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β-D-XYLOSIDASE FROM *Bacillus pumilus* PRL B12: HYDROLYSIS OF ARYL β-D-XYLOPYRANOSIDES

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ABSTRACT

The influence of substituents on the binding and hydrolysis of several substituted β-D-xylopyranosides by β-D-xylosidase from *Bacillus pumilus* PRL B12 has been investigated. From a comparison of the inhibition constants of 1-thio-β-D-xylopyranosides with the apparent Michaelis–Menten constants of the substrates, it followed that the latter constants are good approximations of the true equilibrium constants. The influence of the substituent on the rate and activation parameters is small. The results are in agreement with, but do not prove, a one-step mechanism without the formation of a glycosyl–enzyme intermediate.

INTRODUCTION

β-D-Xylosidases commonly occur in fungi[1–3], *Bacillus* species[4,5], yeasts[6], and other organisms. However, little is known about their mechanism of action. We have reported[5] on the purification and some properties of an induced β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) from *Bacillus pumilus 12*. The enzyme displays an absolute glycon specificity: only β-D-xylopyranosides are hydrolyzed and the aglycon group has to be either another xylose residue or an aryl group. In contrast to many other glycosidases, the β-D-xylosidase does not transfer the xylose residue to alcohols, and, as the reaction product with water is α-D-xylose[7], it operates with inversion of configuration. Since no use could be made of the nucleophilic competition method, it is not known whether the reaction occurs through the two-step mechanism found with many other glycosidases[8–11] (formation of an intermediate enzyme–glycosyl complex) or through a one-step mechanism (without the formation of this intermediate). Although the absence of a transfer reaction and the inversion of configuration both suggest a one-step mechanism, these findings alone are insufficient to prove the mechanism. We have now sought further evidence by investigating the effect of substituent groups on the binding and enzymic hydrolysis of substituted phenyl β-D-xylopyranosides.

RESULTS AND DISCUSSION

Influence of the substituent on the reaction rate

Initial experiments were aimed at determining the effect of substituent groups on the maximal rate of the β-D-xylosidase-catalyzed hydrolysis of substituted phenyl β-D-xylopyranosides. At 25° and pH 7.17, the initial velocities (rate of phenol release) for the enzymic hydrolysis of seventeen aryl β-D-xylopyranosides were measured at 10–15 concentrations of substrate. Each substrate (except the *p*- and *m*-nitro derivatives, which showed substrate inhibition) followed formal Michaelis–Menten kinetics. The calculated values of the maximal rate per unit of enzyme activity (V) and of the apparent association constant ($K_a = 1/K_m$) are summarized in Table I.

TABLE I

RATE AND ACTIVATION PARAMETERS FOR SUBSTITUTED PHENYL β-D-XYLOPYRANOSIDES (pH 7.17)

No.	*Substituent*	10^{10} V[a] ($mol.sec^{-1}.u^{-1}$)		K_a[b] (M^{-1})	$\Delta H^{\ddagger}$ ($kJ.mol^{-1}$)	$\Delta S^{\ddagger}$ (25°) ($J.K^{-1}.mol^{-1}$)	k_2 (25°) (sec^{-1})
		25°	15°				
1	*p*-Iodo	138	72	645	43.1 ±1.8	−89 ±6	3.92
2	*p*-Bromo	67	36	680	42.2 ±3.1	−98 ±10	1.90
3	*p*-Chloro	42	21	833	42.2 ±1.5	−101 ±5	1.19
4	*p*-Fluoro	12	6	800	42.2 ±2.2	−112 ±7	0.34
5	*p*-Methyl	37	22	345	42.4 ±2.1	−102 ±7	1.05
6	*p*-Ethyl	65	35	469	41.4 ±2.2	−97 ±7	1.85
7	*p*-Propyl	46	26	725	41.4 ±1.7	−102 ±6	1.31
8	*p*-Methoxy	20	10	526	53.9 ±1.9	−69 ±6	0.57
9	*p*-Ethoxy	29	14	455	48.5 ±1.6	−84 ±5	0.82
10	*p*-Butoxy	33	16	543	47.7 ±1.8	−85 ±6	0.94
11	*p*-Acetyl	180	91	422	41.8 ±1.4	−90 ±5	5.11
12	*p*-Cyano	197	104	392	39.7 ±1.5	−97 ±5	5.59
13	None	24	12	521	46.8 ±1.0	−91 ±3	0.68
14	*p*-Nitro	268	125	667	51.0 ±1.3	−57 ±4	7.61
15	*o*-Nitro	467	234	1429	42.6 ±1.0	−79 ±3	13.3
16	*m*-Nitro	46	18	1670	48.1 ±1.0	−79 ±3	1.30
17	2,4-Dinitro	1868	470	568	73.2 ±3.0	+33 ±10	53.1

[a]Estimated standard error: ~2%. [b]Estimated standard error: $\lesssim$ 7%.

