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Mechanistic Study of β-Xylosidase from *Trichoderma koningii* G-39[1]

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The catalytic mechanism of the β-xylosidase purified from the culture filtrate of *Trichoderma koningii* G-39 was investigated. By NMR spectroscopy, the stereochemistry of the enzyme catalyzing the hydrolysis of 2,4-dinitrophenyl and *p*-nitrophenyl-β-D-xylosides was found unequivocally to involve retention of the anomeric configuration. Based on the k_{cat} values of a series of arylxylosides with leaving group pK_as in the range of 4–10, an extended Bronsted plot was constructed with a slope (β_{lg}) near zero. Enzymatic hydrolysis of aryl-β-D-xylosides in acetate buffer (pH 4.0) containing 3 or 5% methanol showed a constant product ratio (methylxyloside/xylose), indicating the presence of a common intermediate, probably the xylosyl-enzyme intermediate. In the presence of DTT, the k_{cat} values of *p*-cyanophenyl-β-D-xylopyranoside and *p*-nitrophenyl-β-D-xylopyranoside increased greatly. A two-step mechanism involving the formation and breakdown of the xylosyl-enzyme intermediate was therefore proposed. The rate-limiting step is the breakdown of the intermediate. The secondary deuterium kinetic isotope effect (k_H/k_D) measured for 2,4-dinitrophenyl-β-D-xyloside was 1.02±0.01, suggesting that the transition state for breakdown of the xylosyl-enzyme intermediate is S_N2-like.

Key words: Bronsted plot, mechanism, secondary deuterium isotope effect, β-xylosidase.

β-Xylosidase [EC 3.2.1.37] is an exoglycosidase that degrades short xylooligosaccharides to liberate xylose. It is believed to relieve the end-product inhibition of xylanase. Many β-xylosidases have been found in fungi (*1–5*) and bacteria (*6–8*). Most studies of these enzymes have focused on their properties and functions in hydrolysis of xylooligosaccharides, with a view to their application in industry. Mechanistic studies have been limited by substrate availability. Two types of mechanism resulting in overall retention or inversion of the anomeric configuration at the C1-position on substrates were identified for β-xylosidase. Based on amino acid sequence similarities, β-xylosidases can be grouped into families 39 and 43 of the general classification in glycosyl hydrolases (*9*). Armand *et al.* suggested that β-xylosidases in family 43 operated by a mechanism involving the inversion of the anomeric configuration, whereas those in family 39 were "retaining" enzymes (*10*). Perhaps, the β-xylosidase from *Bacillus pumilus*, classified as a member of family 43, was the one studied much more extensively (*11*). This enzyme hydrolyzes xylooligosaccharides, most effectively xylobiose (*12*), and aryl xylosides but not alkyl xylosides (*13*). It is an "inverting" enzyme, catalyzing the reaction with the inversion of anomeric configuration. For aryl xyloside hydrolysis, the extended Bronsted constant (β_{lg}) was between –0.2 and –0.3 (*14*), and the secondary deteuterium isotope effect was in the range of 1.03–1.09 (*15*), indicating the presence of an oxocarbonium ion character in the transition state. The β-xylosidases from *Clostridium cellulolyticum* (*16*) and *Butyrivibrio fibrisolvens* (*17*) were also identified as "inverting" enzymes, whereas that from *Thermoanaerobacterium saccharolyticum* is a retaining enzyme (*10*). Here, we report an investigation into the catalytic mechanism of β-xylosidase from *Trichoderma koningii* G-39 by kinetic study. Since the amino acid sequence of the purified enzyme is not known, it is not possible to group the enzyme into the appropriate family. However, Gebler *et al.* have suggested that the catalytic mechanism of glucanases and xylanases is conserved within the same family (*18*). This study, therefore, not only demonstrates the catalytic features of a specific enzyme, it also provides valuable data for verification of general viewpoints in glycosyl hydrolases of future.

MATERIALS AND METHODS

Buffers were purchased from either Sigma or E. Merck. The buffer system usually consisted of 100 mM NaCl and 50 mM buffer: NaOAc (pH 3.5–5.6); MES (pH 5.6–7.0); MOPS (pH 6.5–7.9); phosphate (pH 1.0–3.0 and 7.0–7.9); HEPES (pH 6.8–8.2); BICINE (pH 7.6–9.0).

