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Cloning, expression and characterization of β-xylosidase from *Aspergillus niger* ASKU28

Khuanjarat Choengpanya [a], Siriphan Arthornthurasuk [b], Pakorn Wattana-amorn [c], Wan-Ting Huang [d], Wandee Plengmuankhae [b], Yaw-Kuen Li [d], Prachumporn T. Kongsaeree [a,b,e,*]

[a] *Interdisciplinary Graduate Program in Genetic Engineering, Faculty of Graduate School, Kasetsart University, Bangkok 10900, Thailand*
[b] *Department of Biochemistry and Special Research Unit for Protein Engineering and Protein Bioinformatics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[c] *Department of Chemistry, Center of Excellence for Innovation in Chemistry and Special Research Unit for Advanced Magnetic Resonance, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[d] *Department of Applied Chemistry, College of Science, National Chiao Tung University, Hsinchu 300, Taiwan*
[e] *Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok 10900, Thailand*

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ABSTRACT

β-Xylosidases catalyze the breakdown of β-1,4-xylooligosaccharides, which are produced from degradation of xylan by xylanases, to fermentable xylose. Due to their important role in xylan degradation, there is an interest in using these enzymes in biofuel production from lignocellulosic biomass. In this study, the coding sequence of a glycoside hydrolase family 3 β-xylosidase from *Aspergillus niger* ASKU28 (AnBX) was cloned and expressed in *Pichia pastoris* as an N-terminal fusion protein with the α-mating factor signal sequence (α-MF) and a poly-histidine tag. The expression level was increased to 5.7 g/l in a fermenter system as a result of optimization of only five codons near the 5′ end of the α-MF sequence. The recombinant AnBX was purified to homogeneity through a single-step Phenyl Sepharose chromatography. The enzyme exhibited an optimal activity at 70 °C and at pH 4.0–4.5, and a very high kinetic efficiency toward a xyloside substrate. AnBX demonstrated an exo-type activity with retention of the β-configuration, and a synergistic action with xylanase in hydrolysis of beechwood xylan. This study provides comprehensive data on characterization of a glycoside hydrolase family 3 β-xylosidase that have not been determined in any prior investigations. Our results suggested that AnBX may be useful for degradation of lignocellulosic biomass in bioethanol production, pulp bleaching process and beverage industry.

1. Introduction

Ethanol production from lignocellulosic biomass has received much interest in recent years. Lignocellulose consists of cellulose, hemicelluloses and lignin. Xylan is one of the major components of hemicellulose. It is also the second most abundant natural polysaccharide after cellulose. Xylan is a complex polysaccharide comprising of 1,4-β-D-xylose backbone. It usually contains a substitution of other sugars, such as D-glucose, L-arabinose, D-mannose, D-fucose and D-glucuronic acid, their amounts and bond linkages depending on plant tissues and plant sources. Besides, its backbone is also substitutes with O-acetyl groups [1]. The complete degradation of xylan requires a number of hydrolytic enzymes including endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) [2]. Endo-β-1,4-xylanases hydrolyze the xylan into smaller xylooligosaccharides. Then, β-xylosidases hydrolyze these small xylo-oligosaccharides and xylobiose from the non-reducing end to release fermentable D-xylose sugar.

Most β-xylosidases are found in fungi, and some in bacteria and plants [3–6]. On the basis of sequence similarity, they are classified into 10 glycoside hydrolase (GH) families, which are 1, 3, 5, 30, 39, 43, 51, 52, 54, 116 and 120 [7]. In particular, most fungal β-xylosidases belong to GH3, with subunit molecular weights of 75–120 kDa, optimal pH ranging from 2.5 to 5.5, and a wide range of optimal temperatures ranging from 30 to 75 °C [8]. The enzymes

Abbreviations: AnBX, *Aspergillus niger* ASKU28 β-xylosidase; 3,4-DNP, 3,4-dinitrophenol; 3,4-DNP-Xyl, 3,4-dinitrophenyl-β-D-xyloside; DNS, 3,5-dinitrosalicylic acid; GH, glycoside hydrolase family; α-MF, *Saccharomyces cerevisiae* α-mating factor secretion signal; *p*NP, *para*-nitrophenol; *p*NP-Ara, *para*-nitrophenyl-α-L-arabinoside; *p*NP-Glc, *para*-nitrophenyl-β-D-glucoside; *p*NP-Xyl, *para*-nitrophenyl-β-D-xyloside.

