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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* (CtXyn30A) 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 CtXyn30A is unique. In this report, we show that CtXyn30A is a GlcA dependent endoxylanase with no significant activity on arabinoxylans, an anticipated result given the amino acid conservation within the substrate binding cleft.

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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 ß-1,4-endoxylanases which specifically hydrolyze xylans decorated with a-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* (*EcXynA*) [5,6] and XynC from the Gram-positive (G+) bacterium *Bacillus subtilis* (*BsXynC*) [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 a-1,3 and/or a-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 EcXynA and BsXynC [10,11].

Exceptions to these functional characteristics have been reported twice. Xyn30A from *Clostridium papyrosolvens* (CpXyn30A) 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 CpXyn30A 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 (CtXyn30A)* 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 CtXyn30A and other GH30-8 enzymes, we could not reconcile the reported unique function of CtXyn30A with the known function of other GH30-8 enzymes.

In this study, we sought to examine the functional characteristics of CtXyn30A with a focus on its mode of action in WAX hydrolysis. Our findings reveal that, in contrast to the previous report, CtXyn30A 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 CtXyn30A was ordered from Eurofins MWG Operon (Louisville, ICY) 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, CtXyn30A was subcloned into pET28 to create five unique expression products. Constructs $CtXyn30A_1$ and $CtXyn30A_3$ were cloned into the NcoI and XhoI sites of pET28, $CtXyn30A_2$ and $CtXyn20A_4$ into the Ndei and XhoI sites of pET28, and $CtXyn30A_5$ 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 mI was used to inoculate 37 °C equilibrated, 250 ml cultures of LB50 K in 2.81 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 $CtXyn30A_1$ and $CtXyn30A_5$ expressed proteins was performed directly from the cell free extract (CFE). For each of the hexahistidine-tagged CtXyn30A construct versions $CtXyn30A_2$, $CtXyn30A_3$ and $CtXyn30A_4$ 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 alongwith 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 50s 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 0–500mM imidazole gradient. The peak fractions were combined and dialyzed against 25 mM Tris HCI, 150 mM NaCI, 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 $CtXyn30A_2$ and $CtXyn30A_3$ constructs, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) was performed at the University of Wisconsin, Biotechnology Center. Purification of the BsXynC 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* (BsXynC, PDB code: 3KL5 A), xylanase 30D from *Paenibacillus barcinonensis* BP-23 (PbXyn30D, PBD code: 4QAWA), xylanase 30A from *Clostridium thermocellum* (CtXyn30A, PDB code: 4UQE) and xylanase A from *Erwinia chrysanthemi* (EcXynA, 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* (CmXyn 10B) 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-a-D-glucuronate substituted oligoxylosides.

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Table 1
Cloning primers, amino acid sequence features and the calculated molecular weight of the expressed CtXyn30A derived protein.

Enzyme	Cloning Primer Sequence[c]	Protein Features[d]	Molecular Weight (Da)
$CtXyn30A_1$[a]	ccatggctgaaaccgcgacca ctcgagttagctttcaaacgtaaaccaatc	CD/CBM	58846
$CtXyn30A_2$	catatggctgaaaccgcgacca ctcgagttagctttcaaacgtaaaccaatc	H/T/CD/CBM	61009[e]
$CtXyn30A_3$	ccatggctgaaaccgcgacca ctcgagtttgctttcaaacgtaaacca	CD/CBM/H	60039[e]
$CtXyn30A_4$	catatggctgaaaccgcgacca ctcgagttaaccaccaacgaaagtgg	H/T/CD	46775
$CtXyn30A_5$[b]	gctagcgcgaccattaatctctcc ctcgagttattcctgtttgctttcaaacg	H/T/CD/CBM	61252

[a] CtXyn30A with no affinity tags for purification and also having trimmed the N-terminal secretion signal sequence and the C-terminal dockerin domain.
[b] This expression product represents the exact protein studied by [12].
[c] Restriction enzyme cloning sites underlined.
[d] CD, GH30-8 catalytic domain; CBM, family 6 carbohydrate binding module; H, hexahistidine tag tag; T, thrombin protease recognition site.
[e] Expression product size confirmed by MALDI-TOFMS.

