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KINETIC ANALYSIS OF THE MECHANISM OF ACTION OF β-GLUCOSIDASE FROM *BOTRYODIPLODIA THEOBROMAE* PAT.

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Summary

1. The kinetic mechanism of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) of *Botryodiplodia theobromae* Pat. has been studied in the presence of competing glucosyl acceptors.

2. Glycerol, fructose, sucrose, cellobiose and to a much lesser extent, maltose can act as glucosyl acceptors, apart from water.

3. Evidence confirming and supporting the kinetic mechanism previously postulated (Umezurike, G.M. (1971) Biochim. Biophys. Acta. 250, 182—191) is presented.

4. A theoretical kinetic analysis of the behaviour of the enzyme in the presence of two alternative glucosyl acceptors in addition to water is found to be consistent with experimental observation, suggesting a system in which both donor and acceptors bind to the enzyme in a random fashion to form ternary complexes.

5. The results are discussed in terms of the mechanism of group-transfer reactions.

Introduction

It was reported in a previous paper [1] that the β-glucosidase (β-D-glucoside glycohydrolase, EC 3.2.1.21) of *Botryodiplodia theobromae* Pat. has distinct donor and acceptor sites. The observed kinetics of the enzyme were compared with equations derived on the assumption that the mechanism was a random type. The results fitted these equations in the presence of maltose and glycerol, but not in the presence of glucose which binds to both donor and acceptor sites. For two-substrate enzymes, a number of different mechanisms can give rise to similar rate expressions [2] and to distinguish between these mechanisms by kinetic methods alone usually presents formidable problems.

Not much is known about the mechanism of action of β-glucosidase.

Abbreviation: Nph-Glc, *p*-nitrophenyl-β-D-glucopyranoside.

However, Jermyn [3,4] has proposed that the aryl-β-glucosidase of *Stachybotrys atra* operates by a random mechanism leading to the formation of a ternary enzyme-donor-acceptor complex. On the contrary, Legler [5] has implicated an enzyme-bound covalent intermediate (i.e. a glycosyl-enzyme intermediate) in the mechanism of action of β-glucosidase from *Aspergillus wentii*. In this paper, more convincing results are presented to strengthen the validity of the mechanism proposed in the earlier paper [1]. Some direct proof of transglucosylation is presented to support deductions based entirely on kinetics.

Materials and Methods

Chemicals

All chemicals used were as described previously [6].

Enzyme

The high molecular weight β-glucosidase (mol. wt about 320 000) [6] used was obtained from culture media in which *Botryodiplodia theobromae* (IMI. 115626, ATCC 26123) was grown, and purified as described previously [6—8]. The enzyme preparations were used within three days of purification.

Enzyme assay

The activity of β-glucosidase was assayed spectrophotometrically with *p*-nitrophenyl-β-D-glucopyranoside (Nph-Glc) as glycosyl donor. Assay mixtures were incubated for 15 min at 40° C, and enzymic activity was expressed as nmol of *p*-nitrophenol liberated per min (refs 1,6—8).

p-Nitrophenol and glucose estimation

Glucose, carbohydrate and *p*-nitrophenol were estimated as previously reported [1,6].

Protein estimation

Protein estimation was carried out by the method of Lowry et al. [9].

Concentration of reactants

The concentrations of the reagents quoted in this paper were final concentrations in the reaction mixtures.

Paper chromatography

Paper chromatography and staining of carbohydrate spots were carried out as previously described [1].

Chromatography on cellulose-charcoal column

The cellulose-charcoal column was prepared from a 3 : 1 (w/w) mixture of Whatman's cellulose powder CF1 and activated charcoal according to the method of Jermyn [10].

Results

Effect of donor concentration on rate of transglucosylation

In an experiment in which the liberation of glucose and *p*-nitrophenol from Nph-Glc (as donor) by β-glucosidase, in the absence or presence of glycerol (as added acceptor), was followed, it was found that equal amounts of glucose and *p*-nitrophenol were liberated in the absence of glycerol within a short period (90 min) when the donor concentration varied from 0.1 mM to 2.0 mM. The results obtained in the absence or presence of 100 mM glycerol when the donor concentration was either 0.1 mM or 2.0 mM are presented in Fig. 1. It is obvious that the ratio *p*-nitrophenol to glucose liberated increased with increase in donor concentration in the presence of glycerol. In a similar experiment, the amount of transglucosylation product formed in the presence of 100 mM glycerol was calculated from the difference between the amounts of *p*-nitrophenol and glucose liberated when the donor (Nph-Glc) concentration was gradually increased. The results are shown in Table I. Had transglucosylation not taken place equimolar amounts of *p*-nitrophenol and glucose would be obtained in the presence of glycerol (cf. ref. 11). A Lineweaver-Burk plot [12] prepared by using the values in the first and last columns of Table I was linear. The K_m value calculated from a straight line fitted to the experimental points by the least-squares method was 0.33 mM Nph-Glc.

