The effect of glycerol on the activity of β -glucosidase from *Botryodiplodia theobromae* Pat.

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1. In the activity of the high- M_r β -glucosidase A (β -D-glucoside glucohydrolase, EC 3.2.1.21) obtained from culture filtrates of *Botryodiplodia theobromae* Pat. on *o*-nitrophenyl β -D-glucopyranoside as substrate, both V_{max} and K_m increased non-linearly with increasing concentration of glycerol, and the $V_{max}/K_{m(app.)}$ ratio decreased non-linearly with increasing concentration of glycerol. 2. No increase in rate was observed with phenyl β -D-glucopyranoside as substrate in the presence of up to 250 mM-glycerol, indicating that glucosylation is rate-limiting with this substrate. 3. With *o*-nitrophenyl β -D-glucopyranoside, *p*-nitrophenyl β -D-glucopyranoside and phenyl β -D-glucopyranoside as substrates, k_{cat} values of 793.7 s⁻¹, 62.8 s⁻¹ and 5.4 s⁻¹ respectively were calculated. 4. With *o*-nitrophenyl β -D-glucopyranoside and phenyl β -D-glucopyranoside as substrate, α -deuterium kinetic isotope effects of 1.9 ± 0.03 and 1.01 ± 0.01 respectively were found; in the presence of 200 mM-glycerol the values were 1.21 ± 0.03 and 1.02 ± 0.01 respectively. 5. In the presence of a large excess of *o*-nitrophenyl β -D-glucopyranoside ($[S] = 35.7 K_m$), the amount of *o*-nitrophenol and also of the transglucosylation product formed by β -glucosidase action increased non-linearly, whereas that of glucose formed decreased non-linearly with increasing glycerol concentration. 6. All these results were found to fit the data calculated from rate equations derived on the basis of the proposed mechanism of enzyme action involving two ion-pair intermediates and a covalent α -D-glucosyl-enzyme in the reaction sequence [Umezurike (1987) *Biochem. J.* **241**, 455–462].

INTRODUCTION

On the basis of studies on the high- M_r β -glucosidase (β -glucosidase A) from *Botryodiplodia theobromae* Pat., a mechanism of enzyme action involving a solvent-separated ion-pair of glucosyl cation and carboxylate anion, an intimate ion-pair and a covalent α -D-glucosyl-enzyme has been proposed (cf. Umezurike, 1987). The involvement of two ion-pair species was confirmed by the observation that the slight stimulation of enzymic activity by low concentrations of dioxan and the rapid decrease in enzymic activity in the presence of higher concentrations of dioxan when a dilute buffer system was used were both mitigated when a concentrated buffer system was used (Umezurike, 1987). The solvent-separated ion-pair was postulated to react with added glycosyl acceptors in transglucosylation, in agreement with the observation that solvent-separated ion-pairs are more susceptible to attack by added nucleophilic reagents than are intimate ion-pairs (cf. Finar, 1975), whereas the intimate ion-pair was postulated to react preferentially with water in hydrolysis (cf. Umezurike, 1981, 1987). The geometry of both ion-pair species, relative to that of the substrate, is inverted at C-1 so that attack by the nucleophile from the opposite direction in either transglucosylation or hydrolysis ensures the retention of the configuration of the anomeric carbon atom relative to that of the substrate.

Double-reciprocal plots of the data obtained in the presence of various concentrations of a number of efficient glucosyl acceptors were linear and intersected to the right of the vertical axis (Umezurike, 1975, 1987).

In the present paper, the effect of glycerol on kinetic parameters and on the partitioning of products, and the α -deuterium kinetic isotope effects in the presence or absence of glycerol, are discussed in relation to the proposed mechanism of enzyme action, and information on the rate-limiting step is provided.

MATERIALS AND METHODS

The purification and the assay of β -glucosidase activity in 50 mM-sodium acetate/acetic acid buffer, pH 5.0, were carried out as reported previously (Umezurike, 1971, 1979, 1981) except that with phenyl β -D-glucopyranoside as substrate the absorbance of assay mixtures was determined at 290 nm (Dahlquist *et al.*, 1969).