2.6. Biochemical assays

Enzyme activities were determined using the Nelson's test [16,17] as previously described [4]. Optimum reaction conditions for CtXyn30A were selected based on the previous report [12]. This included reactions performed at 70°C and pH 6.5 in sodium phosphate buffer for 10 min. The control xylanases BsXynC and CmXyn10B were measured using their reported buffer systems [4,18] (https://secure.megazyme.com/files/Booklet/E-XYNBCM_DATA.pdf) and at 37°C for 10 min. For these studies the enzymes were used at the concentrations of $CtXyn30A_2$, 24.4 pmol/ml; $CtXyn30A_3$, 18.8 pmol/ml; $CtXyn30A_4$, 21.0 pmol/ml; BsXynC, 23.2 pmol/ml and CmXyn10B 47.6 pmol/ml. To verify no detectable activity the reactions were rerun with increased enzyme and a 12-fold increase in reaction time. The enzyme concentrations were increased 10-fold except for $CtXyn30A_3$ for which the initial dilution was less than two-fold so we opted to utilize the initial strength. The increased reaction protein concentrations for this more severe hydrolysis were: CtXyn30A2, 244 pmol/ml; $CtXyn30A_3$, 320 pmol/ml; $CtXyn30A_4$, 210 pmol/ml; BsXynC, 232 pmol/ml and CmXyn10B 476 pmol/ml. Analysis of CtXyn30A hydrolysis of other alterative polysaccharide substrates was performed using $CtXyn30A_3$ which was used under the exact same conditions as for hydrolysis of WAX described above (320 pmol/ml). Thin layer chromatography (TLC) was performed as previously described [19,20] with overnight reactions performed at 60 °C to prevent loss of activity for CtXyn30A enzymes [12]. The control xylanases BsXynC and CmXyn10B were measured using their recommended buffer systems at 37 °C overnight. All TLC reactions were performed in 30 ul volumes. The enzyme concentration for these reactions were: $CtXyn30A_2$, 240 pmol/ml; $CtXyn30A_3$, 250 pmol/ml; $CtXyn30A_4$, 253 pmol/ml; BsXynC, 260 pmol/ml and CmXyn10B 253 pmol/ml.

2.7. Oligosaccharide mass analysis

Hydrolysis products of SGX by $CtXyn30A_5$ and BsXynC were analyzed by Matrix-Assisted Laser Desorption/Ionization Time-of-Fight mass spectrometer (MALDI-TOFMS) [4]. Reactions consisted of 500 ul in the conditions described above in which $CtXyn30A_5$ was used at a final CFE dilution of 500-fold and BsXynC at 220 pmol/ml for a reaction time of two hours. The reactions were heat killed and a 5-fold dilution with water was prepared which then was treated with Dowex 50W in hydrogen form to reduce interference from cations. Alpha-cyclodextran (972.84 Da) was used to calibrate the MALDI-TOF. Samples were prepared using a 10 mg/ml stock of 2,5-dyhydroxybenzoic acid (DHBA) in 30% acetonitrile. A volume of 10 ul of the diluted SGX hydrolysates was mixed with 90 ul of the 10 mg/ml DHBA matrix stock solution and 1 ul of this was spotted on the sample plate. Analysis was performed using a Sciex 4800 MALDI TOF/TOF mass spectrophotometer in positive ion mode at the University of Wisconsin Biotechnology Center.

3. Results

3.1. CtXyn30A protein expression constructs, protein products and purification

The protein coding sequence for CtXyn30A encodes a three domain protein consisting of a GH30-8 catalytic domain, a centrally positioned family 6 carbohydrate binding module followed by a C-terminal dockerin domain. For this study we subcloned several different forms of CtXyn30A in consideration of different purification and functional assessment possibilities (Table 1). In all cases, the region cloned removed the N-terminal secretion signal sequence and the C-terminal dockerin domain. $CtXyn30A_1$ was prepared to represent the function of CtXyn30A as an unaltered wild-type expression product having no affinity tags; $CtXyn30A_2$, is a version having an N-terminal hexahistidine tag followed by a thrombin protease cleavage site; $CtXyn30A_3$, is an expression product having a C-terminal hexahistidine tag; $CtXyn30A_4$, a version composed of only the catalytic domain having an N-terminal hexahistidine tag and a thrombin protease cleavage site; and finally $CtXyn30A_5$, a version having an N-terminal hexahistidine tag followed by a thrombin protease cleavage site (very similar to $CtXyn30A_2$) which was constructed to the exact specifications of the previously published CtXyn30A xylanase (Table 1) [12]. $CtXyn30A_2$, $CtXyn30A_3$, and $CtXyn30A_4$ were readily purified by nickel IMAC chromatography and resulted in well-defined single peaks by gel filtration purification. The proteins were sized by gel filtration and proved to run significantly smaller than their expected sizes indicating that CtXyn30A has affinity for Superose resin. Purification was followed by SDS-PAGE. Although pure, the $CtXyn30A_2$ and $CtXyn30A_3$ proteins which are only different by 1 kDa in size were not distinguishable via SDS-PAGE analysis. The sizes of these two proteins were confirmed by MALDI-TOF MS. Although, $CtXyn30A_5$ contained an N-terminal hexahistidine tag, expression and activity testing of this construct was done using only the CFE to align the observed overall function of this GH30-8 endoxylanase to the exact form characterized in the previous report [12].

