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β-D-GLUCOSIDASE-CATALYSED TRANSFER OF THE GLYCOSYL GROUP FROM ARYL β-D-GLUCO- AND β-D-XYLO-PYRANOSIDES TO PHENOLS

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ABSTRACT

The effect of phenols on the hydrolysis of substituted phenyl β-D-gluco- and β-D-xylo-pyranosides by β-D-glucosidase from *Stachybotrys atra* has been investigated. Depending on the glycon part of the substrate and on the phenol substituent, the hydrolysis is either inhibited or activated. With aryl β-D-xylopyranosides, transfer of the xylosyl residue to the phenol, with the formation of new phenyl β-D-xylopyranosides, is observed. With aryl β-D-glucopyranosides, such transfer does not occur when phenols are used as acceptors, but it does occur with anilines. A two-step mechanism, in which the first step is partially reversible, is proposed to explain these observations. A qualitative analysis of the various factors determining the overall effect of the phenol is given.

INTRODUCTION

We have reported[1–3] on the hydrolysis of aryl β-D-glucopyranosides and β-D-xylopyranosides by β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from *Stachybotrys atra*. This enzyme is capable of transferring the glycosyl residue of the substrate to alcohols with the formation of alkyl β-D-glycopyranosides[3]. The observation that addition of phenols to the enzymic reaction mixtures strongly affected the reaction rate prompted a study of these effects in more detail.

THEORETICAL SECTION

The minimal reaction scheme given previously[2,3] is insufficient to explain the effect of added phenols. As illustrated in Scheme 1, the step leading from the enzyme–substrate complex (ESP) to the glycosyl–enzyme complex (ES′) has now been split into two separate steps. Step 2 is the bond-fission step leading to a complex (ES′P) in which the aglycon group (P) of the substrate (SP) is still non-covalently bound to the enzyme. Step 6′ represents the desorption of P. Both steps are, at least theoretically, reversible and the retro-constants represent bond formation (k_{-2}) and adsorption (k'_{-6}) of P to ES′. The sequence of reactions leading from ES′ to the new glycoside SN [which is in fact a new substrate, formed by transfer of the glycosyl

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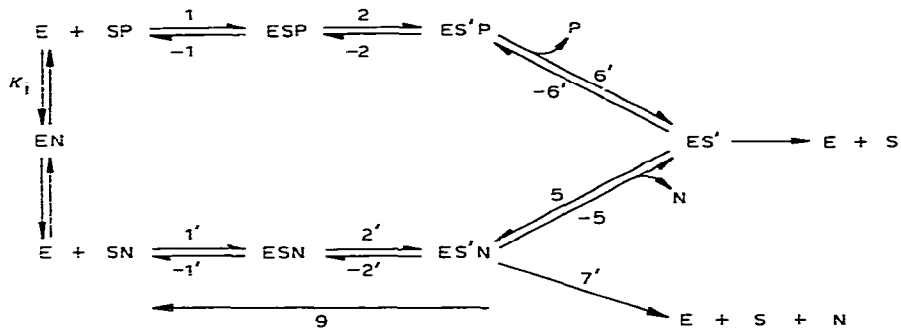

Scheme 1

residue (S') to the added nucleophile (N)] is symbolised by the same steps as used for the hydrolysis of the original substrate (SP).

However, since it is impossible to calculate individually the rate constants for the sequence ES'N→E + SN, the overall rate-coefficient k_9 will be used to simplify the rate equations. If only initial rates (P ~0) are measured, step $-6'$ (but not step -2) can be neglected. By defining the overall coefficient $k_6 = k_2k_6'/(k_2 + k_{-2} + k_6')$, the rate equations and kinetic parameters resulting from the steady-state treatment of Scheme 1 and also the numbering of the steps (or constants) become identical with those given in our previous papers[2,3]. However, to explain *qualitatively* the effects of phenols, the more complete Scheme 1 in this paper is necessary.

EXPERIMENTAL

N-p-Methylphenyl-β-D-glucopyranosylamine was a gift from Professor G. Legler (Köln, B.R.D.). Uniformly labelled [U-^{14}C]phenol was purchased from the Radiochemical Centre (Amersham, England). 4-Methylumbelliferyl β-D-xylopyranoside was synthesised as described[4]. T.l.c. was performed on Silica Gel G (Merck) with ethyl acetate–methanol (9:1). Autoradiograms were made on Structurix DU (Gevaert, Belgium). U.v. spectra were obtained with a Zeiss PMQII-M4QIII spectrophotometer using 1-cm cuvettes. Fluorescence emission spectra (excitation at 313 nm) were recorded with a Zeiss ZMF_4 spectrofluorimeter equipped with two M4QIII monochromators and an Osram 450W Xe lamp. In the presence of anilines, D-glucose was measured with the hexokinase–dehydrogenase[5] enzyme system (Boehringer). All other experimental methods were as described[1–3]. The rate equations, rate coefficients, and methods of data analysis have been reported[2,3]. For the sake of clarity, the meaning of the most important rate-coefficients is given in Table II. All rates and rate constants are calculated per unit (u) of enzyme activity[1,2].

