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Rehabilitation of faulty kinetic determinations and misassigned glycoside hydrolase family of retaining mechanism β-xylosidases

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ABSTRACT

We obtained Cx1 from a commercial supplier, whose catalog listed it as a β-xylosidase of glycoside hydrolase family 43. NMR experiments indicate retention of anomeric configuration in its reaction stereochemistry, opposing the assignment of GH43, which follows an inverting mechanism. Partial protein sequencing indicates Cx1 is similar to but not identical to β-xylosidases of GH52, including Q09LZ0, that have retaining mechanisms. Q09LZ0 β-xylosidase had been characterized biochemically in kinetic reactions that contained Tris. We overproduced Q09LZ0 and demonstrated that Tris is a competitive inhibitor of the β-xylosidase. Also, the previous work used grossly incorrect extinction coefficients for product 4-nitrophenol. We redetermined kinetic parameters using reactions that omitted Tris and using correct extinction coefficients for 4-nitrophenol. Cx1 and Q09LZ0 β-xylosidases were thus shown to possess similar kinetic properties when acting on 4-nitrophenyl-β-D-xylopyranoside and xylobiose. k_{cat} pH profiles of Cx1 and Q09LZ0 acting on 4-nitrophenyl-β-D-xylopyranoside and xylobiose have patterns containing two rate increases with increasing acidity, not reported before for glycoside hydrolases. The dexylosylation step of 4-nitrophenyl-β-D-xylopyranoside hydrolysis mediated by Q09LZ0 is not rate determining for k_{cat}^{4NPX}.

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Introduction

Large quantities of plant cell wall polymers are destined for conversion to ethanol and other biofuels in abrogating our dependence on fossil fuels [1,2]. Deconstruction of the biopolymers to monosaccharides will be catalyzed enzymatically and improvement of the catalytic performance of the relevant glycoside hydrolases will be a continuing effort, driven by economics of the large scale endeavor [2]. β-xylosidase is one of the enzymes needed to catalyze hydrolysis of the hemicellulose xylan, the second most abundant plant polymer after cellulose. The enzyme is targeted for improvement by searching natural sources and through manipulation by genetic means [3–5]. Glycoside hydrolase family 43 has produced the most active β-xylosidases discovered to date [2,5], prompting us to examine additional members of the family. The β-xylosidase from *Lactobacillus brevis* ATCC 367 is the most active β-xylosidase acting on xylooligosaccharides reported to date: for example, $k_{cat}^{xylobiose}$ 407 ± 9 s^{-1} at pH 6.0 and 25 °C. GH43 β-xylosidases have been reported on extensively [3–24].

In this work we set out to biochemically characterize another GH43 β-xylosidase in a similar manner that we have pursued on other GH43 β-xylosidases [2,5,9,19]. The GH43 β-xylosidase of unknown species origin was obtained from a commercial source. Work on the commercial GH43 β-xylosidase led to work on the *Geobacillus stearothermophilus* T-6 β-xylosidase, which has a similar amino acid sequence [25–27].

Materials and methods

Materials and general methods

4-nitrophenol (4NP[1]), D-xylose and D-glucose were obtained from Sigma–Aldrich. 4-nitrophenyl-α-L-arabinofuranoside (4NPA) was obtained from Carbosynth Limited (Compton, Berkshire, United Kingdom). 4-nitrophenyl-β-D-xylopyranoside (4NPX) was obtained from Carbosynth Limited (Compton, Berkshire, United Kingdom) and Gold Biotechnology (St. Louis, MO). Xylobiose (X2) was obtained from Wako Chemicals. Water was purified by a Milli-Q Academic A10 unit (Millipore; Billerica, MA). UV/VIS absorbances were

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[1] *Abbreviations used*: 4NP, 4-nitrophenol; 4NPA, 4-nitrophenyl-α-L-arabinofuranoside; 4NPX, 4-nitrophenyl-β-D-xylopyranoside; Cx1, β-xylosidase studied here; GSXynB2, β-xylosidase of *Geobacillus stearothermophilus* T-6 studied here; I, ionic strength; MuX, 4-methylumbelliferyl-β-D-xylopyranoside; X2, xylobiose.