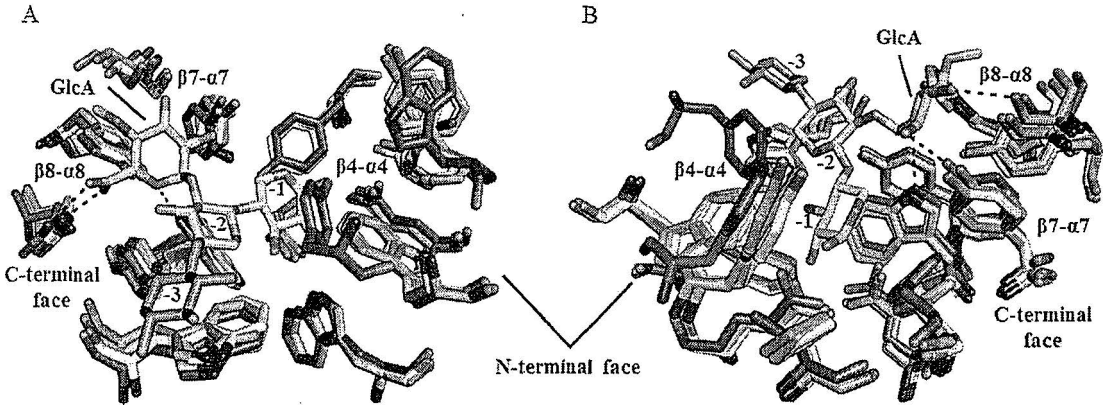

Fig. 1. Superposed views ofstructurally characterized Gram-positive (C+. shades of green) and the Gram-negative (G–,magenta) GH30-8 enzyme substrate binding clefts. (A) A view from the nonreducing terminal xylose (dark grey, –3subsite) toward the catalytic center (light grey, –1subsite). (B) A view from the aglycone region of the reducing terminal xylose at the catalytic center toward the nonreducing terminus. The G+ structures used for this comparison include xylanase C from *Bacillus subtilis* (BsXynC. PDB code: 3KL5 A), xylanase 30D from *Paenibacillus barcinonensis* BP-23 (PbXyn30D, PBD code: 4QAW A), xylanase 30A from Clostridium thermocellum (CtXyn30A, PDB code: 4UQE). The only available G-structure is xylanase A from *Erwinia chrysanthemi* (EcXynA, PDB code: 2Y24).

3.2. Structural substrate binding cleft comparison

Comparison of the protein structures of the biochemically characterized, GlcA-dependent GH30-8 xylanases reveals only slight differences between the G+ and G–versions of these enzymes (Fig. l a and b). The differences that are observed, are conserved throughout these evolutionarily distinct bacterial groups [14]. Among the G+ enzymes, amino acids involving substrate binding in the active site cleft are completely conserved supporting the hypothesis that these enzymes should function identically. The amino acid sequence differences that do exist between the G+ and G– versions of the GH30-8 xylanases involve only the N-terminal face of the substrate binding cleft that is not involved in GlcA recognition (Fig. l a and b). The C-terminal face of the substrate binding cleft is where the GlcA is recognized and bound and is, therefore, the region most important to functional specificity. This region is effectively identical in both C+ and G- enzymes with all of those amino acids which interact with the xylan substrate being perfectly conserved. All of these enzymes, with the exception of CtXyn30A, have previously been characterized as GlcA-dependent enzymes.

3.3. Functional characterization of CtXyn30A

While developing this study, the initial expression and functional assessment of the E. coli CFE containing the CtXyn30A$_1$ protein product indicated that it functions strictly as a glucuronoxylan xylanohydrolase showing no detectable activity on arabinoxylans. We then cloned the CtXyn30A$_2$, CtXyn30A$_3$, and CtXyn30A$_4$ versions of the CtXyn30A enzyme with the consideration that some cloning aspect or unique functional characteristic of the C-terminal CBM6 domain may have endowed this enzyme with the reported novel WAX hydrolysis capability. When these CtXyn30A proteins were compared with BsXynC and CmXyn10B they showed levels of activity expected for this type of xylanase with a glucuronoxylan substrate [4,12,18]. Even when the amount of enzyme was increased 10-fold (for the GH30-8 xylanases, relative to BX activity) and the reaction time increased 12-fold, hydrolysis of WAX was not appreciable (Table 2). The control enzyme CmXyn10B confirmed that the reactions were performed correctly verifying that these 'generic' GH10 endoxylanases show activity on both glucuronoxylans and cereal arabinoxylans.

