Enzyme and Microbial Technology 114 (2018) 29–32

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Absence or presence of metal ion activation in two structurally similar GH43 β-xylosidases

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ARTICLE INFO

Keywords:
GH43
Metal ion activation
Xylosidase
BoXA
Biofuels
Xylan

ABSTRACT

Two GH43 β-xylosidases, RS223-BX from a rice straw metagenomic library, and BoXA from *Bacteroides ovatus*, that share similar amino acid sequences (81% identical) and 19 of 20 active-site residues, were compared by using site-directed mutagenesis of Asp and His residues implicated in metal binding. Thus, RS223-BX is strongly activated by divalent-metal cations and the previously published X-ray structure of this enzyme shows that a Ca^{2+} cation is chelated by an active-site Asp carboxyl group and an active-site His. Mutation to Ala causes 90% loss of activity for the Asp mutant and 98% loss of activity for the His mutant, indicating their importance to catalysis. For the other enzyme (BoXA), mutation to Ala causes 20% loss of activity for the His mutant and 40% gain of activity for the Asp mutant, indicating the lack of importance for activity of the native residues and the lack of metal-dependency, given that the Asp residue occupies the active site to secure the metal cation in known metal ion dependent GH43 xylosidases. The high activity of the BoXA mutants compared to that of the analogous RS223-BX mutants further undermines the possibility that BoXA maintains a tightly bound metal cofactor resistant to EDTA extraction. The results strengthen our conclusion that the very similar proteins differ in one being metal ion dependent and one not.

1. Introduction

Hemicelluloses including xylan, a β-D-(1 → 4)-linked xylopyranose polysaccharide, are structural carbohydrate components of the plant cell wall [1]. Enzymes that are involved in the breakdown of carbohydrate polymers in plant biomass known as glycosyl hydrolases (GH's) have been grouped into a number of families based on amino acid sequence similarity in the Carbohydrate Active Enzyme Database (CAZy; www.cazy.org) [2]. Xylosidases (E.C. 3.2.1.37) hydrolyze the terminal xylopyranoside moiety from the non-reducing end of xylan polymer chains, and are found in nature in bacteria from various ecological niches, with the xylosidase BoXA investigated here being a member of GH family 43 (GH43) from *Bacteroides ovatus* which resides in the mammalian colon (GenBank accession AAB08024) [3]. The breakdown and partial digestion of ingested plant material in the gut by microbial GH's is important to our daily caloric intake from the breakdown of carbohydrate polymers by microbes [4–6], and moreover the various oligosaccharides produced are thought to contribute to colonic health [7]. In addition, xylosidases are critical enzymes for industrial biorefinery conversion of biomass to biofuels [8]. Concerning the enzymatic reaction mechanism of this important group of biocatalysts, GH43 β-xylosidases contain 3 conserved amino acids with carboxylate side chains in the active site, that stabilize a single transition state in an SN1-type reaction that results in inversion of configuration at the anomeric carbon. Thus, the catalytic base (D16, Table 1) activates a water molecule for nucleophilic attack of the anomeric carbon of the scissile bond, the catalytic acid (E224) protonates the leaving group, and D137 functions as a xylose ligand to help stabilize the distorted pyranosyl ring that develops in the transition state, as well as modulating the pK_a of the general acid and maintaining it in the correct orientation [9]. In previous studies we have revealed that divalent metal cations activate two GH43 β-xylosidases by as much as 84-fold (k_{cat}/K_m, [10]) and 14-fold (k_{cat}, [11]): the respective enzymes are a β-xylosidase cloned from an anaerobic mixed microbial culture (RS223-BX; PDB accession 4MLG) and a β-xylosidase/arabinofuranosidase from a cow rumen metagenomic library (RUM630-BX; GenBank accession AFE48532). Metal cations did not affect either the pH or temperature stability of RS223-BX.