* Corresponding author at: Department of Biochemistry, Faculty of Science, Kasetsart University, 50 Paholyothin Road, Chatuchak, Bangkok 10900, Thailand.
E-mail address: fscippt@ku.ac.th (P.T. Kongsaeree).

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from *Aspergillus awamori* [9], *A. japonicus* [5], *A. nidulans* [10], *A. niger* [11–14], *A. oryzae* [15], and *Talaromyces emersonii* [16] have been cloned and characterized. However, only few studies have characterized their enzymatic properties in complete details [17]. The filamentous fungus *A. niger* is known as a good source of food, pharmaceutical products and a wide range of commercial enzymes, including β-xylosidases. Previously, our laboratory have purified a β-xylosidase from *A. niger* ASKU28 (AnBX) with a high catalytic efficiency toward *para*-nitrophenyl-β-D-xyloside (*p*NP-Xyl) of 650 s^{-1} mM^{-1}, and a high K_i value of 1.3 M for glucose [18]. In addition, its tolerance for xylose was about 20- to 50-folds higher than the values reported for other *Aspergillus* β-xylosidases. However, only 5.1 mg (or 0.6 units) of this enzyme was obtained after purification from 1 l of *A. niger* ASKU28 culture. Therefore, an effective production and purification strategy is needed to provide a large amount of this enzyme, which would enable detailed analysis of its structure–function relationships and further improvement of its properties via protein engineering techniques.

The methylotrophic yeast *Pichia pastoris* has been used extensively as an expression host for the production of various heterologous proteins, as it carries out similar posttranslational modifications to higher eukaryotes [19]. The promoter of alcohol oxidase 1, which governs the expression of alcohol oxidase 1 for utilization of methanol as a sole carbon source, has been used to induce high level expression of foreign proteins in *P. pastoris* [19–21]. Moreover, extracellular expression can be successfully driven by constructing an N-terminal fusion of recombinant protein with a signal sequence, such as the *Saccharomyces cerevisiae* α-mating factor signal sequence (α-MF) [22]. However, the levels of protein expression may be variable depending on the nature of the protein sequences, especially the suitability of the codon usage for protein expression needs of *P. pastoris* [23]. In many cases, the expression of synthetic genes with codon optimization to suit particular expression hosts has been able to improve protein yields in bacterial [24,25], yeast [23] and mammalian hosts [26,27].

In this study, we reported the cloning and expression of mature AnBX, and improvement of the recombinant enzyme production via codon optimization and fermentation. As a result, a large amount of the recombinant AnBX was secreted into the culture media, which enabled a single-step protein purification. The purified recombinant AnBX showed comparable enzymatic properties to those reported for the enzyme purified from the natural source. The enzymatic properties of AnBX were characterized in full details and showed AnBX with a good potential to be used in the degradation of lignocellulosic biomass.

2. Materials and methods

2.1. Cells, plasmid and chemicals

Escherichia coli strains TOP10 and DH5α were used for plasmid propagation. *P. pastoris* strains Y11430 and KM71H were used for protein expression in a shake-flask system and in a fermenter, respectively. *A. niger* ASKU28 was obtained from an existing stock collection at the Department of Microbiology, Faculty of Science, Kasetsart University, Thailand. A modified *Pichia* expression vector, pPICZαBNH8, which contained the coding sequence of eight histidine residues following the sequence of the α-MF [20], was used for protein expression in *P. pastoris*. All aryl-glycoside substrates, *para*-nitrophenol (*p*NP), beechwood xylan, and *Thermomyces lanuginosus* xylanase were obtained from Sigma–Aldrich (St. Louis, MO, USA). The xylooligosaccharides (xylobiose, xylotriose and xylotetraose) were purchased from Megazyme (Wicklow, Ireland). 3,4-Dinitrophenyl-β-D-xyloside (3,4-DNP-Xyl) was synthesized as described previously [28]. All other chemicals were of analytical grade.

