Cellulases are essential enzymes that are widely used in various industrial fields, including the bioethanol, textile, detergent, feedstuff, food, forage, beer-brewing and pulp-and-paper industries [5,6]. During the production of bioethanol, which is one of the most significant alternative energy sources, the release of glucose from cellulose is a crucial step governed by cellulolytic enzymes derived from fungi or bacteria [7]. The current limitations on the cellulosic bioconversion of lignocellulosic biomass include suboptimal enzyme stability and the sensitivities of some cellulases to reaction byproducts and other inhibitory agents [8]. Thus, researchers continue to search for novel and industrially useful enzymes, such as pH-stable, thermostable, high-activity, and/or multifunctional cellulases [9,10].

In this study, we used an high-throughput screening (HTS)-based robotic system to screen metagenomic resources for exocellulase activity, identified a novel gene that appeared to encode such an enzyme (*CelEx-BR12*), overexpressed a codon-optimized CelEx-BR12 in *Escherichia coli*, and characterized this novel multifunctional cellulolytic enzyme.

2. Materials and methods

2.1. Chemicals

The 4-methylumbelliferyl-β-D-cellobioside ($MeUmbG_2$), polysaccharide substrates (avicel, birch wood xylan, α-cellulose, carboxymethyl cellulose, curdlan, 2-hydroxyethyl-cellulose, laminarin, oat spelt xylan, salicin, D-gluconic acid, and starch), and cello-oligosaccharides were all purchased from Sigma–Aldrich

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(St. Louis, MO). All other chemicals were of the highest purity that was commercially available.

2.2. Screening the metagenomic library using a robotic, HTS system, and shotgun cloning of hit clones for exocellulase

Metagenomic DNA was isolated from the microorganisms of rumen fluid from a rumen-fistulated Korean cow (Hanwoo) using a modified sodium dodecyl sulfate (SDS)-based DNA extraction protocol [11] and a metagenomic library was constructed [12]. Positive samples ("hits") for novel exocellulases were identified using a robotic, HTS system running a protocol for screening metagenomic resources [12]. A potential exocellulase-encoding clone was isolated from the metagenomic library and cloned using a shotgun method [13,14]. The relevant plasmids were purified using a Plasmid Midi kit (Qiagen, Valencia, CA), recombinant fragments were sequenced by SolGent (Daejeon, Korea), and BLAST analyses were performed using the NCBI server [15]. The obtained sequences were aligned using the multialignment program of BioEdit version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the predicted amino acid sequence was analyzed for potential functional domains using the InterProScan tool (http://www.ebi.ac.uk/InterProScan/).

2.3. Construction of a vector expressing a codon-optimized CelEx-BR12 gene in E. coli

The amino acid sequence of CelEx-BR12 (GenBank Accession No. KC963960) was subjected to codon optimization, by GenoTech (Daejeon, Korea), using a proprietary algorithm, and the optimized gene (*CelEx-BR12opti*) was directionally cloned into the His-tagging vector, pET-22b(+) (Novagen, Madison, WI). DNA sequence of codon-optimized *CelEx-BR12opti* was shown in Supplementary data. More specifically, oligonucleotide primers (CBR12opti-F, 5′-GGA ATT CCA T**AT G**CG GAA GAA TTC CTT TAA A-3′ and CBR12opti-R, 5′-CCG CTC GAG TTT CTC TAG GGG CTT TCC T-3′; underlining indicates an *Nde*I or *Xho*I site, and the bold text shows the start codon) amplified a ~1.2 kb fragment when used for *Taq* polymerase chain reaction (PCR). The amplified DNA fragments were digested with *Nde*I and *Not*I, and ligated into the corresponding sites of pET-22b(+) to generate pET-CBR12. *E. coli* XL1-Blue was used as a host strain for the cloning and maintenance of plasmids. The recombinant plasmid was introduced into *E. coli* BL21 (DE3) for gene characterization and recombinant protein production.

