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Rate-limiting steps of a stereochemistry retaining β-D-xylosidase from *Geobacillus stearothermophilus* acting on four substrates

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ABSTRACT

Kinetic experiments of GSXynB2, a GH52 retaining β-xylosidase, acting on 2-nitrophenyl-β-D-xylopyranoside (2NPX), 4-nitrophenyl-β-D-xylopyranoside (4NPX), 4-methylumbelliferyl-β-D-xylopyranoside (MuX) and xylobiose (X2) were conducted at pH 7.0 and 25 °C. Catalysis proceeds in two steps (xylodidation followed by dexylosidation): E + substrate TO E-xylose + leaving group TO E + xylose. k_{cat} falls into two groups: 4NPX (1.95 s^{-1}) and 2NPX, MuX and X2 (15.8 s^{-1}, 12.6 s^{-1}, 12.8 s^{-1}, respectively). Dexylosylation (E-xylose to E + xylose), the common step for the enzymatic hydrolysis of the four substrates, must exceed 15.8 s^{-1}. k_{cat} of 4NPX would seem mainly limited by xylosylation (step 1) and the other three substrates would seem mainly limited by dexylosylation (step 2) – a conclusion that critically lacks chemical justification (compare 4NPX and 2NPX). Presteady-state rates indicate rapid xylosidation rates for all substrates so a later step (not dexylosidation) is rate-limiting for 4NPX. That 2NPX is an onlier and 4NPX is an outlier (both leaving group pK_a of 7.2) of the Brønsted plot pattern (logk_{cat} vs pK_a of phenol leaving group) is thus possibly explained by 4NP release. The pH dependency of k_{cat} 2NPX encompasses 2 bell-shaped curves with peaks of pH 3 and pH 7.

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1. Introduction

GSXynB2, a β-D-xylosidase of *Geobacillus stearothermophilus* T-6, belongs to glycoside hydrolase family 52 (GH52) [1]. Like other members of GH52, it catalyzes hydrolysis of substrates by a stereochemistry retaining, double displacement mechanism that proceeds through two transition states via xylosylation and dexylosylation reactions (Scheme 1). β-D-xylosidases are of practical interest owing to their critical role in the breakdown of xylan, the second most abundant biomass after cellulose [2,3]. Thus it can serve a role in the enzymatic conversion of xylan to simple sugars for subsequent fermentation to biofuels and other bioproducts.

Recently, we reported that the GH52 β-xylosidase from *G. stearothermophilus* T-6 exhibits two salient features [4]: 1. Its pH dependence of k_{cat} demonstrates a new pH curve never before reported for a glycoside hydrolase and 2. The rate-limiting step of GSXynB2 acting on 4-nitrophenyl-β-D-xylopyranoside (4NPX) appears unlikely that of glycosylation or deglycosylation; instead, substrate/product binding may limit. More recently we have found literature data that requires reinterpretation of the previous reports [1,5] and we have conducted experiments that lend support to what now becomes definitive results.

2. Materials and methods

2.1. Materials and general methods

4-Nitrophenol (4NP), 2-nitrophenol (2NP), 2-nitrophenyl-β-D-xylopyranoside (2NPX), 4-methylumbelliferyl-β-D-xylopyranoside (MuX), 4-methylumbelliferone (Mu), D-xylose and D-glucose were obtained from Sigma–Aldrich (St. Louis, MO). 4-Nitrophenyl-β-D-xylopyranoside (4NPX) was obtained from Gold Biotechnology (St. Louis, MO) and xylobiose (X2) was obtained from Wako Chemicals (Richmond, VA). 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–Visible spectrophotometer (Agilent; Santa Clara, CA). The extinction coefficient used for 4NP at different pH was determined as described [6]. A DX500 Dionex

Abbreviations: 2NP, 2-nitrophenol; 2NPX, 2-nitrophenyl-β-D-xylopyranoside; Mu, 4-methylumbelliferone; MuX, 4-methylumbelliferyl-β-D-xylopyranoside; 4NP, 4-nitrophenol; 4NPX, 4-nitrophenyl-β-D-xylopyranoside; GH, glycoside hydrolase family; X2, xylobiose.

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Scheme 1. Double displacement glycoside hydrolase mechanism. In the case of GSXynB2, S is a substrate molecule comprising xylose and a leaving group linked through a glycosidic bond.

HPLC system equipped with an ED40 electrochemical detector (pulsed amperometry) was used for saccharide separation and detection (Dionex; Sunnyvale, CA). An upgraded model SX.18MV-R stopped-flow (Applied Photophysics), with a thermostated compartment for syringes and reaction chamber and 2 mm or 10 mm pathlengths for absorbance measurements, was used for rapid kinetic studies with absorbance monitoring. Rapid mixing of solutions without absorbance monitoring was accomplished with a rapid-quench flow unit (KinTek; Austin, TX).

The GSXynB2 gene, including a 6X-Histag on the C-terminus, was synthesized and its protein purified as described [4]. The protein concentration was determined using the calculated 280 nm extinction coefficient (135,460 M^{-1} cm^{-1}). Enzyme was flash frozen in liquid nitrogen in small aliquots and stored at −80 °C until used.

2.2. Steady-state kinetic parameters of GSXynB2 acting on 2NPX and MuX

Initial rates for determination of kinetic parameters for GSXynB2 acting on MuX were collected by adding enzyme to 1-mL reactions containing substrate equilibrated at 25 °C and continuously monitoring the 360 nm absorbance change over time. The buffer at 0.3 M ionic strength (adjusted with NaCl) was 100 mM sodium phosphate pH 7.0. The extinction coefficient at 360 nm pH 7.0 used was 2.97 mM^{-1} cm^{-1}.

