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Xylanase 30 A from *Clostridium thermocellum* functions as a glucuronoxylan xylanohydrolase

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ABSTRACT

Endoxylanases classified into glycoside hydrolase family 30 subfamily 8 (GH30-8) have been shown to hydrolyze glucuronoxylan with dependence upon the glucuronic acid (GlcA) appendage. In a recent report, the GH30-8 xylanase from *Clostridium thermocellum* (*Ct*Xyn30A) was shown to hydrolyze arabinoxylan which contains no GlcA. Protein structure comparison with the originally characterized GH30-8 enzymes from *Bacillus subtilis* and *Erwinia chrysanthemi* provided no insight to hypothesize why the function of *Ct*Xyn30A is unique. In this report, we show that *Ct*Xyn30A is a GlcA dependent endoxylanase with no significant activity on arabinoxylans, an anticipated result given the amino acid conservation within the substrate binding cleft.

1. Introduction

Classification of glycoside hydrolases (GH) is maintained through the ongoing efforts of the curators of the Carbohydrate Active Enzymes (CAZy) Database [1]. Organization of the database is based on primary amino acid sequence comparisons with proteins sharing sequence homology being grouped together to form GH families. Given a representative protein structure, GH families can be further categorized into Clans defined by a generalized protein fold. A few GH families have subsequently been organized into subfamilies based on biochemical function and amino acid based phylogenetic analyses [2,3]. Eight subfamilies of GH family 30 (GH30) enzymes have currently been defined. Enzymes classified in subfamily 8 (GH30-8) have been shown to be GlcA-dependent β-1,4-endoxylanases which specifically hydrolyze xylans decorated with α-1,2-linked glucuronic acid moieties (GlcA) to yield GlcA containing xylooligosaccharides or aldouronates. The resulting limit hydrolysis product of glucuronoxylan by this type of xylanase has been shown to be a mixture of aldouronates, each substituted with a single GlcA penultimate to the reducing terminal xylose [4,5].

Original studies characterizing GH30-8 xylanases focused on XynA from the Gram-negative (G−) bacterium *Erwinia chrysanthemi* (*Ec*XynA) [5,6] and XynC from the Gram-positive (G+) bacterium *Bacillus subtilis* (*Bs*XynC) [4]. While only sharing a 40% amino acid sequence identity, these enzymes proved to be functionally identical to an enzyme that had been previously been referred to as a glucuronoxylan xylanohydrolase [7]. A notable functional similarity of these two distinct GH30-8 endoxylanases is their inability to hydrolyze neutral xylooligosaccharide at a significant rate. Both enzymes have been characterized as hydrolyzing xylohexaose (X_6) 3-orders of magnitude slower than glucuronoxylan and to have no detectable activity on wheat arabinoxylan, which contains greater than 1/3 of its mass as α-1,3 and/or α-1,2/1,3-double substituted arabinofuranose moieties [5,8,9] and contains no GlcA substitutions. Several other GH30-8 endoxylanases from G+ bacteria have also been characterized and were found to possess biochemical properties identical to *Ec*XynA and *Bs*XynC [10,11].

Exceptions to these functional characteristics have been reported twice. Xyn30A from *Clostridium papyrosolvens* (*Cp*Xyn30A) was selected for study based on a loss of amino acid sequence conservation in a region known to be specifically involved in GlcA recognition. Functional characterization revealed it to hydrolyze xylan substrates including glucuronoxylan, neutral xylooligosaccharides larger than xylotetraose and wheat arabinoxylan. The low rate of hydrolysis observed for all these substrates suggested that *Cp*Xyn30A may either be a 'broken' GlcA-dependent endoxylanase or that it has evolved an alternative

Abbreviations: GH, glycoside hydrolase; BX, beechwood xylan; WAX, wheat arabinoxylan; SGX, sweetgum wood glucuronoxylan.

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role in xylan degradation and utilization [8]. In contrast, a recent publication concerning the GH30-8 xylanase from *Clostridium thermocellum* (*Ct*Xyn30A) reported this xylanase to have a preference for GlcA containing substrates such as glucuronoxylan, in addition to possessing a significantly high activity on wheat arabinoxylan (WAX) and several other biomass derived polysaccharides that do not contain GlcA [12]. Given the high degree of amino acid sequence conservation in the amino acids comprising the xylan binding cleft shared by *Ct*Xyn30A and other GH30-8 enzymes, we could not reconcile the reported unique function of *Ct*Xyn30A with the known function of other GH30-8 enzymes.

In this study, we sought to examine the functional characteristics of *Ct*Xyn30A with a focus on its mode of action in WAX hydrolysis. Our findings reveal that, in contrast to the previous report, *Ct*Xyn30A functions as a canonical GlcA-dependent GH30-8 glucuronoxylan xylanohydrolase having no significant activity on arabinoxylans.

2. Methods

2.1. Enzyme cloning

DNA encoding the recombinant, expression-optimized sequence for *Ct*Xyn30A was ordered from Eurofins MWG Operon (Louisville, KY) as two large fragments which were stitched together using overlap extension PCR. Following the sequencing of several clones an error free sequence was obtained. From this, *Ct*Xyn30A was subcloned into pET28 to create five unique expression products. Constructs *Ct*Xyn30A$_1$ and *Ct*Xyn30A$_3$ were cloned into the NcoI and XhoI sites of pET28, *Ct*Xyn30A$_2$ and *Ct*Xyn20A$_4$ into the NdeI and XhoI sites of pET28, and *Ct*Xyn30A$_5$ into the NheI and XhoI sites of pET28 (Table 1).

