Biochem. J. (1978) **175**, 455–459
Printed in Great Britain

The β-Glucosidase from *Botryodiplodia theobromae* Pat.

KINETICS OF ENZYME-CATALYSED HYDROLYSIS OF *o*-NITROPHENYL β-D-GLUCOPYRANOSIDE IN DIOXAN/WATER

By GABRIEL M. UMEZURIKE
Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

(*Received* 26 *January* 1978)

1. The hydrolysis of *o*-nitrophenyl β-D-glucopyranoside by the high-molecular-weight β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from *Botryodiplodia theobromae* Pat. has been studied in the presence of added dioxan. 2. At donor saturation, the maximum rate of hydrolysis in the presence of up to 50% (v/v) dioxan was at pH 4.3–4.5 (pH of the buffer system in water) in McIlvaine's buffer. 3. Increasing dioxan concentrations progressively decreased the maximum rate of hydrolysis. 4. The rate of enzyme-catalysed reaction was enhanced at high donor concentrations, but inhibited at low donor concentrations, in the presence of glycerol, methanol, fructose or sucrose. 5. The hydrolytic reaction was found to proceed with retention of configuration at the anomeric carbon atom. 6. The kinetics of the enzyme-catalysed process in the presence of added acceptors indicated that water was necessary for the maintenance of the active enzyme conformation apart from its acceptor function.

Findlay *et al.* (1962*b*) have outlined possible effects of inert solvents on enzyme-catalysed reactions. Studies on the hydrolytic reaction catalysed by the enzyme β-glucosidase from the fungus *Botryodiplodia theobromae* Pat. have been carried out in the presence of added dioxan with a view to identifying the functional groups at the active site of this enzyme (Umezurike, 1977). The effects of dioxan addition on β-glucosidase-catalysed hydrolysis of *o*-nitrophenyl β-D-glucopyranoside in neutral acid buffers and cationic acid buffers indicated that changes in the p*K* values of the groups at the active site of the enzyme and changes in the concentration of water are prominent factors in the activity of this enzyme (Umezurike, 1977). In this paper, studies of the effect of dioxan on β-glucosidase activity under conditions that permit the evaluation of the effect of changes in water concentration are reported. The results are discussed in terms of the mechanism of the hydrolytic reaction catalysed by β-glucosidase as not much is known of the mechanism of action of this enzyme, although the possible involvement of a glucosyl-enzyme (Legler, 1968) or of a ternary enzyme–donor–acceptor complex (Jermyn, 1957, 1962) has been proposed.

Experimental

Materials

Organism. B. theobromae (I.M.I. 115626, A.T.C.C. 26123) was originally isolated from decaying wood (Umezurike, 1969).

Chemicals. o-Nitrophenyl β-D-glucopyranoside was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K.

Buffers. The buffer used for initial-velocity studies was McIlvaine's 0.1 M-citric acid/0.2 M-Na_2HPO_4 buffer (McIlvaine, 1921). The pH values quoted for buffer mixtures containing dioxan were the pH of the same buffer systems in water alone (cf. Findlay *et al.*, 1962*b*).

Concentration of reagents. The reagent concentrations quoted were final concentrations in the reaction mixtures.

Enzyme assay, preparation and purification

β-Glucosidase activity was determined as previously described (Umezurike, 1971*a*) with *o*-nitrophenyl β-D-glucopyranoside as substrate (glucosyl donor). Activity was expressed as nmol of *o*-nitrophenol liberated/min at 40°C.

The high-molecular-weight β-glucosidase used in this work was obtained from culture filtrates of *B. theobromae* and purified as previously described (Umezurike, 1971*a*, 1975*b*).

General methods

Polarimetric determination of the anomeric product. Optical rotation was measured with a Bellingham and Stanley polarimeter model A type P1, by using a 20cm-path-length cell and β-D-glucopyranosyl fluoride (10mM) in 0.05M-sodium acetate/acetic acid buffer (pH 5.0) as substrate for β-glucosidase (Barnett, 1971).

Gel filtration. Gel filtration on a column of Sephadex G-200 was carried out as described previously (Umezurike, 1976).

Paper chromatography. Paper chromatography and detection of carbohydrate spots were carried out as described previously (Umezurike, 1971*b*).

