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Isolation and divalent-metal activation of a β-xylosidase, RUM630-BX

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ABSTRACT

The gene encoding RUM630-BX, a β-xylosidase/arabinofuranosidase, was identified from activity-based screening of a cow rumen metagenomic library. The recombinant enzyme is activated as much as 14-fold (k_{cat}) by divalent metals Mg^{2+}, Mn^{2+} and Co^{2+} but not by Ca^{2+}, Ni^{2+}, and Zn^{2+}. Activation of RUM630-BX by Mg^{2+} ($t_{0.5}$ 144 s) is slowed two-fold by prior incubation with substrate, consistent with the X-ray structure of closely related xylosidase RS223-BX that shows the divalent-metal activator is at the back of the active-site pocket so that bound substrate could block its entrance. The enzyme is considerably more active on natural substrates than artificial substrates, with activity (k_{cat}/K_m) of 299 s^{-1} mM^{-1} on xylotetraose being the highest reported.

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1. Introduction

Plant biomass both extant and converted to fossil fuels is the source of the vast majority of stored chemical energy forms and feedstocks consumed by humankind, with the uncertain availability of prehistorically fixed carbon leading to long term energy insecurity issues, and its extraction and release causing environmental damage and climate change. To address these problems the enzymatic breakdown of biomass for fuels and platform chemicals will play an increasingly important mitigating role as we progress towards more environmentally benign, carbon neutral energy infrastructures. Hemicelluloses comprise the second most prevalent plant polymer type after cellulose, where they are integral plant cell wall components. One of the most common hemicellulose types are termed xylans, comprising a diverse group of heteropolysaccharides that have in common a backbone consisting of β-(1 → 4)-linked xylose [1]. A biomass source-dependent variety of side chains and substituents, including L-arabinofuranose, D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid, and ferulic acid [2], modify the xylan backbone and are removed before the xylan backbone can be enzymatically depolymerized, thus necessitating a suite of enzyme activities known collectively as glycosyl hydrolases (GH's) for complete saccharification by microorganisms [3]. Ultimately endo-(1 → 4)-β-xylanase (EC 3.2.1.8) releases xylooligosaccharides from xylan, which are hydrolyzed from the non-reducing end to xylose by β-xylosidase (EC 3.2.1.37) [4]. The CAZY database (http://www.cazy.org/) groups GH's based on amino acid sequence similarity [5], and β-xylosidases can be found in 10 GH families. The mechanism of hydrolysis by β-xylosidases has been shown to occur either through the formation of an enzyme-bound intermediate which results in retention of anomeric configuration, or by a single displacement mechanism resulting in inversion [6]. The β-xylosidases belonging to family GH43 are alone in having an inverting mechanism, where 3 active site carboxylate residues have been identified to serve the catalytic mechanism [7]. Enzymes having a single displacement mechanism are advantageous for industrial saccharification since, unlike retaining GH enzymes, they are not prone to transglycosylation side reactions. More importantly β-xylosidases GH43 members thus far display the highest catalytic turnovers [4].

Previously, we reported on RS223-BX, a GH43 β-xylosidase/arabinofuranosidase prepared from a metagenomic library [8,9]. This is a divalent-metal-requiring enzyme whose k_{cat}/K_m activity is enhanced as much as 84-fold by the addition of divalent metal cations: Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+}. More recently we have reported on this enzyme's X-ray structure [10]. Notably, the divalent metal cation (Ca^{2+}) of the structure is located at the back end of the active-site pocket. It is chelated in the active site by an aspartate carboxyl group that is not found in non-divalent-metal-activated GH43 enzymes. The divalent metal of RS223-BX is also proximal to an imidazole of a histidine group. It is considered that divalent-metal activation proceeds by transferring positive charge from the Ca^{2+} through the imidazole to stabilize the nearby catalytic base (aspartate carboxyl group)

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in its catalytically active anionic form. RS223-BX is currently the most active β-xylosidase (k_{cat}/Km) acting on xylooligosaccharides. Having insight into the value of metagenomic libraries in finding new β-xylosidases, we further pursued this discovery strategy and report here the cloning and biochemical characterization of a new divalent-metal-activated β-xylosidase which we designate RUM630-BX.

2. Materials and methods

2.1. Bacterial strains and reagents

Escherichia coli SOLR and BL21(DE3) pLysE strains were purchased from Agilent (CA, USA) and Novagen (WI, USA), respectively. Reagents were obtained as follows: 4-nitrophenol (4NP) from Sigma–Aldrich (St. Louis, MO, USA), 4-nitrophenyl-β-D-xylopyranoside (4NPX) from Gold Biotechnology (St. Louis, MO, USA), 4-nitrophenyl-α-L-arabinofuranoside (4NPA) from Carbosynth Limited, xylobiose (X2) and xylotriose (X3) from Wako Chemicals (Richmond, VA, USA) and xylotetraose (X4) and aldouronic acid from Megazyme (Wicklow, Ireland), and DNA modification enzymes from New England Biolabs (MA, USA). Water was purified by a Milli-Q Academic A10 unit (Millipore; Billerica, MA, USA). All other chemical reagents were obtained from Sigma–Aldrich unless otherwise specified.