As V depends on the substituent, at least one of the rate-determining steps must be one in which the aglycon group is still present at the active site. Such a step may be the heterolysis of the glycosidic bond or the desorption of the aglycon group. Therefore, we tried to correlate log V with electronic[12] (σ_H) or hydrophobic[13] (π) substituent parameters. Whereas a regression of log V on π is non-significant ($r = 0.3$), a significant regression of log V on σ_H could be calculated when the point for the *m*-nitro derivative was omitted. Calculation yields the equation:

$$\log 10^{10} V = 1.64 + 0.84\sigma_H,$$

with standard error of the estimate $s_{y.x} = 0.23$, standard error on the slope $s_b = 0.1$, correlation coefficient $r = 0.92$, number of points $n = 16$, and level of significance $\geqslant 98\%$.

For the simple halogen-substituted sub-series, a significant regression can be calculated as follows,

$$\log 10^{10}\, V = 0.768 + 4.46\sigma_H, \quad \text{with} \quad s_{y.x} = 0.14,\ s_b = 0.81,\ r = 0.97, \quad \text{and} \quad n = 4.$$

These regression equations strongly indicate that electron-withdrawing groups increase the rate. However, they only indicate a trend, not an exact proportionality as in Hammett-type linear free-energy relations (LFER). The insignificant correlations for the alkyl or alkoxyl series, and the exceptional V-value of the *m*-nitro compound, *etc.*, clearly prove that electronic factors alone cannot explain the dependence of V on the substituent.

Activation parameters

V and K_a were determined at 5–8 temperatures in the range 10–30°. Up to 30°, log V was linear with 1/T; above 30°, deviations from linearity were observed. The activation parameters calculated (see Experimental) from the V values are summarized in Table I. Except for the 2,4-dinitro derivative, no large differences between the activation parameters are found.

Most substrates belong to an isoenthalpic series ($\Delta H^{\ddagger} \sim 42$ kJ.mol^{-1}), the small differences in the rate being caused by the entropy factor. Within the halogen sub-series, $\Delta S^{\ddagger}$ is proportional to the electron-withdrawing power of the substituent, whereas this proportionality is not found for the alkyl or alkoxyl series (*cf.* log V dependence).

The other substrates form a less regular series with higher enthalpy of activation but less negative entropy. In general, higher rates are caused by a more favorable entropy (*cf.* nitro and dinitro derivatives). However, there is no strict parallelism between the maximal rate and either $\Delta H^{\ddagger}$ or $\Delta S^{\ddagger}$, both quantities change in an irregular manner. When, according to the Exner[14] method, log V (25°) is plotted against log V (15°), a linear relation (with slope ~ 1) between the two quantities is demonstrated (Fig. 1). Linear regression yields the equation:

$$\log 10^{10}\, V(25°) = 0.209 + 1.06 \log 10^{10}\, V(15°), \quad \text{with} \quad s_{y.x} = 0.08,\ s_b = 0.04,\ r = 0.99, \quad \text{and} \quad n = 17.$$

Although this equation cannot be used to prove a real, Leffler[15] isokinetic relationship, because the temperature interval and the differences between the $\Delta G^{\ddagger}$ values are too small, it indicates that the basic reaction mechanism remains unchanged[14] within the series and that some partial compensation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ takes place.

For the calculation of $\Delta S^{\ddagger}$, the reaction constant k_2 is needed. Since k_2 is calculated from $k_2 = V/E_t$ (with E_t = "mol" active site per unit), the possible error on $\Delta S^{\ddagger}$ is a function of the error on E_t (ΔE_t) and equals $\pm R \ln(\Delta E_t)$. In this work, a "molar concentration" of 3.5×10^9 active sites per unit was used (see Experimental). If the actual "concentration" had been 10 times lower, the true $\Delta S^{\ddagger}$ values would