Determination of glucose and the transglucosylation product formed in the presence of glycerol, and the syntheses of phenyl β -D-[1-²H]glucopyranoside and *o*-nitrophenyl β -D-[1-²H]glucopyranoside, were carried out as described previously (Umezurike, 1981).

RESULTS AND DISCUSSION

Effect of glycerol on kinetic parameters

In the presence of increasing concentrations of glycerol both V_{max} and K_m increased non-linearly when *o*-nitrophenyl β -D-glucopyranoside was used as substrate (Fig. 1). The experimentally determined values of V_{max} and K_m were found to fit satisfactorily the data calculated from eqn. (1) which was derived from Scheme 1 (Fig. 1):

$$\frac{v}{[E]_0} = \frac{\left(k_H + \frac{k_{+2}[A]}{K_{A1}}\right) / \left(1 + \frac{[A]}{K_{A1}}\right)}{1 + \frac{K_m}{[S]} \left(\frac{(1 + [A]/K_{A2})}{(1 + [A]/K_{A1})}\right)} \quad (1)$$

Similar effects have been observed when the substrate was *p*-nitrophenyl β -D-glucopyranoside (Umezurike, 1981, 1987), and in β -galactosidase-catalysed reactions in the presence of increasing concentrations of methanol (Sinnott & Viratelle, 1973). The non-linear increases in V_{max} and K_m in the presence of methanol in β -galactosidase-catalysed reactions have been attributed to

Fig. 1. Plots of $V_{\max}$ and $K_{m(\text{app.})}$ against the concentration of glycerol as added glucosyl acceptor

The points for $V_{\max}$ (●) and K_m (○) were obtained experimentally whereas the lines were calculated from eqn. (1), assuming that $k_H = 33$ nmol/min, $k_{+2} = 62$ nmol/min, $K_{A1} = 300$ mM, $K_{A2} = 85$ mM and $K_m = 0.3$ mM.

Scheme 1. Reaction scheme for β -glucosidase-catalysed hydrolysis and transglucosylation in the presence of an added efficient glucosyl acceptor (A)

E, $EG^+ \parallel C^-$, $EG^+ C^-$ and $EG-C$ represent the free enzyme, the solvent-separated glucosyl cation-carboxylate ion-pair, the intimate ion-pair and the covalent α -D-glucosyl-enzyme respectively. The rate constant $k'_{+6} = k_{+6}[\text{H}_2\text{O}]$, but since the concentration of water is very high (55 M) $k_{+6}[\text{H}_2\text{O}]$ is regarded as a constant, k'_{+6} .

'medium effect' (Sinnott & Viratelle, 1973). The equations for k_H , K_{A1} , K_{A2} and K_m have been given previously (Umezurike, 1987). These parameters and k_{+2} can be calculated as described previously (Umezurike, 1987). The equation for $V_{\max}$ (eqn. 2) shows that the effect of any glucosyl acceptor will depend on the ratio k_{+2}/k_H :

$$V_{\max} = \frac{k_H(1 + k_{+2}[A]/k_H K_{A1})}{(1 + [A]/K_{A1})} \quad (2)$$

Since k_H is the maximum rate in the absence of the added glucosyl acceptor A, $k_{+2} > k_H$ (and thus $k_{+2} > k'_{+6}$) if deglucosylation is the rate-limiting step, and $V_{\max}$ will increase non-linearly with increasing concentration of the added effective glucosyl acceptor A. On the other hand, if glucosylation is rate-limiting, $k_{+2} = k_H$ (and thus $k_{+2} \leq k'_{+6}$) and $V_{\max}$ will remain unaffected in the presence of increasing concentrations of the added glucosyl acceptor. Eqn. (1) also predicts that, irrespective of whichever step is rate-limiting (glucosylation or deglucosylation), K_m is increased non-linearly in the presence of

added glucosyl acceptor as shown in eqn. (3) since $K_{A1} > K_{A2}$ (cf. Umezurike, 1987):

$$K_{m(\text{app.})} = \frac{K_m(1 + [A]/K_{A2})}{(1 + [A]/K_{A1})} \quad (3)$$

A plot of $V_{\max}/K_{m(\text{app.})}$ against the corresponding concentrations of glycerol shows a non-linear decrease in the ratio $V_{\max}/K_{m(\text{app.})}$ with increasing concentration of glycerol (Fig. 2), in consonance with eqn. (4) since $K_{A1} > K_{A2}$:

$$\frac{V_{\max}}{K_{m(\text{app.})}} = \frac{k_H}{K_m} \left(\frac{(1 + k_{+2}[A]/k_H K_{A1})}{(1 + [A]/K_{A2})} \right) \quad (4)$$