Mechanism I, where it is assumed that steps 1—4 (with dissociation constants K_1—K_4) attain rapid equilibrium and that k_1 and k_2 are rate limiting, shows the model proposed for the β-glucosidase of *B. theobromae* [1].

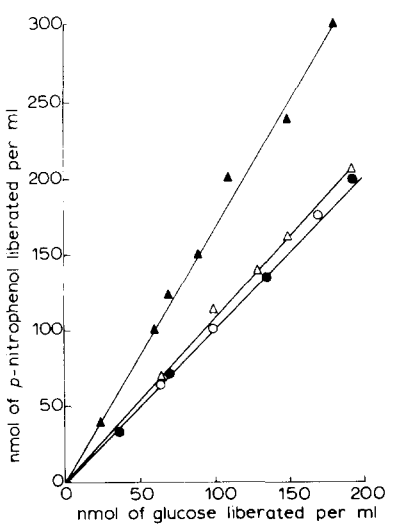


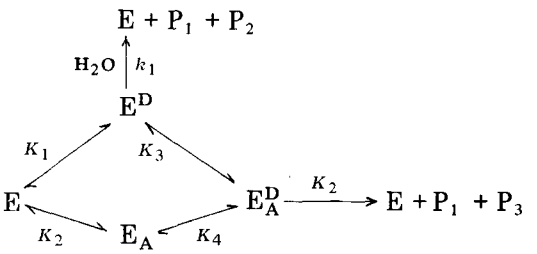
Fig. 1. Course of liberation of *p*-nitrophenol and glucose by β-glucosidase from 0.1 mM (○) and 2.0 mM Nph-Glc (●) in the absence of glycerol; from 0.1 mM Nph-Glc in the presence of 100 mM glycerol (△); and from 2.0 mM Nph-Glc in the presence of 100 mM glycerol (▲). Reaction mixtures were incubated at 40° C and the amount of *p*-nitrophenol or glucose liberated determined at regular time intervals.

TABLE I

STOICHIOMETRY OF PRODUCTS RELEASED BY β-GLUCOSIDASE IN THE PRESENCE OF 100 mM GLYCEROL AND INCREASING CONCENTRATIONS OF DONOR (Nph-Glc)

Assay mixtures were incubated at 40°C for 15 min.

[Nph-Glc] (mM)	[Glycerol] (mM)	$\frac{[\text{Nitrophenol}]}{[\text{Glucose}]}$ ratio	[Transglucosylation product] (nmol/min)
0.1	100	1.13	0.37
0.2	100	1.15	0.57
0.5	100	1.20	0.88
1.0	100	1.35	1.23
2.0	100	1.67	1.50



Mechanism I

On the basis of this model it is obvious that the amount of *p*-nitrophenol (P_1) liberated in the presence of glycerol (A) is equal to the sum of the amount of glucose (P_2) released and the amount of transglucosylation product (P_3) formed from the donor (D).

The transglucosylation product formed in the presence of glycerol as acceptor has been isolated from a concentrated reaction mixture incubated for 15 h by chromatography on cellulose-charcoal column (Fig. 2). The non-reducing substance (i.e. B in Fig. 2), when concentrated in vacuo, gave a syrup which on paper chromatography had a similar R_F value (R_F = 0.58) to that of glucose (R_F = 0.59). The specific optical rotation was found to be $[\alpha]_D^{20}$ = −26.7°. These properties are similar to those of 1-glyceryl-β-D-glucoside (ref. 11). Paper chromatography of the other peaks in Fig. 2 showed that peak A contained free glucose and glycerol, C contained cellobiose and D contained the residual donor (Nph-Glc) and cellotriose. The material eluted after D was *p*-nitrophenol.

Initial velocities in the presence of maltose

Lineweaver-Burk plots [12] (i.e. $1/v$ versus $1/[\text{Nph-Glc}]$) of results obtained in the presence of maltose were linear but Dixon plots ($1/v$ versus [maltose]) of the same results were curved. The inhibition by maltose was