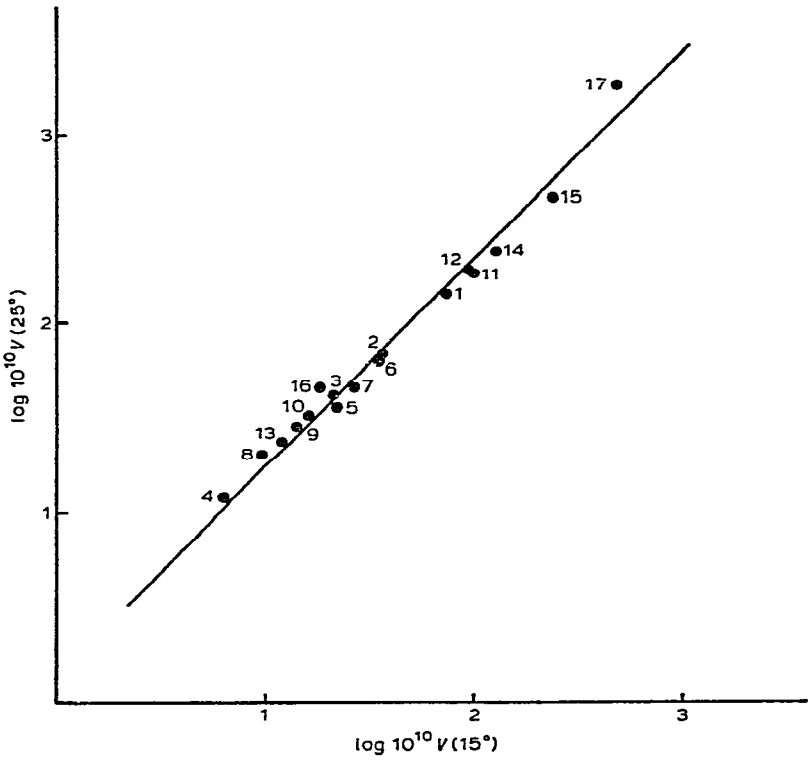

Fig. 1. Exner plot: numbers refer to Table I.

have been 19 $J.K^{-1}.mol^{-1}$ more positive. Thus, even if this large uncertainty in E_t is assumed, the data still indicate a significant decrease of the entropy (except for the 2,4-dinitro derivative). On the relative differences within the series, a constant error in E_t has no effect.

From the foregoing results, regrettably little information about the reaction mechanism can be gained. Neither the V values nor the activation parameters allow a clear-cut choice between the one- and two-step mechanisms. Consequently, all that can be said about k_2 (or V) and the activation parameters is that they refer to the energy changes occurring during the transformation of the Michaelis–Menten complex (ES) to the highest transition-state complex (ES‡) of the reaction pathway.

The change from ES to ES‡ is (*a*) accompanied by a relatively large decrease of entropy, and (*b*) must occur in such a way that electron-withdrawing groups decrease the free energy of activation.

A tentative explanation would be that the enzymic hydrolysis proceeds *via* the one-step pathway by a bimolecular nucleophilic substitution (S_N2). Attack at C-1 of the glycon moiety by a water molecule would occur simultaneously with breaking of the glycosidic bond. As the transition state contains a water molecule, a negative $\Delta S^{\ddagger}$

can be expected. This mechanism would also explain why the reaction product is α-D-xylose. Assuming further that the enzyme catalyzes the reaction by proton transfer to the exocyclic oxygen atom, the small effect of the substituent group can be understood. Electron-withdrawing groups will enhance the rate of heterolysis, but at the same time they will decrease the extent of proton transfer by lowering the electron density around the exocyclic oxygen atom. Such opposed effects are also found in the acid-catalyzed hydrolysis of β-D-xylopyranosides[16]. The less negative (or even positive) values of $\Delta S^{\ddagger}$ for compounds with strong electron-withdrawing nitro groups could be interpreted as being due to a larger extent of bond breaking in the transition complex. This process would then resemble the S_N1 reaction of 2,4-dinitrophenyl β-D-galactopyranoside[17].

Influence of the substituents on K_a

Since the apparent association constant K_a may be a complex constant, also containing rate constants ($K_a = k_1/(k_2 + k_{-1})$, the influence of substituent groups on the binding of aryl β-D-xylopyranosides was first studied by using the corresponding 1-thio-β-D-xylopyranosides and β-D-glucopyranosides, which behave as competitive inhibitors of the β-D-xylosidase reaction. The calculated inhibition constants (association; 25°; pH 7.17) and thermodynamic equilibrium parameters (for glucosides) are collected in Tables II and III.