As is apparent in eqns. (2) and (3), the effect of 250 mM-glycerol was to increase $V_{\max}$ by a factor of 1.4-fold and K_m by a factor of 2.2-fold (Fig. 1).

The increases in $V_{\max}$ and $K_{m(\text{app.})}$ calculable from eqn. (1), as is apparent in Fig. 1, indicate that deglucosylation is predominantly rate-limiting in β -glucosidase activity with *o*-nitrophenyl β -D-glucopyranoside as substrate, as is also the case with *p*-nitrophenyl β -D-glucopyranoside as substrate (Umezurike, 1981, 1987). The Michaelis constants (K_m) for both substrates are similar (0.30 ± 0.03 mM) and the aglycone pK_a values are also similar (7.17 for the former and 7.15 for the latter; Sinnott & Souhard, 1973). It is against chemical expectation that the mere hydrolysis of a glucosyl cation-carboxylate ion-pair should be the slowest step in the hydrolysis of the substrate unless, as is shown in the sequence of reactions in Scheme 1, there is a reversible collapse of the glucosyl cation-carboxylate ion-pair into a preponderance of the unreactive covalent glucosyl-enzyme (cf. Sinnott & Souhard, 1973; Umezurike, 1987). This is consistent with the effect of dioxan on the hydrolysis of *o*-nitrophenyl β -D-glucopyranoside, which indicates that dioxan causes the collapse of the glucosyl cation-carboxylate ion-pair into an unreactive covalent glucosyl-enzyme (Umezurike, 1979).

Contrary to the effect of glycerol on the activity of β -glucosidase on *o*-nitrophenyl β -D-glucopyranoside and *p*-nitrophenyl β -D-glucopyranoside, no increase in rate was observed in the presence of up to 250 mM-glycerol

Fig. 2. Plot of $V_{\max}/K_{m(\text{app.})}$ against the concentration of glycerol

The points were derived from experimental data whereas the line was calculated from eqn. (1) with the data presented in the legend to Fig. 1.

when the substrate was phenyl β -D-glucopyranoside, indicating that with this substrate $k_{+2} = k_H^-$ and thus glucosylation is rate-limiting. The values of k_{cat} calculated from the results obtained with various substrates were 793.7 s^{-1} , 62.8 s^{-1} and 5.4 s^{-1} for *o*-nitrophenyl β -D-glucopyranoside, *p*-nitrophenyl β -D-glucopyranoside and phenyl β -D-glucopyranoside ($K_m = 1.1 \text{ mM}$) respectively. The much larger value of k_{cat} for the *o*-substituted substrate in comparison with the other substrates and the much lower value for phenyl β -D-glucopyranoside in relation to the others provide sustaining evidence for the deductions made from the effect of glycerol on rate. It can be inferred from eqn. (2) that, if k_{+2} is only slightly larger than k_H^- , the increase in V_{max} with increasing concentration of glycerol would be small as was observed in studies with *p*-nitrophenyl β -D-glucopyranoside (cf. Umezurike, 1981). This effect indicates that deglucosylation is only partly rate-limiting. There will thus be a progressive change in the rate-limiting step from deglucosylation to glucosylation as the value of the ratio k_{+2}/k_H^- decreases towards unity (i.e. as the rate of expulsion of the aglycone of substrates becomes slower). Indeed, the plot of V_{max} against [glycerol] shown in Fig. 1 is that characterized by a process in which $k_{+2}/k_H^- = 1.88$.