RESULTS

Qualitative analysis of transfer to phenols

To a solution (0.3 ml) of [U-^{14}C]phenol (0.74 MBq; 0.67 μmol; 2.2mM) and

p-nitrophenyl β-D-xylopyranoside (5mM) or phenyl β-D-xylopyranoside (10mM) in phosphate buffer (pH 6.7) was added β-D-glucosidase (0.8 mu). At intervals, aliquots (5 μL, 12 KBq) were subjected to t.l.c. The autoradiograms showed that, as a function of time, increasing amounts of a radioactive product were formed. Comparison with an authentic sample showed that the product was radioactive phenyl D-xylopyranoside.

When the enzyme was omitted from the mixtures or when the enzyme was added to a solution containing D-xylose (10mM) and [U-^{14}C]phenol, no radioactive phenyl xyloside (or other newly formed radioactive product) could be detected. Consequently, the radioactive xyloside was formed during the hydrolysis of the substrate by transfer of the xylosyl moiety to the added phenol.

After longer incubation periods, the radioactive transfer-product disappeared from the reaction mixture, unless the enzyme was destroyed (by briefly boiling the solution) before the hydrolysis of the substrate was complete. Since the enzyme does not hydrolyse α-D-xylopyranosides, the transfer product must have the β configuration.

In further experiments, the fluorescent 7-hydroxy-4-methylcoumarone (4-methylumbelliferone) was used as acceptor. To a solution (5 mL) of *p*-nitrophenyl β-D-xylopyranoside (5mM) and 4-methylumbelliferone (54μM) in buffer (pH 6.7) was added β-D-glucosidase (14 mu). After 2 h at 30°, the mixture was analysed by t.l.c. Two fluorescent spots, corresponding to 4-methylumbelliferone and 4-methylumbelliferyl D-xylopyranoside, were detected. When the enzyme was omitted from the mixture or when the nitrophenyl xyloside was replaced by xylose, no fluorescent transfer-product could be detected. A small amount of the transfer product was isolated by preparative t.l.c. Both the absorption (maximum at 318 nm; shoulder at 290 nm) and fluorescence (excitation at 313 nm; maximum at 380 nm) spectra were identical with those of authentic 4-methylumbelliferyl β-D-xylopyranoside[6,7]. Treatment of a solution of the transfer product, showing an absorbance at 318 nm equivalent to 11.6μM 4-methylumbelliferyl β-D-xylopyranoside, with β-D-glucosidase resulted in an increase of the fluorescence at 440 nm (maximum for 4-methylumbelliferone) and a decrease at 380 nm. An identical pattern was obtained when 4-methylumbelliferyl β-D-xylopyranoside was hydrolysed by the enzyme under comparable conditions. The final fluorescence (440 nm) of the transfer product (complete hydrolysis) was equivalent to 11.6μM 4-methylumbelliferone. Thus, only the β anomer of 4-methylumbelliferyl β-D-xylopyranoside is formed by the transfer of the xylosyl residue to 4-methylumbelliferone.

When the above experiments were repeated with aryl β-D-glucopyranosides, no radioactive or fluorescent transfer-products could be detected, although the substrates were completely hydrolysed.

Thus, it is concluded that the xylosyl, but not the glucosyl, residue in ES′ can be transferred to phenols, although both residues can be transferred to alcohols[3].

Influence of p-*chlorophenol and* p-*methoxyphenol on the hydrolysis of* p-*nitrophenyl* β-D-*xylopyranoside*

With a constant concentration of either *p*-chlorophenol or *p*-methoxyphenol

TABLE I

INFLUENCE OF *p*-CHLOROPHENOL ON THE HYDROLYSIS OF *p*-NITROPHENYL β-D-XYLOPYRANOSIDE AT pH 6.7 AND 30°

p-Chlorophenol (mM)	10^6 $k_{cat,1}$ *(mol.min^{-1}.u^{-1})*	K_{app} (M^{-1})
0	1.01 ±0.02	5 760 ±650
0.4	0.96 ±0.01	5 090 ±200
1.0	0.88 ±0.01	4 730 ±200
2.0	0.81 ±0.02	3 575 ±170
3.0	0.74 ±0.02	3 350 ±300
5.0	0.68 ±0.01	2 589 ±300

and at least ten different concentrations of *p*-nitrophenyl β-D-xylopyranoside, the initial rate of *p*-nitrophenol release (v_1) was measured (pH 6.7, 30°) and k_{cat1} and K_{app} were calculated. Repeating these measurements with other concentrations of the phenol yielded the values collected in Table I (for *p*-chlorophenol). From the linear dependence of $(k_{cat1} \times K_{app})^{-1}$ on the concentration of the phenol, the competitive inhibition constant (K_i) could be calculated (460 ±26 M^{-1} for *p*-chlorophenol and 70 ±5 M^{-1} for *p*-methoxyphenol). Thus, both phenols bind (EN) to the free enzyme, and the substituent has an effect on the free energy of binding.