The substituent has only a very limited influence on the binding constants. For the 1-thioxylopyranosides, it is possible to calculate a regression equation with σ_H and π, but only if the nitro derivative is omitted from the calculations:

$\Delta G°(K_i) = -3.26 - 0.59\sigma_H - 0.07\pi$, with $s_{y.x} = 0.10$, multiple correlation coefficient $R = 0.85$, and $n = 6$.

However, because the equation is not very significant (~95%), and only six points were used, it only indicates a possible trend. No linear correlation between log K_i (thioxylosides) and log K_a (xylosides) can be demonstrated and the ratio K_a/K_i is not constant (*cf.* Table II), so that there is no proof that K_a represents a true association

TABLE II

EQUILIBRIUM BINDING PARAMETERS FOR SUBSTITUTED PHENYL 1-THIO-β-D-XYLOPYRANOSIDES[a]

Substituent	K_i (M^{-1})	$-\Delta G°$ ($kJ.mol^{-1}$)	K_a/K_i
p-Bromo	410 ±30	14.90 ±0.18	1.7
p-Chloro	295 ±23	14.10 ±0.19	2.8
p-Fluoro	231 ±18	13.50 ±0.20	3.5
p-Methyl	216 ±13	13.31 ±0.15	1.6
p-Methoxy	188 ±14	13.02 ±0.18	2.8
p-Nitro	299 ±20	14.11 ±0.17	2.2
None	307 ±23	14.17 ±0.19	1.7

[a]pH 7.17; 25°.

TABLE III

EQUILIBRIUM BINDING PARAMETERS FOR SUBSTITUTED PHENYL β-D-GLUCOPYRANOSIDES[a]

Substituent	K_i (25°)[b] (M^{-1})	$-\Delta G°$ (25°)[c] ($kJ.mol^{-1}$)	$-\Delta H°$[c] ($kJ.mol^{-1}$)	$-\Delta S°$ (25°)[c] ($J.K^{-1}.mol^{-1}$)
None	12.1 ±4	6.2 ±2.6	22.3 ±1.8	54 ±6
p-Methoxy	17.0 ±6	7.0 ±3.3	20.3 ±2.3	45 ±8
p-Methyl	18.8 ±5	7.3 ±3.3	19.6 ±2.3	42 ±8
p-Acetyl	26.4 ±5	8.1 ±1.7	16.2 ±1.2	27 ±3
p-Nitro	38.2 ±4	9.0 ±1.2	15.3 ±1.0	21 ±2

[a]pH 7.17; 25°. [b]Standard error calculated from the estimated standard errors on K_a' and K_a. [c]Standard error calculated from the regression of log K_i on 1/T.

constant. However, since both constants are of the same order of magnitude and a non-constant ratio is also found when true association constants are compared (see later), we consider that K_a' is at least a good approximation of the true association constant. Exceptions are perhaps the *o*- and *m*-nitro compounds. Thus, the standard free-energy of binding would be -14.6 to -16.7 kJ.mol^{-1} for aryl β-D-xylopyranosides, and somewhat less (-13 to -15 kJ.mol^{-1}) for aryl 1-thio-β-D-xylopyranosides.

For β-D-glucopyranosides (Table III), the free energy of binding is significantly lower (-6.3 to -9.2 kJ.mol^{-1}). The substituent has a small effect on K_i; with the exception of the unsubstituted derivative, K_i seems to increase with increasing electron-withdrawing power of the substituent (σ_H). However, it is impossible to ascertain whether this is more than a coincidence.

The ratio K_i(thioxylosides)/K_i(glucosides) is not constant, but varies from 25 for the unsubstituted derivative to 8 for the *p*-nitro compound, although both constants are true equilibrium constants. Also, the ratio K_a(xylosides)/K_i(glucosides) varies from 43 (H) to 17 (*p*-nitro). The reason for the inconstancy of the ratios could probably be found (next to experimental errors on the K values) in the altered orientation of the aglycon group resulting in a modified influence of the substituent group on the binding of the aglycon group.

The thermodynamic parameters for aryl β-D-glucopyranosides, calculated from K_i values at five different temperatures (10° to 30°), are given in Table III. The near constancy of $\Delta H°$ suggests that direct and specific interactions between the substituent group and amino acid side-chains on the enzyme must be very small or absent.

