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A highly active beta-glucanase from a new strain of rumen fungus *Orpinomyces* sp.Y102 exhibits cellobiohydrolase and cellotriohydrolase activities

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HIGHLIGHTS

- A new strain of rumen fungus was isolated and designated *Orpinomyces* sp.Y102.
- *CelC7* from the cDNA library of *Orpinomyces* sp.Y102 encodes an exocellulase.
- The N-terminus of CelC7 may be a dockerin-containing domain.
- The N-terminal truncated forms of CelC7 have relatively high activities.
- Truncated CelC7 has cellobiohydrolase and cellotriohydrolase activities.

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ABSTRACT

A new strain of rumen fungus was isolated from *Bos taurus*, identified and designated *Orpinomyces* sp.Y102. A clone, *celC7*, isolated from the cDNA library of *Orpinomyces* sp.Y102, was predicted to encode a protein containing a signal peptide (Residues 1–17), an N-terminal dockerin-containing domain, and a C-terminal cellobiohydrolase catalytic domain of glycoside hydrolase family 6. CelC7 was insoluble when expressed in *Escherichia coli*. Deletion of 17 or 105 residues from the N-terminus significantly improved its solubility. The resulting enzymes, CelC7(-17) and CelC7(-105), were highly active to β-glucan substrates and were stable between pH 5.0 and 11.0. CelC7(-105) worked as an exocellulase releasing cellobiose and cellotriose from acid-swollen Avicel and cellooligosaccharides, and displayed a V_{max} of 6321.64 μmole/min/mg and a K_m of 2.18 mg/ml to barley β-glucan. Further, the crude extract of CelC7(-105) facilitated ethanol fermentation from cellulose. Thus, CelC7(-105) is a good candidate for industrial applications such as biofuel production.

1. Introduction

Cellulose, a polymer of D-glucose connected by β-1,4 linkage, is the major component of plant cell wall. To release glucose from lignocellulose as feedstock to produce ethanol is the idea for the second generation bioethanol (Hasunuma et al., 2013; Zhang et al., 2006), in which the efficient decomposition of cellulose is a key determining factor for the feasibility of this strategy. Therefore, highly effective cellulases, enzymes catalyzing the hydrolysis of cellulose, are required. Cellulase is also applied in several other industries, such as textile, detergent, and pulp/paper industries (Ko et al., 2010; Miao et al., 2014; Zhao et al., 2012). The utilization of enzymes in these industries to replace the traditional chemical processing is friendlier to the environment. Thus, the market of cellulase is expected to grow (Cherry and Fidantsef, 2003; Zhang et al., 2006).

At least three types of cellulases are required to work in synergy to convert cellulose into glucose monomer. Endocellulase or endoglucanase (endo-β-1,4-glucanase; EC 3.2.1.4) randomly cleaves the internal β-1,4 bonds of cellulose, generating shorter

Abbreviations: CBM, carbohydrate-binding module; CMC, carboxymethyl cellulose; DNS, 3,5-dinitrosalicylic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; ITS1, internal transcribed spacer 1 region; LB, Luria–Bertani; NCBI, National Center for Biotechnology Information; TLC, thin-layer chromatography.

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chains or oligosaccharides. Exocellulase or exoglucanase is usually referred to enzymes that hydrolyze cellulose by releasing cellobiose successively from the reducing end (EC 3.2.1.176; cellobiohydrolase I) or non-reducing end (EC 3.2.1.91; cellobiohydrolase II) of cellulose. Meanwhile, exocellulases that liberate glucose successively from the termini of cellulose are also reported (EC 3.2.1.74; glucan 1,4-β-glucosidase or exo-1,4-β-glucosidase). Finally, β-glucosidase (EC 3.2.1.21) dissects cellobiose or oligoglucosaccharides into glucose (Zhang et al., 2006). The work of endocellulase and exocellulase is considered the rate limiting step for the degradation of cellulose (Zhang et al., 2006). In the classification of glycoside hydrolase (EC 3.2.1.-) (Henrissat and Bairoch, 1996), endocellulases are found in 17 families; type I cellobiohydrolases are classified in families 7, 9, and 48, whereas type II cellobiohydrolases are in families 5, 6, 7, and 9.

The most common sources for exploring cellulases were bacteria and fungi (Kumar et al., 2008), and the available cellulases in the market were mostly cloned from *Trichoderma* (e.g. *Trichoderma reesei*, *Trichoderma viride*) and *Aspergillus* (Zhang et al., 2006). Evidence suggested that rumen fungi contain cellulases with much higher activities than those from *Trichoderma* (Chen et al., 2003; Harhangi et al., 2003; Nagy et al., 2007; Qiu et al., 2000). Thus, rumen fungi are regarded as a potential source for seeking highly efficient cellulases. Since cultivation of rumen fungi on a large scale is not easy due to their unique growth conditions, the number of cellulases cloned from rumen fungi is limited.

In this study, a new strain of the fungus *Orpinomyces* was isolated from the rumen of cattle (*Bos taurus*), and a cDNA library was constructed from its total RNA. The library was subjected to screening for β-glucan- or carboxymethyl cellulose (CMC)-degrading activity. The biochemical properties of the enzyme encoded by one of the positive clones, designated *celC7*, were investigated.