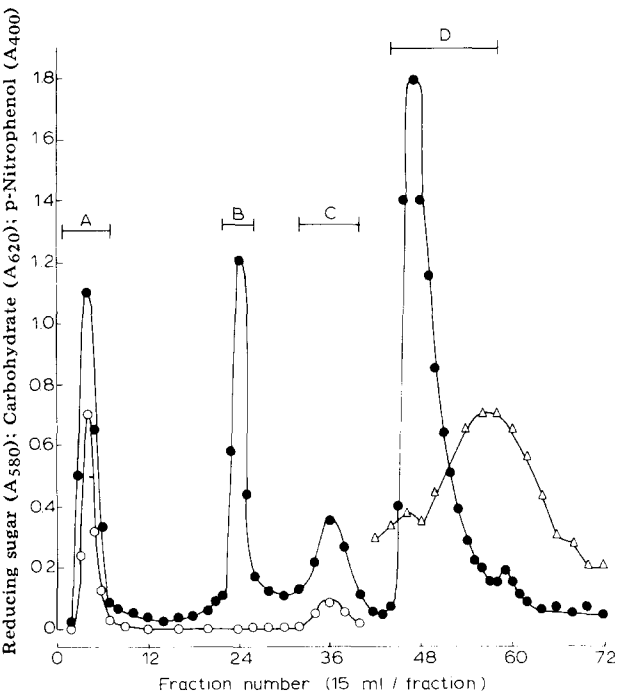


Fig. 2. Elution from a cellulose-charcoal column (13 cm × 2 cm) of a concentrated reaction mixture which consisted or 2.0 mM Nph-Glc, 100 mM glycerol and β-glucosidase in 0.05 M sodium acetate buffer, pH 5.0, (total volume 50 ml) and which had been incubated at 40 °C for 15 h in the presence of a few drops of toluene to prevent microbial contamination. ○, reducing sugar as glucose determined with Folin-Wu reagents (cf. ref. 1); ●, carbohydrate determined with anthrone-H_2SO_4 reagent (cf. ref. 6); △, *p*-nitrophenol determined spectrophotometrically (ref. 1). Elution was with distilled water until 7 fractions were collected and then with a linear gradient of ethanol made from 500 ml of distilled water and 500 ml of 95% ethanol. Fraction size was 15 ml.

found to be non-competitive with respect to the donor (Nph-Glc) [1]. The replots of slope and intercept values from similar primary double-reciprocal plots against the concentration of maltose are shown in Fig. 3a. Both the replots of slopes and intercepts are hyperbolic. When the results were replotted as $1/(\text{slope}_x - \text{slope}_o)$ or $1/(\text{intercept}_x - \text{intercept}_o)$ against the reciprocal of maltose concentration (where the subscripts x and o denote the values of slope or intercept in the presence and absence of maltose, respectively) as suggested by Cleland [13] for hyperbolic inhibition, the lines were linear (Fig. 3b). Both lines crossed the horizontal axis at the same point corresponding to 50 mM maltose.

The effect of donor (Nph-Glc) concentration on the inhibition by maltose is shown in Fig. 4. It is note-worthy that maltose is a partial inhibitor since increasing its concentration did not lead to total inhibition even in the presence of low donor concentrations. These results and the fact that data obtained in the presence of maltose fitted the equation derived on the assumption that the mechanism of action of β-glucosidase was as shown in Mechanism I (ref. 1) suggest that enzyme complexes containing maltose undergo the same reactions as in the absence of maltose but at a reduced rate. Maltose was, therefore,

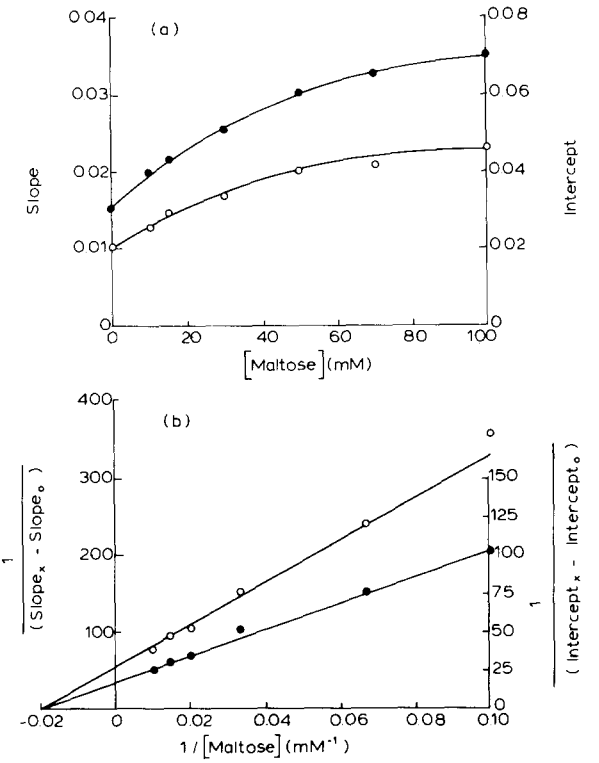
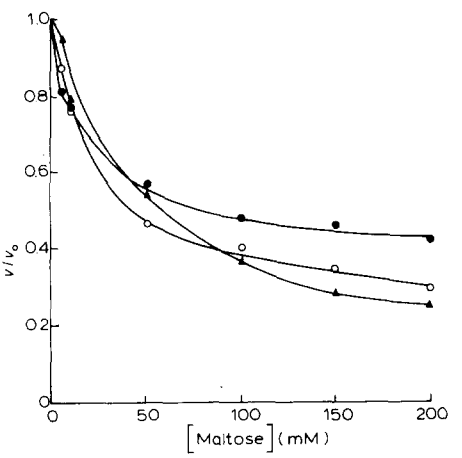


Fig. 3. Replots of slope and intercept values obtained from primary double-reciprocal plots ($1/v$ versus 1/[Nph-Glc]) in the absence and presence of various constant concentrations of maltose. (a), replots of slopes (○) and intercepts (●) against maltose concentration. (b), replots of the same slope (○) and intercept (●) values as $1/(\text{slope}_x - \text{slope}_o)$ and $1/(\text{intercept}_x - \text{intercept}_o)$ versus the reciprocal of maltose concentration. The subscripts x and o denote the slope or intercept values in the presence and absence of maltose, respectively.

probably behaving as a very poor glucosyl acceptor, partially diverting the reaction flux to an alternate pathway at the high concentrations required for inhibition to occur. Since earlier results indicated that K_3 equals K_2 in the presence of maltose [1] it follows that each of these two parameters equals 50 mM maltose.