More recently, we reported that a search for enzymes similar to RS223-BX in amino acid sequence identified a GH43 β-xylosidase for which an X-ray crystal structure is not known to exist, that is 81%

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https://doi.org/10.1016/j.enzmictec.2018.03.007
Received 8 November 2017; Received in revised form 20 March 2018; Accepted 22 March 2018
Available online 24 March 2018

Table 1
Comparison of predicted active site residue alignment of BoXA to RS223-BX, RUM630-BX and CoXyl43[a].

Residue #[b]	15	16[c]	31	32	85	86[d]	110	136	137	168	222	224[e]	233	244	245	275[f]	290
BoXA	A	D	S	H	W	D	R	I	D	A	F	E	D	T	H	H	H
RS223-BX	A	D	S	H	W	D	R	M	D	A	F	E	D	T	H	H	H
RUM630-BX	A	D	S	H	W	D	H	I	D	D	F	E	D	S	H	H	H
CoXyl43	A	D	S	H	W	A	R	I	D	D	F	E	D	T	H	H	H

[a] Adapted from Jordan et al. [11].
[b] Residue number is from BoXA.
[c] Catalytic base.
[d] Binds Ca^{2+} in RS223-BX.
[e] catalytic acid.
[f] Binds Ca^{2+} in RS223-BX and CoXyl43 [13,18].

identical in amino acid sequence to the full enzyme and shares 19 of 20 active-site residues, with the difference being an Ile residue in BoXA instead of a Met residue in RS223-BX, where the S atom of the Met residue in the X-ray structure of RS223-BX is > 8 Å from D85, the residue that binds metal ion (*vide infra*), and > 9 Å from the D15, the catalytic base, and thus the Met residue is not involved in metal ion binding (Table 1): this enzyme is a β-xylosidase from *Bacteroides ovatus* (BoXA) [12]. Thus, it would seem predictable that BoXA would mirror the divalent-metal activation properties reported for RS223-BX. However, we found this is not the case. Using the same experimental conditions that we used for RS223-BX that showed extensive loss of activity when treated with EDTA to remove divalent metal cations, BoXA lost only 3% activity. Also, in the case of RS223-BX, the addition of divalent metal cations to the enzyme resulted in as much as an 84-fold increase in k_{cat}/K_m and a 32-fold increase in k_{cat}, whereas addition of divalent metal cations to BoXA resulted in no increase in activity. The X-ray crystal structure of RS223-BX was previously determined complexed with Ca^{2+} [13], which is a known activator of this enzyme, showing calcium cation proximal to Asp and His residues (corresponding to D86 and H275, Table 1), whereby the metal ion positive charge can be transferred to and stabilize the catalytically active carboxylate anion of the catalytic base Asp residue in the transition state and thereby accelerate the hydrolysis rate. In the current work, we examine these two active-site residues of RS223-BX and BoXA by site-directed mutagenesis.

2. Materials and method

2.1. Materials

4-Nitrophenyl-β-D-xylopyranoside (4NPX) was obtained from Gold Biotechnology (St. Louis, MO). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Water was purified by a Milli-Q Academic A10 unit (Millipore; Billerica, MA). UV–vis absorbances were determined using a thermostated Cary 50 Bio UV–vis spectrophotometer (Agilent; Santa Clara, CA). The calculated 280 nm extinction coefficient [14] of wild-type RS223-BX is 80.8 $mM^{-1}\,cm^{-1}$ and of wild-type BoXA is 82.02 $mM^{-1}\,cm^{-1}$.