In consideration of these results. we prepared the protein expression construct CtXyn30A$_5$, a version of CtXyn30A having the exact amino acid sequence previously reported [12]. Characterization of the CtXyn30A enzymes by TLC showed that all of the constructs including this newly prepared CtXyn30A$_5$, which was used in its unpurified form functioned as GlcA-dependent endoxylanases. A hallmark of this activity is that each limit product contains one GlcA and that no neutral xylooligosaccharides are generated during prolonged hydrolysis (Fig. 2). The enzymes BsXynC and CmXyn10B were included in this study as a negative and positive control, respectively, as related to WAX hydrolysis. As expected, the control GH30-8 endoxylanase BsXynC efficiently hydrolyzed BX while a much greater amount of enzyme and longer reaction times did not result in any appreciable hydrolysis of WAX (Fig. 2). Hydrolysis of both substrates by the well-studied GH10 xylanase CmXyn10B confirmed that the reactions were performed properly. Other biomass derived polysaccharide substrates including arabinogalactan, arabinan, galactomannan, xyloglucan and glucomannan where tested in consideration of the previous report. Our findings indicate that CtXyn30A does not display significant activity on any of these other polysaccharides (Table 3)

Table 2
Activity determination with the Nelson's test using BX and WAX.

Enzyme	BX[a]	WAX[b]
CtXyn30A$_2$	15.77±1.59	0.049±0.002
CtXyn30A$_3$	17.01±3.06	0.043±0.001
CtXyn30A$_4$	12.78±0.76	0.040±0.004
BsXynC	10.62±12.80	0.009±0.001
CmXyn10B	52.41±0.11	14.1 6 ± 0.60

[a] Reactions performed for 10 min under optimum conditions as previously defined.
[b] Except for CmXyn10B, reactions performed using at least a 10-fold increase in enzyme and 12-fold increase in incubation time relative to BX reaction conditions.

Table 3
Activity determination with the Nelson's test with alternative biomass derived polysaccharides.

Substrate	Specific Activity of CtXyn3OA$_3$[a]
Arabinogalactan, larch wood	0.0391±0.0038
Arabinan (debranched),beet	0.085±0.008
Calactomannan, carob	0.0049±0.0011
Xyloglucan. tamarind	ND[b]
Glucomannan, konjac	0.0090±0.001 6

[a] Reactions performed using CtXyn30A$_3$ as described for the WAX hydrolysis.
[b] ND, none detected.