In view of the principle of microscopic reversibility, it is apparent in Scheme 1 that the nucleophilic attack of an alcohol on the solvent-separated glucosyl cation-carboxylate ion-pair or of water on the intimate ion-pair would be general-base-catalysed. Thus the active-site functional group (aspartate β -carboxylate group, $pK_a = 3.91$) would be easily displaced from the ion-pair by strongly basic nucleophilic groups in S_N1 reactions *ipso facto*.

If the covalent α -glucosyl-enzyme intermediate (being an ester) were susceptible to nucleophilic hydrolysis, this could involve the nucleophilic attack on the carbonyl carbon atom and thus would introduce some complications into the proposed mechanism (cf. Legler, 1973). In consonance with the proposed mechanism, therefore, the tendency of the enzyme to revert to the free-enzyme conformation (relief of steric strain) coupled with the stabilizing effect of the ring oxygen atom on the developing carbonium ion would ensure the production of the intimate ion-pair from the preponderant covalent α -glucosyl-enzyme for the deglucosylation process in hydrolysis.

Kinetic isotope effect

The α -deuterium kinetic isotope effects (k_H/k_{2H}) of 1.09 ± 0.03 and 1.01 ± 0.01 have been observed in the present study for *o*-nitrophenyl β -D-glucopyranoside and phenyl β -D-glucopyranoside respectively. In the presence of 200 mM-glycerol the α -deuterium kinetic isotope effects of 1.21 ± 0.03 and 1.02 ± 0.01 were found for *o*-nitrophenyl β -D-glucopyranoside and phenyl β -D-glucopyranoside respectively. These results are consistent with the involvement in the deglucosylation process of a solvent-separated glucosyl cation-carboxylate ion-pair that reacts with glycerol, an intimate ion-pair that reacts with water and a preponderance of an unreactive covalent α -glucosyl-enzyme in equilibrium as shown in Scheme 1. The observed isotope effects would thus be a product of two processes: a direct isotope effect on the equilibrium and an inverse isotope effect on the hydrolysis or glycerolysis of the ion-pairs (cf. Sinnott *et al.*, 1978).

Thus the high value of the α -deuterium kinetic isotope effect observed in the presence of glycerol also reflects the effect of a rapid reaction of glycerol with the solvent-separated ion-pair as it is generated, as this would diminish the magnitude of ion-pair collapse since glycerol is much more nucleophilic than water towards the glucosyl cation (cf. Umezurike, 1981, 1987). The virtual constancy of the α -deuterium kinetic isotope effect close to unity in the absence and in the presence of glycerol when the substrate was phenyl β -D-glucopyranoside reflects the significance of electrostatic stabilization (by the carboxylate group) of the developing glucosyl cation in the hydrolysis of substrates with poor leaving groups.

Dahlquist *et al.* (1969) have also reported an α -deuterium kinetic isotope effect of 1.01 ± 0.01 for the hydrolysis of phenyl β -D-glucopyranoside by sweet-almond β -glucosidase, and this, taken together with the results obtained by Nath & Rydon (1954), indicates a random variation of k_{cat} with aglycone acidity for the same enzyme. An α -deuterium kinetic isotope effect of around unity for the 'slower' substrates and the substantially random variation of k_{cat} values with aglycone acidity are in consonance with the view that substrate binding is followed by a fast-equilibrium protonation step and by a rate-determining loss of an alcohol to generate a cyclic carbonium ion (glucosyl cation) that is stabilized by a carboxylate counter-ion, the protonation step being facilitated by a protein conformation change that ensures the correct orientation of catalytic groups (cf. Sinnott & Souchart, 1973). Bond-breaking may thus be assisted by the 'electrostatic strain' imposed on the substrate in the hydrolysis of substrates with poor leaving groups (aglycones).