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determined using a thermostated Cary 50 Bio UV-Visible spectrophotometer (Agilent; Santa Clara, CA). The extinction coefficient used for 4NP at different pH was determined as described previously [9]. A DX500 Dionex HPLC system equipped with an ED40 electrochemical detector (pulsed amperometry) was used for saccharide separation and detection (Dionex; Sunnyvale, CA).

CAZyme™ Xylosidase 1 (Cx1) was obtained from Lucigen (Middleton, WI). The enzyme was pelleted from ammonium sulfate by centrifugation, resolubilized in 10 mM sodium phosphate pH 7.0 and then gel filtered through a P-6 DG column (Bio-Rad) equilibrated with 10 mM sodium phosphate pH 7.0. The protein concentration was determined using the 280 nm extinction coefficient (141,400 M^{-1} cm^{-1}) provided by the manufacturer. The enzyme was flash frozen and stored at −80 °C until needed. When used, the enzyme was appropriately diluted with 10 mM sodium phosphate pH 7.0.

Cx1 was partially sequenced by the Wistar Proteomics Facility of Philadelphia, PA [5,28–31]. Wistar used trypsin digestion, HPLC separation of the peptides, sequencing of the peptides by mass spectroscopy and a BLAST search of the peptides in attempting to identify the protein.

The GSXynB2 gene (Q09LZ0), including a 6×-Histag on the C-terminus, was synthesized by GenScript USA Inc. (Piscataway, NJ) and placed into the pET-29a(+) (EMD Chemicals; Philadelphia, PA) vector. The pET-29a(+) vector containing the GSXynB2 gene was transformed into BL21(DE3)*Escherichia coli*. Two 500 mL shake flask cultures (Terrific Broth supplemented with 30 μg/mL kanamycin) were grown at 25 °C to an OD_{600nm} of 1, induced with 1 mM IPTG (final concentration), allowed to grow overnight, and harvested by centrifugation. Cells were lysed using a BeadBeater and 0.1 mm glass beads (BioSpec Products; Bartlesville, OK). Purification steps included a HisPrep FF 16/10 column (GE Healthcare; Piscataway, NJ), desalting through a column of Bio-Gel P-6 DG resin (Bio-Rad; Hercules, CA) equilibrated in 10 mM sodium phosphate pH 7.0 and concentration using Amicon Ultra-15 mL spin filters (EMD Millipore; Billerica, MA). The protein concentration was determined using the calculated 280 nm extinction coefficient (135,460 M^{-1} cm^{-1}). Enzyme was flash frozen in small aliquots and stored at −80 °C until used.

pH and temperature stabilities of Cx1 and GSXynB2

pH stabilities were determined in buffers of constant ionic strength (0.3 M, adjusted with NaCl) at 25 °C: 100 mM sodium succinate (pH 4–6), 100 mM sodium phosphate (pH 7), 30 mM sodium pyrophosphate (pH 8–10), 82 mM sodium phosphate (pH 11) and 58 mM sodium phosphate (pH 12). Buffer without enzyme was placed in a 0.5 mL tube and preincubated at 25 °C for 10 min prior to enzyme addition. After incubating for 60 min, an aliquot of pH-treated enzyme was added directly to a hydrolysis assay mixture containing 5.0 mM 4NPX, 100 mM sodium phosphate, ionic strength 0.3 M at pH 7.0 and 25 °C. Absorbance was monitored continuously at 400 nm to determine initial rates. Initial-rate data for preincubated enzyme was compared to control reactions, enzyme preincubated at pH 7.0 on wet ice for 60 min, to determine relative remaining activity.

Thermal stabilities of enzymes were determined in 100 mM sodium phosphate, ionic strength 0.3 M at pH 7.0. Enzyme was placed in 0.2 mL or 0.5 mL tubes and incubated in a thermocycler at different temperatures (30–80 °C); 25 °C samples were incubated in a water bath. After incubating for 60 min, enzyme tubes were placed on wet ice. Temperature-treated enzyme was incubated at room temperature for at least 10 min before assaying in 5 mM 4NPX, 100 mM sodium phosphate, ionic strength 0.3 M at pH 7.0 and 25 °C. Absorbance was monitored continuously at 400 nm to determine initial rates. Initial-rate data for preincubated enzyme were compared to that of a control reaction, enzyme preincubated in pH 7.0 buffer on ice for 60 min, to determine relative remaining activity.

