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Characterization of a thermostable, specific GH10 xylanase from *Caldicellulosiruptor bescii* with high catalytic activity

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ABSTRACT

Xylanase (EC 3.2.1.8) is one of the most important enzymes for the biodegradation of xylan. Since many industrial processes utilizing xylanase are operated at elevated temperatures, thermostable xylanases are highly desirable. In the present study, *xyn10B* gene from thermophilic bacterium *Caldicellulosiruptor bescii* that encodes a glycoside hydrolase (GH) family 10 xylanase was overexpressed in *Escherichia coli* and systematically characterized. CbXyn10B exhibited optimal activity at pH 7.2 and 70 °C. It had a half-life of about 7.7 h at 60 °C, and retained over 85% of maximal activity after incubation at pH 4.0–12.0. The activity of this xylanase was not affected by most divalent cations, but inhibited by Fe^{3+} and Zn^{2+}. CbXyn10B exhibited high activity on beech wood xylan, oat spelt xylan, and birch wood xylan, with specific activities of about 450 U mg^{-1}. Compared with other GH10 xylanases, CbXyn10B was highly specific for xylan and showed low catalytic efficiency toward sodium carboxymethyl cellulose and *p*-nitrophenyl-β-D-xylopyranoside. HPLC analysis of the products released from xylo-oligosaccharides and xylan revealed that xylobiose was the predominant hydrolytic product. The action mode of the enzyme was studied by product analysis, homology modeling and molecular docking to gain an insight into the structural basis for its substrate recognition mechanism.

1. Introduction

Xylan, as the major constituent of plant cell walls, is the second most abundant polysaccharide in nature and accounts for approximately one-third of all renewable organic carbon on earth [1]. Biodegradation of xylan requires the coordinate action of several enzymes, such as endo-β-1,4-xylanase (xylanase for short), β-D-xylosidase, α-L-arabinofuranosidase, α-D-glucuronidase, acetylxylan esterase and feruloyl esterases [2]. Among these, xylanases (EC 3.2.1.8) that cleave internal linkages on the β-1,4-xylose backbone play a leading role. Xylanases are produced by many microorganisms, including bacteria, actinomycetes, protozoa and fungi [3,4]. These enzymes vary in primary sequences, structure folds, substrate specificities and catalytic mechanism [5,6]. They have been mainly classified into glycoside hydrolase (GH) families 5, 7, 8, 10, 11 and 43 [7]. GH10 xylanases, as one of the most well studied family, typically also have considerable activity toward glucose-derived substrates such as aryl-cello-oligosaccharides [8–10].

Xylanases have immense potential in various biotechnological industries, including the food, feed, textile, waste treatment, bioethanol production and pulp bleaching [11]. However, most known xylanases show maximal activity at temperatures between 40 °C and 60 °C [12], whereas industrial processes favor higher temperatures. In addition, thermostable xylanases that function at elevated temperatures lower the likelihood of microbial contamination, increase reaction rates and substrate solubility and simplify the downstream protein purification [13]. Thus, the requirement for thermostable xylanases is progressively increased.

Caldicellulosiruptor bescii DSM 6725 is an anaerobic thermophilic bacterium that can efficiently utilize various types of untreated plant biomass. This strain can grow at temperatures as high as 90 °C, which makes it the most thermophilic

Abbreviations: *p*NPX, p-nitrophenyl-β-D-xylopyranoside; GH, glycoside hydrolase; CMC-Na, sodium carboxymethyl cellulose; XOS, xylo-oligosaccharides; X1–X6, xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose.

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cellulose-degrading organism known to date [14]. Based on its genome information, *C. bescii* DSM 6725 produces three types of xylanases, including two GH10 xylanases (CbXyn10A and CbXyn10B) and a GH11 xylanase [15]. CbXyn10A is composed of an N-terminal signal-peptide, two carbohydrate-binding domains and a C-terminal catalytic domain, while CbXyn10B possesses only a catalytic domain. It has recently been reported that these two GH10 xylanases are highly thermostable and could work synergistically with other xylan-degrading enzymes from *C. bescii* to hydrolyzed xylan at 65–80 °C [16]. To enable CbXyn10B to function under well-defined conditions, a fine characterization of CbXyn10B appears to be desirable.

Here, the gene encoding CbXyn10B was overexpressed in *Escherichia coli*, and recombinant enzyme was purified and further characterized. The mode of action of the enzyme on xylo-oligosaccharides (XOS) was analyzed in detail. In addition, homology modeling and molecular docking were performed to further dissect the structural basis for CbXyn10B action manner.

2. Materials and methods

2.1. Chemicals, strains and plasmids

Xylose (X1), *p*-nitrophenyl-β-xylopyranoside (*p*NPX), *p*-nitrophenyl-β-glucopyranoside (*p*NPG), beech wood xylan, birch wood xylan and oat spelt xylan were purchased from Sigma–Aldrich (St. Louis, USA). Beta-1,4-D-xylooligosaccharides from xylobiose to xylohexaose (X2–X6) were obtained from Megazyme International Ireland Ltd. The pET-28a vector was purchased from Novagen (Darmstadt, Germany). *E. coli* TOP10 and *E. coli* BL21-CodonPlus (DE3)-RIL strains were purchased from Invitrogen (Carlsbad, CA, USA) and they were used for DNA manipulation and recombinant protein production, respectively. *C. bescii* DSM 6725 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

2.2. Multiple sequence alignment and phylogenetic analysis

Homology search was performed with BLAST program at the NCBI web server (http://www.ncbi.nlm.nih.gov/BLAST) [17]. The phylogenetic tree was constructed by Mega 5 software using the neighbor joining method [18]. Sequence alignments were performed with ClustalX [19].