Initial rates for determination of kinetic parameters for GSXynB2 acting on 2NPX were conducted by adding enzyme to 1-mL reactions containing substrate equilibrated at 25 °C and continuously monitoring the 400 nm absorbance change over time. pH 4.0 and 4.5 enzyme slowly precipitated causing light scattering and interfered with measuring rates by the continuous method so pH 4 and pH 4.5 reactions were run using a discontinuous method by quenching aliquots of 2NPX reaction mixtures in 100 mM sodium carbonate pH 11.5 at various time points and reading the absorbance at 420 nm. The high pH of the quench completely solubilized the precipitated protein and eliminated light scattering. Buffers of 0.3 M ionic strength (adjusted with NaCl) were used: 100 mM sodium citrate (pH 3.5), 100 mM succinate (pH 4–6), 100 mM sodium phosphate (pH 7) and 30 mM sodium pyrophosphate (pH 8–9). The following 400 nm delta extinction coefficients (product − substrate) were determined from newly purchased and freshly opened bottles of 2NPX and 2NP (mM^{-1} cm^{-1}): 0.764 (pH 3.5), 0.785 (pH 5), 1.012 (pH 6), 2.366 (pH 7), 3.727 (pH 8) and 4.049 (pH 9). The 420 nm delta extinction coefficient used for pH 11.5 was 4.5 mM^{-1} cm^{-1}. Substrate absorbance subtracted about 1% from the delta extinction coefficients. A similar delta extinction coefficient has been reported for pH 6.0 (1.07) [7].

2.3. Stopped-flow at pH 7.0 and 25 °C

The following setup was used for stopped-flow experiments for GSXynB2 acting on 2NPX. One syringe contained GSXynB2 (59.6 μM) and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M and the other syringe contained 2NPX (6.91 mM) and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M. After mixing equal volumes, the absorbance was monitored at 420 nm. The background absorbance was measured (6 repeats) by replacing the enzyme in the syringe with buffer; then the average background absorbance was subtracted (0.0043) from each run along with the absorbance due to enzyme (0.026). All 9 runs were fit to Eq. (3), where the amplitude and y-intercept are set equal. The 420 nm extinction coefficient for 2NP used was 2.27 mM^{-1} cm^{-1}.

For GSXynB2 acting on 4NPX at pH 7.0 and 25 °C, one syringe contained 60 μM GSXynB2 and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M and the other syringe contained 7.61 mM 4NPX and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M. Equal volumes were mixed and absorbance was monitored at 400 nm. The background absorbance due to substrate (0.0835) was determined by pushing 7.61 mM 4NPX into cell, dividing by 2 for reaction dilution and then subtracting it from each run, along with the absorbance due to enzyme (0.03). All 4 runs were fit to Eq. (3), where the amplitude and y-intercept are set equal. The 400 nm extinction coefficient for 4NP used was 9.41 mM^{-1} cm^{-1}.

For GSXynB2 acting on MuX at pH 7.0 and 25 °C, one syringe contained 134 μM GSXynB2 and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M and the other syringe contained 2.62 mM MuX and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M. Equal volumes were mixed and absorbance was monitored at 360 nm. The background absorbance was measured (4 repeats) by replacing the enzyme in the syringe with water; then the average background absorbance (0.0306) was subtracted from each run along with the absorbance due to enzyme (0.085). All 5 runs were fit to Eq. (3), where the amplitude and y-intercept are set equal. The 360 nm extinction coefficient for Mu used was 2.97 mM^{-1} cm^{-1}.

2.4. Rapid quench-flow at pH 7.0 and 25 °C

Rapid quench flow reactions for GSXynB2 acting on X2 used the following setup. One syringe (16 μL) contained 100 μM GSXynB2 and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M and the other syringe (16 μL) contained 40.0 mM X2 and 100 mM sodium phosphate pH 7.0 ionic strength 0.3 M. Equal volumes of the syringes were combined and allowed to age for 2–300 ms before quenching with 100 mM sodium phosphate pH 11.3. Time zero reactions (3 repeats) contained water instead of substrate in one syringe; after the enzyme had been quenched, substrate was added. The amount of xylose present in each sample was determined using high-performance anion-exchange chromatography with pulsed amperometric detection. The data was corrected for the time 0 background and fit to Eq. (3), which has the amplitude and the y-intercept set equal.

2.5. Equations

Kinetic equations were fit using GraFit [8]. Data were fit to the following equations where p is the determined parameter at a single pH; P is the pH-independent value of the parameter; H^+ is the proton concentration; K_{a1} is the proton concentration where p is half of P for the first group(s) affecting P; K_{a2} is the proton concentration where p is half of P for the second group(s) affecting P; K_{a3} is the proton concentration where p is half of P for the third group(s) affecting P; *Abs* is the observed absorbance; *amp* is the amplitude/intercept; k is the first-order rate constant; t is the time; v_s is the steady-state rate; K_s is the dissociation constant of S from ES (Scheme 1); K_m is the Michaelis constant; k_2 is the rate of glycosylation (Scheme 1); k_3 is the rate of deglycosylation (Scheme 1); F_{bound} is the fraction of enzyme occupied by substrate; S is the substrate concentration. Note that Eq. (4) assumes $k_{-1} >> k_2$, where k_{-1}/k_1 equals K_s (Scheme 1).