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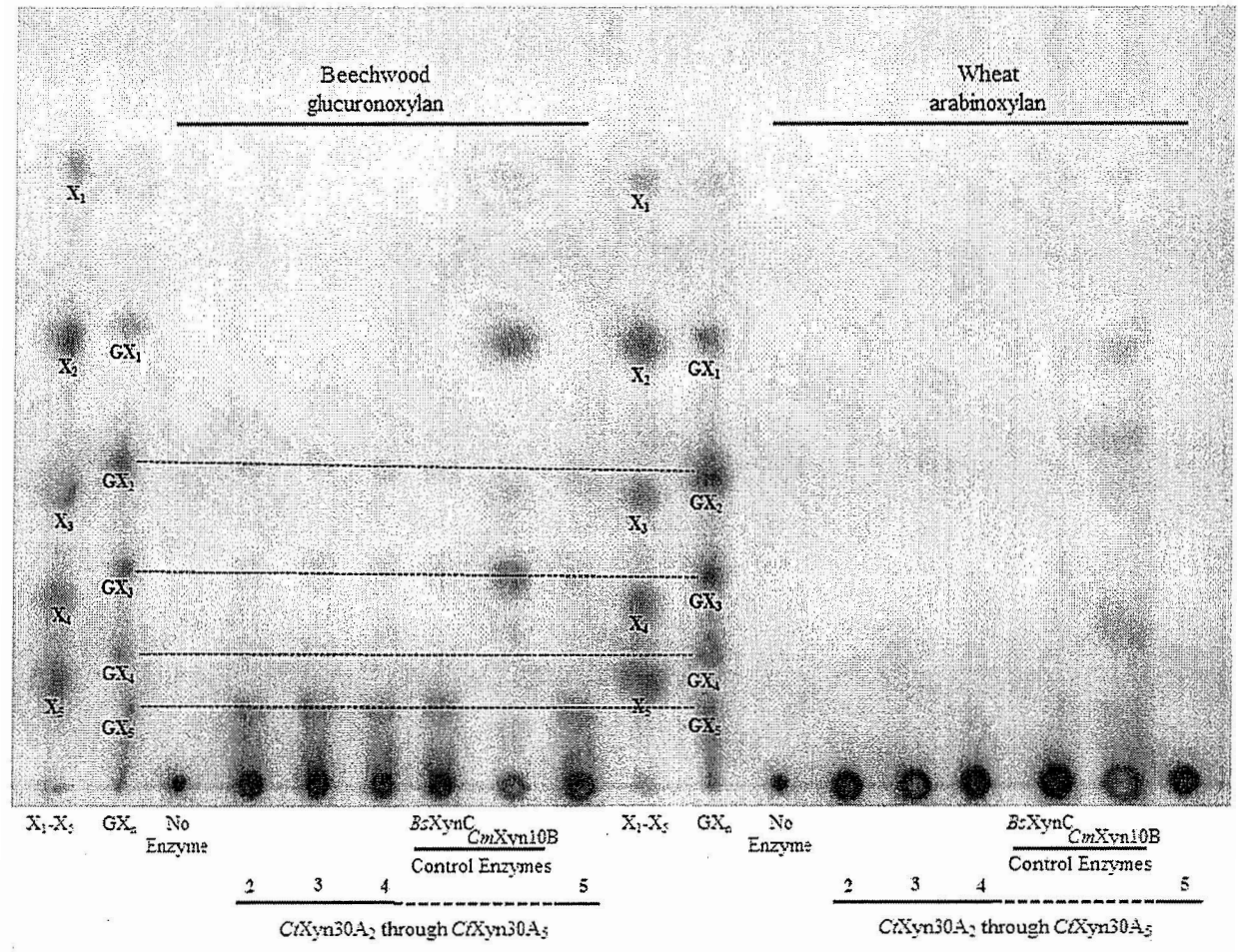

Fig. 2. TLC analysis of CtXyn30A activity compared to the control xylanases BsXynC and CmXyn10B. All reactions were digested overnight with a minimum of240 pmole/ml of enzyme except for the unpurified CtXyn30A$_5$ used as a dilution of the CFE. Standards are denoted as X_1 through X_5 representing xylose through xylopentaose and as GX_1 through GX_5 representing the range of 4-*O*-methyl-a-D-glucuronate substituted xylooligosaccharides or aldouronates.

3.4. Hydrolysis product analysis by MALDI-TOF

Analysis of hydrolysis products by MALDI-TOF MS confirmed the observations made by TLC and that of all previous reports concerning these enzymes [4,9]. Each aldouronate species is separated from its one larger species by the mass of an anhydroxylose (132.13 Da). Other than those labeled, two other peaks stood out in the low mass range. The peak at 438.02 Da is present in both the BsXynC and CtXyn30A hydrolysates and is near to the expected size of X_3-Na adduct, however this peak also was observed in the a-cylodextran control calibration standard. The peak at 549.72 Da which also occurs in both BsXynC and CtXyn30A hydrolysates does not match the mass of any considered xylooligosaccharide species and was also present in both a-cylodextran calibration standard and the water blank samples. Lastly, the analysis for BsXynC resulted in two other peaks (578.67 Da and 606.75 Da, respectively) which did not match to any calculated xylooligosaccharide or appear in any other spectrum (Fig. 3).

4. Discussion

The GH30-8 endoxylanases, originally named for their unique specificity as glucuronoxylan xylanohydrolases, have been well defined with respect to both their structure and function. All of the structurally and biochemically characterized enzymes, including the recently reported CtXyn30A, contain a highly conserved motif allowing coordination of the GlcA appendage of glucuronoxylans. In the recent report regarding this enzyme [12], results indicated that CtXyn30A functions preferably as a glucuronoxylan xylanohydrolase while also displaying approximately one-third of its maximal activity on wheat arabinoxylan. Notably, from that study, the measured activities on diverse non-xylan hemicellulose substrates, such as arabinogalactan, were also significantly high, with reported activities within a few Units/mg of those measured for arabinoxylan. This report caught our interest leading us to further investigate CtXyn30A and verify the reported results.