2.2. Cloning of AnBX in a Pichia expression vector

A. niger ASKU28 was grown in minimal medium [18] supplemented with 2% (w/v) beechwood xylan for 2 days at 30 °C 200 rpm. The total RNA was extracted from the fungal mycelia by using RNeasy Mini Kit (Qiagen, Hilden, Germany), and the first-strand cDNA was then generated according to protocol for first-strand cDNA synthesis with oligo $(dT)_{18}$ as a primer (Fermentas, Glen Burnie, MD, USA). The coding sequence of mature AnBX was amplified from the first strand cDNA by using 3 units of *Pfu* Ultra HF DNA polymerase (Promega, Madison, WI, USA) and specific primers, XlnD-For (5′-CATGCTGCAGCTAA CACCAGCTATGT CGATTACAACG-3′) and XlnD-Rev (5′-TTGTTCTAGACTACTCCT TCCCAGGCCACTTC-3′), which were designed based on the DNA sequence of *A. niger xlnD* gene (GenBank accession No. AF108944) [11]. The PCR product was ligated into pZErO™-2 cloning vector (Invitrogen, Carlsbad, CA, USA) at the *Eco*RV site, and then transformed into *E. coli* TOP10 by electroporation. The positive transformants were selected on LB-agar containing 25 μg/ml kanamycin at 37 °C. The nucleotide sequence of AnBX was checked by DNA sequencing.

The coding sequence of mature AnBX was then sub-cloned into pPICZαBNH8, at the *Pst*I-*Xba*I sites. The resultant expression plasmid was designated as pPICZαBNH8-AnBX, and was transformed into *E. coli* DH5α by electroporation. The positive transformants were selected on LB-agar containing 25 μg/ml zeocin at 37 °C. After that, the pPICZαBNH8-AnBX plasmid was extracted, sequenced and linearized by *Sac*I, before transforming into *P. pastoris* Y11430 and KM71H by electroporation. The transformants were plated onto YPDS-agar containing 100 μg/ml zeocin, and grown for 3–5 days at 30 °C.

2.3. Cloning of AnBX in an optimized Pichia expression vector

The codon usage frequency in the recombinant construct of pPICZαBNH8-AnBX plasmid was analyzed by using Graphical Codon Usage Analyzer, which is available at http://gcua.schoedl.de/sequential_v2.html [29]. The first five codons in the coding sequence of the α-MF that showed low codon preference (⩽20%) for expression in *P. pastoris* were changed to the codons with the highest usage frequency for the same amino acids by site-directed mutagenesis method using 3 units of *Pfu* Ultra HF DNA polymerase according to the method published previously [30]. The sequence of the sense mutagenic primer was 5′-GATT TCCTTCTATTTTTACTGCTGTTTTGTTCGCAGCATCTTCTGCATTGGCT-GCTCCAG-3′ (the sites of mutations are underlined), and the sequence of the antisense mutagenic primer was the reverse complement of the sequence shown above. The PCR reaction was treated with 10 units of *Dpn*I at 37 °C overnight to remove the parental pPICZαBNH8-AnBX plasmid, and the resulting modified plasmid, called Opt-pPICZαBNH8-AnBX, was then transformed into *E. coli* DH5α by electroporation. The positive transformants were selected on LB-agar containing 25 μg/ml zeocin at 37 °C, checked by restriction digestion, and confirmed by DNA sequencing. The Opt-pPICZαBNH8-AnBX plasmid was linearized by *Sac*I, before transforming into *P. pastoris* Y11430 and KM71H by electroporation. The transformants were plated onto YPDS-agar containing 100 μg/ml zeocin, and grown for 3–5 days at 30 °C.

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