Steady-state kinetic parameters of Cx1 and GSXynB2 acting on natural and artificial substrates

Initial-rates for determination of kinetic parameters acting on 4NPX and 4NPA reactions were conducted by adding enzyme to reactions containing substrate equilibrated at 25 °C and continuously monitoring the 400 nm absorbance change over time (380 nm for pH 3.5). pH 4 and 4.5 reactions were run using a discontinuous method; adding aliquots of 4NPX reaction mixtures to 100 mM sodium hydroxide and aliquots of 4NPA reaction mixtures to 100 mM sodium carbonate pH 11 at various time points and reading the absorbance at 400 nm.

Enzyme-catalyzed reactions of X2 hydrolysis were run and analyzed by a Dionex HPLC system using a CarboPac PA-100 column, similarly to that described [13], except the linear sodium acetate gradient was replaced with an isocratic 100 mM sodium hydroxide development step, followed by a column washing step consisting of 250 mM sodium acetate in 100 mM sodium hydroxide and an equilibration step of 100 mM sodium hydroxide before the next injection. Alternatively, a CarboPac PA-1 column was used for some of the analysis using the following setup: isocratic 15 mM sodium hydroxide development step, followed by a column washing step consisting of 250 mM sodium acetate in 100 mM sodium hydroxide and an equilibration step of 15 mM sodium hydroxide before the next injection. Briefly, 0.5 mL reactions were prepared without enzyme. For time 0 determinations, two 100 μL aliquots were removed and combined with 100 μL 200 mM sodium phosphate adjusted so the quenched reaction pH was ~10.5. The catalyzed reactions were initiated by adding 7 μL enzyme to the remaining 300 μL. At each time point, the reactions were quenched with an equal volume of 200 mM sodium phosphate adjusted so that the quenched reaction pH was ~10.5, and then placed on wet ice until analysis by HPLC.

Buffers at 0.3 M ionic strength (adjusted with NaCl) were used: 100 mM succinate (pH 4–6), 100 mM sodium phosphate (pH 7–7.5) and 30 mM sodium pyrophosphate (pH 8–10). Inhibition of Cx1 and GSXynB2 by D-xylose or D-glucose were run at pH 6.0 and 25 °C. Reactions contained 3 concentrations of inhibitor and varied concentrations of 4NPX. pH-dependent inhibition by Tris of GSXynB2 utilized buffers at 0.3 M ionic strength (adjusted with NaCl): 100 mM succinate (pH 4–6), 100 mM sodium phosphate (pH 7) and 30 mM sodium pyrophosphate (pH 8–9).

NMR experiments

Aqueous solutions of 4NPX; X2; 100 mM sodium phosphate; and enzyme were lyophilized to dryness. They were solubilized in a minimum volume of D_2O and lyophilized to dryness. 60 μL of 100 mM D-xylose was added to directly to 1 mL D_2O and lyophilized to dryness. Finally, the sodium phosphate was solubilized in D_2O and used to solubilize the remaining samples, except the enzyme, to provide the following stocks in 100 mM sodium phosphate (D_2O) pD 6.6: 6 mM D-xylose, 3 mM 4NPX, 3 mM X2. The enzyme was solubilized in D_2O to give a stock of 179 μM enzyme in 20 mM sodium phosphate pD ~7. For the 4NPX and the X2 reactions (1-mL), an NMR spectrum was recorded prior to enzyme addition (5 μL of 179 μM enzyme from above), followed by spectra taken every 3 min and finally a 24 h spectrum was recorded. ^1H NMR spectra were collected using a Brüker Avance 500 MHz NMR operating Topspin 1.3 patch level 8 at 27 °C. All chemicals shifts reported are relative to the referenced lock solvent.