Journal of Molecular Catalysis B: Enzymatic 73 (2011) 104–110

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Screening and characterization of a cellulase with endocellulase and exocellulase activity from yak rumen metagenome

Lei Bao, Qiang Huang, Lei Chang, Jungang Zhou*, Hong Lu*

State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, No. 220 Handan Road, Shanghai 200433, China

ARTICLE INFO

Article history:
Received 26 March 2011
Received in revised form 1 August 2011
Accepted 13 August 2011
Available online 22 August 2011

Keywords:
Cellulose saccharification
Function-based screening
Endocellulase
Exocellulase
Uncultured yak rumen microorganism

ABSTRACT

Cellulose saccharification is an important process in conversion from lignocellulosic biomass to biofuels and other chemicals, and requires concerted action of endocellulase, exocellulase and β-glucosidase. Thus, it is very interesting to discover and develop multifunctional cellulase in order to convert cellulose to glucose more efficiently. Here we report an endo/exocellulase Rucel5B with 336 amino acids cloned from yak rumen uncultured microorganism, and its recombinant expression in *Escherichia coli*. This cellulase possesses endo-β-1,4-glucanase activity of 220 U mg^{-1} against carboxymethylcellulose and exo-β-1,4-glucanase activity of 52.9 U mg^{-1} against 4-nitrophenyl-β-D-cellobioside, and is able to hydrolyze not only amorphous cellulose (carboxymethylcellulose, barley glucan, lichenan, phosphate acid swollen cellulose, etc.), but also crystalline cellulose (filter paper, avicel, etc.). The exo-type action mode of Rucel5B was confirmed by its release of cellobiose from cellooligosaccharides and crystalline cellulose, and its endo-type action mode was confirmed by a time-dependent decrease in the polymerization degree of hydrolysates when Rucel5B was incubated with soluble amorphous cellulose. Therefore, the enzymatic activities, the endo/exo-mode of action and the ability in saccharification of both amorphous and crystalline cellulose make Recul5B a very interesting candidate for efficient saccharification of cellulose.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Cellulose, the linear polymer of glucose residues linked by β-D-(1-4)-glucopyranosidic bonds, is the main constitute of plants and is the most abundant renewable biomass on earth [1]. Cellulose can be converted to glucose through concerted action of three types of cellulases: endocellulase (endo-β-1,4-glucanases, EC 3.2.1.4), exocellulase (exo-β-1.4-cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [2,3]. The first step of cellulose hydrolysis, a critical one, is that the endocellulase randomly attacks the amorphous regions in cellulose chains to generate cellooligosaccharides and release free ends [4]. Then the exocellulase cleaves cellobiose from the free ends in both amorphous and crystalline regions through exo-mode of action. Exocellulase is very important for degradation of crystalline cellulose because exocellulase is the only enzyme that can degrade highly ordered crystalline regions [4]. Finally the released cellobiose is converted to glucose by β-glucosidase.

Cellulases are important industrial enzymes that are broadly utilized in bioethanol, textile, paper, detergent, forage, and beer-brewing industries [5,6]. In the conversion from cellulose biomass into bioethanol and other chemicals, saccharification of cellulose to glucose by cellulases is the key process [2,3]. Although a number of cellulases have been discovered from fungi and bacteria, it is still unable to meet the needs of industrial application because of certain limitations, such as stability, activity, sensitivity to byproducts [2,5]. So researchers are still making efforts to isolate novel cellulases, such as thermostable cellulases, halostable cellulases, high activity cellulases, and multifunctional cellulases [5–10]. Most cellulases were found to be monofunctional, however, there also exist bifunctional/multifunctional cellulases, which could be used as two/more kinds of monofunctional cellulases [5,11,12]. Several artificially constructed bifunctional/multifunctional cellulases have also been reported [13]. Hence, discovery and development of multifunctional cellulases are very useful for potential applications in efficient conversion from cellulose to glucose.

Microorganisms from various environments provide potential sources for novel cellulases. Traditional explore for novel cellulase is mainly based on the isolation and re-cultivation of

Abbreviations: GH, glycoside hydrolase; CMC, carboxymethylcellulose sodium; MuC, 4-methylumbelliferyl-β-D-cellobiopyranoside; PASC, phosphate acid swollen cellulose; pNPC, 4-nitrophenyl-β-D-cellobioside; pNPG, 4-nitrophenyl-β-D-glucopyranside; T_m, melting temperature; DP, degree of polymerization; TLC, thin-layer chromatography; HPAEC-PAD, high performance anion-exchange chromatography with pulsed amperometric detection.