However, since k_{cat1} decreases with increasing concentration of the phenol (Table I), competitive inhibition is not the only way in which the phenols inhibit the reaction. The observation that the xylosyl residue is transferred to added phenols, with the formation of new aryl β-D-xylopyranosides, suggests that the phenols (N) bind to the glycosyl–enzyme complex (ES′) yielding the ternary glycosyl–enzyme–phenol complex (ES′N). Bond formation (k'_2) takes place in this complex and yields the new β-D-xylopyranoside (SN).

If the model is correct, the rate of the reaction must obey the rate equations derived from Scheme 1. Therefore the initial rate of *p*-nitrophenol (v_1) or xylose (v_2) release was measured (pH 6.7, 30°) for a constant concentration (5mM) of *p*-nitrophenyl β-D-xylopyranoside and about 20 different concentrations of either *p*-chlorophenol (Fig. 1) or *p*-methoxyphenol (Fig. 2). Data analysis, using the formulae and methods described previously[2,3], yielded the kinetic parameters collected in Table II. The solid lines in Figs. 1–2, representing the theoretical curves generated by these parameters, show that the rates can be described by the equations used. A second series of measurements with 4mM *p*-nitrophenyl β-D-xylopyranoside yielded the same values (independent of the substrate concentration) for α_0, β_1, and β_2. This suggests that $k'_3 \ll (k'_3 + k_6)K_1(SP)$ so that $\alpha_0 = k'_3k_6/(k'_3 + k_6)$ and therefore $\alpha_1 = K_3k_6(k'_7 + k_9)/(k'_3 + k_6)$.

For *p*-chlorophenol, it follows from Fig. 1 that, whereas both v_1 and v_2 decrease with increasing concentration of *p*-chlorophenol, the rate of the transfer reaction ($v_3 = v_1 - v_2$) first increases, but thereafter decreases. This decrease is due

TABLE II

INFLUENCE OF *p*-CHLOROPHENOL OR *p*-METHOXYPHENOL ON THE HYDROLYSIS OF *p*-NITROPHENYL β-D-XYLOPYRANOSIDE (pH 6.7, 30°)

	p-Chlorophenol	*p-Methoxyphenol*
$\alpha_0{}^a = \dfrac{K_1 k_3' k_0\,(\mathrm{SP})}{k_3' + (k_3' + k_0)\,K_1\,(\mathrm{SP})}$	$(0.248 \pm 0.003) \times 10^{-6}$	$(0.243 \pm 0.003) \times 10^{-6}$
$\alpha_1 = \dfrac{K_1 K_n k_0 (k_7' + k_0)\,(\mathrm{SP})}{k_3' + (k_3' + k_0)\,K_1\,(\mathrm{SP})}$	$(1.60 \pm 0.3) \times 10^{-3}$	$(0.305 \pm 0.025) \times 10^{-3}$
$\alpha_1' = \dfrac{K_1 K_n k_0 k_7'\,(\mathrm{SP})}{k_3' + (k_3' + k_0)\,K_1\,(\mathrm{SP})}$	$(0.61 \pm 0.11) \times 10^{-3}$	$(0.116 \pm 0.010) \times 10^{-3}$
$\beta_1 = \dfrac{k_3' K_1 + K_n(k_7' + k_0) + K_1 K_n(k_0 + k_7' + k_0)\,(\mathrm{SP})}{k_3' + (k_3' + k_0)\,K_1\,(\mathrm{SP})}$	7400 M^{-1}	880 M^{-1}
$\beta_2 = \dfrac{K_1 K_n (k_7' + k_0)}{k_3' + (k_3' + k_0)\,K_1\,(\mathrm{SP})}$	$(4.73 \pm 0.35) \times 10^5$ M^{-2}	—

[a]Dimensions of α_0, α_1, α_1': $mol.min^{-1}.u^{-1}$.

