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EFFECTS OF ALCOHOLS ON HYDROLYSIS CATALYZED BY β-D-GLUCOSIDASE FROM *STACHYBOTRYS ATRA*

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The interaction of alcohols in the hydrolysis of aryl β-D-glucopyranosides and aryl β-D-xylopyranosides by β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3 2 1 21) from *Stachybotrys atra* has been investigated The results constitute support for the presence of a glycosyl-enzyme intermediate, formed during the first step (glycosylation) of the proposed two-step mechanism Transfer of the glycosyl group to an alcohol, with the formation of an alkyl glycopyranoside, can take place in parallel to the transfer to a water molecule (second or deglycosylation step) The alcohol binds to the free enzyme and to the glycosyl-enzyme intermediate The glycosyl-enzyme-alcohol complex undergoes hydrolysis in addition to the alcoholysis For aryl β-D-glucopyranosides the deglycosylation step is rate-limiting For aryl β-D-xylopyranosides two kinds of substrate behaviour can be observed Depending on the substituent group on the phenyl ring, either both steps are rate-controlling or the first step is rate-limiting Electron-withdrawing substituents increase the rate at which the substrate aglycon group is released

Introduction

In a previous paper [1] we have reported on the hydrolysis of aryl β-D-glucopyranosides and aryl β-D-xylopyranosides by an induced β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3 2 1 21) [2] from *Stachybotrys atra*

For both glucosidase and xylosidase activities, the hydrolysis of aryl glycosides proceeds via a common glycosyl-enzyme intermediate In a first step the glycosidic bond between the aglycon group (P) and the sugar moiety (S) in the substrate (SP) is broken, P is released and the glycosyl-enzyme (ES′) is formed This intermediate can bind (i) a second substrate molecule thereby yielding a glycosyl-enzyme-substrate (ES′SP) complex or (ii) an inhibitor (e g , aryl 1-thio-glycopyranosides) thereby yielding a glycosyl-enzyme-inhibitor complex (ES′-I) Both these ternary complexes can still react with water with the formation of glucose or xylose [1]

However, a difference could be observed between glucosidase and xylosidase activity as regards the kinetics of the reaction Substantial evidence indicates that for the β-D-glucosidase activity the breakdown (deglycosylation) of the ES′ intermediate is rate-limiting, whereas for the β-D-xylosidase activity both the formation (glycosylation) and breakdown of the ES′ complex are rate-controlling

Since we observed that the glycon moiety of aryl β-D-glycopyranosides can be transferred to nucleophiles other than water (e g , alcohols and phenols) we extended our study to the kinetics of this transfer reaction Such nucleophilic competition studies under steady-state conditions can yield valuable information about the intermediate glycosyl-enzyme complex, its formation and breakdown [3,4] In this work the effect of added alcohols has been investigated

The same minimal reaction scheme as proposed in our previous study [1] will be used (Scheme I) In many respects it is very similar to the mechanisms proposed for several glycosidases and esterases β-D-galactosidase [4], β-D-mannosidase [5], bovine-liver esterase [6], pig-liver esterase [7], papain [8] and others The nucleophile N (e g , an alcohol) binds to

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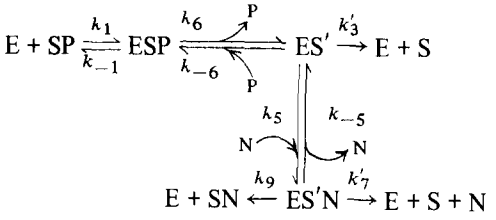
the glycosyl-enzyme (ES') with the formation of a ternary glycosyl-enzyme-nucleophile (ES'-N) intermediate The latter can react either with water (k'_7) to yield glucose (S) or xylose, the nucleophile and the free enzyme, or with the nucleophile (k_9) producing a new glycoside (SN)



Scheme I The rate equations derived from this scheme are given in the Appendix

Materials and Methods

The enzyme was isolated, purified and standardized as reported [2] Thesynthesis of the substrates used has been described *p*-nitrophenyl β-D-glucopyranoside [9], *p*-nitrophenyl β-D-xylopyranoside [10], aryl β-D-glucopyranosides [11], aryl β-D-xylopyranosides [12,13]

Qualitative analysis of transfer products was performed by TLC on silica gel G (Merck) plates using acetic acid/water/ethyl acetate (1 1 3), v/v), spray reagent, 5% sulfuric acid in ethanol (10 min at 120°C)

