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The β-Glucosidase from *Botryodiplodia theobromae*

MECHANISM OF ENZYME-CATALYSED REACTIONS

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1. The effects of pH and temperature on Michaelis constant (K_m) and maximum velocity ($V_{max.}$) and of NaCl on the activity of the high-molecular-weight β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from cultures of *Botryodiplodia theobromae* Pat. have been studied. 2. Donor binding and inhibition of activity by glucose were dependent on the ionization of a group (pK6.0) that appeared to be an imidazole group. 3. Catalytic activity and the stimulation of activity by glycerol were dependent on the ionization of two groups, which appeared to be a carboxy group and an imidazole group. 4. The Arrhenius activation energy (E_a) calculated from results obtained at pH4.0 and 5.0 was about 45–46kJ·mol^{-1}. 5. The enthalpies (ΔH^0) calculated from results obtained at pH4.0 and 5.0 were similar (about −4kJ·mol^{-1}), whereas at pH6.5 the value was about −33kJ·mol^{-1}. 6. The entropies (ΔS^0) calculated from these results at 37°C were −21, −22 and −118J·K^{-1}·mol^{-1} at pH4.0, 5.0 and 6.5 respectively. A low concentration of NaCl (16.6mM) stimulated enzymic activity and decreased the K_m for the donor, whereas high concentrations (up to 500mM) inhibited enzymic activity, increased the K_m and had no effect on $V_{max.}$. 8. Plots of initial velocity data obtained in the presence of dioxan as $1/v$ against the ratio of the molar concentration of dioxan to that of water were linear. 9. These results are discussed in terms of the enzyme mechanism.

The retention of configuration at the anomeric carbon atom in the glucopyranose ring in hydrolytic and transferase activities of β-glucosidase of *Botryodiplodia theobromae* Pat. indicates two substitution reactions and consequently a sequential liberation of products (Umezurike, 1978). This is in accord with the postulation that the action of glycosidases involves an initial general-acid catalysis by an acidic group on the enzyme to generate a glycosyl cation stabilized by an ionized acidic group (Wallenfels & Malhotra, 1961). Previous studies on the β-glucosidase of *B. theobromae* have indicated that the enzyme is dependent on a carboxylate group and an imidazolium group for its activity (Umezurike, 1977) and it appears that the carboxylate group is involved in the stabilization of the glucosyl cation (Umezurike, 1978). The effects of dioxan and a number of glucosyl acceptors have been studied, and effects on Michaelis constant (K_m) and maximum velocity ($V_{max.}$) were observed (Umezurike, 1977, 1978). Some of these effects, particularly the increase in K_m caused by these substances and the decrease in $V_{max.}$ in the presence of dioxan, were attributed to 'medium effect' envisaged to be caused by changes in the hydration of some groups on the enzyme (Umezurike, 1978), indicating that water is required for the maintenance of the active enzyme conformation. In the present paper, results are presented that appear to throw some more light on the mechanism of β-glucosidase action.

Materials and Methods

o-Nitrophenyl β-D-glucopyranoside was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. All other chemicals were of analytical grade.

The high-molecular-weight β-glucosidase used in the present work was obtained from culture filtrates of *B. theobromae* (I.M.I. 115626, A.T.C.C. 26123) and purified as described previously (Umezurike, 1971*a*, 1975).

β-Glucosidase activity was determined as described previously (Umezurike, 1971*a*) with *o*-nitrophenyl β-D-glucopyranoside in 0.05M-sodium acetate/acetic acid buffer (pH5.0) unless otherwise specified. Michaelis constants (K_m) and maximum velocities ($V_{max.}$) were calculated from plots of v against v/[donor].

Initial-velocity studies in the presence of up to 50% (v/v) dioxan were carried out at pH4.5 (value in water) as has been reported previously (Umezurike, 1978).