2.2. Site-directed mutagenesis, preparation, and purification of enzymes

Four highly conserved residues in the RS2223-BX (Asp85, His274) and BoXA (Asp86, His275) enzymes were mutated to alanines. The native genes subcloned in the pET29b expression vector (EMD Millipore; MA, USA) were modified with oligonucleotides encoding the desired mutations using the QuikChange II kit (Agilent; CA, USA). The targeted codons encoding aspartate (GAC) and histidine (CAC) were all mutated to alanine (GCC). The resulting vectors encoding the modified enzymes were p29-RS223BX-D85A, p29-RS223BX-H274A, p29-BoXA-D86A, and p29-BoXA-H275A. For expression, 400 mL Terrific Broth containing 30 μg/mL kanamycin (TB_{kan}) was inoculated with 10 mL of a TB_{kan} culture grown overnight at 30 °C, grown to log phase, induced with 1 mM IPTG, and grown overnight at 30 °C. Centrifugation provided cell pellets which were flash-frozen with liquid nitrogen and stored at −80 °C. The ~8 g cell pellets obtained were lysed using 65 mL/5 g cell pellet Cellytic B reagent, amended with 0.5 mg/mL hen egg white lysozyme, 5 U/mL Benzonase, 1 μL/mL protease inhibitor catalog #P8849, and 2 mM β-mercaptoethanol (all lysis reagents from Sigma, St. Louis, MO). The cells were stirred slowly at room temperature for 30 min, centrifuged to provide a soluble enzyme containing supernatant that was adjusted to 300 mM NaCl, 12 mM imidazole, and 50 mM sodium phosphate pH 8 for binding to Superflow Ni-NTA resin (0.5 mL resin slurry/50 mL; Qiagen, Valencia, CA) and rotated at 4 °C for 2 h. The resin was collected in 12 mL BioRad fritted columns (BioRad Laboratories, Hercules, CA), and washed with 20 mL wash buffer: 300 mM NaCl, 12.5 mM imidazole, 50 mM sodium phosphate, pH 8.0, and 10% glycerol. Protein was eluted using the same buffer with 250 mM imidazole, and the enzymes further purified by size-exclusion chromatography using a Superdex 200 16/60 column (GE Life Sciences) equilibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0, and protein concentrations were obtained spectrophotometrically using OD_{280} [14].

2.3. Relative activities of BoXA wild-type and mutants

Initial-rate reactions (1-mL) at 25 °C contained 5.0 mM 4NPX in 20 mM MOPS pH 7.0, ionic strength (I) = 0.3 M. Reactions were run in duplicate and were initiated by the addition of a 10 μL aliquot of BoXA wild-type or mutant enzyme. Production of 4-nitrophenol (4NP) was monitored continuously at 400 nm for 0.5 min. The 400 nm extinction coefficient of 4NP is 18.3 $mM^{-1}\,cm^{-1}$ in 0.1 M sodium hydroxide [15] and 8.5 $mM^{-1}\,cm^{-1}$ at pH 7.0.

2.4. Relative activities and Mg^{2+}-activation of RS223-BX wild-type and mutants

The relative activities of the RS223-BX wild-type enzyme and two mutants were determined as above except reactions also contained 10.0 mM Mg^{2+}, and enzyme was preincubated with Mg^{2+} for 0.7 h at 25 °C prior to initiation of reactions by enzyme addition. To determine the Mg^{2+}-activation parameters of the RS223-BX wild-type and mutant enzymes, the concentration of Mg^{2+} was varied (0–10.0 mM) in the reaction mixture as well as in the enzyme preincubation while all other conditions remained as described previously.

2.5. Equations

Data were fit to the following equation where ν is the observed initial (steady-state) rate of catalysis; E_T is the total enzyme concentration; k_{cat} is the turnover number of catalysis; M is the divalent metal concentration; $K_{0.5}$ is the half-activation concentration; C is the activity in absence of added divalent metal. Data were fit to the nonlinear equation using GraFit [16].

$$\frac{v}{E_t} = \frac{k_{cat} M}{K_{0.5} + M} + C \quad (1)$$

3. Results

The two RS223-BX mutants (D85A and H274A) have reduced activity compared to wild-type RS223-BX (Table 2). The greatest reduction of activity was observed for the RS223-BX H274A mutant, with only 1.8% of activity remaining compared to wild-type RS223-BX. The D85A RS223-BX mutant retains 10% of the wild-type RS223-BX