Initial velocities in the presence of glycerol

Lineweaver-Burk plots ($1/v$ versus 1/[Nph-Glc]) of the results obtained in the presence of glycerol were linear and were found to intersect at a point corresponding to 0.6 mM Nph-Glc, but the same results plotted as $1/v$ versus 1/[glycerol] were non-linear particularly in the presence of high concentrations of glycerol [1]. Both slopes and intercepts of double-reciprocal plots in the presence of glycerol were affected [1]. Replots of slope and intercept values from similar primary double-reciprocal plots (i.e. $1/v$ versus 1/[Nph-Glc]) in the presence of glycerol are shown in Fig. 5. According to the terminology of Cleland [13] the results presented in Fig. 5 suggest slope-parabolic inhibition and intercept-linear activation. The line for the replot of intercepts cuts the



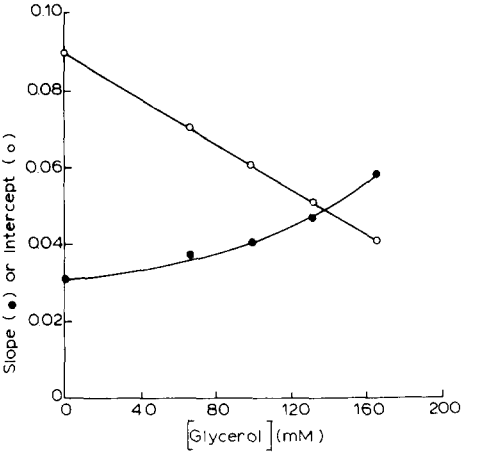


Fig. 4. Inhibition of rate of *p*-nitrophenol liberation by increasing concentrations of maltose in the presence of 0.1 mM (▲), 0.5 mM (○) and 2.0 mM Nph-Glc (●). The final concentrations of maltose are indicated. v_0 and v = nmol of *p*-nitrophenol liberated per min at 40°C in the absence and presence of maltose, respectively.

Fig. 5. Replots of slope (●) and intercept (○) values from primary double-reciprocal plots ($1/v$ versus $1/[\text{Nph-Glc}]$) in the absence and presence of various fixed concentrations of glycerol against glycerol concentration.

horizontal axis at a point corresponding to 300 mM glycerol. It should be noted that the intercept values represent the reciprocal of the velocities in the presence of glycerol when the donor (Nph-Glc) is saturating. If β-glucosidase action follows the pathways shown in Mechanism I, then the pathway through the E_A complex will be eliminated in the presence of saturating Nph-Glc concentrations and the value of 300 mM glycerol calculated from Fig. 5 represents the dissociation constant of the reaction between E^D and A to form E^D_A (i.e. K_3). This seems to correct the earlier figure of 200 mM (ref. 1) calculated from inadequate experimental data. With the value of K_3 known, the value of K_2 can be calculated as will be shown later. The value for K_2 was calculated to be approximately 85 mM glycerol. This indicates that with glycerol as acceptor K_3 is about 3.5 times K_2.

Further insight into the applicability of Mechanism I to the kinetics of β-glucosidase was obtained by following the liberation of *p*-nitrophenol in the presence of various fixed levels of donor (Nph-Glc) when the concentration of glycerol was varied. The results are shown in Fig. 6. In the presence of 1.0 mM or 2.0 mM Nph-Glc, the enhancement in enzymic activity appeared to be sigmoidal with increasing concentration of glycerol (up to 250 mM). When the concentration of Nph-Glc was 0.1 mM there was inhibition of enzymic activity. These results are consistent with the idea that glycerol was binding to two different enzyme species as shown in Mechanism I. Increasing the concentration of donor reduced the sigmoidicity of the plots in Fig. 6 and the shape of the curve approached a hyperbola. This effect, and the inhibition of enzymic activity when the donor concentration was 0.1 mM suggest the diversion of reaction flux to one or the other pathway of Mechanism I depending on the relative concentrations of donor and added acceptor.