2.2. Activity screening and gene cloning

A mixed population of microorganisms was isolated from rumen fluid that was collected from a fistulated cow. The genomic DNA from the entire microbial population was prepared using the FastDNA kit (Qbiogene, CA, USA). The cells were lysed according to manufacturer's instructions using CLS-TC buffer and Matrix E (ceramic, silica, and glass beads). The isolated DNA was partially digested with *Apo*I DNA restriction enzyme and resolved on an agarose gel. Fragments sized 4–10 kb were excised from the agarose gel and ligated to a lambda phage vector to create a phage DNA library (Lambda ZAP II Vector and Gigapack III Packaging Extract; Agilent). The library was subjected to *in vivo* excision to produce a genomic DNA library in the pBluescript plasmid that was carried in the *E. coli* SOLR strain. The genomic DNA library was spread on nylon membranes and screened for activity against the aldouronic acid substrate as previously described [11].

The plasmid from a transformed bacterium which gave a positive signal in the activity assay was sequenced, and a putative β-xylosidase gene (*rum630-bx*) was detected. The gene was amplified by PCR with the following primers:

630BX-5: GACGCACATATGAAGCCACGCTATCTTTATCCCAG
630BX-3: GACCTCGAGCTCATTCACAACCTTCTGGATGGTG

The PCR product and the pET29b plasmid (Novagen) were digested with the DNA restriction enzymes *Nde*I and *Xho*I and ligated. The resulting expression vector encoded the *rum630-bx* gene fused to a C-terminal 6x-histidine tag and was transformed into *E. coli* BL21(DE3) pLysE cells. The bacteria were grown in ZYM5052 autoinduction media [12] for 24 h at 37 °C. The cells were harvested, resuspended in lysis solution (50 mM sodium phosphate, 100 mg/ml lysozyme, 25 U/μl Benzonase, pH 8), sonicated, and centrifuged. The supernatant was applied to a nickel sepharose column (HisTrap; GE Healthcare, PA, USA), and the enzyme was eluted with imidazole in 50 mM sodium phosphate, 300 mM sodium chloride, pH 7. The fractions containing purified enzyme were pooled and buffer-exchanged on a Superdex 200-16/30 sizing column equilibrated with 10 mM HEPES-NaOH, 150 mM sodium chloride, pH 7.

2.3. Enzyme assays

All enzyme reactions were conducted at pH 7.0 (either 10 mM or 100 mM HEPES-NaOH) and 25 °C. The delta extinction coefficient (4NP–4NPX or 4NP–4NPA) at 400 nm and pH 7.0 is 8.18 cm^{-1} mM^{-1}. UV/vis absorbances were determined using a thermostated Cary 50 Bio UV Visible spectrophotometer (Agilent; Santa Clara, CA, USA). A DX500 Dionex HPLC system equipped with an ED40 electrochemical detector (pulsed amperometry) was used for saccharide separation and detection (Dionex; Sunnyvale, CA, USA) as previously described [13]. Initial rate (s^{-1}) is μM product produced/s/μM enzyme active sites.

2.4. Stripping divalent metal from RUM630BX using EDTA

RUM630-BX was incubated 5 h at 25 °C and overnight at 4 °C in 10 mM HEPES-NaOH, 10 mM EDTA at pH 7.0. The incubated enzyme was gel filtered through a BioRad P-6 column (Bio-Rad; Hercules, CA) equilibrated and developed with 10 mM HEPES-NaOH pH 7.0 as had been done before with the RS223-BX enzyme [8]. The enzyme was flash-frozen in liquid N_2 until use.

2.5. Steady-state kinetics of non-divalent-metal-activated, Mg^{2+}-activated, Mn^{2+}-activated RUM630BX-catalyzed reactions with substrates 4NPX and 4NPA

Enzyme was preincubated in 100 mM HEPES-NaOH, 1.0 mM Mg^{2+} (or no divalent metal or 0.5 mM Mn^{2+}) for at least 15 min at ~25 °C. Reactions (1 mL) containing 100 mM HEPES-NaOH, 1.0 mM Mg^{2+} (or no divalent metal or 0.5 mM Mn^{2+}) and various concentrations of 4NPX or 4NPA at pH 7.0 and 25 °C were initiated with 7 μL preincubated enzyme. Progress of the reactions was continuously monitored (0.3–1.0 min) at 400 nm.

2.6. Equations

Data were fit to the following equations where *A*0 is the initial value; *k* is the first-order rate constant; *t* is time; *C* is the endpoint; *v* is the observed initial (steady-state) rate of catalysis; *ET* is the total enzyme concentration; k_{cat} is the turnover number of catalysis; *S* is the substrate concentration; K_m is the Michaelis constant; and K_{si} is the substrate inhibition constant, the dissociation constant for *S* from ESS. Data were fit to equations using GraFit [14].

$$v = (A_0 - C) \times e^{-k \times t} + C \quad (1)$$

$$\frac{v}{E_t} = \frac{k_{cat} \times S}{K_m + S} \quad (2)$$

$$\frac{v}{E_t} = \frac{k_{cat} \times S}{K_m + S \times \left(1 + \frac{S}{K_{si}}\right)} \quad (3)$$

$$v = A_0 \times (1 - e^{-k \times t}) + C \quad (4)$$

3. Results and discussion

A gene (*rum630-bx*) encoding a putative 333-amino acid β-xylosidase enzyme was isolated from a genomic library using an activity screen originally designed to discover new α-glucuronidase enzymes. The α-glucuronidase enzymes hydrolyze the substrate, aldouronic acid, and release xylose and xylooligomers. Because the detection assay relies on the further conversion of the xylose to xylonate, the presence of the β-xylosidase gene on the same genomic DNA fragment resulted in elevated xylose levels and a high positive signal from the bacteria harboring this genomic fragment (GenBank JN684207) [15]. The *rum630-bx* gene was then subcloned into an expression