Enzyme and Microbial Technology 72 (2015) 1–9

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Paenibacillus curdlanolyticus B-6 xylanase Xyn10C capable of producing a doubly arabinose-substituted xylose, α-L-Ara*f*-(1 → 2)-[α-L-Ara*f*-(1 → 3)]-D-Xyl*p*, from rye arabinoxylan

Siriluck Imjongjairak[a], Pattaporn Jommuengbout[b], Pirin Karpilanondh[c], Hirotaka Katsuzaki[d], Makiko Sakka[d], Tetsuya Kimura[d], Patthra Pason[a], Chakrit Tachaapaikoon[e], Jariya Romsaiyud[f], Khanok Ratanakhanokchai[a,**], Kazuo Sakka[d,*]

[a] *School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkuntien, Bangkok 10150, Thailand*
[b] *Faculty of Science, Chandrakasem Rajabhat University, 39/1 Ratchadaphisek Road, Bangkok 10900, Thailand*
[c] *Department of Agro-Industrial Technology, King Mongkut's University of Technology North Bangkok, 1518 Pibulsongkram Road, Bangsue, Bangkok 10800, Thailand*
[d] *Graduated School of Bioresources, Mie University, 1577 Kurimamachiya-cho, Tsu 514-8507, Japan*
[e] *Pilot Plant Development and Training Institute, King Mongkut's University of Technology, Thonburi, Bangkok 10150, Thailand*
[f] *Department of Chemistry, Ramkhamhaeng University, Bangkok 10241, Thailand*

ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form 31 January 2015
Accepted 5 February 2015
Available online 13 February 2015

Keywords:
Xylanase
Paenibacillus curdlanolyticus
Carbohydrate-binding module
Arabinoxylan
Arabinoxylooligosaccharides
Molecular docking

ABSTRACT

Paenibacillus curdlanolyticus B-6 Xyn10C is a single module xylanase consisting of a glycoside hydrolase family-10 catalytic module. The recombinant enzyme, rXyn10C, was produced by *Escherichia coli* and characterized. rXyn10C was highly active toward soluble xylans derived from rye, birchwood, and oat spelt, and slightly active toward insoluble wheat arabinoxylan. It hydrolyzed xylooligosaccharides larger than xylotetraose to produce xylotriose, xylobiose, and xylose. When rye arabinoxylan and oat spelt xylan were treated with the enzyme and the hydrolysis products were analyzed by thin layer chromatography (TLC), two unknown hydrolysis products, U1 and U2, were detected in the upper position of xylose on a TLC plate. Electrospray ionization mass spectrometry and enzymatic analysis using *Bacillus licheniformis* α-L-arabinofuranosidase Axh43A indicated that U1 was α-L-Ara*f*-(1 → 2)-[α-L-Ara*f*-(1 → 3)]-D-Xyl*p* and U2 was α-L-Ara*f*-(1 → 2)-D-Xyl*p*, suggesting that rXyn10C had strong activity toward a xylosidic linkage before and after a doubly arabinose-substituted xylose residue and was able to accommodate an α-1,2- and α-1,3-linked arabinose-substituted xylose unit in both the −1 and +1 subsites. A molecular docking study suggested that rXyn10C could accommodate a doubly arabinose-substituted xylose residue in its catalytic site, at subsite −1. This is the first report of a xylanase capable of producing α-L-Ara*f*-(1 → 2)-[α-L-Ara*f*-(1 → 3)]-D-Xyl*p* from highly arabinosylated xylan.

1. Introduction

Plant cell wall polysaccharides, including cellulose, hemicelluloses, and pectin, are the important renewable resources of biofuels [1] and also are prebiotic oligosaccharides [2]. Among hemicelluloses, xylans are the second most common biomass next to cellulose. Xylans, heterogeneous polysaccharides, consist of homopolymeric backbone chains of β-1,4 linked D-xylopyranose units and substituted groups such as arabinose, glucuronic acids, ferulic acid, and *p*-coumaric acid [3]. The structures of xylans vary depending on their origin. For example, xylopyranosyl residues (*X*) of rye arabinoxylans are mainly substituted with α-L-arabinofuranosyl residues (*A*) to varying degrees at the O-2 position, the O-3 position, or both. The A/X ratio of total arabinoxylans is 0.49–0.82 depending on the study [4]. Arabinoxylans also contain small amounts of ferulate residues bound to arabinose as esters at its O-5 position [4]. Arabinoxylan from oat spelt is less

* Corresponding author at: Graduated School of Bioresources, Mie University, 1577 Kurimamachiya-cho, Tsu 514-8507, Japan. Tel.: +81 59 231 9621.
** Corresponding author. Tel.: +66 2 470 7755.
E-mail addresses: khanok.rat@kmutt.ac.th (K. Ratanakhanokchai), sakka@bio.mie-u.ac.jp (K. Sakka).

http://dx.doi.org/10.1016/j.enzmictec.2015.02.002
0141-0229/

arabinosylated compared with rye arabinoxylan, *i.e.*, its *A*/*X* ratio is about 0.1 [5]. Birchwood xylan was found to consist of *O*-acetyl-(4-*O*-methylglucurono)xylans containing one 4-*O*-methylglucuronic acid substituent for approximately every 15 D-xylose residues [6]. Therefore, enzymatic hydrolysis of xylan molecules requires endo-β-1,4-xylanase (xylanase; EC 3.2.1.8), β-xylosidase, and a number of accessory enzymes such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase [7].