The enzyme was induced and purified from the culture filtrate of *Trichoderma koningii* G-39 as described below. The basal medium used for growth and enzyme induction was minimum medium (MM) containing 1% xylan and 0.1% xylose. Spores for inoculation were obtained by culturing at 28°C in Petri dishes, each containing 15 ml of MM with 3.9% PDA. After one week of incubation, the spores

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Abbreviations: 2,4-DNPX, 2,4-dinitrophenyl-β-D-xylopyranoside; 2,5-DNPX, 2,5-dinitrophenyl-β-D-xylopyranoside; 3,4-DNPX, 3,4-dinitrophenyl-β-D-xylopyranoside; CNPX, 4-Chloro-2-nitrophenyl-β-D-xylopyranoside; PNPX, *p*-nitrophenyl-β-D-xylopyranoside; ONPX, *o*-nitrophenyl-β-D-xylopyranoside; MNPX, *m*-nitrophenyl-β-D-xylopyranoside; PCPX, *p*-cyanophenyl-β-D-xylopyranoside; PX, phenyl-β-D-xylopyranoside.

from four PDA plates were suspended in the basal MM with 2% glucose and inoculated into a 1-liter culture flask containing 800 ml of medium. The flask was shaken at 180 rpm for 24 h at 28°C. The seed culture was then filtered. The mycelia were washed twice with sterile water. Enzymes were then induced by incubating the clean mycelia in 400 ml of MM containing 1% xylan and 0.1% xylose for 24 h at 28°C. After fermentation, the culture was centrifuged to obtain a yellowish supernatant. Crude protein was obtained by slowly adding ice-cold enthanol (200 ml) to the supernatant (200 ml). After resuspension (in 25 ml NaOAc buffer, 100 mM, pH 4.5) and desalting of the crude protein solution, the low-salt sample solution was ready for chromatographic separation. A 15-ml sample solution was applied to a HiTrap Q column (Pharmacia, 5 ml) equilibrated with 20 mM NaOAc buffer, pH 4.5. Elution was performed with a 2.5 mM/min linear gradient of NaCl (from 50 to 150 mM) in the same buffer at a flow rate of 2.5 ml/min. Fractions of 2.5 ml were collected, and elution was monitored at 280 nm. Active fractions were pooled, concentrated, and used for most of the kinetic studies.

Substrate Syntheses—Except for PNPX and ONPX (obtained from Sigma), the substrates used in this study were synthesized from β-D-xylose tetraacetate *via* 2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl bromide (*19*), 2,3,4-tri-*O*-acetyl-aryl-β-D-xylopyranoside, and deacetylation to form product (*20*). DNPX was prepared in one step by Sharma's method (*21*). The C1-deuterated 2,4-DNPX was synthesized by reducing δ-xylonolactone to deuterated xylose with sodium amalgam (*15*) and followed by Sharma's method. Each product was purified by silica gel chromatography and obtained in crystalline form. All products were identified by NMR spectroscopy and characterized by melting point. Melting points determined for substrates are as follows: 2,5-DNPX, 162–163°C; 3,4-DNPX, 156–157°C; CNPX, 142–143°C; MNPX, 156–158°C; PCPX, 194–196°C. The ^{1}H-NMR spectrum of C1-deuterated 2,4-DNPX is identical to that of protiated 2,4-DNPX except for the absence of the anomeric proton and related coupling. Contamination of the deuterated substrate with protiated compound was estimated to be within 5% by integration of the anomeric proton region.

Protein Determination—The protein content of enzyme preparations was determined either by the bicinchoninic acid (BCA) method as described in the manufacturer's protocol (Sigma, BCA-1, Kit for protein determination).