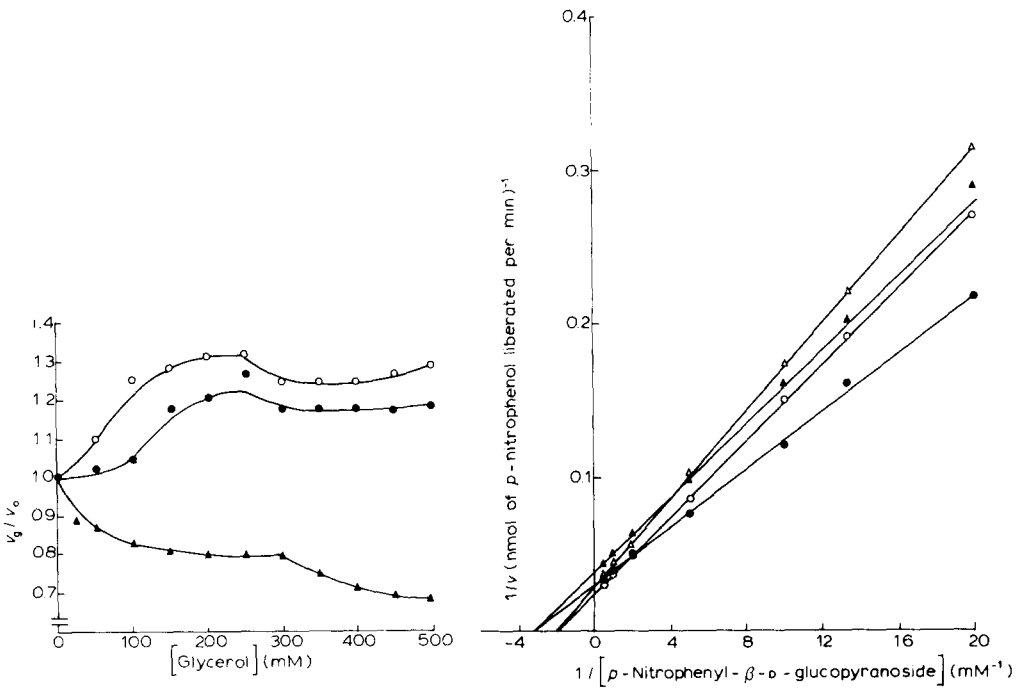


Fig. 6. Dependence on glycerol concentration of *p*-nitrophenol liberation in the presence of 0.1 mM (▲), 1.0 mM (●) and 2.0 mM Nph-Glc (○). v_g and v_0 = nmol of *p*-nitrophenol liberated per min at 40°C in the presence and absence of glycerol, respectively.

Fig. 7. Double-reciprocal plots showing the effects of 100 mM glycerol (○), 14.3 mM maltose (▲) or both 100 mM glycerol and 14.3 mM maltose (△) on the rate of liberation of *p*-nitrophenol by β-glucosidase, relative to a control which contained neither maltose nor glycerol (●). Initial rate, v = nmol of *p*-nitrophenol liberated per min at 40°C. The points on the plots were obtained experimentally but the lines were calculated from Eqn 3 (see later section) with $K_1 = 0.3125$, $K_2 = 85$, $K_3 = 300$, $K_5 = K_6 = 50$, $k_{15} = 33.33$, $k_{16} = 62.01$, $k_{17} = 0.2538$, $[A_1] = 100$, $[A_2] = 14.3$. In the absence of any particular acceptor, the parameters associated with that acceptor were taken to be zero.

In all cases, an apparent inhibition occurred in the presence of about 250 mM glycerol. This apparent inhibition was gradually reduced with further increase in glycerol concentration when the concentration of Nph-Glc was 1.0 mM or 2.0 mM but not when it was 0.1 mM. The glycerol concentration in this experiment (see Fig. 6) approached 5% (w/v) and at the higher concentrations of glycerol the solution properties and structure of the enzyme could be altered. This explanation for the inhibition in the presence of more than 250 mM glycerol cannot be positively ruled out since it has been found that the high-molecular-weight enzyme can dissociate into active species under certain conditions [6].

Inhibition by maltose in the presence of glycerol

The results obtained in an experiment in which velocity was determined as a function of Nph-Glc concentration in the absence or presence of maltose alone, glycerol alone or both glycerol and maltose are presented in Fig. 7. Maltose did not have any effect on the apparent Michaelis constant for the

