The Active Site of β -Glucosidase from *Botryodiplodia theobromae* EFFECTS OF pH AND DIOXAN ON ENZYME-CATALYSED REACTIONS

By GABRIEL M. UMEZURIKE

Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

(Received 12 August 1977)

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) of *Botryodiplodia theobromae* Pat in the absence or presence of added dioxan was found to be dependent on the ionization of two groups, which appeared to be a carboxyl group and an imidazole group.
2. Dioxan increased the Michaelis constant, K_m , but decreased the maximum velocity, V .

Although some properties of the enzyme β -glucosidase from the fungus *Botryodiplodia theobromae* have been reported (Umezurike, 1971a,b, 1975a,b), nothing is known about the identity of the functional groups at the active site of this enzyme. Studies of the hydrolytic process catalysed by this enzyme have therefore been carried out. In this paper, aspects of these studies aimed at identifying the functional groups at the active sites of this enzyme are reported.

Materials and Methods

Botryodiplodia theobromae Pat (I.M.I. 115624, A.T.C.C. 26123) was originally isolated from decaying wood (Umezurike, 1969).

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.

β -Glucosidase activity was determined as previously described (Umezurike, 1971a) with *o*-nitro-

phenyl β -D-glucopyranoside as substrate (glucosyl donor). Activity is 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, 1971a, 1975b).

Enzymic hydrolysis of *o*-nitrophenyl β -D-glucopyranoside was measured under the standard assay conditions with various substrate concentrations, and maximum velocities (V) and Michaelis constants (K_m) were determined by using the least-squares method from double-reciprocal plots (Lineweaver & Burk, 1934).

Photo-oxidation of the enzyme was carried out as described by Dey (1969).

The buffer solutions used were 0.05M-sodium acetate/acetic acid, McIlvaine's 0.1M-citric acid/0.2M- Na_2HPO_4 buffer (McIlvaine, 1921), 0.2M-imidazole/HCl and 0.15M-pyridine/acetic acid (Canfield, 1963). The pH values of the dioxan/buffer

Fig. 1. Determination of the ionization constants, K_A and K_B , of functional groups at the active site of β -glucosidase from data for the pH-dependence of the hydrolysis of *o*-nitrophenyl β -D-glucopyranoside in McIlvaine's buffer. V^0 and V' are the extrapolated maximum velocities obtained from primary double-reciprocal plots at the pH optimum (pH 5.0) and at other pH values respectively. (a) Plot of data at pH values lower than pH 5.0 and (b) plot of data at pH values higher than 5.0.

mixtures quoted were the same as the buffer systems in water alone (cf. Findlay *et al.*, 1962).

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

Results and Discussion

Effect of pH

The purified high-molecular-weight β -glucosidase is stable in the pH range 2.7–8.0, since the enzyme that was incubated at 40°C for 30 min in McIlvaine's buffer at pH values within this range showed full activity after adjustment of the pH to 5.0. A plot of $\log V$ versus pH suggested that the catalytic process was affected by the ionization of two groups at the enzyme active site. Fig. 1 shows the maximum velocity (V) plotted in terms of ionization of both a basic and an acidic group by reference to eqn. (1) as suggested by Hammond & Gutfreund (1959), where V^0 is the rate of hydrolysis at the optimum pH and V' is the rate of hydrolysis at other pH values.

$$\frac{V^0}{V'} = 1 + \frac{[H^+]}{K_B} + \frac{K_A}{[H^+]} \quad (1)$$

The two pK values calculated from the plots of Fig. 1 are 3.65 (pK_B) and 6.75 (pK_A), where the subscripts denote whether the ionizing group is a Brönsted base or acid at the pH optimum. The pK values found for V in Fig. 1 are molecular (macroscopic) rather than group (microscopic) values. Since the two pK values differ appreciably from each other, one of the molecular constants may be almost entirely due to one group and the other molecular constant to another group.