All enzymic assays were performed in 0 1 M phosphate buffer (pH 6 7) at 30°C All reaction rates have been calculated per unit (U) of enzyme activity The dimensions of $v_i = (v'_i/E_t)$, V_i $(=V'_i/E_t)$ and the first-order or pseudo-first-order rate coefficients are mol min^{-1} U^{-1} Since 1 unit equals 5 10^{-10} 'mol' active site [1], an estimated of the rate constants (as min^{-1}) can be calculated by dividing the given figures (mol min^{-1} U^{-1}) bu 5 10^{-10} Real and apparent binding constants are expressed as association constants (M^{-1})

Further experimental conditions, methods and data analysis were as described in our previous paper [1]

Results and Discussion

Transfer from aryl β-D-glucopyranosides to n-propanol

Qualitative analysis To a solution of *p*-nitrophenyl β-D-glucopyranoside (5 mM) in buffer (pH 6 7)/1 M methanol (or ethanol, or *n*-propanol) β-D-glucosidase (50 munit/ml) was added and the mixture kept at 30°C for 2 h Acetic acid was added and nitrophenol extracted with chloroform The aqueous solution was deionized, concentrated in vacuo and analyzed by TLC Comparison with authentic samples showed that significant amounts of the corresponding alkyl D-glucopyranosides were formed When the enzyme was omitted, or when the glucoside was replaced by D-glucose no alkyl glucosides could be detected Consequently the alkyl derivatives must be formed during the enzymic hydrolysis of the glucoside This qualitative analysis showed *n*-propanol to be the best acceptor This alcohol was thus used in all further experiments

Competitive inhibition With a constant concentration of *n*-propanol and at least ten different concentrations of *p*-nitrophenyl β-D-glucopyranoside, the initial rate of phenol release (v_1) was measured (pH 6 7, 30°C) and $k_{cat,1}$ and K_{app} were calculated from

TABLE I

EFFECT OF *n*-PROPANOL ON THE HYDROLYSIS OF *p*-NITROPHENYL β-D-GLUCOPYRANOSIDE AT pH 6 7 AND 30°C

The coefficients $k_{cat,1}$ and K_{app}, at given *n*-propanol concentration, were calculated from Eqn 17

n-Propanol (M)	10^6 $k_{cat,1}$ (mol min^{-1} u^{-1})	K_{app} (M^{-1})	$[k_{cat,1}$ $K_{app}]^{-1}$ (l min^{-1} u^{-1})
0	1 07 ± 0 07	17 900 ± 500	52
0 05	1 21 ± 0 03	14 900 ± 730	55
0 1	1 23 ± 0 02	13 560 ± 520	60
0 2	1 26 ± 0 02	11 000 ± 340	72

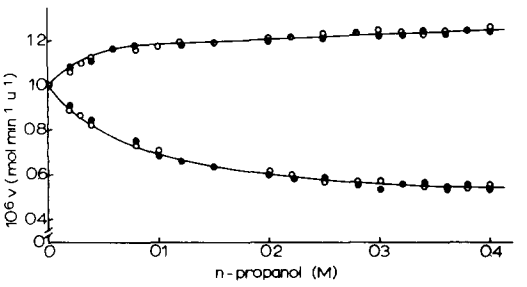


Fig 1 Effect of *n*-propanol on the rate of aglycon (v_1) or glucose (v_2) release for *p*-nitrophenyl β-D-glucopyranoside (●——●) and *p*-methoxyphenyl β-D-glucopyranoside (○——○) Initial rates at pH 6 7 and 30°C Solid lines represent the recalculated (Eqn 11) curves

Eqn 17 The measurements were then repeated with another concentration of *n*-propanol (Table I)

The increase in $k_{cat,1}$ indicates that the alcohol reacts with some intermediate enzyme complex, thereby increasing the rate of phenol release The increase in $k_{cat,1}$ (Eqn 18) will result in a decrease of K_{app} (Eqn 19) even if K_i is zero However, since the quantity $(k_{cat,1}\ K_{app})^{-1}$ is not constant but a linear function of [N], K_i is non-zero, From Eqn 20 $K_1k_6 = (19\,4 \pm 0\,4)\ 10^{-3}$ l min^{-1} U^{-1} and K_i approx 2 M^{-1} can be calculated A tentative mechanism (Scheme I) is thus proposed in which the alcohol, (1) behaves as a (weak) competitive inhibitor (EN) and (11) binds to the intermediate ES′ with the formation of ES′-N In the latter complex the glycosyl moiety can react with the alcohol yielding *n*-propyl (α/β)-D-glycopyranoside, or with water yielding glucose or xylose