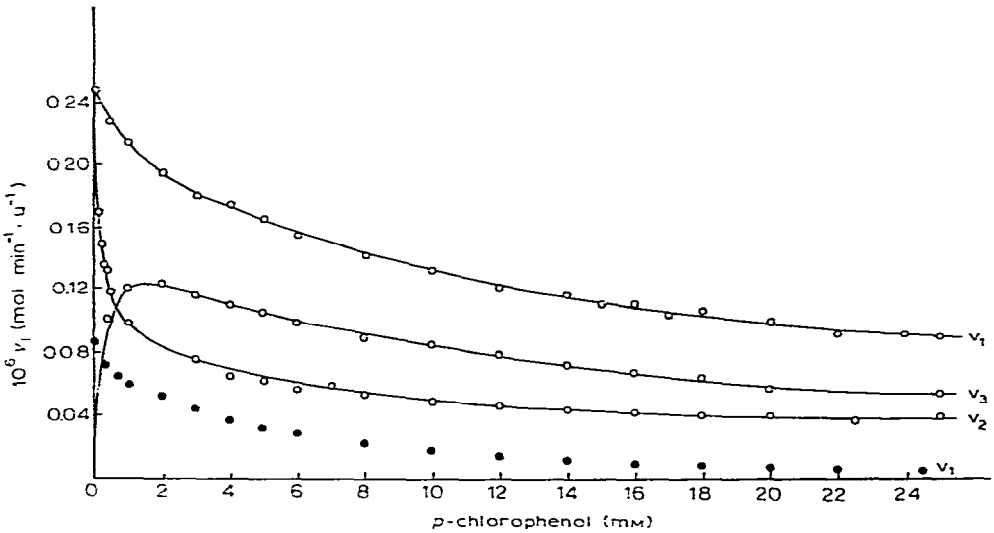

Fig. 1. Influence of *p*-chlorophenol on the hydrolysis of *p*-nitrophenyl β-D-xylopyranoside. Initial rate of nitrophenol (v_1) or xylose (v_2) release and of transfer reaction (v_3). Substrate concentration is 5mM (○) or 0.1 mM (●).

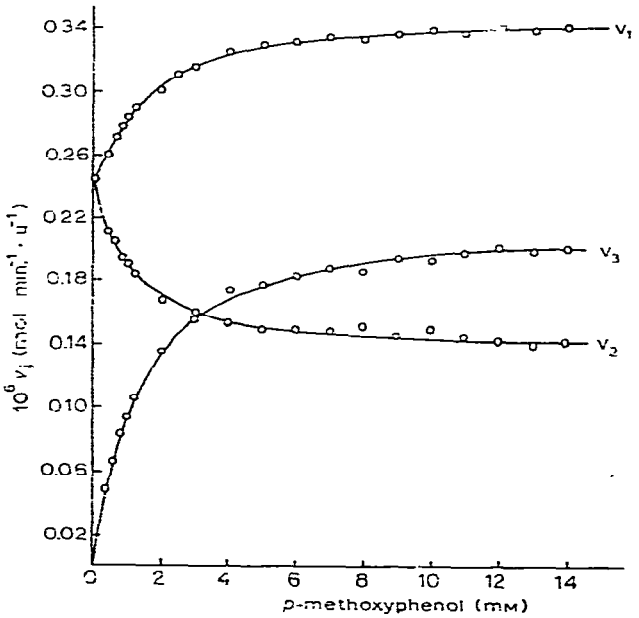

Fig. 2. Influence of *p*-methoxyphenol on the hydrolysis of *p*-nitrophenyl β-D-xylopyranoside (5mM). For v_1, v_2, and v_3: see Fig. 1.

to the competitive inhibition (K_i = 460M^{-1}) by the phenol. The lowest series of points in Fig. 1 illustrates that, at sufficiently low concentration of substrate (0.1mM), the rate v_1 approaches zero because of this type of inhibition. Fig. 2 shows that, whereas v_2 decreases, both v_1 and v_3 increase with increasing concentration of *p*-

methoxyphenol. The K_i value of this phenol is too low to cause an experimentally detectable (up to 14mM) decrease of v_3. Consequently, the term $\beta_2(N)^2$ does not significantly contribute to the rate equations, and β_2 cannot be calculated.

Influence of p-*methoxyphenol on the hydrolysis of* p-*nitrophenyl* β-D-*glucopyranoside or* p-*methoxyphenyl* β-D-*glucopyranoside*