Results and Discussion

Effect of dioxan

The effects of dioxan on the pH (in water)–activity curves of β-glucosidase-catalysed hydrolysis of *o*-nitrophenyl β-D-glucopyranoside (donor) at donor saturation in the presence of neutral acid and cationic acid buffers have been reported (Umezurike, 1977). Further evidence indicated that at donor saturation ($[S] = 50K_m$) the maximum rate of β-glucosidase activity in the presence of up to 50% (v/v) dioxan was always at pH (in water) 4.3–4.5 in McIlvaine's buffer. The effect of dioxan on the activity of β-glucosidase in McIlvaine's buffer at pH4.5 (value in water alone) in the presence of saturating concentration of *o*-nitrophenyl β-D-glucopyranoside ($[S] = 50K_m$) is shown in Fig. 1. When the concentration of water in the assay mixtures was calculated on the assumption that in the absence of added dioxan the concentration of water was 55M, and the results were plotted as initial velocity (v) against the concentration of water, the plot obtained was non-linear (concave up).

Under these assay conditions, β-glucosidase activity appeared to be more sensitive to dioxan than was found in trypsin-catalysed hydrolysis of benzoyl-L-arginine ethyl ester, in which 68% of the rate in water was found in the presence of 88% (v/v) dioxan (Inagami & Sturtevant, 1960).

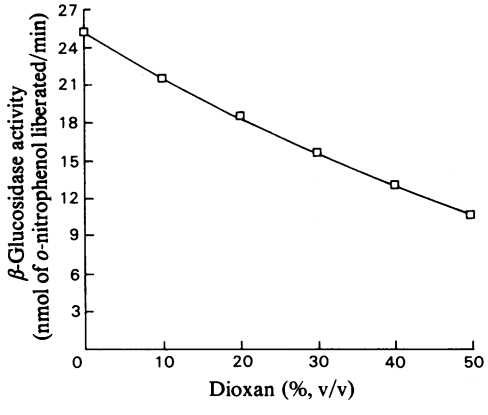


Fig. 1. *Effect of dioxan concentration on the rate of β-glucosidase-catalysed hydrolysis of o-nitrophenyl β-D-glucopyranoside*

The final substrate concentration in assay mixtures was such that $[S] = 50K_m$. Enzyme activity was determined at pH4.5 (pH of the buffer in water) in McIlvaine's buffer.

The possibility that β-glucosidase may dissociate into lower-molecular-weight species with different kinetic parameters in the presence of dioxan, as the enzyme can exist in differently aggregated molecular species (Umezurike, 1975*b*), was also examined. Incubation of the high-molecular-weight species of the enzyme at 30°C for 30min at pH4.5 in the presence of 50% (v/v) dioxan did not lead to the formation of lower-molecular-weight species of the enzyme on gel filtration on Sephadex G-200. The polymerization of lower-molecular-weight species into the high-molecular-weight species of β-glucosidase from the giant African snail (*Achatina achatina*) has been found to be energy-dependent (Umezurike, 1976).

The data obtained from initial-velocity studies carried out in the presence of up to 30% (v/v) dioxan at pH4.5 (value in water) gave non-linear (concave up) Lineweaver–Burk plots (Lineweaver & Burk, 1934) when plotted as $1/v$ against 1/[water], but gave linear plots that intersected at a point to the right of the vertical axis when replotted as the reciprocal of velocity against the reciprocal of the cube of water concentration ($1/v$ against $1/[water]^3$). Plots of the same data as $1/v$ against 1/[donor] were linear and intersected to the left of the vertical axis. With respect to the donor, dioxan addition thus increased the Michaelis constant (K_m), but decreased the maximum velocity ($V_{max.}$), as was reported previously (Umezurike, 1977). Apart from the direct effect of changes in water concentration on velocity, some of these effects may be conveniently attributed to 'medium effect'. The 'medium effect' may be pictured as resulting from a decrease in the hydration of a site on the enzyme protein as the concentration of dioxan is increased.

Effect of added acceptors

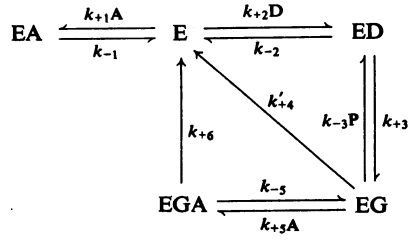
Double-reciprocal plots of the results obtained when glycerol, fructose and sucrose, which have been shown to be efficient glucosyl acceptors in β-glucosidase-catalysed reactions (Umezurike, 1975*a*), were tested for their effects on β-glucosidase activity in the absence of dioxan, were linear, and intersected to the right of the vertical axis, as was found previously with glycerol as the added acceptor (Umezurike, 1971*b*). These results show that enzyme activity is inhibited by each of these compounds in the presence of low donor concentrations, but enhanced in the presence of high donor concentrations. Methanol showed the same effect. The Michaelis constant (K_m) for the donor increased from a value of 0.28mM in the absence of added acceptors to 0.56, 1.0 and 1.67mM in the presence of 0.5mM-sucrose, 1.0mM-fructose

and 4.1M-methanol respectively. The maximum velocity ($V_{max.}$) also increased with increase in the concentration of added acceptors. The variation of both K_m and $V_{max.}$ with the concentration of added acceptors was found to be non-linear (convex). A similar effect was found in β-galactosidase-catalysed reactions in the presence of methanol (Sinnott & Viratelle, 1973).