Transfer reaction With a constant concentration of *p*-nitrophenyl β-D-glucopyranoside (2 mM) or *p*-methoxyphenyl β-D-glucopyranoside (5 mM) and 20 different concentrations (0--0 4 M) of *n*-propanol the initial rates (pH 6 7, 30°C) v_1 (phenol release) and v_2 (glucose release) were measured Fig 1 illustrates that the influence of *n*-propanol on the rates (v_1 and v_2) is independent of the substrate used

Without any assumption it follows from Eqn 11 that $v_1/v_2 = (1 + a[N])/(1 + b[N])$ with $a = \alpha_1/\alpha_0$ and $b = \alpha'_1/\alpha_0$ Thus, the data pairs (v_1/v_2 and [N]) were used to calculate *a* and *b* (Table II) As required by Scheme I the value of these parameters is independent of the aglycon part of the substrate From *a* and *b* the ratio $k_9/k'_7 = 2$ can be calculated Since $k'_7 = k_7W$ and W is approx 55 M the ratio k_9/k_7 is about 100 M

For both substrates α_0 is the initial rate ($v_1 = v_2$) at [N] = 0 and [SP] = 2 or [SP] = 5 mM For aryl β-D-glucopyranosides the initial rate at high substrate concentration is independent of the substituent on the phenyl ring because $k_6 \gg k'_3$ [1], and thus $\alpha_0 \simeq k'_3 = 1\ 10^{-6}$ mol min^{-1} U^{-1} This enables the calculation of α_1 and α'_1 from *a* and *b* (Table II)

TABLE II

EFFECT OF *n*-PROPANOL ON v_1 AND v_2 FOR *p*-NITROPHENYL β-D-GLUCOPYRANOSIDE AND *p*-METHOXYPHENYL β-D-GLUCOPYRANOSIDE AT pH 6 7 AND 30°C

Kinetic parameters calculated from the experimental data illustrated in Fig 1 Dimension of parameters α_0, mol min^{-1} u^{-1}, α_1, α'_1 and A, l min^{-1} u^{-1}

	p-Nitrophenyl glucoside	*p*-Methoxyphenyl glucoside
$a = \alpha_1/\alpha_0 = K_a(k_9 + k'_7)/k'_3$	15 6 ±1 7 M^{-1}	18 4 ±2 0 M^{-1}
$b = \alpha'_1/\alpha_0 = K_a k'_7/k'_3$	5 3 ±0 8 M^{-1}	6 7 ±1 0 M^{-1}
$[a/b] - 1 = k_9/k'_7$	2	1 8
α_1	(0 156 ± 0 017) 10^{-4}	(0 184 ± 0 020) 10^{-4}
α'_1	(0 053 ± 0 008) 10^{-4}	(0 067 ± 0 010) 10^{-4}
A } from v_3	(0 106 ± 0 007) 10^{-4}	(0 128 ± 0 01) 10^{-4}
β_1 } from v_3	12 3 ±1 1 M^{-1}	16 9 ±2 4 M^{-1}
α_0 } from Eqn 11 with v_1	(1 02 ±0 01) 10^{-6}	(1 03 ±0 01) 10^{-6}
β_1 } from Eqn 11 with v_1	12 3 ±1 0 M^{-1}	12 4 ±1 0 M^{-1}
α_0 } from Eqn 11 with v_2	(1 02 ±0 01) 10^{-6}	(1 04 ±0 02) 10^{-6}
β_1 } from Eqn 11 with v_2	13 3 ±1 1 M^{-1}	13 6 ±1 0 M^{-1}

According to Scheme I and Eqns 13 and 13bis, both α_1 and α'_1 depend upon the substituent (K_1, k_6) and upon the substrate concentration [SP] However, the data in Table II show the experimental values for the nitroglucoside and methoxyglucoside to be the same within the estimated standard error Again this indicates that $k_6 \gg k'_3$ and, for the substrate concentration used, $k'_3 < (k'_3 + k_6)K_1$ [SP] so that $\alpha_1 = K_a(k'_7 + k_9)$ and $\alpha'_1 = K_a k'_7$ Hence $\alpha_1/\alpha'_1 = (k'_7 + k_9)/k'_7$ and $k_9/k'_7 \simeq 2$