With a constant concentration (2mM) of the nitrophenyl glucoside and various concentrations of *p*-methoxyphenol, v_1 and v_2 were measured at pH 6.7 and 30°. Under the same conditions, v_2 was measured for 6mM *p*-methoxyphenyl glucoside. Fig. 3 shows that (*a*) in agreement with the fact that no radioactive transfer-products could be detected during aryl β-D-glucopyranoside hydrolysis, no transfer reaction can be observed kinetically; (*b*) the curves for the nitrophenyl and methoxyphenyl derivative are identical; (*c*) both v_1 and v_2 decrease with increasing concentration of *p*-methoxyphenol, but are still non-zero at the highest concentration of phenol used. Thus, competitive inhibition is not predominant (and β_2 cannot be calculated). The calculated rate-parameters were (*1*) for *p*-nitrophenyl glucoside: $\alpha_0 = (1.00 \pm 0.01) \times 10^{-6}$ mol.min^{-1}.u^{-1}, $\alpha_1 = (1.2 \pm 0.2) \times 10^{-6}$ mol.min^{-1}.u^{-1}, $\beta_1 = 400 \pm 44$M^{-1}, and $\alpha_1' = (1.5 \pm 0.2) \times 10^{-6}$ mol.min^{-1}.u^{-1}; and (*2*) for *p*-methoxyphenyl glucoside: $\alpha_0 = (1.00 \pm 0.02) \times 10^{-6}$ mol.min^{-1}.u^{-1}, $\alpha_1' = (2.0 \pm 0.5) \times 10^{-6}$ mol.min^{-1}.u^{-1}, and $\beta_1 = 490 \pm 92$M^{-1}. The solid lines in Fig. 3, representing the curves generated by these parameters, show the agreement between theoretical and experimental points. For aryl glucopyranosides[2,3] at the concentrations used, $k_3' \ll (k_3' + k_6)K_1(SP)$ and $k_6 \gg k_3'$, so that $\alpha_0 = k_3'$, $\alpha_1 = K_a(k_7' + k_9)$, and $\alpha_1' = K_a k_7'$. Since one unit (u) of the enzyme equals 5×10^{-10} "mol" of active site[1,2], the following estimates can

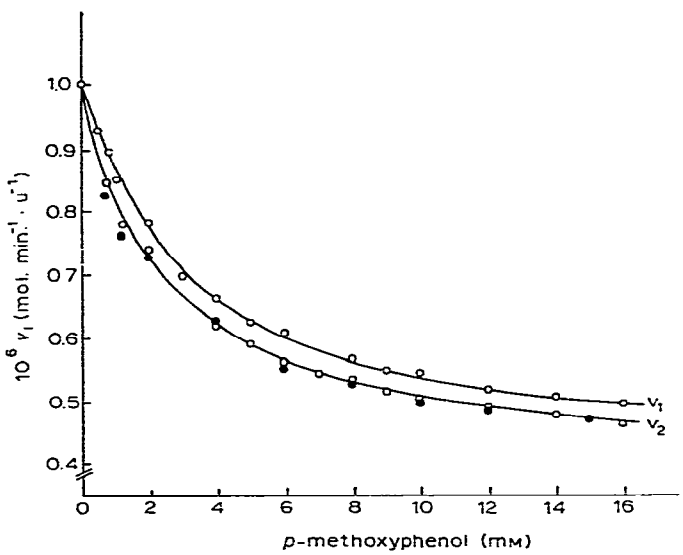

Fig. 3. Influence of *p*-methoxyphenol on the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (○) and *p*-methoxyphenyl β-D-glucopyranoside (●). For v_1 and v_2: see Fig. 1.

be calculated from the above parameters: $k_3' = 2 \times 10^3$ min^{-1}, $K_a(k_7' + k_9) = (0.24 \pm 0.06) \times 10^6$M^{-1}.min^{-1}, and $K_a k_7' = (0.3 \pm 0.07) \times 10^6$M^{-1}.min^{-1} for the nitrophenyl glucoside, and $K_a k_7' = (0.4 \pm 0.1) \times 10^6$M^{-1}.min^{-1} for the methoxyphenyl glucoside. Under the assumptions $k_3' \ll (k_3' + k_6)K_1(\mathrm{SP})$ and $k_6 \gg (k_7' + k_9)$, the parameter β_1 simplifies to $\beta_1 \simeq [k_3' K_i + K_a(k_7' + k_9) + K_1 K_a k_6(\mathrm{SP})]/k_6 K_1(\mathrm{SP})$. Since the numerical value of $K_1 k_6$ for both substrates is also known[2], the value of K_a can be estimated from β_1. For $\beta_1 = 313$M^{-1}, calculation yields $K_a \sim 300$M^{-1}. Once it is known that K_a is ~ 300M^{-1} (or larger), calculation becomes superfluous, since then $K_1 K_a(k_6 + k_7' + k_9)(\mathrm{SP}) > k_3' K_i + K_a(k_7' + k_9)$, and the equation for β_1 simplifies to $\beta_1 \simeq K_a$. For $K_a \sim 300$M^{-1}, k_7' is $\sim 10^3$ min^{-1}. Of course, only the order of magnitude of the constants k_7' and K_a is significant and no conclusion about the magnitude of k_7' relative to k_9 can be drawn. However, Fig. 3 shows that k_9 must be small ($v_3 \sim 0$). Moreover, since α_1' is significantly different from zero (Table II), k_7' cannot be zero and thus water can still react with the glucosyl–enzyme–phenol complex (ES'N).

Transfer of the glucosyl residue to anilines

Theoretically, the absence of detectable transfer reactions to phenols of glucopyranosides could mean that the basic reaction mechanism is different from that for xylosides. Therefore, some preliminary tests, using anilines as the nucleophile (N), were performed.