Effect of dioxan

Information about the charge types of the pair of ionizing groups at the active site of an enzyme can be obtained by varying the solvent composition under suitable conditions (Findlay *et al.*, 1962). A study of the effect of increasing dioxan concentration (up to 50%, v/v) on the activity of *B. theobromae* β -glucosidase in 0.05 M-sodium acetate buffer (neutral acid buffer) at pH 5.0 with $[S] = 38 K_m$ showed that the relative initial velocity in 50% (v/v) dioxan was approx. 2% of its value at pH 5.0 in the absence of dioxan. Fig. 2(a) shows the pH (in water)–activity curves in water and in 28.6% (v/v) dioxan when activity was determined in McIlvaine's buffer at saturating substrate concentration ($[S] = 38 K_m$). In Fig. 2(a) enzyme activity at any pH (i.e. pH of the buffer in water) is expressed relative to the activity at pH 5.0 to make comparison possible, since activity was markedly decreased in the presence of dioxan. The pH values at the mid-points of the ascending arms of the pH–relative-activity curves in water

Fig. 2. pH–activity curves for β -glucosidase-catalysed hydrolysis of *o*-nitrophenyl β -D-glucopyranoside in (a) McIlvaine's buffer in the absence (■) and presence (□) of 28.6% (v/v) dioxan and (b) cationic buffers in the absence (■) and presence (□) of 28.6% (v/v) dioxan

The final substrate concentration in assay mixtures was such that $[S] = 38 K_m$. Enzyme activity at any pH (pH of the buffer in water) is expressed relative to the activity at the pH optimum. The buffers used in (b) were 0.2 M-imidazole/HCl buffers (pH 6.1–7.6) and 0.15 M-pyridine/acetic acid buffers (pH 3.5–6.0).

and in 28.6% (v/v) dioxan/water differ by about 0.2 pH unit, whereas the pH values at the mid-points of the descending arms of the curves in water and in dioxan/water differ by about 1.2 pH units. These results indicate that one of the ionizing groups at the active site of the enzyme is a neutral acid and the other is a cationic acid (cf. Findlay *et al.*, 1962). The results of an experiment in which cationic acid buffers were used are shown in Fig. 2(b). The pH values at the mid-points of the ascending arms of the pH–relative-activity curves in water and in 28.6% (v/v)

dioxan/water differ by about 1.6pH units, whereas the pH values at the mid-points of the descending arms of the curves differ by about 0.2pH unit. These results confirm the involvement of a neutral acid group and a cationic acid group at the active site of β -glucosidase (cf. Findlay *et al.*, 1962). With reference to the pK values listed by Dixon & Webb (1964, p. 149), it is possible to argue from these results that, since the group with the lower constant (pK 3.65) is unaffected by dioxan addition in neutral acid buffers but its ionization constant is increased by dioxan addition in cationic acid buffers, it is probably a carboxylic acid. Similarly, the upper constant (pK 6.75) can be ascribed to an imidazole group, particularly as photo-oxidation of the enzyme led to loss of activity. It has been reported that the related enzyme α -galactosidase from sweet almond (Dey, 1969) and that from *Vicia faba* (Dey & Pridham, 1969) require a protonated imidazole group and an unprotonated carboxyl group.

The marked inhibitory effect of dioxan and the effect of dioxan on the ionization of the functional groups at the active site of *B. theobromae* β -glucosidase (Figs. 2a and 2b) suggest that the concentration of water is an important factor in β -glucosidase-catalysed hydrolysis of *o*-nitrophenyl β -D-glucopyranoside, and can be interpreted as a dielectric effect. From Lineweaver-Burk plots (Lineweaver &

Burk, 1934) showing the effect of dioxan on enzyme-catalysed hydrolysis of the substrate (donor) at pH 5.0, K_m was found to increase, whereas V decreased with increasing dioxan concentration. Similar effects have been reported for β -galactosidase (Sinnott & Viratelle, 1973).

References

- Canfield, R. E. (1963) *J. Biol. Chem.* **238**, 2691–2697
Dey, P. M. (1969) *Biochim. Biophys. Acta* **191**, 644–652
Dey, P. M. & Pridham, J. B. (1969) *Biochem. J.* **115**, 47–54
Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., Longmans Green and Co., London
Findlay, D., Mathias, A. P. & Rabin, B. R. (1962) *Biochem. J.* **85**, 139–144
Hammond, B. R. & Gutfreund, H. (1959) *Biochem. J.* **72**, 349–357
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. (1971a) *Biochim. Biophys. Acta* **227**, 419–428
Umezurike, G. M. (1971b) *Biochim. Biophys. Acta* **250**, 182–191
Umezurike, G. M. (1975a) *Biochim. Biophys. Acta* **397**, 164–178
Umezurike, G. M. (1975b) *Biochem. J.* **145**, 361–368