2.2. Protein expression

All constructs were expressed according to methods outlined in the pET System Manual 10th Edition and as previously described [8,13,14]. Chemically competent *Escherichia coli* BL21 (DE3) was transformed with expression construct plasmid DNA and selected for on Luria Broth (LB) agar plates containing 50 ug/ml kanamycin. Single colonies were used to inoculate 25 ml overnight cultures of LB broth containing 50 ug/ml kanamycin (LB50 K). A volume of 5 ml was used to inoculate 37 °C equilibrated, 250 ml cultures of LB50 K in 2.8 l Fernbach flasks. For growth and induction, standard procedures were followed [13]. Expression cultures were centrifuged at 8000 rpm in a JLA.250 rotor (Beckman, Brea, CA) for 10 min at 4 °C and the pellets were frozen at −80 °C.

2.3. Protein purification and verification

Functional characterization of the *Ct*Xyn30A$_1$ and *Ct*Xyn30A$_5$ expressed proteins was performed directly from the cell free extract (CFE). For each of the hexahistidine-tagged *Ct*Xyn30A construct versions *Ct*Xyn30A$_2$, CtXyn30A$_3$ and *Ct*Xyn30A$_4$ the proteins were purified from the CFE. Briefly, the pellet resulting from 250 ml of expression culture were thawed on ice and resuspended in 12 ml of Tris-based cell processing buffer (CPB) consisting of 20 mM Tris base pH 7.4, to a fluid state using a glass rod and a 5 ml pipet. An aliquot of EDTA free protease inhibitor cocktail was added to the suspension along with lysozyme to a final concentration of 10 ug/ml and the cells were lysed by sonication (Heat Systems, Ultrasonic processor XL) using a procedure employing 12 cycles each consisting of a 10 s pulse followed by a 50 s rest, while on ice at 20% power. Magnesium chloride was then added to a final concentration of 2 mM followed by the addition of 150 units of Benzonase (EMD Millipore, Billerica, MA) and the cell lysate was incubated at room temperature for 30 min. The lysate was then centrifuged at 28,000 × *g* for 30 min at 15 °C. The supernatant was filtered (0.22 um pore size) to generate a cell free extract (CFE) which was subsequently amended by the addition of 5 M sodium chloride to a final concentration of 500 mM prior to purification by immobilized metal affinity chromatography (IMAC). Proteins were purified from the CFE using a 1 ml His Trap column (GE Healthcare Biosciences, Pittsburgh, PA) with a 0–500 mM imidazole gradient. The peak fractions were combined and dialyzed against 25 mM Tris HCl, 150 mM NaCl, pH 7.5. Proteins were further purified and had their approximate molecular weights determined on a molecular weight standardized Superose 6 gel filtration column equilibrated in the same buffer. For protein sizing, the column was calibrated with a set of low-range gel filtration protein standards purchased from Sigma (St. Louis, MO). The collected fractions containing purified protein were combined and the volume concentrated to less than 1 ml. Protein concentration was determined using absorbance at 280 nm with an extinction coefficient provided using the program ProtParam (http://web.expasy.org/protparam/). SDS-PAGE was used to assess purity and verify the mass of the expected protein product. To verify the molecular masses of the similarly sized *Ct*Xyn30A$_2$ and *Ct*Xyn30A$_3$ constructs, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was performed at the University of Wisconsin, Biotechnology Center. Purification of the *Bs*XynC protein was performed as previously described [14].

2.4. Structure analysis

Structures were obtained from the Protein Data Bank and analyzed and figure images prepared in the software package PyMOL [15]. Structures used for comparison include xylanase C from *Bacillus subtilis* (*Bs*XynC, PDB code: 3KL5 A), xylanase 30D from *Paenibacillus barcinonensis* BP-23 (*Pb*Xyn30D, PBD code: 4QAW A), xylanase 30A from *Clostridium thermocellum* (*Ct*Xyn30A, PDB code: 4UQE) and xylanase A from *Erwinia chrysanthemi* (*Ec*XynA, PDB code: 2Y24).

2.5. Biochemical substrates

Beechwood xylan (BX) was obtained from Sigma-Aldrich (St. Louis, MO) and a soluble fraction was prepared at 20 mg/ml to use as a stock solution [4]. Sweetgum wood glucuronoxylan (SGX) was a gift from the laboratory of Dr. James F. Preston at the University of Florida [6]. This fully soluble glucuronoxylan was prepared to 20 mg/ml. Low viscosity WAX was obtained from Megazyme International (Wicklow, Ireland) and prepared to 10 mg/ml as the product literature describes. All studies were performed using the glucuronoxylans BX and SGX at 10 mg/ml and WAX at 5 mg/ml. The glycoside hydrolase family 10 xylanase 10B from *Cellvibrio mixtus* (*Cm*Xyn10B) was purchased from Megazyme International for use as a positive control xylanase for both substrates. This xylanase was desalted from the ammonium sulfate preparation provided by the manufacturer and protein concentration was quantified at 280 nm using the ProtParam reported extinction coefficient. Xylanase activity was determined as described below. Larch wood arabinogalactan (Sigma-Aldrich), debranched beet arabinan (Megazyme), carob galactomannan (Megazyme), tamarind xyloglucan (FPL collection) and konjac glucomannan (Megazyme) were all used as substrates at 5 mg/ml. For thin layer chromatography (TLC) analysis, the range of neutral xylooligosaccharides used for standards included xylose through xylopentaose (X, X_2 – X_5) and the aggregate aldouronate standard (GX_n = GX_2, GX_3, GX_4, GX_5. . . . .etc) which were obtained from Megazyme International. These aldouronate standards are composed of 4-*O*-methyl-α-D-glucuronate substituted oligoxylosides.

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