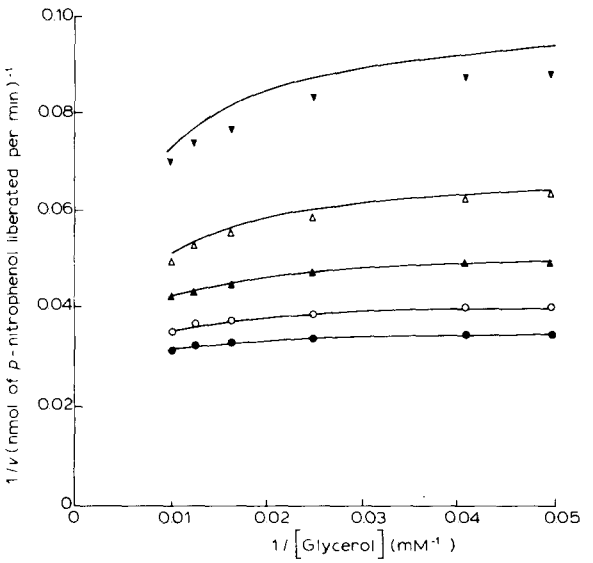


Fig. 8.(a) Double-reciprocal plots of $1/v$ versus the reciprocal of glycerol concentration in the presence of a fixed high concentration of Nph-Glc (2.0 mM) and in the absence (●) and presence of 10 mM (○), 25 mM (▲), 50 mM (△) and 100 mM maltose (▼). The points on the plots were obtained experimentally but the lines were calculated from Eqn 3 (see later section) with $K_1 = 0.3125$, $K_2 = 85$, $K_3 = 300$, $K_5 = K_6 = 50$, $k_{15} = 33.33$, $k_{16} = 62.01$, $k_{17} = 0.2538$. The same enzyme solution was used for the experiments the results of which are presented in Figs 7 and 8.

donor in the absence of glycerol or on that in the presence of glycerol. In another experiment, velocity was determined in the presence of a fixed high concentration of Nph-Glc (2.0 mM) and in the absence or presence of various concentrations of maltose as a function of glycerol concentration. The results are plotted as the reciprocal of velocity against the reciprocal of glycerol concentration in Fig. 8. These results will be discussed later.

Sugars as acceptors

A reaction mixture containing only Nph-Glc (1 mM) and β-glucosidase was incubated for 15 h at 40°C and concentrated under reduced pressure. Paper chromatography of the material gave spots for glucose, cellobiose and cellotriose. Obviously, secondary reactions had taken place during the prolonged incubation. Only glucose was detected in reaction mixtures incubated for a shorter period (15 min). With Nph-Glc (1.0 mM) as donor, cellobiose (0.01 mM) was found to be an efficient glycosyl acceptor; paper chromatography showed a large cellotriose spot. This is consistent with the observed stimulatory effect of Nph-Glc breakdown by cellobiose at low cellubiose concentrations (cf. ref. 1). Only a trace amount of an unidentified transglucosylation product, which behaved like a trisaccharide on paper chromatography, was found when the reaction mixture contained Nph-Glc (1 mM) as donor and maltose (50 mM) as acceptor. Fructose and sucrose were found to be efficient accpetors. Transglucosylation products obtained by paper chromatography of reaction mixtures which contained fructose or sucrose have not been identified but are probably a glucosyl fructose or a glucosyl-sucrose, respectively. That sucrose was found

to be an efficient glucosyl acceptor is in accord with the observed stimulatory effect of sucrose on the release of *p*-nitrophenol from Nph-Glc by β-glucosidase [1]. These results show that the acceptor site on β-glucosidase has a broad specificity. Maltose is, however, a very poor glucosyl acceptor compared to water and the other substances tested. This may be because the acceptor group on the maltose molecule is not orientated in a suitable manner for rapid transfer reactions.

Effect of aglycone on enzymic hydrolysis

The same concentration (1 mM) of Nph-Glc, cellobiose and methyl-β-D-glucoside in 0.05 M sodium acetate buffer (pH 5.0) were incubated at 40° C for 15 min in the presence of the same amount of β-glucosidase. The reaction was then terminated by the addition of 1 ml of 1 M Na_2CO_3 solution and the amount of glucose liberated estimated. The order of decreasing of hydrolysis by β-glucosidase was found to be Nph-Glc, cellobiose, methyl glucoside. The hydrolysis of methyl glucoside was barely detectable under these conditions.

Theoretical considerations

The kinetics of β-glucosidase have been shown to fit Mechanism I (Umezurike [1]), and the rate equation for this mechanism, derived under the rapid equilibrium assumption, is:

$$\frac{v}{e_0} = \frac{k_1(1 + k_2[A]/k_1K_3)/(1 + [A]/K_3)}{1 + \frac{K_1}{[D]}\left\{(1 + \frac{[A]}{K_2})/(1 + \frac{[A]}{K_3})\right\}} \quad (1)$$

If K_2 equals K_3, and k_1 is greater than k_2 Eqn 1 reduces to:

$$\frac{v}{e_0} = \frac{k_1(1 + k_2[A]/k_1K_3)/(1 + [A]/K_3)}{1 + K_1/[D]} \quad (2)$$

Eqn 2 is identical to the equation for the classical non-competitive inhibition where the enzyme-substrate-inhibitor complex breaks down at a slower rate than the enzyme-substrate complex [14]. Inhibition of β-glucosidase by maltose was found to fit Eqn 2. These kinetic features have been fully considered previously [1].