When 1.5 u.ml^{-1} of β-D-glucosidase was added to *p*-chlorophenyl β-D-glucopyranoside (0.5mM), hydrolysis was complete (pH 6.7, 30°) within 2 min, and the theoretical amounts of *p*-chlorophenol and D-glucose were formed. When anilines or methanol were added, hydrolysis was still complete after 2 min (100% phenol), but far less than the theoretical amount of D-glucose was found (Table III). This strongly suggests the formation of *N*-aryl-D-glucopyranosylamines by transfer of the glucosyl residue to the aniline. Comparison of the data in Table III shows that (*a*) anilines are far better acceptors than methanol, (*b*) the acceptor capacity depends

TABLE III

INFLUENCE OF ANILINES ON THE HYDROLYSIS OF *p*-CHLOROPHENYL β-D-GLUCOPYRANOSIDE AT pH 6.7 AND 30°

Acceptor	*Concentration (mM)*	p-*Chlorophenol (% after 2 min)*	D-*Glucose (%)* *3 min*	*8 min*	*12 min*	*20 min*
None	—	100	100	100	100	100
Aniline	1.2	100	40	51	58	73
p-Methylaniline	1.0	101	28	30	33	36
p-Chloroaniline	1.0	99	16	17	20	26
p-Bromoaniline	1.0	100	13	20	21	30
Methanol	1,220	100	80	82	81	82

upon the substituent group, and (*c*) the transfer products are slowly hydrolysed.

It is known that *N*-aryl-D-glucopyranosylamines mutarotate and hydrolyse spontaneously[8,9]. However, a test showed that the increase in percent glucose (Table III) is mainly due to the enzymic hydrolysis of the transfer product. To a solution (0.1mM) of authentic *N*-*p*-methylphenyl-β-D-glucopyranosylamine (pH 6.7, 30°) was added β-D-glucosidase (970 mu.ml^{-1}), whereafter the absorbance at 248 nm (at this wavelength, the absorbance of the liberated aniline is negligible) was monitored. Whereas the absorbance of a reference solution without enzyme remained constant for several minutes (~10% hydrolysis after 3 h), the absorbance of the enzymic mixture decreased significantly. The initial rate of this enzymic reaction was $\sim 7 \times 10^{-9}$ mol.min^{-1}.u^{-1}, which is more than 100 times lower than the initial rate for aryl β-D-glucopyranosides under comparable conditions[2]. Thus, whereas anilines are better entering-groups than phenols in the transfer reactions, phenoxy groups are better leaving-groups than aniline aglycons in the hydrolysis reaction.

The experiments with anilines indicate that the absence of transfer reactions to phenols does not mean that the basic mechanism for glucosides is different from that for xylosides. When anilines, which are better nucleophiles than phenols[10,11], are used, transfer activity can be detected for glucosides.

Product inhibition during the hydrolysis of p-*nitrophenyl* β-D-*xylopyranosides*

Since added phenols inhibit the enzymic hydrolysis of aryl β-D-gluco- and β-D-xylo-pyranosides, the phenols liberated during hydrolysis of the substrate must inhibit the reaction. The degree of this product inhibition depends on the substituent group and is very strong for nitrophenyl glycosides. Table IV shows the effect of various concentrations of *p*-nitrophenol on the enzymic hydrolysis of *p*-nitrophenyl β-D-xylopyranoside. The initial rates v_1 and v_2 refer to a 5mM substrate concentration. From the dependence of $[k_{cat1} \times K_{app}]$ on *p*-nitrophenol, the competitive inhibition constant $K_i = 3000\text{M}^{-1}$ was calculated. The affinity of *p*-nitrophenol for the free enzyme is much higher than that of *p*-chlorophenol ($K_i = 460\text{M}^{-1}$) or *p*-methoxy-

TABLE IV

INFLUENCE OF *p*-NITROPHENOL ON THE HYDROLYSIS OF *p*-NITROPHENYL β-D-XYLOPYRANOSIDE AT pH 6.7 AND 30°

p-*Nitrophenol* (mM)	10^6 v_1 (mol.min^{-1}.u^{-1})	10^6 v_2 (mol.min^{-1}.u^{-1})	10^6 k_{cat1} (mol.min^{-1}.u^{-1})	K_{app} (M^{-1})
0	0.244	0.242	0.245 ±0.006	5,760 ±650
0.02	0.221	0.200	0.223 ±0.003	6,400 ±450
0.03	—	—	0.210 ±0.001	5,755 ±215
0.04	0.189	0.188	0.195 ±0.004	6,490 ±675
0.06	0.173	0.177	0.179 ±0.003	6,013 ±600
0.08	0.161	0.164	0.165 ±0.004	6,238 ±830
0.10	0.146	0.144	0.152 ±0.002	7,350 ±560

phenol (K_i = 70M^{-1}). Again, the decrease in k_{cat1} indicates that *p*-nitrophenol also inhibits the reaction by binding to ES′ (and probably more strongly than the two other phenols). Formally, Scheme 1 can be used to explain product inhibition; in the special cases when added and liberated phenol are the same (N = P) or when no extra phenol is added, the lower part of Scheme 1 is superfluous, as it is then merely the reverse of the upper part.