Gel filtration on Sephadex G-200 was carried out as described previously (Umezurike, 1976). In some cases the buffer used for swelling the gel and for elution (0.1M-citrate/0.2M-Na_2HPO_4, pH5.0; McIlvaine, 1921) contained 0.5M-NaCl.

The reagent concentrations quoted were final concentrations in the assay mixtures.

Results and Discussion

Effect of pH

The purified high-molecular-weight β-glucosidase has been found to be stable in the pH range 2.7–8.0, and two ionizing groups, a carboxylate group and a protonated imidazolium group, have been implicated in catalysis (Umezurike, 1977). The effect of pH on the values of Michaelis constant (K_m) and maximum velocity ($V_{max.}$) has now been studied in McIlvaine's buffer (McIlvaine, 1921). A plot of pK_m ($-\log K_m$) against pH indicated that donor binding involved an ionizing group with pK 6.0. Another ionizing group with pK4.1 in the free enzyme and 3.5 in the enzyme–substrate complex did not appear to be involved directly in the link between enzyme and donor. A plot of $\log V_{max.}$ against pH indicated that the catalytic process was dependent on the ionization of two groups at the enzyme active site. The pK values for these groups were calculated to be 3.65 and 6.75. These results are in agreement with previous results (Umezurike, 1977). The same groups, identified as a carboxylate group and a protonated nitrogen atom of an imidazole group, have also been implicated in catalysis by the β-glucosidase of *Trichoderma viride* (Maguire, 1977).

The effect of pH on the stimulation of the activity of β-glucosidase from *B. theobromae* by glycerol has also been studied. The effect of pH on the stimulation of enzyme activity at saturating donor concentration ([donor] = $100K_m$) is shown in Fig. 1(*a*) as a plot of logarithm of percentage stimulation against pH. The results indicate that the stimulatory effect of glycerol at saturating donor concentration is affected by the ionization of two groups, presumably a carboxylate group (pK3.5) and an imidazolium group (pK6.0). Fig. 1(*b*) shows the effect of pH on the inhibition of β-glucosidase activity by the competitive inhibitor glucose (Umezurike, 1971*b*). The results indicate that inhibition by glucose is facilitated by protonation of a group, apparently a protonated imidazolium group on the free enzyme (pK6.0). This group is involved in glucose binding as well as in donor binding. The 'medium effect' found in the presence of glycerol (Umezurike, 1978) was not found in the presence of glucose, which elicits its effect at much lower concentrations (0.1–2.0mM), since a plot of slope values calculated from double-reciprocal plots ($1/v$ against $1/[S]$) against glucose concentration was linear. However, at pH5.0 the enzyme has more affinity for the donor *o*-nitrophenyl β-D-glucopyranoside (K_m 0.27mM) than for glucose (K_i 1.28mM).

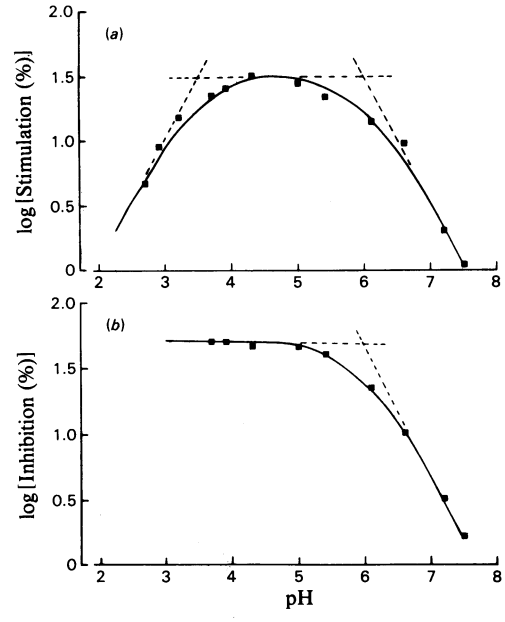


Fig. 1. *Effect of pH on the stimulation or inhibition of β-glucosidase activity by glycerol or glucose respectively in McIlvaine's buffer, pH*5.0