From Eqns 11, 12 and 13bis it follows that the rate of the transfer reaction (v_3) is given by the following equation

$$v_3 = v_1 - v_2 = \frac{A[\mathrm{N}]}{1 + \beta_1[\mathrm{N}] + \beta_2[\mathrm{N}]^2}$$

with

$$A = \alpha_1 - \alpha'_1 = \frac{K_1 K_a k_6 k_9 [\mathrm{SP}]}{k'_3 + (k'_3 + k_6) K_1 [\mathrm{SP}]}$$

However, since K_i is very small it can be expected that, at least up to 0 4 M *n*-propanol, the term $\beta_2[\mathrm{N}]^2$ will be negligible This also follows from the shapes of the curves in Fig 1 (neither v_1 nor v_2 approaches zero) or from a plot of $[\mathrm{N}]/v_3$ against [N] in Fig 2 The parameters A and β_1 calculated from v_3 (with $\beta_2 = 0$) are given in Table II The value of parameter A is equal to the difference between α_1 and α'_1 Since α_1 and α'_1 have been calculated from

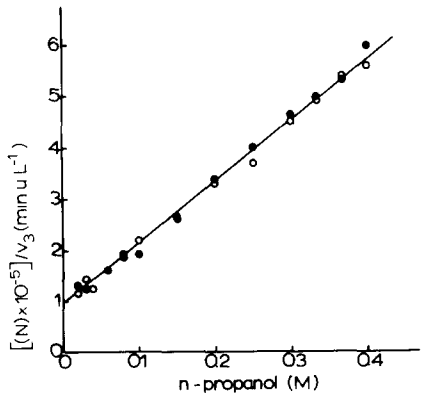


Fig 2 Effect of *n*-propanol on the transfer reaction ($v_3 = v_1 - v_2$) at pH 6 7 and 30°C for *p*-nitrophenyl β-D-glucopyranoside (○———○) and *p*-methoxyphenyl β-D-glucopyranoside (●———●) The plot illustrates the linear dependence of $[\mathrm{N}]/v_3$ upon the *n*-propanol concentration

v_1/v_2 without any assumption, the agreement is further evidence that the term $\beta_2[\mathrm{N}]^2$ is negligible

The estimates $\alpha_1 = 0\,17\ 10^{-4}$ l min^{-1} U^{-1} (mean value) and $\alpha'_1 = 0\,06\ 10^{-4}$ l min^{-1} U^{-1}, the data pairs (v_1 and v_2, [SP]) and Eqn 11 (with $\beta_2 = 0$) were then used to recalculate α_0 and β_1 All kinetic coefficients remained stable (Table II) The solid lines in Fig 1, represent the theoretical curves generated by the coefficients $\alpha_0 = 1\,02\ 10^{-6}$ mol min^{-1} U^{-1}, $\alpha_1 = 0\,17\ 10^{-4}$ l min^{-1} U^{-1} (for v_1) or $\alpha'_1 = 0\,06\ 10^{-4}$ l min^{-1} U^{-1} (for v_2), and $\beta_1 = 13\,3$ M^{-1}

It follows from Eqn 14 that β_1 is a composite function of rate and equilibrium (or steady-state) constants, amongst which the substituent-dependent constants k_6 and K_1 However, the finding that different glucosides yield the same value for β_1 (Table II) suggests that Eqn 14 can be simplified The assumptions $k_6 \gg k'_3$ and $k'_3 < (k'_3 + k_6)K_1$[SP] have already been discussed If also $K_1K_a(k_6 + k'_7 + k_9)$[SP] $\gg k'_3K_i + K_a(k'_7 + k_9)$ Eqn 14 simplifies to $\beta_1 \simeq K_a(k_6 + k'_7 + k_9)/k_6$, an equation that still contains the substrate-dependent parameter k_6 as denominator If $k_6 \gg k'_7 + k_9$ further simplification yields $\beta_1 \simeq K_a$ However, the main question remains as to whether the assumption $K_1K_a(k_6 + k'_7 + k_9)$-[SP] $\gg k'_3K_i + K_a(k'_7 + k_9)$ is correct With $k'_3 = 1\ 10^{-6}$ mol min^{-1} U^{-1}, 1 unit = 5 10^{-10} 'mol' active site, $K_1k_6 = 19\ 10^{-3}$ l min^{-1} U^{-1}, $K_i = 2$ M^{-1} and $a = 16$ M^{-1} (Table II, mean value) calculation yields for the nitrophenyl glucoside $k'_3K_1 + K_a(k'_7 + k_9) = 36\ 10^3$ l min^{-1} U^{-1}