That these compounds behaved like more efficient glucosyl acceptors than water is probably due to the presence of a functional terminal primary hydroxy group in each of the compounds. Terminal primary hydroxy groups have been shown to be better glucosyl acceptors than penultimate secondary hydroxy groups in many polyhydroxylic alcohols in reactions catalysed by aryl-β-glucosidase from *Stachybotrys atra* (Jermyn, 1966). The rate augmentation observed in the presence of glycerol, methanol, sucrose or fructose when the concentration of donor is high suggests that the functional alcoholic groups in these compounds are more reactive as nucleophiles than water.

Alcohols appear to be more reactive than water towards glycosyl cations, but less reactive than water towards saturated carbon atoms (cf. Sinnott & Viratelle, 1973). It thus appears that the generation of the second product, leading to regeneration of the free-enzyme conformation (deglucosylation), is partly rate-limiting in β-glucosidase-catalysed reactions. These results therefore are consistent with the involvement of a glucosyl cation as an intermediate of the reaction scheme in β-glucosidase-catalysed reactions (cf. Sinnott & Viratelle, 1973).

The addition of propan-2-ol (up to 2M) to the reaction mixtures gave double-reciprocal plots ($1/v$ against 1/[donor]) that indicated competitive inhibition with respect to the donor. The difference in the effects of propan-2-ol and methanol is due to the absence of alcoholysis in the presence of propan-2-ol, as was also found in ribonuclease-catalysed reactions (Findlay *et al.*, 1962*a*). Paper chromatography of concentrated reaction mixtures containing methanol, unlike those containing propan-2-ol, revealed the presence of a transfer product that behaved like methyl β-D-glucopyranoside on chromatography. The apparent competitive inhibition by propan-2-ol a can be attributed to a 'medium effect'.



Scheme 1. *Reaction scheme for the hydrolytic and transferase activities of β-glucosidase in the presence of an added acceptor (A) capable of inducing a 'medium effect'*

D is donor, EG is glucosyl-enzyme, k_{+1}–k_{+5} are rate constants for reactions in the forward direction, and k_{-1}–k_{-5} are rate constants for reactions in the reverse direction; $k'_{+4} = k_{+4}[H_2O]$, but since the concentration of water is very high $k_{+4}[H_2O]$ may be regarded as a constant (k'_{+4}). See the text for further details.

It has been pointed out that when the concentration of water is very high several hydrolytic enzymes that catalyse reactions involving glycosyl-enzyme intermediates give apparent Ordered Uni Bi kinetics (Cleland, 1963). Thus the kinetics could be treated as if water were not involved. Under this condition, the free enzyme would be saturated with water. In the presence of an alternative glycosyl acceptor (A) that can bind to the free enzyme (E) to form an unreactive enzyme species, in addition to its acceptor function on its interaction with the glycosyl-enzyme (EG), the reaction scheme can be represented as shown in Scheme 1. If the alternative acceptor does not bind to the free enzyme in competition with the donor, but induces a 'medium effect' as discussed above, the effect of the addition of the alternative acceptor would be similar to that in which the alternative acceptor binds to the free enzyme to form an unreactive enzyme species (cf. Scheme 1). A 'medium effect' resulting from the addition of methanol to the assay mixtures has been observed in β-galactosidase-catalysed hydrolysis (Sinnott & Viratelle, 1973).