Consequently, we suppose that the aglycon moiety of the substrates (or substrate analogues) is "bound" through rather unspecific, hydrophobic forces arising from the transfer of the aromatic ring from the bulk-water phase to a more hydrophobic micro-region on the enzyme. The return of some of the water molecules from the highly ordered water-structure around the aromatic ring to the less organized bulk-water is characterized, in itself, by positive ΔH and ΔS values. The negative change of enthalpy resulting from the active and specific binding of the glycon part of the molecule will be partially neutralized, so that the experimentally attainable, overall value of $\Delta H°$ will become less negative. The same will be true for the expected,

large negative change of entropy accompanying the formation of the enzyme–inhibitor complex. Because the main driving force for the "binding" of the phenyl ring stems from a common process, the partial compensation between ΔH° and ΔS°, which is clear from the data in Table III, is more easily understood.

The free energy of transfer of a phenyl ring from water to benzene[18] is $-19.4\ \mathrm{kJ.mol^{-1}}$, whereas ΔG° for the binding of phenyl 1-thio-β-D-xylopyranoside is only $-14.2\ \mathrm{kJ.mol^{-1}}$. ΔG° values for the other derivatives are of the same order of magnitude or even less. Thus, the phenyl ring is only very partially transferred out of the water phase. Consequently, there will be no LFER with π, and the influence of the substituent will be very small and probably unpredictable.

From the foregoing results, it may be concluded that the "binding" of aryl aglycon groups occurs merely as the consequence of the active binding of the glycon moiety. The binding forces are small and very unspecific, and they result from a partial restructuring of the water layer around the aromatic aglycon group. Similar conclusions were also drawn from our previous study on the binding of para-substituted phenyl glycosides to Concanavalin A[19] and of substituted phenyl β-D-galactopyranosides to β-D-galactosidase from *E. coli*[20].

EXPERIMENTAL

The substrates and inhibitors were synthesized as indicated: substituted phenyl β-D-xylopyranosides[21–23], *p*-substituted phenyl β-D-glucopyranosides[19], *p*-substituted 1-thio-β-D-xylopyranosides[24], *o*-nitrophenyl β-D-xylopyranoside[25]. 2,4-Dinitrophenyl β-D-xylopyranoside was a gift from Dr. M. L. Sinnott (Bristol). The isolation, purification, and standardization of the enzyme have been described[5,26]. Hydrolysis of nitrophenyl xylopyranosides was followed continuously at 400 (*p*- and *m*-nitrophenol), 420 (*o*-nitrophenol), or 380 nm (2,4-dinitrophenol). To prevent spontaneous hydrolysis of the 2,4-dinitrophenyl β-D-xylopyranoside, fresh solutions were prepared and used immediately. Hydrolysis of non-chromogenic phenyl β-D-xylopyranosides was followed discontinuously by measuring the liberated phenol[27,28] or D-xylose[29]. All experiments were carried out in 0.01M phosphate buffer (pH 7.15) containing mM EDTA.

For the determination of V and K_a, the classical methods[30,31] were used. For at least ten concentrations of substrate, the initial velocity (v_i) was measured, and V and K_a were estimated from Hanes[32] plots and then calculated by the method of Wilkinson[33] on a Wang 2200 table-computer. Each value of V and K_a is the arithmetical mean of at least three determinations, and is always calculated on the same enzyme-activity basis (1 unit). If necessary, the measurements were repeated until the standard error on V was $<2\%$, and that on K_a $<7\%$. The rate constant k_2 was calculated from $V = E_t k_2$, assuming a "molar concentration" of 3.5×10^{-9} active sites per unit. This figure is based on a specific activity of 4732 units per gram of protein, a dimeric structure having molecular weight[34] of 120 000, and one active site per monomer (60 000). For the calculation of the activation parameters, V was

estimated (as described above) at 5–8 temperatures between 10° and 30°. The Arrhenius activation energy was then calculated from least-squares, straight-line fits of the plot log V *versus* 1/T. The calculations of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were based on absolute-reaction rate theory[35].

The inhibition constants K_i were determined by repeating the measurement of V and K' for *p*-nitrophenyl β-D-xylopyranoside in the presence of a constant concentration of inhibitor (I). The inhibition constant K_i was then calculated from[31] $K_a = K'_a[1 + (I)K_i]$, with K'_a the value with, and K_a the one without, inhibitor. The process was then repeated with a different concentration of inhibitor. Each K_i is the arithmetical mean of three determinations. The standard enthalpy of binding ($\Delta H°$) was calculated from the K_i values at five different temperatures by the method of least-squares. $\Delta S°$ was then calculated from $\Delta G° = \Delta H° - T\Delta S°$. Because of the uncertainty in K_i, only large differences in $\Delta H°$ and $\Delta S°$ are meaningful.

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