2.3. Cloning and plasmid construction

The *CbXyn10B* gene (GenBank No. ACM59337) was amplified from genomic DNA of *C. bescii* DSM 6725 using the primers *CbXyn10B*_F (5′-CCAGTCCCATGGAGAGCGAAGATTATTATGAAAA-3′) and *CbXyn10B*_R (5′-CGACGACTCGAGAAAGTCAATTATTCTGAAAAATGCC-3′). The PCR products were digested with FD *Nco*I and FD *Xho*I (Fermentas, Shenzhen, China) and inserted into pET-28a expression vector.

2.4. Overexpression and purification of CbXyn10B

The pET28-*CbXyn10B* was expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells. Cells were grown at 37 °C in 2YT medium supplemented with 100 μg/ml kanamycin until the optical density at 600 nm (OD600) reached 0.6–0.8. Gene expression was induced for 16 h at 24 °C by the addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested and suspended in 30 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl and 30 mM imidazole, and then disrupted by sonication. The cell lysates were heated at 60 °C for 30 min, and the resulting precipitate was removed by centrifugation. The recombinant protein was purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany). Protein concentration was measured by the Bradford method with a Bovine Serum Albumin standard set (Fermentas, Shenzhen, China).

2.5. Determination of enzyme activities and properties

The standard assay for xylanase activity was performed at 65 °C in 40 mM pH 7.2 sodium phosphate buffer in the presence of 1.0% (w/v) xylans for 5 min. For CMCase activity, the assay was performed in the same buffer containing 1.0% (w/v) CMC-Na for 30 min. The amount of reducing sugars released was determined with the 3,5-dinitrosalicylic acid (DNS) reagent, using xylose or glucose as standard. After incubation, DNS reagent was added and the samples were heated in a boiling water bath for 5 min followed by cooling on ice. The absorbance was then measured at 540 nm. Each assay was performed in triplicate. One unit of xylanase activity is defined as the amount of enzyme required to release one μmole of xylose/glucose reducing-sugar equivalents per minute at 65 °C and pH 7.2. For *p*NPX and *p*NPG, the assay was performed with a final concentration of 0.2 mM at 65 °C for 30 min and the absorbance was measured at 405 nm. One unit of xylanase activity is defined as the amount of enzyme required to release one μmole of *p*-nitrophenol per minute at 65 °C and pH 7.2.

The effect of temperature on enzyme activities was determined by assaying the enzymes at temperatures from 40 to 85 °C using 1% (w/v) beech wood xylan as substrate. Thermal stability of xylanase was assessed by incubated enzyme solutions (0.2 mg/ml) for 4 h at 60 °C, 65 °C, 70 °C and 75 °C, respectively. The relative activities of heat-treated CbXyn10B were measured at definite time intervals.

The effects of pH on enzyme activity were determined at 60 °C under pH ranging from 4 to 11 using 1% (w/v) beech wood xylan as substrate. The reaction buffer contained 30 mM each of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid (TAPS), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(N-morpholino)ethanesulfonic acid (MES) and acetic acid [20], and was adjusted to the appropriate pH with 1 M NaOH. To exclude the possibility of thermo-inactivation at high temperature, the pH stability of the purified enzyme was diluted 100-fold using respective buffers having pH values ranging from 3.0 to 13.0 as described above and were incubated for 4 h at 37 °C [21,22]. The relative activity of the incubated enzyme was estimated following the procedure described above.

The kinetic parameters were determined by incubating CbXyn10B in 40 mM sodium phosphate buffer (pH 7.2) with birch wood xylan, beech wood xylan and oat spelt xylan at concentrations ranging from 0.2 to 10 mg/ml. All constants were determined at 65 °C to avoid the activity loss during the reaction process. The extent of hydrolysis for each substrate was kept consistent, so that the change of the inhomogeneous substrate was minimized. Kinetic parameters V_{max} (app) and K_m (app) were acquired by fitting enzymatic activities as a function of substrate concentrations to the Michaelis–Menten equation using non-linear regression of the software Origin 8.0. The k_{cat} (app) was obtained by using the equation k_{cat} (app) = V_{max} (app)/[E], where [E] was the molar concentration of the enzymes.

The effects of metal ions on purified xylanase enzyme activity were determined by incubating the enzyme with a 1, 5 or 10 mM solution of Ca^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+} or EDTA for 1 h at 25 °C. Xylanase activity was measured using 1% (w/v) beech wood xylan as substrate under the standard condition.

2.6. Reaction product analysis

To analyze the hydrolysis mode of xylo-oligosaccharides (XOS), 0.15 mg/ml XOS were incubated with 0.06 μM recombinant