(*a*) The data are presented as the logarithm of the percentage stimulation by glycerol (200mM) in the presence of saturating concentrations of *o*-nitrophenyl β-D-glucopyranoside ([donor] = $100K_m$) as a function of pH, and the curve fitted to the experimental points was calculated by assuming pK values of 3.5 and 6.0 for the ionizing groups. (*b*) The data are presented as the logarithm of the percentage inhibition by 2.0mM-glucose in the presence of donor (0.15mM) as a function of pH, and the curve fitted to the experimental points was calculated by assuming a pK value of 6.0 for the ionizing group.

Effect of temperature

The variation of the Michaelis constant (K_m) and maximum velocity ($V_{max.}$) with temperature in enzyme-catalysed hydrolysis of *o*-nitrophenyl β-D-glucopyranoside has been investigated in the temperature range 20–50°C and in buffers at three pH values (i.e. at a pH near the pK value of the carboxylate group, at a pH around the optimum pH and at a pH near the pK of the imidazolium group). The enzyme was found to be stable in this temperature range. The Arrhenius plots of the data as $\log V_{max.}$ against $1/T$ were linear at pH 4.0 and 5.0 in 0.05M-sodium acetate buffer. The values of the Arrhenius activation energy (E_a) calculated from the plots of the data obtained at pH4.0 and 5.0 were similar (45–46kJ·mol^{-1}). The plot of the data obtained at

pH6.5 in McIlvaine's buffer (McIlvaine, 1921) was non-linear. At pH6.5 the imidazolium group exists in two ionic forms, and a possible interpretation of the type of non-linearity observed (convex down) has been discussed by Gutfreund (1972).

Plots of pK_m (−log K_m) against 1/T of the data obtained at pH4.0, 5.0 and 6.5 were linear. The enthalpies (ΔH^0) calculated from the plots at pH4.0 and 5.0 were similar (about $-4\,kJ\cdot mol^{-1}$), whereas that at pH6.5 was about $-33\,kJ\cdot mol^{-1}$. The difference between the ΔH^0 values at pH4.0 and 5.0 and that at pH6.5 appears to be due to the heat of ionization of the imidazolium group (about $29\,kJ\cdot mol^{-1}$). The entropies (ΔS^0) calculated from these results at 37°C were −21, −22 and $-118\,J\cdot K^{-1}mol^{-1}$ at pH4.0, 5.0 and 6.5 respectively. Since ΔH^0 and ΔS^0 values would represent the changes occurring during the formation of the Michaelis complex, there thus appears to be a more considerable conformational change in the enzyme during the course of the reaction at pH6.5 than at pH4.0 and 5.0. These results are consistent with the involvement of an imidazolium group in donor binding.

Effect of NaCl

The effect of various concentrations of NaCl on β-glucosidase activity is shown in Fig. 2. A low concentration of NaCl (16.6mM) had a stimulatory effect and decreased the apparent Michaelis constant for the donor. On the contrary, higher concentrations of NaCl (up to 500mM) inhibited enzymic activity, and the apparent K_m for donor increased with increasing NaCl concentration, but there was apparently no effect on $V_{max.}$, indicating that it was the binding of the donor that was affected. This can be attributed to 'medium effect', as has been defined previously (Umezurike, 1978). It appears that high NaCl concentrations alter the critical spacing between some charged groups, possibly the functional groups at the enzyme active site, thus affecting donor binding.