DISCUSSION

The aglycon group of aryl β-D-glucopyranosides and β-D-xylopyranosides binds through hydrophobic forces to an "aglycon site" on β-D-glucosidase from *Stachybotrys atra*[2,3]. The observed, competitive inhibition by phenols indicates that phenols ("free aglycon groups") also bind to that site. The binding constants (K_i) of these hydrophobic aromatic compounds are much larger than those of lower alcohols[3] and depend upon the substituent group in the order *p*-nitro > *p*-chloro > *p*-methoxy. Since the aglycon site of the glycosyl–enzyme complex (ES′) is unoccupied, phenols can also bind to this complex. However, this type of binding (ES′N) will be detectable only if the reaction step 3′ is sufficiently slow. Otherwise, the complex ES′ would react immediately with water and binding of the phenol would be impossible. The overall step 3′ consists of several steps (not shown in Scheme 1, but analogous to the sequence from ES′ to E + SN): binding of a water molecule (ES′W), transfer of the glycosyl residue to water (ESW), and desorption of glucose (or xylose). It is not known which of these steps determines the overall rate of step 3′. However, since the concentration of water is always much larger than that of the phenol, it is difficult to see how a phenol, in competition with water, could bind to ES′ if the aglycon site were freely accessible for water. Thus, we think that the main reason for the low rate of step 3′ is the fact that the equilibrium ES′ + W ⇄ ES′W lies to the left, because of the hydrophobic nature of the aglycon site. Only then can hydrophobic molecules, even when present in low concentration, compete favourably with water. These two basic assumptions, namely, (*a*) the high affinity of the phenols for the aglycon site and (*b*) the slow 3′-step, are sufficient to explain the effects of phenols on the enzymic reaction.

When the concentration of P increases, the quasi-equilibrium ES′P ⇄ ES′ + P will shift to the left and decrease the net rate at which P is formed. The magnitude of this effect depends on the difference in Gibbs free-energy $\Delta G^0_{-6'} = G°(ES'P) - G°(ES' + P)$ and thus mainly on the hydrophobicity of the phenol. If, for *p*-methoxyphenol, K_a = 300M^{-1} is taken as a good estimate of the association constant $k_{-6'}/k_{6'}$, then $\Delta G^0_{-6'} \simeq -14$ kJ.mol^{-1}. Since even small concentrations (~mM) of phenol are always larger than the actual concentration of ES′, step 6′ will be reversible. The overall reaction constant k_3' for the transfer of a glucosyl residue in ES′ to water is 33 sec^{-1} (see Results) and thus the experimental free-energy of activation ($\Delta G^{\ddagger}$ from ES′ to a theoretical transition-complex) of step 3′ is +64 kJ.mol^{-1}. Consequently, product inhibition can be caused, for glucosides as well as for xylosides, by the binding

of the phenol, liberated during the hydrolysis, to (*a*) the free enzyme (EP equivalent with EN) and (*b*) the ES′ complex. It is the back-formation of ES′P (equivalent with ES′N) that causes the decrease in k_{cat1} (Tables I and IV).

However, for xylopyranosides, the experiments also indicate that steps 2 and 1 must be reversible. The reversibility of step 2 is determined by the ratio k_2/k_{-2}: the smaller the ratio, the more step 2 will be reversible (*i.e.*, proceed to the left in scheme 1). If the ratio is large enough to make step 2 virtually irreversible, the liberated phenol will still inhibit the reaction (because of ES′N and EN), but no radioactive substrate will be detected when a radioactive phenol is added. For aryl glucosides, the net rate of the glycosylation step (E + SN to ES′ + P) is higher than for aryl xylosides[2,3]. Since it is improbable that the desorption/adsorption step (k'_6/k'_{-6}) depends on the glycon structure, the ratio k_2/k_{-2} must also be larger for glucosides than for xylosides. Thus, the absence of radioactive glucosides is probably a consequence of this larger ratio.

Since, for *p*-nitrophenyl β-D-xylopyranoside and all aryl β-D-glucopyranosides tested, the glycosylation step is not rate-limiting[2,3], the reaction of an added phenol with ES′ may result in either an increase or a decrease in the initial rate (v_1) of these substrates. Again, the net effect will depend on (*a*) the affinity (K_i and k_5/k_{-5}) of the phenol for the aglycon site and (*b*) the ratios k'_{-2}/k'_2 and k'_{-1}/k'_1 (Scheme 1). The higher the affinity and the smaller the ratios (especially k'_{-2}/k'_2), the more the phenol will behave as an inhibitor and block enzyme molecules as EN and ES′N complexes. If the ratio k'_{-2}/k'_2 is small enough, inhibition but not transfer will be observed. However, the larger the ratio, the more the net rate for the formation of SN (thus also for the regeneration of free enzyme) will increase and the more the steady-state concentration of ES′N will decrease. When the ratio is sufficiently large, the rate v_1 will increase with increasing concentration of the phenol.