Our efforts to reproduce the reported WAX hydrolysis function of CtXyn30A and characterize its mode of action on WAX were not successful. Given the structural likeness to homologous GH30-8 enzymes and their reported function, we surmised that, if CtXyn30A did have activity on WAX, then it must be due to some other feature such as a novel function for the C-terminal CBM6 domain or the N-terminal His-Tag/Thrombin protease recognition site which was not reported as being cleaved during purification of CtXyn30A [12]. We pursued this by creating several expression constructs consisting of variations between a no affinity tag/wild-type form, various hexahistidine-tagged forms and a form lacking the CBM6 domain. Finally, after not being able to observe the WAX hydrolysis function in any of the CtXyn30A variants, we created the exact CtXyn30A version previously reported (CtXyn30A$_5$) [12]. In all cases the measured activity and functional assessment confirmed that CtXyn30A strictly is a GlcA-dependent glucuronoxylan

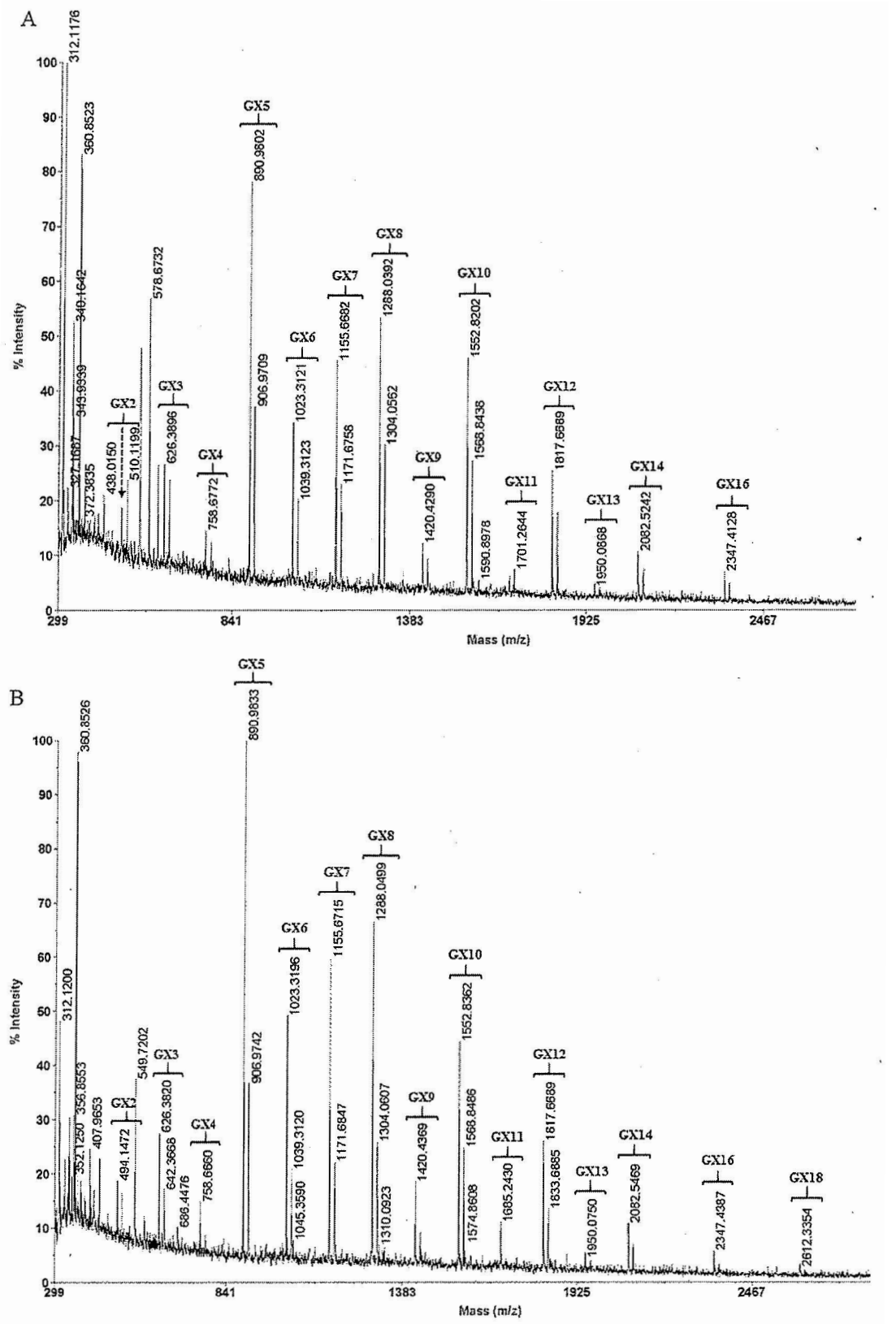

Fig. 3. MALDI-TOF MS spectrums of hydrolysis products of SGX by BsXynC (A) and CtXyn30A$_5$ (6). Each cluster of peaks represents at least a sodium adduct form and in many cases a second potassium adduct of the given aldouronate [4]. Peaks represent a series of singly substituted aldouronates in either a Na+ or K+ adduct form which differs from the next cluster of peaks by the size of a single anhydroxylose.

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xylanohydrolase. Importantly, following the observation of an expected level of activity for CtXyn30A on glucuronoxylan, but no detectable activity with the same enzyme level on WAX, all subsequent studies were performed with a massive excess of enzyme. The enzyme concentration used in the previous CtXyn30A study was approximately 28 pmol/ml; Our initial studies employed a similar level of enzyme, but in order to verify the reported WAX hydrolysis behavior of CtXyn30A, we increased the amount of enzyme by at least 10-fold with a concomitant 12-fold increase in reaction time but nevertheless observed no significant activity for WAX or the alternative biomass related polysaccharides.