An illuminating kinetic result was observed when Mechanism I was extended to include another competing acceptor. Under this condition Mechanism I is modified to Mechanism II.

$$E + D \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ED \quad K_1 = k_2/k_1$$

$$E + A_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA_1 \quad K_2 = k_4/k_3$$

$$ED + A_1 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EA_1D \quad K_3 = k_6/k_5$$

$$EA_1 + D \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EA_1D \quad K_4 = K_8/k_7$$

$$E + A_2 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EA_2 \quad K_5 = k_{10}/k_9$$

$$ED + A_2 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} EA_2D \quad K_6 = k_{12}/k_{11}$$

$$EA_2 + D \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} EA_2D \quad K_7 = k_{14}/k_{13}$$

$$ED \underset{+H_2O}{\overset{k_{15}}{\longrightarrow}} E + P_1 + P_2$$

$$EA_1D \overset{k_{16}}{\longrightarrow} E + P_1 + P_3$$

$$EA_2D \overset{k_{17}}{\longrightarrow} E + P_1 + P_4$$

Mechanism II

where P_1 is *p*-nitrophenol, P_2 is glucose, P_3 and P_4 are transglucosylation products in the case where the donor (D) is Nph-Glc, and A_1 and A_2 are added competing acceptors (e.g. glycerol and maltose). Assuming that the rate-limiting steps are those denoted by k_{15}, k_{16} and k_{17}, and that all other steps are fast, the rate equation for Mechanism II derived by the method of Cha [15] is:

$$\frac{v}{e_0} = \frac{k_{15}\left\{1 + \frac{k_{16}[A_1]}{k_{15}K_3} + \frac{k_{17}[A_2]}{k_{15}K_6}\right\} \Big/ \left\{1 + \frac{[A_1]}{K_3} + \frac{[A_2]}{K_6}\right\}}{1 + \frac{K_1}{[D]}\left\{\frac{(1 + [A_1]/K_2 + [A_2]/K_5)}{(1 + [A_1]/K_3 + [A_2]/K_6)}\right\}} \tag{3}$$

The assumptions which give rise to Eqn 3 are similar to the conditions that obtained in the experiments which gave the results presented in Figs 7 and 8.

It should be noted that Eqn 3 reduces to the simple Michaelis-Menten equation (Eqn 4) in the absence of any added acceptor (i.e. in the absence of A_1 and A_2).

$$\frac{v}{e_0} = \frac{V}{1 + K_1/[D]} \tag{4}$$

On the other hand, Eqn 3 reduces to Eqn 1 in the presence of only one added acceptor (A_1 or A_2). Under V conditions (i.e. when donor concentration is much more than K_1) Eqn 1 reduces to Eqn 5.

$$\frac{V}{e_0} = k_1(1 + k_2[A]/k_1K_3)/(1 + [A]/K_3) \tag{5}$$

In the absence of an added acceptor Eqn 5 reduces to Eqn 6.

$$\frac{V}{e_0} = k_1 \tag{6}$$

The parameter k_1 is thus the experimentally determined maximum velocity in the absence of added acceptors. Eqn 5 can be rearranged to Eqn 7.

$$\frac{V}{e_0} = \frac{k_1}{1 + [A]/K_3} + \frac{k_2}{1 + K_3/[A]} \tag{7}$$

In Eqn 7 the term containing k_1 is the hydrolytic component and that containing k_2 is the transglucosylation component. It is clear that k_2 can be calculated from Eqn 7 by substituting the various experimentally determined parameters in Eqn 7.

If Eqn 1 applies, all the primary double reciprocal plots (i.e. $1/v$ vs. $1/[\text{Nph-Glc}]$) in the presence of various concentrations of added acceptor intersect at a particular point as observed (cf. ref. 1). The negative value of the donor concentration at the point of intersection ($-[D]_x$) is related to other parameters as shown in Eqn 8 (cf. ref. 16).

$$-[D]_x = \frac{K_1(K_3/K_2 - k_2/k_1)}{1 - k_2/k_1} \tag{8}$$

Thus K_2 can be easily calculated from Eqn 8 if the other parameters are known.

It should be noted that K_3 in Eqn 7 is the same as K_3 or K_6 in Eqn 3 depending on whether A_1 or A_2, respectively, is being considered alone. Similarly, k_2 in Eqn 7 is the same as k_{16} or k_{17} in Eqn 3 depending on whether A_1 or A_2 is being considered alone.

If the various parameters for two different added acceptors (e.g. glycerol and maltose) are calculated from experimental data and from Eqns 6—8, the applicability of Eqn 3 to the kinetics of β-glucosidase can be tested. Plots of data obtained from theoretical calculations using Eqn 3 by applying, in all cases, the conditions that $K_5 = K_6$, $K_3 \gg K_2$ and $k_{16} > k_{15} \gg k_{17}$ gave results which fitted the experimental data plotted in Figs 7 and 8 if A_1 was glycerol and A_2 was maltose. That is, by using $K_5 = K_6 = 50$, $K_3 = 300$, $K_2 = 85$, $K_1 = 0.3125$ (i.e. K_m for Nph-Glc in the absence of added acceptor) and other parameters calculated from the experimental data presented in Figs 7 and 8. Indeed all the lines in Figs 7 and 8 were calculated from Eqn 3 with the parameters shown in the legends to Figs 7 and 8 whereas the points were obtained experimentally.