Enzyme Assays—β-Xylosidase activity was assayed with 2,4-dinitrophenyl-β-D-xylopyranoside as substrate by determining the amount of 2,4-dinitrophenol released. In all assays, an enzyme unit was defined as the amount of enzyme that released 1 μmol of 2,4-dinitrophenol from substrate in 1 min. In the activity assay, a suitable amount of purified protein was added in 0.5 ml of acetate buffer (50 mM, pH 4.2) containing 1 mM 2,4-dinitrophenyl-β-D-xylopyranoside. The absorbance of 2,4-dinitrophenolate was measured at 400 nm. The extinction coefficient of 2,4-nitrophenolate/2,4-nitrophenol was determined to be 6,100 M^{-1}•cm^{-1} at pH 4.2. Kinetic studies were performed by monitoring the production of phenols on a Hewlett Packard Model 8452A Diode Array Spectrophotometer with a circulating water bath set at 25°C.

Kinetics—The Michaelis constant of each synthetic substrate was determined at pH 6.8 and analyzed with the Michaelis-Menten equation fitted by least squares non-linear regression analysis (*22*). Rates were determined at 7–10 different substrate concentrations ranging from approximately 0.15 times the K_m value ultimately determined to 6–8 times its value. The wavelengths employed and extinction coefficient differences ($\Delta\varepsilon$, M^{-1}•cm^{-1}, at pH 6.8) obtained at that wavelength for each xyloside were adapted from Withers' previous work (*23*), as follows: 2,4-DNPX, 400 nm, 10,910; 2,5-DNPX, 440 nm, 4,288; 3,4-DNPX, 400 nm, 11,009; CNPX, 425 nm, 3,546; PNPX, 400 nm, 7,280; ONPX, 400 nm, 2,170; MNPX, 380 nm, 385; PCPX, 270 nm, 3,101; PX, 277 nm, 778.

NMR Experiments—^{1}H-NMR spectra in D_2O solution were obtained at 300 MHz on a Varian UNITY-300FT spectrometer. The enzyme for NMR study was prepared by exchanging the buffer system for deuterioacetate (100 mM, pD = 4.5) by ultrafiltration. A 5 mM solution of DNPX was prepared in deuterioacetate buffer (100 mM, pD = 4.5). After recording the spectrum of the substrate (500 μl in a 5-mm NMR tube), 50 μl of deuterioacetate-exchanged enzyme was added. The spectra were recorded subsequently.

Product Partition—PNPX, ONPX, and CNPX were enzymatically hydrolyzed in acetate buffer (50 mM, pH 4.0) containing 3% and 5% methanol. All products and reactants could be resolved by Nucleosil 10 NH2 column (4.6 × 250 mm) with 78% acetonitrile as eluent. A Waters 626 HPLC system integrated with a PL-ELS 1000 detector (Polymer Lab, UK) was employed. For quantitative analysis, glucose was used as internal standard.

RESULTS AND DISCUSSION

Substrate Specificity—The purified β-xylosidase was characterized as a monomeric glycoprotein with an estimated M_r value of 104 kDa and pI 4.6. It possessed significant α-L-arabinosidase activity (13% of that of β-xylosidase), but no other glycosidase activity, such as β-glucosidase, β-galactosidase, or β-*N*-acetylglucosaminidase, was detected. More properties of this enzyme will be published elsewhere. For better understanding of substrate specificity and the structure-activity relationship, a series of xylosides, including 2,4-DNPX, 2,5-DNPX, 3,4-DNPX, CNPX, PNPX, ONPX, MNPX, PX, and PCPX, was synthesized and tested as substrates. Kinetic parameters for these substrates are summarized in Table I. The purified enzyme showed a broad tolerance for the aglycone portion of xylosides. It accommodated various arylxylosides. The K_m values were in the range 0.02–0.08 mM, with the exception of pheny-β-D-xyloside (K_m = 0.73 mM). The k_{cat} values for all tested substrates were similar.

Extented Bronsted Plot—The extended Bronsted plot has

TABLE I. **K_m and k_{cat} values for a series of aryl-β-D-xylopyranosides.**[a]

Phenols on substrates	pK_a	K_m (mM)	k_{cat} (s^{-1})	log k_{cat}
2,4-dinitro	3.96	0.024	3.89	0.59
2,5-dinitro	5.15	0.067	4.72	0.67
3,4-dinitro	5.36	0.073	4.80	0.68
4-chloro-2-nitro	6.45	0.02	3.9	0.59
4-nitro	7.18	0.042	3.04	0.48
2-nitro	7.22	0.028	3.48	0.54
3-nitro	8.39	0.065	5.22	0.72
4-cyano	8.49	0.08	3.28	0.52
H	9.99	0.73	5.13	0.71