Both TLC and MALDI-TOF analyses confirm the yield of strictly aldouronate products. For each of these analyses, enzymatic hydrolysis of xylans to yield small neutral oligoxylosides, would result in an increase of these terminal small sugars over time and a reduction in the amount of larger aldouronates down to the size of the aldouronate, GX_3 or GX_4. No small neutral sugars were detected in either TLC or MALDI-TOF, rather, both enzymes created a range of larger aldouronates as anticipated. These findings directly support all previous reports concerning these enzymes except the recent characterization of CtXyn30A [12].

The published report by Verma and Goyal [12] contains several identified problems which could not be reconciled through rigorous review of the reported methods. One reason for this, and the possible reason the authors came to the conclusion they did, is that the data obtained from the reducing terminus assays and TLC analysis were internally consistent. Given the authors experience in bioinformatics and the large body of knowledge available concerning the behavior of these enzymes, it is unexpected that they did not reconcile their results with what is currently known regarding these enzymes [21]. A follow-up publication involving the same authors which describes ligand bound structures of CtXyn30A has recently became available [22]. This more recent manuscript identifies the structural conservation of these enzymes as it relates to the reported biochemical function and provides several important ligand bound structures. Notably, the work reported the first G+ GH30-8 (CtXyn30A) having a ligand in the glycone subsites [22]. However, it did not correct the original reported biochemical results for CtXyn30A and provided as supplemental material a similar dataset (http://scripts.iucr.org/cgi-bin/sendsupfiles?gi5009&file=gi5009sup1.pdf&mime=application/pdf). In the work we describe here, even when using considerably elevated amounts of enzyme, we believe it has been made clear that CtXyn30A does not display any appreciable activity on non-glucuronoxylan substrates nor does it possess activity toward any other biomass-derived polysaccharides, an observation consistent with the conserved structure of the xylan binding cleft [9,14,22,23].