One the other hand $K_1K_a(k_6 + k'_7 + k_9)$[SP] = $K_1K_ak_6$[SP] + $K_1K_a(k'_7 + k_9)$[SP] = (80 $10^3 K_a$ + 64 K_1) l min^{-1} U^{-1} Thus, with reasonable values for K_1 and K_a (e g , $K_a > 10$ M^{-1}) the assumption will be correct Similar calculations leading to the same conclusion can be made for the methoxyphenyl glucoside If it is accepted that $\beta_1 \simeq K_a = 13\,5$ M^{-1} (mean value) estimates of k'_3, k'_7 and k_9 can be calculated from the data in Table II With 1 unit = 5 10^{-10} 'mol' active site this yields $k'_3 = 2\ 10^3$ min^{-1}, $k'_7 = 0\,8\ 10^3$ min^{-1} and $k_9 = 1\,4\ 10^3$ min^{-1} Because of the assumptions made and the large standard deviations on the values of Table II, only the order of magnitude of these constants is significant The reaction of the glucosyl moiety with water proceeds at the same rate ($k'_3 \simeq k'_7$) in ES' and ES'N, but the reaction with *n*-propanol (ES'N) is much

faster than that with water ($k_9/k_7 \simeq 100$ M)

If the influence of *n*-propanol on v_1 and v_2 is correctly described by Eqn 11 with $\beta_2 \simeq 0$, $k'_3 < k_6$, $k'_3 < (k'_3 + k_6)K_1[SP]$ and $\beta_1 \simeq K_a$ both rates must approach a constant non-zero value given by the ratio $\alpha_1/\beta_1 = (k'_7 + k_9)$ for v_1 and $\alpha'_1/\beta_1 = k'_7$ for v_2 Using the data in Table II calculation yields the estimates $\alpha_1/\beta_1 = (1\ 19 \pm 0\ 17)\ 10^{-6}$ mol min^{-1} U^{-1} and $\alpha'_1/\beta_1 = (0\ 44 \pm 0\ 08)\ 10^{-6}$ mol min^{-1} U^{-1} (compare Fig 1)

For aryl β-D-glucopyranosides $k_6 \gg k'_3$ so that, for [N] = 0 and high substrate concentration the k'_3 step, i e , the regeneration of the free enzyme, is rate-limiting and determines v_1 When *n*-propanol, reacting with ES′ according to Scheme I, increases the rate of the regeneration reaction, v_1 will also increase Fig 1 shows that v_1 (and also v_2) approaches a constant value at high concentration of *n*-propanol A first explanation would be that the regeneration reaction (k'_3, k'_7 and k_9) is now so fast that step 6 is rate-limiting However, this seems very unlikely since then $v_1 = k_6 \simeq 1\ 2\ \ 10^{-6}$ mol min^{-1} U^{-1} and thus $k_6 \simeq k_3$ A more plausible explanation is that, at the high alcohol concentrations, nearly all the enzyme is in the form ES′N (saturation kinetics of the alcohol nucleophile) This implies that, prior to reaction, the alcohol 'binds' to ES′ with the formation of a ternary glucosyl-enzyme-nucleophile complex (ES′N) The shape of the curves in Fig 1 and the fact that α'_1 is non-zero (thus $k'_7 = 0$) indicate that water molecules can still react with the glycosyl residue in the complex ES′N

Transfer from aryl β-D-xylopyranosides to n-propanol

The same measurements and calculations as described for the arylglucosides were repeated using *p*-nitrophenyl (5 mM) and *p*-methoxyphenyl β-D-xylopyranoside (20 mM) as substrates, and *n*-propanol (up to 0 17 M) as nucleophile The results are collected in Table II and illustrated in Fig 3 The solid lines in Fig 3 represent the theoretical curves generated by the parameters α_0, α_1 or α'_1 and β_1 (given in Table III) and Eqn 11 (with $\beta_2 = 0$)