If $k_{-3}P = 0$ in Scheme 1, the steady-state rate equation for Scheme 1 derived by the method of King & Altman (1956), where the rate of the reaction is determined by the release of the first product (P), is:

$$\frac{v}{[E]_0} = \frac{\dfrac{k_{+3}k'_{+4}}{(k_{+3}+k'_{+4})}\left(1+\dfrac{k_{+5}k_{+6}[A]}{k'_{+4}(k_{-5}+k_{+6})}\right)\Big/\left(1+\dfrac{[A]}{K_{a2}}\right)}{1+\dfrac{(k_{-2}+k_{+3})\,[k'_{+4}(k_{-5}+k_{+6})+k_{+5}k_{+6}]}{[S]k_{+2}(k_{-5}+k_{+6})\,(k_{+3}+k'_{+4})}\left(\dfrac{(1+[A]/K_{a1})}{(1+[A]/K_{a2})}\right)} \quad (1)$$

where $K_{a1} = k_{-1}/k_{+1}$

and $K_{a2} = \dfrac{(k_{-5}+k_{+6})\,(k_{+3}+k'_{+4})}{k_{+5}(k_{+3}+k_{+6})}$

It can be shown from eqn. (1) that, if the transglucosylation rate (where the alternative acceptor, A, is involved) is more than the hydrolytic rate, the intercepts of double-reciprocal plots ($1/v$ against $1/[\text{donor}]$) will be given by eqn. (2):

$$\text{Intercept} = \frac{\left[\frac{(k_{+3}+k'_{+4})}{k_{+3}k'_{+4}} + \frac{k_{+5}(k_{+3}+k_6)[A]}{k_{+3}k'_{+4}(k_{-5}+k_{+6})}\right]}{1+k_{+5}k_{+6}[A]/k'_{+4}(k_{-5}+k_{+6})} \quad (2)$$

If, on the other hand, the transglucosylation rate is less than the hydrolytic rate, the intercept of double-reciprocal plots will be given by eqn. (3):

$$\text{Intercept} = \frac{\left[\frac{(k_{+3}+k_{+6})}{k_{+3}k_{+6}} + \frac{(k_{+3}+k'_{+4})(k_{-5}+k_{+6})}{k_{+3}k_{+5}k_{+6}[A]}\right]}{1+k'_{+4}(k_{-5}+k_{+6})/k_{+5}k_{+6}[A]} \quad (3)$$

In either case (see eqns. 2 and 3), a plot of intercept values against [A] will be non-linear. A plot of intercept values, calculated from results obtained in an experiment in which the effect of various concentrations of added glycerol was studied, against glycerol concentration is shown in Fig. 2. A similar plot of data obtained when the effect of maltose, which has been shown to be a very poor glucosyl acceptor (Umezurike, 1975*a*), was studied is shown in Fig. 3. The plots of Figs. 2 and 3 are consistent with eqns. (2) and (3) respectively. Double-reciprocal plots ($1/v$ against $1/[\text{donor}]$) of the results obtained when various concentrations of maltose were added to the assay mixtures intersected on the horizontal axis, as is expected from eqn. (1) if $K_{a1} = K_{a2}$. A similar non-competitive effect has been reported previously (Umezurike, 1971*b*).

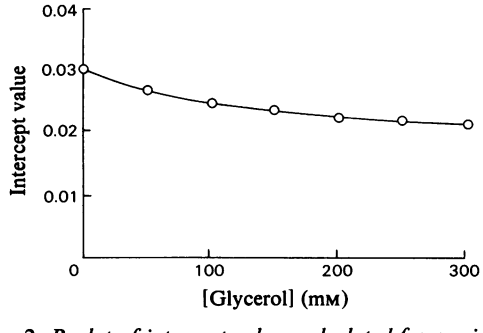


Fig. 2. *Replot of intercept values calculated from primary double-reciprocal plots* ($1/v$ *against* $1/$[*o-nitrophenyl β-D-glucopyranoside*]) *of data obtained in the absence and presence of various fixed concentrations of glycerol against the concentration of glycerol*

Retention of configuration

It is implied in Scheme 1 that β-glucosidase-catalysed reactions proceed with retention of configuration at the anomeric carbon atom. The retention of configuration of the pyranose ring in β-glucosidase-catalysed reactions can be deduced from the transferase activity of the enzyme (Umezurike, 1971*a*, 1975*a*). That the hydrolytic reaction catalysed by this enzyme also proceeds with retention of configuration at the anomeric carbon atom was indicated by the results of a polarimetric experiment in which β-D-glucopyranosyl fluoride was used as substrate by the method of Barnett (1971). The β-glucosidase from other sources also functions with retention of configuration at the anomeric carbon atom (Koshland, 1954; Jermyn, 1958; Legler, 1968: Barnett, 1971).