[a]All measurements are within 5% standard deviation.

been shown to be a valuable tool in investigating the action mechanisms of enzymes (*24–27*). Based on the k_{cat} values of arylxylosides (in Table I), an extended Bronsted plot was constructed by plotting the logarithmic form of the k_{cat} values against the pK_as of the leaving phenols (Fig. 1). The slope of the plot (β_{lg} value) was nearly zero. Clearly, the reaction rate–limiting step of the hydrolysis catalyzed by this enzyme is independent of the leaving aryl group (pK_a 4–10) on the substrate. A similar result can be derived from the data of *T. viride* β-xylosidase for substrates with a more basic leaving group (pK_a 7–11) (*5*). This is different from the case of β-glucosidases from sweet almond (*26*), *A. faecalis* (*23*), and *F. meningosepticum* (*25*), which exhibited a biphasic plot with concave downward shape (see Fig. 1, inset). The slope of the plot (*i.e.*, the Bronsted constant, β) is about zero for good substrates (with the pK_as of leaving phenols < 7) and –0.7 – –1.0 for poor substrates (with the pK_as of leaving phenols > 7). A two-step mechanism involving the formation and breakdown of a glucosyl-enzyme intermediate, as shown in Scheme 1, could readily explain these results. One step depends strongly on the substituent group (β_{lg} = –0.7 – –1.0) and is the rate-limiting step for substrates with the more basic leaving groups. As the acidity of the leaving phenol increases, the rate-limiting step changes so that the whole process becomes independent of subsitutent effects (β_{lg} = ~0). For such a mechanism, k_{cat} can be derived as the term $k_2k_3/(k_2 + k_3)$. For good substrates, $k_2 \gg k_3$, and therefore, $k_{cat} = k_3$ and is independent of the leaving group. Thus, the rate-limiting step of the reaction is the breakdown of the intermediate. For poor substrates, $k_3 \gg k_2$, and therefore, $k_{cat} = k_2$ and the formation of glucosyl-enzyme intermediate is the slow step. In the case of our enzyme, however, even though the Bronsted plot exhibited only a single phase with β_{lg} = ~0, it will still be likely that this enzyme catalyzes the reaction by a two-step mechanism if an intermediate can be directly or indirectly shown to exist. Besides, the breakdown of the intermediate is the slowest step, *i.e.*, $k_2 \gg k_3$, for all tested substrates.

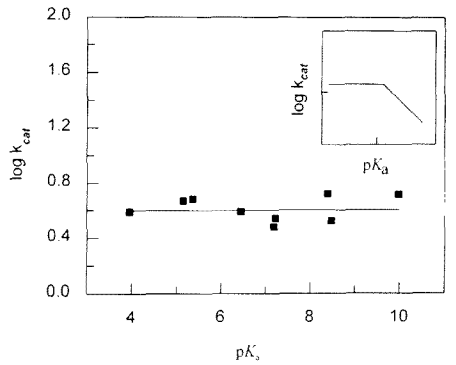
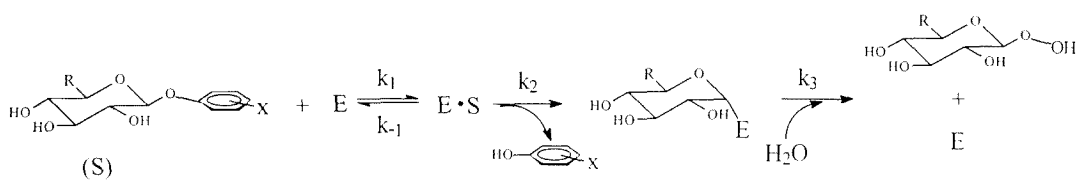


Fig. 1. **A Bronsted plot of β-xylosidase from *Trichoderma koningii* G-39.** The figure was constructed by plotting the logarithmic form of the k_{cat} values against the pK_as of the leaving phenols. The line (drawn arbitrarily) indicates that the Bronsted constant (β_{lg}) is nearly zero. The inset shows a regular Bronsted plot seen in several glycosidases.