The results now clearly depend on the substituent group For both substrates v_2 decreases, but, whereas v_1 increases for the nitrophenyl derivative it remains constant for the methoxyphenyl xyloside In agreement with Scheme I, the values of *a* and *b* (Table III) are independent of the glycon part of the substrate (Table II) The ratio k_9/k'_7 is greater for xylosides than for glucosides ($k_9/k_7 \simeq 270$ M)

For these xylosides both α_1 and α'_1 (thus also $A = [\alpha_1 - \alpha'_1]$) are dependent on the substrate This is not unexpected since for xylosides the assumption $k_6 \gg k'_3$ is incorrect [1] so that, even if the substrate concentration is so high that $k'_3 < (k'_3 + k_6)K_1[SP]$,

TABLE III

EFFECT OF *n*-PROPANOL ON THE RATE OF AGLYCON (v_1) AND XYLOSE (v_2) RELEASE FOR *p*-NITROPHENYL β-D-XYLOPYRANOSIDE AND *p*-METHOXYPHENYL β-D-XYLOPYRANOSIDE AT pH 6 7 AND 30°C

Kinetic parameters calculated from the experimental data illustrated in Fig 3 Dimension of parameters α_0, mol min^{-1} u^{-1}, α_1, α'_1 and A, 1 min^{-1} u^{-1}

	Nitrophenyl xyloside	Methoxyphenyl xyloside
$a = \alpha_1/\alpha_0 = K_a(k_9 + k'_7)/k'_3$	51 ± 4 M^{-1}	47 ± 2 M^{-1}
$b = \alpha'_1/\alpha_0 = K_a k'_7/k'_3$	9 ± 1 M^{-1}	7 6 ± 2 M^{-1}
$[a/b] - 1 = k_9/k'_7$	4 7 ± 0 6	5 1 ± 1 3
α_1	(0 124 ± 0 010) 10^{-4}	(0 059 ± 0 002) 10^{-4}
α'_1	(0 022 ± 0 002) 10^{-4}	(0 009 ± 0 002) 10^{-4}
A from v_3	(0 1 ± 0 01) 10^{-4}	(0 049 ± 0 003) 10^{-4}
β_1 from v_3	17 6 ± 1 6 M^{-1}	46 ± 4 M^{-1}
α_0 from Eqn 11 with v_1	(0 235 ± 0 005) 10^{-6}	(0 126 ± 0 002) 10^{-6}
β_1 from Eqn 11 with v_1	17 5 ± 0 2 M^{-1}	46 ± 0 6 M^{-1}
α_0 from Eqn 11 with v_2	(0 237 ± 0 004) 10^{-6}	(0 127 ± 0 002) 10^{-6}
β_1 from Eqn 11 with v_2	17 8 ± 0 4 M^{-1}	46 ± 1 M^{-1}

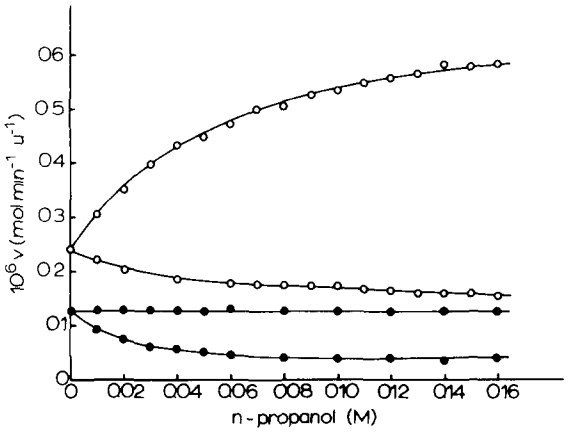


Fig 3 Effect of *n*-propanol on the rate of aglycon (v_1) or xylose (v_2) release for *p*-nitrophenyl β-D-xylopyranoside (○——○) and *p*-methoxyphenyl β-D-xylopyranoside (●——●) Initial rates at pH 6 7 and 30°C Solid lines represent the curves generated (Eqn 11) by the coefficients given in Table III

Eqns 13 and 13bis can only be simplified to $\alpha_1 = K_a k_6(k'_7 + k_9)/(k'_3 + k_6)$ and $\alpha'_1 = K_a k_6 k'_7/(k'_3 + k_6)$ Both parameters still contain the substituent-dependent k_6-coefficient

However, Scheme I requires that

α_1(nitro-xyl)/α_1(Methoxy-xyl) = α'_1(Nitro-xyl)/α'_1-(Methoxy-xyl)