Analysis of products

With a large excess ($[S] = 35.7 K_m$) of *o*-nitrophenyl β -D-glucopyranoside as substrate, the amount of *o*-nitrophenol and glucose liberated by β -glucosidase action in the presence of increasing concentrations of glycerol were determined, and the amount of the transglucosylation product formed (1-glyceryl β -D-glucoside; Umezurike, 1975) was calculated from the difference. As shown in Fig. 3, the results are similar to the data calculated from eqn. (1). The profiles of the products in Fig. 3 are different from those expected if glucosylation is rate-limiting in the absence of ion-pair collapse, since the liberation of *o*-nitrophenol increased with increase in glycerol concentration. Also, the results are different from those expected if deglucosylation is rate-limiting in the absence of ion-pair collapse, since the amount of glucose liberated decreased with increasing glycerol concentration (cf. Fersht, 1977) and both *o*-nitrophenol and the transglucosylation product increased in amount non-linearly with increasing glycerol concentration. Thus the results of Fig. 3 indicate that deglucosylation is rate-limiting with the substrate used in a system involving ion-pair collapse as shown in Scheme 1, but with k_{+2} being only slightly larger than k_H^- (cf. eqn. 2).

Jermyn (1966, 1969) has observed that polyhydric substances containing non-polar groups (e.g. benzyl β -D-fructopyranoside, methyl β -D-fructopyranoside, 6-*O*-methylglucose and methyl 6-*O*-methylglucoside) bind only at the acceptor centre of β -glucosidase from the fungus *Stachybotrys atra* without being able to function

Fig. 3. Effect of increasing concentration of glycerol on the rate of production of *o*-nitrophenol (●), glucose (○) and a transglucosylation product (△) by β -glucosidase

The assay mixture contained 10 mM-*o*-nitrophenyl β -D-glucopyranoside (i.e. $[S] = 35.7 K_m$). The points were experimental data whereas the lines were calculated from eqn. (1) assuming that $k_H = 6.56$ nmol/min, $k_{+2} = 13$ nmol/min, $K_m = 0.28$ mM, $K_{A1} = 300$ mM and $K_{A2} = 85$ mM.

as glucosyl acceptors and thus give rise to uncompetitive inhibition. With reference to Scheme 1, the interaction of a dead-end inhibitor (I) with the solvent-separated ion-pair intermediate can be represented as:

where $EG^+(I) \parallel C^-$ is the dead-end intermediate. The modified scheme (cf. Scheme 1) gives a rate equation showing that both V_{max} and K_m are divided by the same factor $(1 + [I]/K_i)$ to give uncompetitive inhibition, where:

$$K_i = \frac{k_{-5}\{k_{+3}k_{-4}k'_{+6} + k_{+2}[k_{-4}(k_{-3} + k'_{+6}) + k_{+3}(k_{-4} + k_{+4})]\}}{k_{+2}k_{-4}k_{+5}(k_{-3} + k'_{+6})} \quad (5)$$

The observed uncompetitive inhibition by substances containing non-polar groups indicates a low polarity of the acceptor site of the enzyme following the release of the leaving group (aglycone). This would favour the subsequent generation of the 'tight' conformation of the intimate ion-pair and its collapse into the covalent α -glucosyl-enzyme intermediate in order to shield the exposed hydrophobic centre from the aqueous medium.

Water would thus become an effective glucosyl acceptor when the hydrophobic centre has been shielded by the generation of the intimate ion-pair. This postulation would explain the much higher nucleophilicity of glycerol (and especially sucrose and fructose, which both elicit their effects at much lower concentrations than glycerol; Umezurike, 1981, 1987) than water, which is present in large excess (55 M).

β -Glucosidase-catalysed hydrolysis of *o*-nitrophenyl β -D-glucopyranoside proceeds with retention of configuration at the anomeric carbon atom (Umezurike, 1978). On this account, the failure of the solvent-separated ion-pair to collapse under the influence of the carboxylate anion to release α -D-glucose (cf. Umezurike, 1987), the formation of the intimate ion-pair and then the covalent α -D-glucosyl-enzyme, and the preference of water for the intimate ion-pair, rather than the solvent-separated ion-pair, would all derive from the same molecular event: the displacement of the water molecules bound to the carboxylate anion by the decrease in the polarity of the micro-environment of the enzyme acceptor site, apparently because of the exposure of a hydrophobic centre following the departure of the aglycone.

These considerations indicate that the mechanism proposed for the action of β -glucosidase from *B. theobromae* can provide satisfactory explanations for the puzzling observations often made in the action of many glucosidases.

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