2. Methods

2.1. Bacterial strains and chemicals

Escherichia coli BL21(DE3) (Novagen, Madison, WI, USA) was used for protein expression and purification and *E. coli* DH5α (Yeastern Biotech, Taipei, Taiwan) for plasmid propagation and isolation. Oat spelt xylan, CMC, glucose, cellobiose, cellotriose, cellooligosaccharides, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-galactopyranoside, and chemicals for buffer preparations were purchased from Sigma–Aldrich (St. Louis, MO, USA); Avicel from Asahi Chemical (Tokyo, Japan); CBH I, barley β-glucan, pachyman, cellopentaose, and cellohexaose from Megazyme (Wicklow, Ireland); restriction enzymes from New England Biolabs (Beverly, MA, USA); and DNA polymerase and T4 DNA ligase from Roche Applied Science (Indianapolis, IN, USA). PCR or sequencing primers were synthesized by MDBio Inc. (Taipei, Taiwan). Acid-swollen Avicel was prepared according to previously published method (Wood and Bhat, 1988). Soluble oat spelt xylan was prepared as described previously (Chen et al., 2012).

2.2. Isolation and phylogenetic analysis of Orpinomyces sp.Y102

Isolate Y102 was obtained from the rumen of male *Bos indicus* bred in the Livestock Research Institute in Hengchun, Taiwan, using previously published method (Chen et al., 2007). Briefly, the rumen fluid was inoculated into a Hungate tube (125 × 16 mm, Bellco Glass Inc., Vineland, NJ, USA) containing 5 ml isolation agar medium. One colony, designated Y102, was observed and inoculated into isolation broth with 0.5% (w/v) dry rice straw as the sole carbon source. Isolate Y102 was maintained by sub-culturing every five days using the same broth. Microscopic slides were prepared at various growth stages of Y102, fixed with glutaraldehyde and stained with phloxin red for observing the zoospores and thallus under a light microscope (CX41, Olympus Imaging Crop., Japan). The images were recorded by the attached digital camera (C-5050, Olympus Imaging Crop.).

Genomic DNA extraction, PCR amplification, and sequencing were performed as described previously (Chen et al., 2007). Sequences of internal transcribed spacer 1 region (ITS1) for Isolate Y102 and the reference sequences obtained from the GenBank were aligned by Bioedit software (Hall, 1999). The aligned sequences were checked by eyes, and corrected manually to minimize the number of gaps. The Neighbor-joining tree featuring 1000 bootstrap replication was performed by MEGA 6.0 software compiled for the Microsoft Windows 7 64-bit compatible PC platform (Chen et al., 2007; Tamura et al., 2013).

2.3. Isolation of celC7 and generation of CelC7 truncated forms

The cDNA library of Isolate Y102 was constructed using the SMART cDNA library construction kit (BD Bioscience, Palo Alto, CA) as described previously (Hung et al., 2012), and screened for β-glucanase activity by growing the plaques in Luria–Bertani (LB) soft agar plates containing 0.1% (wt/vol) CMC and 0.1% (wt/vol) barley β-glucan. Positive plaques were revealed by Congo red staining (Teather and Wood, 1982). The insert in the vector from one of the positive clones was sequenced (Mission Biotech, Taipei, Taiwan) and designated *celC7*. The sequence of *celC7* was submitted to GenBank [accession number KF682138].

The coding region of *celC7* was cloned into the *Bam* HI-*Xho* I site of vector pET21a(+) (Novagen), which provides a 6xHis tag in the C-terminus and a T7 tag in the N-terminus of the recombinant protein. Oligonucleotide primers were designed to amplify the desired region of *celC7* cDNA sequence by PCR to create the N-terminal truncated forms, using the vector harboring *celC7* as the template. The reverse primer was identical for both truncated forms: 5′-CCGCTCGAGCGGAAATGGGGGGTTAGCATTTTC-3′. The forward primer for *celC7(-17)* was 5′-CGCGGATCCGCGTCCAGATGT CATCCAAGTTACC-3; for *celC7(-105)* was 5′-CGCGGATCCGCGACT AGTGCTAGAACCACTACCAGAAC-3′. The PCR reaction mixture was adjusted to 50 μl and contained 50 ng of template DNA, 360 μM each dNTP, 300 nM each of the primers, and 3.75 units of Expand long DNA polymerase in reaction buffer supplied by the manufacturer. PCR was performed on a thermal cycler (GeneAmp® PCR System 2700, Applied Biosystems, Foster City, CA, USA) with a thermocycling program of 2 min at 94 °C, 25 cycles of 30 s at 52 °C, 90 s at 72 °C, and 30 s at 94 °C, followed by 5 min at 72 °C. The desired PCR fragment was purified using Gel/PCR DNA Fragment Extraction kit (Geneaid, Taipei, Taiwan) per the manufacturer's instruction, and digested with *Bam* HI and *Xho* I. The resulting fragment was purified via the same kit, and ligated with vector pET21a(+), also digested with *Bam* HI and *Xho* I and purified, using T4 DNA ligase in the supplied buffer at 4 °C for 16 h. The product was transformed into *E. coli* DH5α by heat shock. Colonies were randomly selected from the resulting plate and inoculated into 5 ml LB/ampicillin (100 μg/ml) liquid culture. Plasmids were purified using a Plasmid Miniprep Kit (Biokit, Taipei, Taiwan). Clones of truncated forms were identified and confirmed by DNA sequencing of the plasmids (Mission Biotech).

2.4. Expression and purification of CelC7 truncated forms

The vector containing the coding region of *celC7*, *celC7(-17)*, or *celC7(-105)* was transformed into *E. coli* BL21(DE3) by heat shock. A colony of the resulting plate was grown in 5 ml LB/ampicillin medium at 37 °C overnight as the seed culture. It was subsequently