^1H Nuclear Magnetic Resonance (^1H-NMR) Analysis of the Stereoselectivity of the Enzymatic Hydrolysis—^{1}H-NMR spectroscopy has been used to investigate the stereoselectivity of various glycohydrolases such as cellulases and xylanase. Studies revealed that glycohydrolases often act *via* a double-displacement mechanism, which involves the retention of the anomeric configuration. However, as mentioned previously, β-xylosidases purified from *C. cellulolyticum*, *B. pumilus*, and *B. fibrisolvens* were shown to act by inverting the β-anomeric configuration. To better understand the catalytic action of the purified *T. koningii* β-xylosidase, an NMR study of it stereoselectivity was carried out. The results are shown in Fig. 2. In a xylose ^{1}H-NMR study, two doublets were found centered at δ = 5.06 ppm (J = 3.3 Hz) and δ = 4.45 ppm (J = 7.8 Hz), corresponding to the α and the β anomeric protons, respectively. The ratio of α/β was 35/65 at equilibrium. The C1 proton of DNPX was shown to be a doublet centered at 5.27 ppm (J = 7.2 Hz). When enzyme was added to DNPX, the β anomeric signal increased. At 12 min, a new doublet centered at δ = 5.06 ppm (J = 3.3 Hz) emerged as a result of mutarotation (data not shown). The ratio of α-xylose to β-xylose was 10:90. At 22 min, the α and β anomeric signals constantly increased to 16:84. The anomeric proton of DNPX was extinguished completely in 32 min and the ratio of α to β was 25:75. This NMR study clearly showed that *T. koningii* β-xylosidase catalyzes the hydrolysis of DNPX with retention of the anomeric configuration. This result strongly indicated that a reaction intermediate is present and a double-displacement mechanism is involved.

Product Partitioning—It is always difficult to prove the formation of covalent enzyme intermediate, due to its short lifetime. However, alternative methods can be applied to search for indirect evidence. A constant product partition has often been used as an indication of the formation of a common intermediate in an enzymatic reaction. In this study, PNPX, ONPX, and CNPX were enzymatically hydrolyzed in acetate buffer (pH 4.0) containing 3 or 5% methanol. Regardless of the substrate, the product ratio (xylose/



R: H or CH_2OH

Scheme 1.

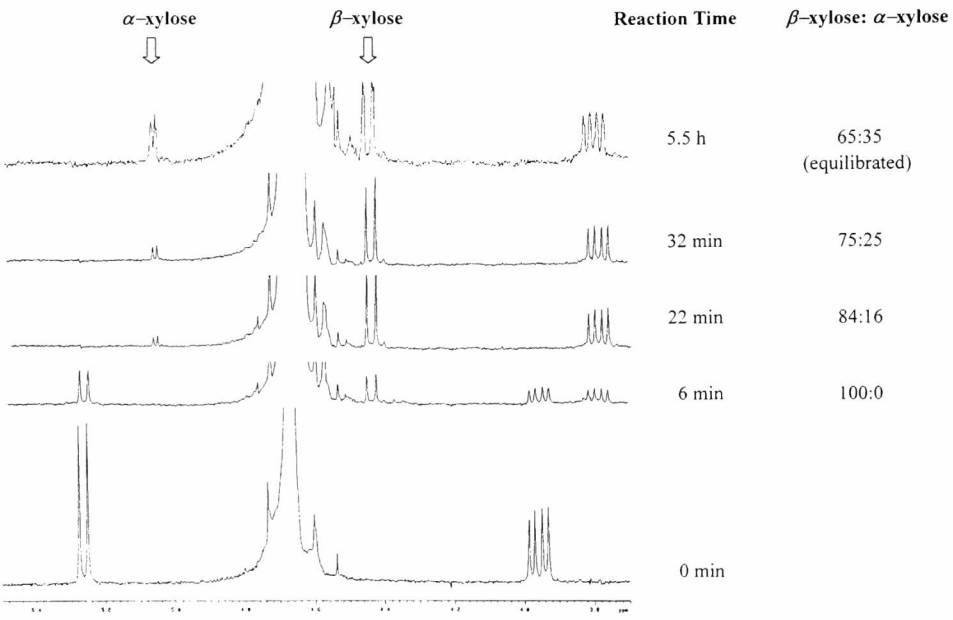


Fig. 2. **^{1}H NMR analysis of the stereoselectivity of the reaction catalyzed by β-xylosidase.** A suitable amount of enzyme was applied on 5 mM DNPX at pD = 4.5. The reaction was monitored about every 8 min after addition of the enzyme.