2.4. Expression and purification of recombinant CelEx-BR12

To prepare recombinant CelEx-BR12 cellulase, the above-described *E. coli* were grown at 30 °C in LB (Luria–Bertani) broth containing 50 μg/ml ampicillin and transcription of the recombinant gene was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After incubation for 4 h at 30 °C, cells were harvested by centrifugation at 7000*g* for 10 min and washed with ice-cold PBS buffer (200 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, and 1 mM Na_2HPO_4; pH 7.5). Cells were disrupted using a VCX750 sonicator (Sonics Materials, Newtown, CT) and the His-tagged fusion protein was purified using a HiTrap chelating HP column (GE Healthcare, Piscataway, NJ) employing a gradient of 0–500 mM imidazole in PBS buffer containing 500 mM NaCl. The purified protein was desalted on a HiPrep 26/10 desalting column (GE Healthcare). All purification steps were performed using a fast protein liquid chromatography (FPLC) system (ÄKTA Explorer; GE Healthcare). The protein composition was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and the protein concentration was measured by the Bradford method (Bio-Rad protein assay kit; Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard [16].

2.5. Substrate specificity and determination of cellulolytic enzyme activity

Substrate specificity was examined using $MeUmbG_2$ and various carbohydrates (e.g., avicel, birch wood xylan, α-cellulose, carboxymethyl cellulose, curdlan, 2-hydroxyethyl-cellulose, laminarin, oat spelt xylan, salicin, and starch).

Enzyme activity was assayed by measuring the release of the MeUmb group when an aliquot of the enzyme was incubated with various MeUmb glycosides (final concentration, 0.1 mM) in 100 mM sodium acetate buffer (pH 5.0) for 20 min at 37 °C, along with the appropriate 4-methylumbelliferylliferone standards (0.05–1 nM). The fluorescence intensities of the released MeUmb groups were determined using a 1420 VICTOR multilabel counter (PerkinElmer Life Sciences, Wallac Finland Oy, Turku, Finland) with $\lambda_{excitation}$ = 365 nm and $\lambda_{emission} \geqslant$ 460 nm. Assays were performed in 100 μl reaction volumes of 100 mM sodium acetate buffer (pH 5.0) containing 0.1 mM of MeUmb glycoside. The reaction was terminated by addition of 100 μl of 500 mM glycine buffer (pH 10.4). One unit of enzyme activity was defined as the amount of enzyme that produced the equivalent of 1 μmol of product (reducing sugar or MeUmb) under optimal conditions in 1 min.

The endocellulase and xylanase activities of CelEx-BR12 for polysaccharide substrates were measured using the 3,5-dinitrosalicylic acid (DNS) reagent, as described by [17]. The reaction mixture consisted of 50 μl of 2% (w/v) carboxymethyl cellulose (molecular weight, 90 kDa; degree of carboxymethyl substitution, 0.7) (Sigma–Aldrich) in 100 mM sodium acetate buffer (pH 5.0); 50 μl of enzyme solution was added to the reaction mixture, followed by incubation at 40 °C for appropriate durations. All natural glycosides were assayed under the same conditions. To stop the reaction, 100 μl of DNS reagent was added and the reaction mixture was heated at 100 °C for 10 min. The mixture was then cooled at room temperature for 10 min and centrifuged at 10,000*g* for 10 min, and the absorbance of the supernatant at 540 nm was measured using a microplate reader (Model 680, Bio-Rad Laboratories).

The Michaelis–Menten constants (K_m values) and the maximal reaction velocities (V_{max} values) of purified CelEx-BR12 were calculated from double-reciprocal plots according to the Lineweaver–Burk method, and the kinetic constants K_m, V_{max}, k_{cat}, and k_{cat}/K_m were estimated using standard formulae [18].

2.6. Characterization of recombinant CelEx-BR12

The optimum pH of CelEx-BR12 was determined by incubation with 0.1 mM $MeUmbG_2$ in various buffers (pH 3.0–13.0) at 37 °C for 20 min. The buffers used to explore the optimum pH were as follows: 100 mM sodium acetate buffer (pH 3.0–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0), 100 mM Tris–HCl buffer (pH 8.0–9.0), 100 mM sodium bicarbonate buffer (pH 9.0–11.0), and 100 mM potassium chloride buffer (pH 11.0–13.0). The pH stability of the enzyme was assessed by incubating the enzyme at 4 °C for 24 h at various pH values, followed by assessment of residual activity. The optimum temperature for hydrolysis of 0.1 mM $MeUmbG_2$ in 100 mM sodium acetate buffer (pH 5.0) was determined by incubating a mixture of cellulase and 1% (w/v) carboxymethyl cellulose (CMC) for 20 min at temperatures ranging from 20 to 70 °C. Thermostability was evaluated by incubating the enzyme in 100 mM sodium acetate buffer (pH 5.0) for 0–120 min at temperatures ranging from 20 to 60 °C. The effects of metal ions were determined at a final ion concentration of 1 mM. All assays were performed at the optimum pH and