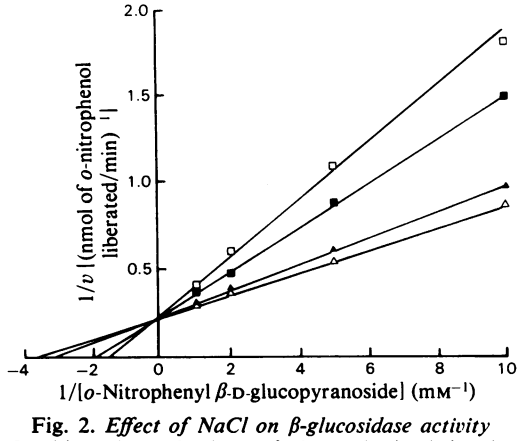


Fig. 2. *Effect of NaCl on β-glucosidase activity*
Double-reciprocal plots of data obtained in the absence (▲) and presence of 16.6mM- (△), 166mM- (■) and 500mM-NaCl (□) with *o*-nitrophenyl β-D-glucopyranoside in 0.05M-sodium acetate buffer (pH5.0) as substrate.

That the effect of NaCl, particularly at high concentrations, may be due to dissociation of the enzyme into active lower-molecular-weight forms can be discounted on the grounds that gel filtration of the enzyme on Sephadex G-200 in the presence of 0.5M-NaCl did not give rise to dissociated forms. The lower-molecular-weight forms of the enzyme have slightly greater affinity for the donor than the high-molecular-weight form used in the present studies (Umezurike, 1975). Thus the apparent K_m value should decrease progressively with increasing NaCl concentration if dissociation was taking place.

Mechanism of action

The acid-catalysed generation of an enzyme-bound glucosyl cation–carboxylate ion-pair in β-glucosidase-catalysed hydrolysis of *o*-nitrophenyl β-D-glucopyranoside can be represented as shown in eqns. (1) and (2), where the generation of the reactive enzyme-bound glucosyl cation–carboxylate ion-pair is the rate-limiting step.

$$EC^-(IMH^+)+GOX \rightleftharpoons EC^-(IMH^+ \ldots GOX) \quad (1)$$

$$EC^-(IMH^+ \ldots GOX) \rightarrow EC^-(IM) \ldots G^+ + HOX \quad (2)$$

In eqns (1) and (2), the free enzyme with its carboxylate group (C^-) and protonated imidazolium group (IMH^+) is denoted as $EC^-(IMH^+)$, GOX is the donor, $EC^-(IMH^+ \ldots GOX)$ is the Michaelis enzyme–substrate complex, $EC^-(IM) \ldots G^+$ is the enzyme-bound glucosyl cation stabilized by intimate ion-pairing with the carboxylate ion, and HOX is the first product. The second substitution reaction to generate the second product can be represented as shown in eqn. (3).

$$EC^-(IM) \ldots G^+ + H_2O \rightarrow EC^-(IMH^+) + GOH \quad (3)$$

In eqn. (3), the glucosyl acceptor (water) transfers a proton to the deprotonated imidazolium group as it (water) attacks the glucosyl cation to form the second product (GOH).

The transferase activity of β-glucosidase in the presence of methanol, glycerol, sucrose or fructose (Umezurike, 1978) indicates that these substances do react in place of water with the glycosyl cation as in eqn. (3). That $V_{max.}$ increases in the presence of the protic solvent methanol and of the glucosyl acceptors (sucrose, fructose and glycerol) that contain functional alcoholic groups (cf. Umezurike, 1978)

suggests that the ion-pair does not collapse into an unreactive covalent glycosyl-enzyme in the presence of these compounds. Since the glucosyl cation can be further stabilized through mesomeric interaction with the ring oxygenation atom (cf. Capon, 1969) and is isoelectronic with a protonated aldehyde (cf. Sinnott & Viratelle, 1973), it has been postulated that the greater nucleophilicity of alcoholic glucosyl acceptors compared with water is consistent with the involvement of a glucosyl cation in β-glucosidase-catalysed reactions (Umezurike, 1978). That the ion-pair does not collapse into an unreactive covalent glucosyl-enzyme in the presence of alcoholic acceptors is partly due to the efficient solvation of the carboxylate anion, as the solvation of anions by protic solvents is generally considerably more efficient than any other form of electrophilic solvation (Alder *et al.*, 1971). Changes in the nature of the solvent are known to change the rates of the reactions of anions with carbonium ions, indicating that specific interactions of the solvent with the transition state of the reactions of anions with the carbonium ions are important factors (cf. Jencks, 1969, p. 95).