Xylanases that catalyze the breakdown of the xylan backbone are divided into two major groups, glycoside hydrolase families 10 and 11 [8]. There are differences in substrate specificity between these two major families judging from the products of their hydrolysis of various xylans, *viz.*, family-11 xylanases prefer relatively unsubstituted regions of xylan, whereas some family-10 enzymes are able to hydrolyze substituted forms of the xylan backbone, producing oligosaccharides that carry substituents at the non-reducing terminal xylopyranose residue [9]. α-L-Ara*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* was identified as the smallest product [10,11]. Many xylanases consist of a catalytic module and one or more non-catalytic carbohydrate-binding modules (CBMs) that are located either at the N- or C-terminus, or both [12]. Currently, based on amino acid sequence similarities, 69 CBMs families are described in the latest update of the CAZy database (http://www.cazy.org) [13]. One established function of CBMs is to bring and maintain the catalytic module close to the surface of the substrate during the hydrolysis.

Paenibacillus curdlanolyticus strain B-6 is characterized by its ability to produce a xylanolytic–cellulolytic multi-enzyme complex-like cellulosome under even aerobic conditions [14–16], although the cellulosome is well known as a high molecular mass cellulolytic complex produced by anaerobic bacteria [17]. Four xylanase genes, *xyn10A* [18], *xyn10B* [19], *xyn10D* [20], and *xyn11A* [21], were cloned from *P. curdlanolyticus* B-6, and the recombinant enzymes produced by *Escherichia coli* were biochemically characterized. Xyn10B is a single module enzyme consisting of a family-10 xylanase module and the others are modular enzymes containing at least one CBM in addition to a catalytic module. Xyn10D has a family-3 CBM (CBM3) that plays an important role in the hydrolysis of insoluble xylan and natural biomass [20].

In the present study, we characterized the single module xylanase Xyn10C whose amino acid sequence is highly similar to that of the catalytic module of Xyn10D. We found that the recombinant Xyn10C (rXyn10C) produced a large amount of a doubly arabinose-substituted xylose, α-L-Ara*f*-(1 → 2)-[α-L-Ara*f*-(1 → 3)]-D-Xyl*p*, from highly arabinosylated xylan. A molecular docking study suggested that rXyn10C could accommodate a doubly arabinose-substituted xylose residue in its catalytic site. We also investigated the artificial addition of CBM3 derived from Xyn10D to Xyn10C.

2. Materials and methods

2.1. Genomic library, bacterial strains, and plasmid and growth conditions

A genomic library of *P. curdlanolyticus* strain B-6 was constructed using a CopyControl™ Fosmid Library Production Kit (Epicenter, Madison, WI, USA) as described previously [19]. Plasmid vectors pBluescript II KS(+) and pET-28a(+) (Novagen, Madison, WI, USA) were used for subcloning and gene expression, respectively. *E. coli* DH5α was used as a host for pBluescript II KS(+). *E. coli* BL21-CodonPlus® (DE3)-RIPL (Novagen) carrying a recombinant plasmid was cultivated in Super Broth (3.5% Bacto™ tryptone [BD Diagnostic, Sparks, MD, USA], 2% Bacto™ yeast extract [BD Diagnostic], 0.5% NaCl, pH 7.5) supplemented with chloramphenicol (50 μg/mL) and kanamycin (25 μg/mL) at 37 °C for protein expression.

2.2. Screening for a new xylanase gene

Among 768 recombinant fosmid clones, we identified 46 clones expressing xylanase activity *via* Congo red plate assays using oat spelt xylan (Sigma-Aldrich Japan, Tokyo, Japan) as a substrate at 37 °C as described previously [22]. Because we have already identified four xylanase genes, *xyn10A* [16], *xyn10B* [17], *xyn10D* [18], and *xyn11A* [19], we detected and excluded recombinant clones carrying these xylanase genes by performing PCR using recombinant fosmid DNA as templates and PCR primer sets designed to detect the known xylanase genes. As a result, we identified more than 20 candidates that had new xylanase genes and chose clone C21 for further investigation. A recombinant fosmid was prepared from clone C21, partially digested with *Sau*3AI, and ligated into the *Bam*HI-digested pBluescript II KS(+) vector. The ligation mixture was used to transform *E. coli* DH5α. A xylanase-active clone was detected using Congo red plate assays, and pBS-Xyn10C carried by the recombinant *E. coli* was sequenced.