The data in Table III shows this to be correct the ratio for $\alpha_1 = 2\ 1 \pm 0\ 2$ and for $\alpha'_1 = 2\ 4 \pm 0\ 5$

The exact meaning of the parameter β_1 is unknown because further simplification is impossible

For aryl β-D-xylopyranosides it has been proposed [1] that both k'_3 and k_6 are partially rate-determining at [N] = 0 and high substrate concentration Fig 3 shows that, whereas v_1 for the nitrophenyl xyloside increases with increasing concentration of *n*-propanol, v_1 for the methoxyphenyl xyloside remains constant This indicates that for the latter compound at [N] = 0 the rate of phenol release (v_1) is limited by the rate of the k_6-step $k_6 \gg k'_3$ and $v_1 = k_6 = 0\ 124\ \ 10_6$ mol min^{-1} U^{-1} For the nitrophenyl xyloside the increase in v_1 proves that the k_6-step was not rate-limiting but the data do not allow the calculation of k_6 However, it is obvious that k_6(nitro-Xyl) > k_6(methoxy-Xyl) This is the first clear indication that the substituent group has an effect on the rate at which the aglycon group is released The electron-withdrawing nitro substituent makes the nitrophenyl group a better leaving-group than the *p*-methoxyphenyl group with its electron-donating substituent Since it would be illogical to accept substituent effects in the aryl xyloside series but not in the aryl glucoside series, the fact that these effects cannot be detected in the latter series [1] does not mean that they are absent As already discussed, the reason is that $k_6 \gg k'_3$ for glucopyranosides

For both substrates v_2 approaches a non-zero constant value (Fig 3) but the limiting value for the nitrophenyl xyloside is higher than that for the methoxyphenyl xyloside When saturation of the enzyme by the alcohol is complete $v_1 = \alpha_1/\beta_1$ and $v_2 = \alpha'_1/c1$ As mentioned before, the parameter β_1 cannot be simplified and thus the ratios α_1/β_1 and α'_1/β_1 will remain composite functions of several rate constants, including the substrate dependent constant k_6

Conclusions

The hydrolysis of aryl β-D-glucopyranosides and aryl β-D-xylopyranosides catalyzed by β-D-glucosidase from *Stachybotrys atra* proceeds through a glycosyl-enzyme intermediate (ES') according to Scheme I In the presence of an alcohol, the glycosyl-enzyme can react with that alcohol thereby yielding an alkyl glycoside The levelling off of the rates indicates an alcohol-binding-site (ES'N) that becomes saturated at high concentrations of alcohol Such a site could also explain the inhibition by a second substrate molecule and by an inhibitor (aryl 1-thioglycoside) with aromatic aglycon group [1] The exact nature of this non-covalent binding is unknown, but it is very probable that the hydrophobicity of the alkyl chains or of the aromatic ring-structures plays an important role If the added nucleophile can bind to the glycosyl-enzyme complex, it would seem reasonable to expect that the nucleophile may also bind to the free enzyme The observation of competitive inhibition proves that this is the case

Since alcohols react with the glycosine residue yielding the alkyl glycopyranoside, alcohols are good entering groups In contrast, alkyl β-D-glucopyranosides and alkyl β-D-xylopyranosides are not (or very slowly) hydrolyzed by the enzyme and thus the

alkoxy group is a very poor leaving group On the basis of the estimated rate parameters, *n*-propanol is a better nucleophile than water towards the glycosine residue However, an exact comparison between the water- and *n*-propanol-reactivity is difficult because it is not known how the water molecules react with the complexes ES′ and ES′N and because the parameters k_3, k_7 and k_9 may be composite functions of several constants For example it is possible that an ES′W complex, involving non-covalently-bound water exists Thus, any conclusion abut the exact nature of the glycosyl group in ES′ would would be too hypothetical

With *p*-nitrophenyl glucoside and *p*-methoxyphenyl glucoside the increase in v_1 with increasing nucleophile concentration shows that glycosylation cannot be rate-limiting The lack of observed substituent effects is further evidence that for aryl β-D-glucopyranosides the k'_3-step is rate-limiting