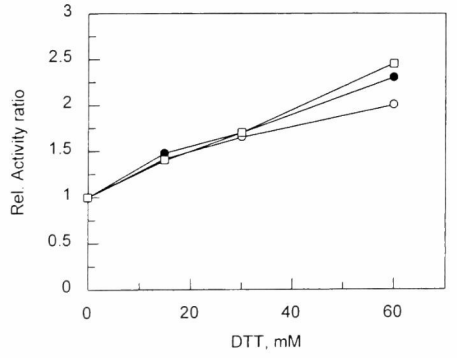
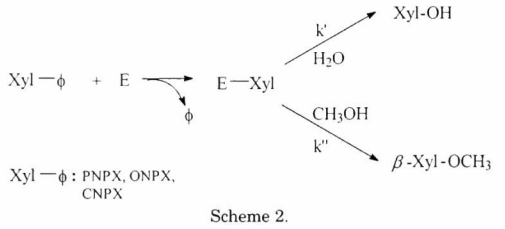


Fig. 3. **Relative activity of β-xylosidase for DNPX (–○–), CNPX (–□–), and PNPX (–●–) at different DTT concentrations.** For each substrate, the activity in the absence of DTT served as control.

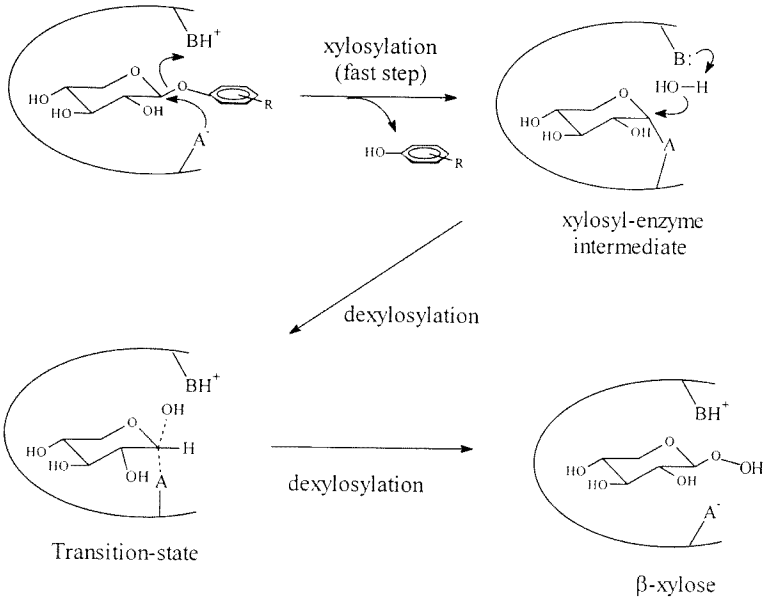


Scheme 2.

methyl-β-D-xyloside), analyzed by HPLC, was found to be nearly constant, 1.55 ± 0.05 (1.50 for PNPX, 1.58 for ONPX, and 1.56 for CNPX) in 3% methanol, and 1.15 ± 0.05 (1.12 for PNPX, 1.14 for ONPX, and 1.20 for CNPX) in 5% methanol. This finding suggests that a common intermediate, probably xylosyl-enzyme intermediate, is present in the reaction pathway.

Nucleophilic Competition—The k_{cat} values of DNPX, CNPX, and PNPX were measured at three different concentrations of DTT (up to 60 mM). For these substrates, the k_{cat} values increased almost linearly with increasing DTT concentration. The ascending slopes for substrates are approximately equal as shown in Fig. 3. Large increases in k_{cat} values can be attributed to the promotion of nucleophilic dexylosylation of xylosyl-enzyme intermediate by DTT. This rate enhancement can be illustrated simply by replacing methanol with DTT and their correspondent products in Scheme 2. In the presence of DTT, the rate constant of the reaction (k) is the sum of k' (H_2O) + k'' (DTT). Therefore, if the dexylosylation step is the slow step, the rate constant will be proportional to the DTT concentration (or at least show a positive trend). The experimental results supported the conclusion that a two-step mechanism is involved in the reaction and the rate-limiting step is the breakdown of xylosyl-enzyme intermediate.