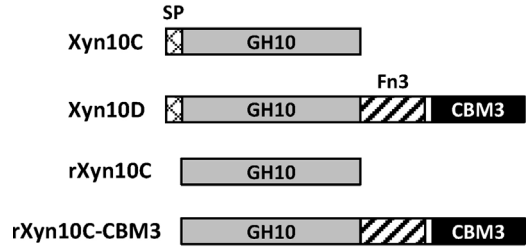

Fig. 1. Module organization of *P. curdlanolyticus* B-6 Xyn10C, Xyn10D, and recombinant enzymes. SP, signal peptide; GH10, glycoside hydrolase family-10 module; Fn3, fibronectin type-3 homology module; CBM3, family-3 CBM of Xyn10D.

2.3. Construction and purification of rXyn10C and rXyn10C-CBM3

A plasmid used to produce rXyn10C (Fig. 1) was constructed. The Xyn10C region devoid of the N-terminal signal peptide region was amplified by PCR from pBS-Xyn10C with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) and a PCR primer set, XynC-BamF, 5′-CCCCGGATCCGCTGATGCTGGGCTGGCCCG-3′, and XynC-HinR, 5′-GGGGAAGCTTTCAATGTGTCGACATATACG-3′, containing a *Bam*HI or *Hin*dIII recognition site (underlined). The resulting PCR fragment was digested with *Bam*HI and *Hin*dIII, ligated between the same restriction sites of pET-28a(+) to yield plasmid p Xyn10C.

A plasmid to produce rXyn10C-CBM3, Xyn10C fused to a family-3 CBM derived from *P. curdlanolyticus* B-6 Xyn10D (Fig. 1), was constructed by using a two-step overlapping PCR procedure. In the first step, the full-length Xyn10C gene and the gene segment encoding family-3 CBM were separately amplified using two primer sets: Xyn10C-BamF and Xyn10C-Rev, 5′-ATGTGTCGACATATACGTCTTC-3′; and CBM3-Fwd, AGACGTATATGTCGACACATGGCAGCACGAATCCGACGGC, and CBM3R-Hin CCCCAAGCTTCTAAGGAGCTACGCCCCATACAATC, containing a *Hin*dIII recognition site. Primers Xyn10C-Rev and CBM3-Fwd contained 20-nucleotide complementary regions. The two amplified fragments were denatured, annealed, and collectively amplified using primers Xyn10C-BamF and CBM3R-Hin in the second step of the overlapping PCR. The chimeric gene encoding rXyn10C-CBM3 was digested with *Bam*HI and *Hin*dIII, and cloned between the same restriction sites of pET-28a(+), yielding plasmid pET-Xyn10C-CBM3. The fidelity of the genes encoding rXyn10C and rXyn10C-CBM3 was confirmed by DNA sequencing.

For production of rXyn10C and rXyn10C-CBM, recombinant *E. coli* BL21-CodonPlus® (DE3)-RIL cells harboring plasmid pET-Xyn10C or pET-Xyn10C-CBM3 were cultivated in 200 mL of Super Broth supplemented with kanamycin (50 μg/mL) at 37 °C. When the absorbance at 600 nm of culture reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to the culture to give a final concentration of 1 mM for induction of gene expression, and incubation was continued at 37 °C for a further 4 h. Cells were harvested by centrifugation, resuspended in 20 mL of 20 mM sodium phosphate buffer (pH 7.4) containing 10 mM imidazole and 300 mM sodium chloride, and disrupted by sonication. Cell debris was removed by centrifugation. The supernatant was used as the enzyme solution for purification of rXyn10C and rXyn10C-CBM3 with a HisTrap™ Chelating HP column (GE Healthcare Japan, Tokyo, Japan) following the supplier's protocol. The imidazole concentrations in wash and elution solutions were 10 and 300 mM, respectively. The purity of each fraction was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli [23]. The protein concentration was measured as described by the method of Bradford [24] with bovine serum albumin as the standard, using the Bio-Rad protein assay kit (Bio-Rad Japan, Tokyo, Japan).

2.4. Xylanase assay

The reaction mixtures were incubated at 50 °C for 10 min with rye arabinoxylan (Megazyme, Wicklow, Ireland), birchwood xylan (Sigma-Aldrich Japan, Tokyo, Japan), or oat spelt xylan (Sigma-Aldrich Japan) as substrates (each 0.5%, w/v) in 50 mM sodium phosphate buffer (pH 7.0 for rXyn10C and pH 6.0 for rXyn10C-CBM3). The activity toward insoluble wheat arabinoxylan (P-WAXYI, megazyme) was also assayed under the same condition except that the incubation time was 15 min. Reducing sugars released from the substrates were measured using a 3,5-dinitrosalicylic acid reagent as described by Miller [25], with xylose as the standard.