For aryl β-D-xylopyranosides two kinds of substrate behaviour can be observed With the *p*-methoxyphenyl xyloside glycosylation is rate-limiting, whereas with the *p*-nitrophenyl xyloside this step cannot be the limiting process The kinetic results cannot determine whether $k_6 \gg k'_3$ or $k_6 \simeq k'_3$ However, it is clear that the substituent has an effect on the rate at which the aglycon group is released and that electron-withdrawing substituent groups increase this rate It may be recalled that aryl 1-thio-β-D-glucopyranosides are hydrolyzed by the enzyme only when a strong electron-withdrawing substituent (e g *p*-nitro group) is present in the aglycon group [2]

Because, even in the presence of large alcohol concentrations, the rate of glucose (or xylose) release never approaches zero, the mode of binding of the alcohol must be such that water can still react with the glycosyl-enzyme-nucleophile complex. This agrees with our previous finding [1] that glycosyl-enzyme-substrate and glycosyl-enzyme-inhibitor complexes can react with water

Appendix

Rate equations

The rate equations derived from Scheme I are very similar to those given in Ref 1 and thus the same numbering will be used in the present paper Only the significant differences will be emphasized here

When the concentration of substrate [SP] is kept constant, the influence of the nucleophile [N] concentration on the initial rate is given by Eqn 1

$$v_i = \frac{v'_i}{E_t} = \frac{\alpha_0 + \alpha_1[N]}{1 + \beta_1[N] + \beta_2[N]^2} \qquad i = 1, 2 \qquad (1)$$

When the rate of aglycon (phenol) release (v_1) is measured, the meaning of the kinetic parameters α_0, α_1, β_1 and β_2 is given by Eqns 12–16 and 6 in Ref 1 However, the following changes have to be made delete E_t, change k_3 to k'_3 and k_7 to $(k'_7 + k_9)$ When the rate of glucose or xylose release is measured (v_2) the meaning of α_0, β_1 and β_2 remains the same as for v_1 However, α_1 must be replaced by α'_1,

$$\alpha'_1 = \frac{K_1 K_a k_6 k'_7(SP)}{k'_3 + (k'_3 + k_6) K_1(SP)} \qquad (12\text{ bis})$$

For a constant concentration of the nucleophile, Eqn 1 rearranges to a formal Michaelis-Menten equation (Eqn 17 in Ref 1) K_{app} and $k_{cat,1}$ (for v_1) are given by Eqns 18–19 However, change [I] to [N], k_3 to k'_3 and k_7 to $(k'_7 + k_9)$ The meaning of $k_{cat,2}$ (for v_2) is given by Eqn 13

$$k_{cat,2} = \frac{k'_3 k_6 + K_a k'_7 k_6[N]}{k'_3 + k_6 + K_a(k_6 + k'_7 + k_9)[N]} \qquad (13)$$

The coeffucients k'_7 and k'_3 are pseudo-first-order coefficients $k'_7 = k_7[W]$ and $k'_3 = k_3[W]$ with [W] the practically constant concentration of water The coefficients k_3, k_7 and k_9 are functions of different rate constants which it is impossible to evaluate individually For example it is possible that a complex ES′W, involving non-covalently bound water, exists

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References

1 De Bruyne, C K , Aerts, G M and De Gussem, R L (1979) Eur J Biochem 102, 257–267
2 De Gussem, R L , Aerts, G M , Claeyssens, M and De Bruyne, C K (1978) Biochim Biophys Acta 525, 142–153

3 Hinberg, I and Laidler, K J (1972) Can J Biochem 50 1334–1359
4 Viratelle, O M and Yon, J M (1973) Eur J Biochem 33, 110–116
5 De Prijcker, J and De Bruyne, C K (1975) Carbohydr Res 43, 173–182
6 Wynne, D and Shalitin, Y (1972) Eur J Biochem 31, 554–560
7 Greenzaid, P and Jencks, W P (1971) Biochemistry 10, 1210–1222
8 Fink, A L and Bender, M L (1969) Biochemistry 8, 5109–5118
9 Glaser, E and Wulwek, W (1924) Biochem Z 145, 514–534
10 Loontiens, F G and De Bruyne, C K (1964) Naturwiss 51, 359–360
11 Loontiens, F G , Van Wauwe, J P , De Gussem, R and De Bruyne, C K (1973) Carbohydr Res 30, 51–62
12 De Bruyne, C K , Versele, H and Claeyssens, M (1965) Nature (London) 205, 900
13 De Bruyne, C K and Vervoort, A (1966) Nature (London) 211, 1292–1293