* Corresponding authors. Tel.: +86 21 65642505; fax: +86 21 65642505.
E-mail addresses: zhoujg@fudan.edu.cn (J. Zhou), honglu0211@yahoo.com (H. Lu).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.08.006

microorganisms, however, more than 90% of microorganisms resist laboratory cultivation [14,15]. Development of metagenomics provides a cultivation independent method to mine wide range of biocatalysts in unculturable microorganisms. Combining metagenomics method and high throughput functional screening, many novel cellulases-encoding genes have been cloned, such as endoglucanase Cel5A from soil metagenome, cellulase CelAM11 from abalone intestine [8,9,14,16–20].

The yak (*Bos grunniens*) is a kind of ruminant living on Qinghai-Tibetan Plateau in China. Their diet of pure grass pasture instead of grain (starch based) makes rumen a residence for highly active lignocellulose hydrolyases [21]. However, more than 85% of rumen microorganisms were uncultured and many novel enzymes present in difficult-to-culture microbes are not characterized yet [21], while metagenomic approach is suitable to resolve this problem. So in this study, we employed metagenome method and function-based screening to explore yak rumen cellulase resources, and isolated a cellulase named Recul5B, which exhibited both endo-β-1,4-glucanase and exo-β-1,4-glucanase activities. Besides, its enzymatic properties, and the endo-/exo-mode of action were fully investigated.

2. Materials and methods

2.1. Library construction and screening

The yak rumen samples were collected in Qinghai-Tibet Plateau, China, January, 2007. Metagenomic DNA was extracted as described previously [21]. Cosmid library was constructed using pWEB Cosmid Cloning Kit (Epicentre, Madison, USA) according to product manual and preserved in 96-well plates at −80 °C [22].

The library clones were incubated in LB medium containing 100 mg L^{-1} ampicillin at 37 °C for 16 h. After centrifugation, cell pellets were collected and resuspended in 30 μL of 50 mM sodium citrate (pH 6.0). The cells were lysed by freezing and thawing [23]. For endo-β-1,4-glucanase screening, the lysates were piped on agar plates containing 0.5% CMC, then the plates were incubated at 37 °C for 1 h, stained with 1% (w/v) Congo red for 20 min and destained with 1 M NaCl [24]. The positive clones would form a halo against a red background. For exo-β-1,4-glucanase screening, 10 μL of 0.1% (w/v) MuC (Sigma, St. Louis, USA) was added into the cell lysates and incubated at 37 °C for 1 h. The positive clones would emit blue florescence under UV light. Then the cosmid DNA of positive clone was extracted to construct subclone library in pGEM11z (Promega, Madison, USA) and also screened for endo-β-1,4-glucanase and exo-β-1,4-glucanase activities. The insert fragments of positive subclones were then separately sequenced.

The BLAST program at the NCBI was used for database searches [25]. Conserved domain analysis was performed by the online software SMART (http://smart.embl-heidelberg.de). Putative signal sequence was identified by SignalP 3.0 server (http://www.cbs.dtu.dk/services/). The phylogenetic tree was constructed with MEGA3 [26].

2.2. Expression and purification

The ORF of *Rucel5B* without signal peptide coding sequence was amplified by PCR with a sense primer (5′-ATAGAATTCCATCATCACCATCATCACGGCAACGGCTGGGTC-3′) and an antisense primer (5′-TGTAAGCTTACCCGCCTGTCCCTG-3′), and then cloned into pET21a (Novagen, Madison, USA). Recombinant Rucel5B was overexpressed in *Escherichia coli* BL21 (DE3) (Novagen, Madison, USA) according to pET System Manual, before the cells were suspended in lysis buffer (20 mM Tris–HCl, 50 mM NaCl, pH7.4) and lysed by sonication. Rucel5B was purified by Ni/NTA resin and analyzed by SDS-PAGE for size and purity. Protein concentration was determined by the BCA assay using bovine serum albumin as standard [27].