Our data do not allow a quantitative separation of the two factors (affinity and ratios) that will determine the overall effect of a phenol, but some qualitative comparisons can be made. For transfer of the xylosyl residue to phenols, two kinds of behaviour have been observed (Figs. 1–2). The competitive inhibition constants indicate that *p*-chlorophenol (K_i = 460M^{-1}) has an affinity for the free enzyme, and thus probably also for the ES′ complex, that is higher than that of *p*-methoxyphenol (K_i = 70M^{-1}). On the other hand, the net rate of the glycosylation step (and thus probably also k_2/k_{-2}) for *p*-chlorophenyl β-D-xylopyranoside is higher than that for *p*-methoxyphenyl β-D-xylopyranoside, because electron-withdrawing substituents increase the rate of that step[2,3]. Thus, when the phenols are used as acceptors, the ratio k'_{-2}/k'_2 ($=k_{-2}/k_2$ for the corresponding xylosides) will be smaller for *p*-chlorophenol (σ_H = 0.24)[12] than for *p*-methoxyphenol (σ_H = −0.11)[12]. The experimental results (Fig. 1) show that, for *p*-chlorophenol, the combination of high affinity and small ratio indeed results in an inhibitory effect of the phenol, although the ratio k'_{-2}/k'_2 is still large enough to allow a detectable transfer reaction. For *p*-methoxyphenol, the lower affinity and larger ratio results in an activating effect. Now the

transfer reaction is predominant and increases the rate at which the free enzyme is regenerated (Fig. 2).

For *p*-nitropheny' and *p*-methoxyphenyl β-D-glucopyranoside, the effect of *p*-methoxyphenol is purely inhibitory (Fig. 3). As mentioned before, the ratio k_2/k_{-2} is larger (and thus k'_{-2}/k'_2 is smaller) for aryl glucosides than for aryl xylosides. Thus, the reason that no transfer reaction can be observed is probably too small a ratio k'_{-2}/k'_2 (Fig. 3). The experiments with anilines (Table III) indicate that transfer of the glucosyl moiety in ES'N does occur if acceptors with sufficiently large k'_{-2}/k'_2 ratios are used

The complex effect of phenols on the hydrolysis of aryl glycopyranosides can be explained by the reaction sequence depicted in Scheme 1. Only two simple and reasonable assumptions must be made: (*a*) ES' is a relatively long-living complex, because step 3' is slow; (*b*) phenols have a much higher affinity for the active site of the enzyme than water. If these assumptions are correct, the glycosylation step must be partially reversible. The extent to which the step is actually reversible is determined by the relative magnitude of rate and binding constants, depending on both the glycon and aglycon parts of the substrate.

The aforementioned effects of phenols, especially the non-competitive inhibition, would be difficult to explain, if kinetically significant amounts of ES'N and ES'P complexes did not exist. This is the reason why these complexes were included in Scheme 1.

Further work is now in progress to study these effects on a more quantitative basis.

APPENDIX

When the concentration of the nucleophile N is kept constant, the steady-state equation derived from Scheme 1 has the form,

$$v_i = v_i/E_t = \frac{k_{cat} K_{app} (SP)}{1 + K_{app} (SP)}.$$

When the initial rate of aglycon release (v_1) is measured, k_{cat} and K_{app} are given by the following equations.

$$k_{cat} = \frac{k'_3 k_6 + K_a k_6 (k'_7 + k_9) (N)}{k'_3 + k_6 + K_a (k_6 + k'_7 + k_9) (N)}$$

$$K_{app} = \frac{K_1 (k'_3 + k_6) + K_1 K_a (k_6 + k'_7 + k_9) (N)}{k'_3 + [K_i k'_3 + K_a (k'_7 + k_9)] (N) + K_i K_a (k'_7 + k_9) (N)^2}$$

When the initial rate of glycon release (v_2) is measured, K_{app} remains unchanged but, in the equation for k_{cat}, the constant k_9 must be deleted in the numerator.

For a constant concentration of substrate (SP), the influence of (N) on the initial rate v_1 is given by an equation of the form,

$$v_1 = v_1'/E_t = \frac{\alpha_0 + \alpha_1 (N)}{1 + \beta_1(N) + \beta_2(N)^2}.$$

When v_2 is measured, α_1 must be substituted by α_1'. The meaning of α_0, α_1, α_1', β_1, and β_2 is given in Table II.

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