It has been suggested that in lysozyme-catalysed reactions, it would be 'difficult to draw a sharp line between a carbonium ion stabilized by an adjacent carboxylate group and a strained bond between C-1 of the sugar and the carboxyl group', and that a fine balance may exist between the two unreactive extremes (Jencks, 1969, p. 228). Anions are poorly solvated in dipolar aprotic solvents and are therefore rather reactive (Alder *et al.*, 1971). If the carboxylate counter-ion is poorly solvated in the environment of the active site of β-glucosidase, owing to the addition of dioxan, its nucleophilic reactivity will be enhanced, thus favouring the collapse of the glucosyl cation–carboxylate ion-pair into an unreactive covalent glucosyl-enzyme as shown in eqn. (4), more so as the solvation of the positively charged glucosyl cation by aprotic solvents would be weak because it is the Lewis basicity or nucleophilicity of the solvent that is important in this type of solvation (Alder *et al.*, 1971).

$$EC^-(IM)\ldots G^+ \underset{\text{water}}{\overset{\text{Dioxan}}{\rightleftharpoons}} EC(IM)\text{–}G \qquad (4)$$

The first term in eqn. (4) is the enzyme-bound glucosyl cation stabilized by the carboxylate counter-ion (C^-) and the second term [EC(IM)–G] is the unreactive covalent glucosyl-enzyme. It has been postulated that an unreactive glycosyl-enzyme is in equilibrium with a reactive glycosyl cation in β-galactosidase-catalysed hydrolysis (Sinnott & Souchard, 1973). In view of the foregoing considerations, the ratio of the molar concentration of dioxan to that of water will thus determine the proportion of the intermediate that exists as the reactive ion-pair or as the unreactive covalent glucosyl-enzyme, as will be shown below.

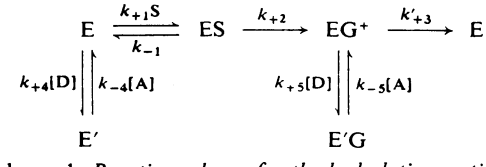
Apart from their effects on $V_{max.}$, all the substances mentioned above increase the apparent Michaelis constant (K_m) for the donor (Umezurike, 1977, 1978). This effect can be attributed to a 'medium effect' caused by changes in the hydration of some groups at or near the enzyme active site. Changes in the degree of hydration (or solvation) of some groups, possibly the carboxylate group and the imidazolium group in close proximity at the active site, may alter the critical spacing of these groups and affect donor binding. The postulated involvement of water in the maintenance of the active enzyme conformation (Umezurike, 1978) may therefore pertain to the hydration of these functional groups to maintain the proper spacing.

A scheme based on the reactions of eqns. (1)–(4) and on the assumption of a 'medium effect' on donor binding in the presence of dioxan is shown in Scheme 1. Two enzyme species, the free enzyme (E) and the enzyme–glucosyl cation intermediate (EG^+), are assumed to be susceptible to dioxan-induced effects. The steady-state rate equation for Scheme 1, derived by the method of King & Altman (1956), and where the rate of reaction is determined by the release of the first product, is:

$$\frac{v}{[E]_0} = \frac{k_{cat.}/(1+[D]/[A]K_b)}{1+\dfrac{K_m}{[S]}\left(\dfrac{(1+[D]/[A]K_a)}{(1+[D]/[A]K_b)}\right)} \qquad (5)$$

where

$$k_{cat.} = k_{+2}k'_{+3}/(k_{+2}+k'_{+3})$$
$$K_m = k'_{+3}(k_{-1}+k_{+2})/k_{+1}(k_{+2}+k'_{+3})$$
$$K_a = k_{-4}/k_{+4}$$
$$K_b = k'_{+3}k_{-5}(k_{+2}+k'_{+3})/k_{+2}k_{+5}$$