2.3. Enzymatic assay

Substrate CMC (Sigma, St. Louis, USA) was used for endoglucanase activity assay and pNPC (Sigma, St. Louis, USA) for exoglucanase activity assay. The standard assay condition for endoglucanase activity was to incubate 1 μg Rucel5B with 300 μL of 1% CMC in 50 mM sodium citrate (pH 6.5) at 60 °C for 2 min. The amount of reducing sugar released was measured with DNS reagent [28]. The standard assay condition for exoglucanase activity was to incubate 1 μg Rucel5B with 300 μL of 1 mM pNPC in 50 mM sodium citrate (pH 5.5) at 65 °C for 2 min. Then 100 μL 1 M Na_2CO_3 was added into the mixture to quench the reactions and visualize yellow color. The amount of *p*-nitrophenyl released was quantified by the absorbance at 405 nm. One unit (U) of endoglucanase or exoglucanase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar from CMC or 1 μmol *p*-nitrophenyl from pNPC per minute. All the assays were performed in triplicate.

The pH optima for endoglucanase and exoglucanase activities were assayed at 60 °C for 2 min from pH 4.0 to 9.0 using appropriate buffers: sodium citrate (50 mM, pH 4.0–6.5), sodium phosphate (50 mM, pH 6.0–8.0), Tris–HCl (50 mM, pH 8.0–9.0). The temperature optima for endoglucanase and exoglucanase activities were determined at an interval of 5 °C from 25 °C to 80 °C in 50 mM sodium citrate, pH 6.0 for 2 min. The thermostability was determined by measuring residual endoglucanase activity under standard condition after treatment of the enzyme at 50, 55 and 60 °C for 10–60 min in the absence of substrates.

Circular dichroism (CD) spectra of Rucel5B were measured using JASCO J-715 CD spectropolarimeter (JASCO, Tokyo, Japan) from 35 °C to 90 °C at the rate of 1 °C min^{-1} over the wavelength range from 200 to 250 nm. The thermal denaturation melting curves were determined by Boltzman fitting of the ellipticity values from 35 °C to 90 °C at 222 nm, and the T_m was calculated according to the thermal melting curves.

Substrate specificity of Rucel5B was assayed using a serial of substrates. Specific activity against 1% (w/v) CMC, barley β-glucan (Megazyme, Bray, Ireland), lichenan (Megazyme, Bray, Ireland), laminarin (Sigma, St. Louis, USA), β-1,6-glucan (Sigma, St. Louis, USA), xylan from birchwood (Sigma, St. Louis, USA) and PASC [29] was assayed at 60 °C in 50 mM sodium citrate (pH 6.5) (CMC, lichenan, laminarin and birchwood xylan were prepared as colloidal suspension in water according to Megazyme's procedures). Activity against 12% (w/v) avicel PH101 (Sigma, St. Louis, USA) and 4% (w/v) Whatman NO. 1 filter paper (Sigma, St. Louis, USA, first smashed to about 1 mm × 1 mm and then prepared as suspension) was assayed in 50 mM sodium citrate (pH 6.0) at 37 °C for 8 h. Activity against 1 mM pNPG (Sigma, St. Louis, USA) and 1 mM pNPC was carried out at 65 °C in 50 mM sodium citrate (pH 5.5). One unit of activity against the above substrates was defined as the amount of enzyme releasing 1 μmol reducing sugar or 1 μmol *p*-nitrophenyl per minute using glucose or pNP as standard. Specific activity was defined as the units of enzyme per milligram of protein.

The values of Michaelis constant (K_m), turnover number (k_{cat}) and specificity constant (k_{cat}/K_m) on CMC, barley glucan, lichenan and pNPC were determined by the activities at 60 °C in 50 mM sodium citrate (pH 6.5) against 0.1–1% polysaccharides substrate, or the activities at 65 °C in 50 mM sodium citrate (pH 5.5) against 0.1–1 mM pNPC through double-reciprocal plot method [30].

Effects of metal ions on the activity of Rucel5B were determined against 1% (w/v) CMC or 1 mM pNPC under standard assay condition with metal ions at final concentrations of 5 mM. The inhibition or

Download English Version:

https://daneshyari.com/en/article/70089

Download Persian Version:

https://daneshyari.com/article/70089

Daneshyari.com