The models of mechanisms for some enzymes involving enzyme-bound covalent intermediates [17—19] gave rate equations, derived by the method of King and Altman [20] by taking the reaction rates as the amount of the aglycone released from the donor, which did not fit the observed kinetics of β-glucosidase. Similarly, rate equations of an ordered mechanism involving a ternary complex did not fit the observed kinetics of β-glucosidase even in the presence of competing acceptors.

Discussion

In common with some other glycosidases (cf. ref. 21) the β-glucosidase of *B. theobromae* catalyses reactions of the type:

$$G\text{-}OX + H\text{-}OR \rightleftharpoons G\text{-}OR + XOH$$

where G (glucosyl unit) is transferred from the donor (GOX) to an acceptor (HOR). Obviously, the difference between hydrolysis (where water is the acceptor) and transglucosylation (where the acceptor is an alcohol or sugar) is only a semantic one. In the absence of an alternative acceptor only hydrolysis takes place and the reaction obeys Michaelis-Menten Kinetics (Umezurike [1]). The Michaelis-Menten model is valid when only one substrate is involved in the reaction (cf. ref. 22). As stated above, the β-glucosidase reaction involves two substrates (the donor and the acceptor). The β-glucosidase reaction, when only water is present as acceptor, fits the above model because water is present in large excess and its (water) concentration will be little affected during the course of hydrolysis. Thus, the kinetics will be treated as if water was not present. Even in the presence of a competing acceptor (or acceptors) when the kinetics fit Eqns 1—3 water is still present in excess, and the conversion of the enzyme-donor complex to products by reactions with water would appear as a spontaneous breakdown of the complex not involving an acceptor (water). That is, this particular reaction would be pseudo-first order. That glycerol and the other acceptors can act as acceptors under these conditions may be, presumably, because the acceptor site has a preference for these acceptors rather than for water. This may be due to the differences in the relative strengths of the conjugate bases of the alcoholic functional groups of these acceptors and the hydroxide ion of water. The conjugate bases of the alcoholic functional groups of these acceptors are apparently more reactive as nucleophiles than the hydroxide ion. That this argument applies is shown by the ability of released glucose to function as an acceptor to form cellobiose after prolonged incubation of a reaction mixture containing only Nph-Glc and β-glucosidase.

The kinetic study of the reaction catalysed by β-glucosidase showed that the mechanism of action of this enzyme in the presence of an added acceptor is consistent with Mechanism I which involves the presence of alternate pathways apparently leading to the formation of ternary complexes. Supporting evidence includes the slope and intercept effects in the presence of maltose (Fig. 3) and of glycerol (Fig. 5). The effect of increasing glycerol concentration on rate as a function of donor concentration (Fig. 6), and the results shown in Figs 7 and 8 which show that the kinetics of β-glucosidase in the presence of two other acceptors apart from water fit theoretical considerations are also in support of the mechanism proposed. Unpublished data obtained in this laboratory from some preliminary experiments indicated that glycerol can bind to β-glucosidase in the absence of donor (Nph-Glc). Apparently, the proposed mechanism is basically similar to the "reciprocal complex formation version of the ternary complex hypothesis" of Jermyn [4,23] proposed for the aryl-β-glucosidase of *Stachybotrys atra* from different results. Mechanisms involving enzyme-bound

covalent intermediates [17—19] in the presence of competing acceptors lead to kinetic features which are different from those of β-glucosidase. The decreasing rate of enzymic hydrolysis in the order Nph-Glc, cellobiose and methyl-β-D-glucoside seems to support the proposed mechanism.

The β-glucosidase of *B. theobromae* can hydrolyse cellobiose [6], *p*-nitrophenyl-β-D-glucopyranoside [6], salicin [7] and methyl-β-D-glucoside and a number of compounds can act as glucosyl acceptors. In each of these substrates (donors) the glycone is a glucosyl moiety but the aglycones differ. In other words, the enzyme can tolerate a wide variety of aglycones provided that the substrate (donor) has the β-configuration. This observation predicts that the enzymic hydrolysis of these glucosides takes place by the fission of the glucosyl-oxygen bond [24]. Indeed, β-glucosidase from other sources have been reported to cleave this particular bond in β-glucosides, and glucose is initially released in the β-form [5,24]. The retention of the configuration at the anomeric carbon atom of the product of hydrolysis can be explained by either the double-displacement or front-side mechanism [24]. However, for glycosidases that behave in this way, the double-displacement mechanism has usually been invoked. Thus, Legler [5] has proposed this mechanism for the β-glucsidase of *Aspergillus wentii*. Koshland [24] has pointed out that the double displacement mechanism should not be construed as showing that the displaced aglycone has necessarily been released from the enzyme surface before the acceptor molecule binds to the enzyme. If the acceptor molecule is required to activate the reaction, then it will be reasonable to assume that both donor and acceptor should be present together on the enzyme surface [24]. The results of kinetic experiments presented in this paper suggest that the acceptor and donor bind to β-glucosidase in a random fashion to form a ternary enzyme-donor-acceptor complex. With the above consideration in view, these results may be taken as being consistent with a double-displacement mechanism. However, a front-side mechanism cannot be ruled out.

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