The retention of configuration at the anomeric carbon atom indicates that there must be two substitution reactions. A group on the active enzyme may be required as a temporary anchorage site, after the first substitution reaction, for the glucosyl group being transferred. The temporary anchorage may not involve covalent bonding, but electrostatic shielding or ion-pairing involving a glucosyl cation intermediate (Vernon & Banks, 1963; Capon, 1969). This mechanism would hold only if the enzyme has no absolute specificity for the aglycone, and the glucosyl group being transferred remains enzyme-bound until it is attacked by water (or any of the alternative acceptors), as the acceptors are required to interact at the agly-

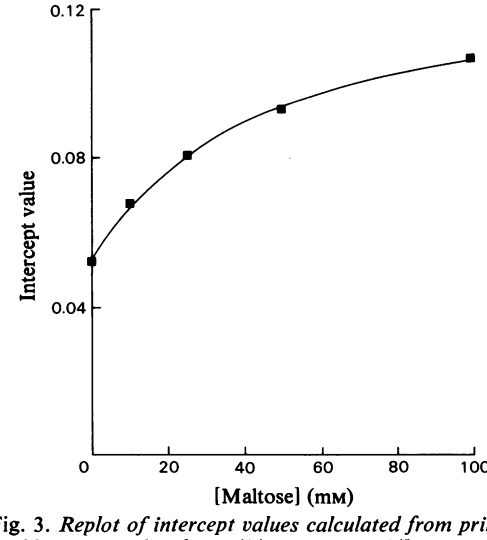


Fig. 3. *Replot of intercept values calculated from primary double-reciprocal plots* ($1/v$ *against* $1/$[*o-nitrophenyl β-D-glucopyranoside*]) *of data obtained in the absence and presence of various fixed concentrations of maltose against the concentration of maltose*

cone-binding site for the second substitution reaction. The first product is thus assumed to be released before the acceptors interact to generate the second product. If this assumption is not made, and the acceptor is assumed to bind to the catalytic site before the release of the first product, the postulation of another binding site for the acceptor, apart from the aglycone-binding site and the so-called site responsible for the 'medium effect', would be necessary. Retention of configuration at the anomeric carbon atom would therefore be difficult to explain in view of the principle of microscopic reversibility.

It thus appears that, when water is saturating, the reaction scheme for β-glucosidase activity approximates to an Ordered Uni Bi mechanism (Cleland, 1963). A 'medium effect' is operative in the presence of alternative acceptors as shown in Scheme 1, suggesting that water is required for the maintenance of the active enzyme conformation in addition to its acceptor function.

I thank Dr. M. L. Sinnott of the School of Chemistry, University of Bristol, U.K., for a gift of some β-D-glucopyranosyl fluoride.

References

Barnett, J. E. G. (1971) *Biochem. J.* **123**, 607–611
Capon, B. (1969) *Chem. Rev.* **69**, 407–498
Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
Findlay, D., Mathias, A. P. & Rabin, B. R. (1962*a*) *Biochem. J.* **85**, 134–139
Findlay, D., Mathias, A. P. & Rabin, B. R. (1962*b*) *Biochem. J.* **85**, 139–144
Inagami, T. & Sturtevant, J. M. (1960) *Biochim. Biophys. Acta* **38**, 64–79
Jermyn, M. A. (1957) *Science* **125**, 12–15
Jermyn, M. A. (1958) *Aust. J. Biol. Sci.* **11**, 114–126
Jermyn, M. A. (1962) *Aust. J. Biol. Sci.* **15**, 248–261
Jermyn, M. A. (1966) *Aust. J. Biol. Sci.* **19**, 1153–1165
King, E. L. & Altman, C. (1956) *J. Phys. Chem.* **60**, 1375–1378
Koshland, D. E. (1954) in *The Mechanism of Enzyme Action* (McElroy, W. D. & Glass, B., eds.), pp. 608–641, Johns Hopkins Press, Baltimore
Legler, G. (1968) *Biochim. Biophys. Acta* **151**, 728–729
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183–186
Sinnott, M. L. & Viratelle, O. M. (1973) *Biochem. J.* **133**, 81–87
Umezurike, G. M. (1969) *Ann. Bot.* **33**, 451–462
Umezurike, G. M. (1971*a*) *Biochim. Biophys. Acta* **227**, 419–428
Umezurike, G. M. (1971*b*) *Biochim. Biophys. Acta* **250**, 182–191
Umezurike, G. M. (1975*a*) *Biochim. Biophys. Acta* **397**, 164–178
Umezurike, G. M. (1975*b*) *Biochem. J.* **145**, 361–368
Umezurike, G. M. (1976) *Biochem. J.* **157**, 381–387
Umezurike, G. M. (1977) *Biochem. J.* **167**, 831–833
Vernon, C. A. & Banks, B. (1963) *Biochem. J.* **86**, 7P