Scheme 1. *Reaction scheme for the hydrolytic reaction of β-glucosidase showing dioxan-induced 'medium effect'* E, ES and EG^+ are free enzyme, enzyme-donor complex and enzyme-bound glucosyl cation respectively; E′ and E′G are modified free enzyme and unreactive glucosyl-enzyme respectively. D and A are dioxan and water respectively. The rate constants for reactions in the forward direction are denoted as k_{+1}–k_{+5}, and those in the reverse direction as k_{-1}–k_{-5}; $k'_{+3} = k_{+3}$[water], but since the concentration of water is very high k_{+3}[water] may be regarded as a constant (k'_{+3}).

This equation (eqn. 5) can be rearranged into:

$$\frac{[E]_0}{v} = \frac{1}{k_{cat.}}\left(1+\frac{K_m}{[S]}\right)+\frac{1}{k_{cat.}}\cdot\frac{[D]}{[A]}\left(\frac{1}{K_b}+\frac{K_m}{K_a[S]}\right) \quad (6)$$

Eqn. (5) predicts that plots of $1/v$ against 1/[donor] will be linear and intersecting as observed (Umezurike, 1978). Eqn. (5) also predicts that increasing concentrations of dioxan will decrease $V_{max.}$ as observed (Umezurike, 1977, 1978). Eqn. (6) predicts that plots of $1/v$ against the ratio of the molar concentration of dioxan to that of water ([D]/[A]) will also be linear and intersecting. Plots of $1/v$ against [D]/[A] of initial velocity data obtained in the presence of up to 50% (v/v) dioxan are shown in Fig. 3. The changes in intercept and slope with donor concentrations ([S]) are as predicted by eqn. (6).

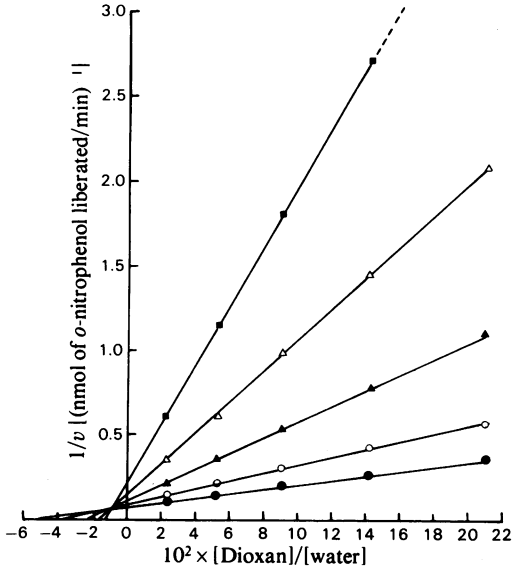


Fig. 3. *Plots of the reciprocal of initial velocity* ($1/v$) *in β-glucosidase-catalysed hydrolysis against the ratio of the molar concentration of dioxan to that of water* ([*dioxan*]/[*water*]) *in the presence of* 1.0 mM- (●), 0.5 mM- (○), 0.25 mM- (▲), 0.125 mM- (△) *and* 0.0625 mM-*o-nitrophenyl β-D-glucopyranoside* (■)

The results reported in the present paper thus appear to indicate that the mechanism of action of β-glucosidase is consistent with acid-catalysed generation of a glucosyl cation stabilized by ion-pairing with an ionized carboxylate group. This is in agreement with the observation that 2-carboxyphenyl β-D-glucopyranoside undergoes non-enzymic hydrolysis with intramolecular general-acid catalysis (Capon, 1963), and the rate of hydrolysis of this compound is several orders of magnitude higher than that of 4-carboxyphenyl β-D-glucopyranoside, in which the acidic group is farther away from the glycosidic bond (cf. Capon, 1969).

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