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References

[1] V. Lombard, H.G. Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res. 42 (D1) (2014) D490–495.
[2] F.J. St John, J.M. González, E. Pozharski, Consolidation of glycosyl hydrolase family 30: a dual domain 4/7 hydrolase family consisting of two structurally distinct groups, FEBS Lett. 584 (21) (2010) 4435–4441.
[3] H. Aspeborg, P.M. Coutinho, Y. Wang, H. Brumer, B. Henrissat, Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5), BMC Evol. Biol. 12 (1) (2012) 186.
[4] F.J. St John, J.D. Rice, J.F. Preston, Characterization of XynC from *Bacillus subtilis* subsp. subtilis strain 168 and analysis of its role in depolymerization of glucuronoxylan, J. Bacteriol. 188 (24) (2006) 8617–8626.
[5] M. Vršanská, K. Kolenová, V. Puchart, P. Biely, Mode of action of glycoside hydrolase family 5 glucuronoxylan xylanohydrolase from *Erwinia chrysanthemi*, FEBS J. 274 (7) (2007) 1666–1677.
[6] J.C. Hurlbert, J.F. Preston, Functional characterization of a novel xylanase from a corn strain of *Erwinia chrysanthemi*, J. Bacteriol. 183 (6) (2001) 2093–2100.
[7] K. Nishitani, D. Nevins, Glucuronoxylan xylanohydrolase. A unique xylanase with the requirement for appendant glucuronosyl units, J. Biol. Chem. 266 (10) (1991) 6539–6543.
[8] F.J. St John, D. Dietrich, C. Crooks, E. Pozharski, J.M. Gonzalez, E. Bales, K. Smith, J.C. Hurlbert, A novel member of glycoside hydrolase family 30 subfamily 8 with altered substrate specificity, Acta Crystallogr. Sect. D 70 (11) (2014) 2950–2958.
[9] L. Urbániková, M. Vršanská, K.B. Morkeberg Krogh, T. Hoff, P. Biely, Structural basis for substrate recognition by *Erwinia chrysanthemi* GH30 glucuronoxylanase, FEBS J. 278 (2011) 2105.
[10] Ó. Gallardo, M. Fernández-Fernández, C. Valls, S.V. Valenzuela, M.B. Roncero, T. Vidal, P. Díaz, F.J. Pastor, Characterization of a family GH5 xylanase with activity on neutral oligosaccharides and evaluation as a pulp bleaching aid, Appl. Environ. Microbiol. 76 (18) (2010) 6290–6294.
[11] S.V. Valenzuela, P. Diaz, F.J. Pastor, Modular glucuronoxylan-specific xylanase with a family CBM35 carbohydrate-binding module, Appl. Environ. Microbiol. 78 (11) (2012) 3923–3931.
[12] A.K. Verma, A. Goyal, A novel member of family 30 glycoside hydrolase subfamily 8 glucuronoxylan endo-β-1, 4-xylanase (CtXynGH30) from *Clostridium thermocellum* orchestrates catalysis on arabinose decorated xylans, J. Mol. Catal. B: Enzym. 129 (2016) 6–14.
[13] Novagen, pET System Manual, 10 ed., Novagen, 2003.
[14] F.J. St John, J.C. Hurlbert, J.D. Rice, J.F. Preston, E. Pozharski, Ligand bound structures of a glycosyl hydrolase family 30 glucuronoxylan xylanohydrolase, J. Mol. Biol. 407 (1) (2011) 92–109.
[15] W.L. DeLano, The PyMOL User's Manual, DeLano Scientific, Palo Alto, 2002.
[16] N. Nelson, A photometric adaptation of the Somogyi method for the determination of glucose, J. Biol. Chem. 153 (2) (1944) 375–379.
[17] B.V. McCleary, P. McGeough, A comparison of polysaccharide substrates and reducing sugar methods for the measurement of endo-1, 4-β-Xylanase, Appl. Biochem. Biotechnol. 177 (5) (2015) 1152–1163.
[18] G. Pell, E.J. Taylor, T.M. Gloster, J.P. Turkenburg, C.M. Fontes, L.M. Ferreira, T. Nagy, S.J. Clark, G.J. Davies, H.J. Gilbert, The mechanisms by which family 10 glycoside hydrolases bind decorated substrates, J. Biol. Chem. 279 (10) (2004) 9597–9605.
[19] M. Bounias, *N*-(1-naphthyl) ethylenediamine dihydrochloride as a new reagent for nanomole quantification of sugars on thin-layer plates by a mathematical calibration process, Anal. Biochem. 106 (2) (1980) 291–295.
[20] F.J. St John, J.D. Rice, J.F. Preston, Paenibacillus sp. strain JDR-2 and XynA1: a novel system for methylglucuronoxylan utilization, Appl. Environ. Microbiol. 72 (2) (2006) 1496–1506.
[21] A.K. Verma, A. Goyal, In silico structural characterization and molecular docking studies of first glucuronoxylan-xylanohydrolase (Xyn30A) from family 30 glycosyl hydrolase (GH30) from *Clostridium thermocellum*, Mol. Biol. 48 (2) (2014) 278–286.
[22] F. Freire, A. Verma, P. Bule, V. Alves, C. Fontes, A. Goyal, S. Najmudin, Conservation in the mechanism of glucuronoxylan hydrolysis revealed by the structure of glucuronoxylan xylanohydrolase (CtXyn30A) from Clostridium thermocellum, Acta Crystallogr. Sect. D: Struct. Biol. 72 (11) (2016).
[23] M.A. Sainz-Polo, S.V. Valenzuela, B. González, F.J. Pastor, J. Sanz-Aparicio, Structural analysis of glucuronoxylan-specific Xyn30D and its attached CBM35 domain gives insights into the role of modularity in specificity, J. Biol. Chem. 289 (45) (2014) 31088–31101.