Inhibition—Inhibitory studies are useful tools in elucidating the mechanistic action of an enzyme. δ-Gluconolactone has been shown to be a very potent inhibitor of β-glucosidase. This compound was considered as a possible transition-state analog due to its sp^2 hybridization at the C1 carbon. The catalytic mechanisms of β-xylosidase and β-



Scheme 3. **Proposed two-step mechanism of β-xylosidase.**

glucosidase are thought to be closely related. If the transition state of β-xylosidase catalysis is a carbocation or its resonance form, an oxocarbonium ion, it would be reasonable to expect strong inhibition of β-xylosidase by δ-xylonolactone. δ-Xylonolactone was shown to inhibit the purified enzyme competitively with a moderate K_i value of 3.5 ± 0.1 mM. This value is much greater than that of δ-gluconolactone on β-glucosidase (normally in the range of 1–10 μM) (*25*) but comparable to that of xylose on the purified β-xylosidase (K_i = 5.0 ± 0.1 mM). Thus, the transition state of the β-xylosidase–catalyzed reaction is inconsistent with a carbocation structure. This suggestion was confirmed by study of the α-deuterium secondary kinetic isotope effect.

Secondary Kinetic Isotope Effect—The kinetic α-deuterium isotope effect is considered to be a useful tool for distinguishing between S_N1 and S_N2 mechanisms. It provides a way to investigate changes in hybridization at the substituted site in proceeding from the ground state to the transition state of the reaction. Isotope effects of $k_H/k_D > 1$ were expected for the S_N1 mechanism, while effects near unity were thought to indicate an S_N2 reaction. Secondary deuterium kinetic isotope effects (k_H/k_D) upon the deglycosylation of various glycosidases, such as β-galactosidase and β-glucosidase, have been shown to range from 1.25 to 1.09 (*15*, *28*, *29*). For these enzymes, the transition states of the reactions were believed to possess substantial carbocation (or alternatively oxocarbonium) ion character, corresponding to late transition-state characteristics. Interestingly, the secondary deuterium kinetic isotope effect of the purified β-xylosidase for 2,4-dinitrophenyl-β-D-xylopyranoside was found to be 1.02 ± 0.01 (average of triplicate data, 1.02, 1.02, and 1.01). This indicates a relatively small amount of carbocation character at the transition state. This strongly suggested that the transition state of the rate-limiting step (breakdown of the xylosyl-enzyme intermediate) is S_N2-like. Due to the availability of C1-deuterated substrates, the k_H/k_D value for other xylosides is not available. However, a reasonable estimation can be made since the rate-limiting step for all tested substrates was independent of the leaving phenols.

Proposed Reaction Mechanism—NMR study unequivocally demonstrated that the enzyme catalyzed hydrolysis with the retention of the anomeric configuration of the substrate. Thus, a double-displacement mechanism is involved. The mechanism involves the initial binding of β-xylosides to the enzyme, followed by a general acid–catalyzed the nucleophilc attack of the essential residue on the anomeric carbon to form xylosyl-enzyme intermediate. This intermediate is then hydrolyzed by general-base catalysis to restore the β-form configuration of xylose. The proposed mechanism is illustrated in Scheme 3. The product partitioning and nucleophilic competition experiment strongly supported the existence of a xylosyl-enzyme intermediate. The extended Bronsted plot with β_{lg}~0 suggests that the rate-limiting step of enzymatic hydrolysis of aryl-β-xylosides was the breakdown of xylosyl-enzyme intermediate for all tested substrates. Secondary deuterium kinetic isotope effect (k_H/k_D) was 1.02 ± 0.01. The transition state for breakdown of the xylosyl-enzyme intermediate is therefore suggested to follow a S_N2-like mechanism. This mechanism is distinguished from that of other glycosidases such as β-glucosidase and β-galactosidase. The weak inhibition of δ-xylonolactone also supported a transition-state structure with very little sp^2 character.

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