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KINETIC PROPERTIES OF β-GLUCOSIDASE FROM *BOTRYODIPLODIA THEOBROMAE* PAT.

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SUMMARY

1. Low concentrations (less than about 10^{-4} M) of D-cellobiose stimulated, but higher concentrations inhibited, the activity of the high molecular weight β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) of *Botryodiplodia theobromae* Pat. when *p*-nitrophenyl-β-D-glucopyranoside (PNPG) was the substrate.

2. In the hydrolytic function of the enzyme, the inhibition by maltose (non-competitive inhibitor) was completely abolished, and that by glucose (a dead-end competitive inhibitor) was partially abolished, in the presence of up to 250 mM D-cellobiose. There was inhibition in the presence of higher concentrations of D-cellobiose.

3. Higher values of both v_{max}^{PNPG} and K_m^{PNPG} were obtained in the presence of glycerol than in the hydrolytic process from which glycerol was absent. Both values increased with increase in the concentration of glycerol, leading to inhibition at low of concentrations of donor (PNPG) (less than 0.5 mM) and to stimulation at higher concentrations.

4. A transglucosylation product was formed in the presence of glycerol.

5. The donor, acceptor and inhibitors bound to the enzyme independently.

6. Kinetic data fitted an equation derived to account for the properties of the enzyme.

7. It is concluded that the enzyme has distinct sites for the donor and acceptors, and that in the presence of glycerol, which is a more efficient glucosyl acceptor than water, hydrolysis and transglucosylation are parallel processes.

INTRODUCTION

JERMYN[1-6] has worked extensively on the aryl-β-glucosidase from *Stachybotrys atra*. This enzyme differs from the high molecular weight β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from *Botryodiplodia theobromae* in that the former does not hydrolyse cellobiose[2,4], whereas the latter does[7]. JERMYN[2] has shown that the

Abbreviation: PNPG, *p*-nitrophenyl-β-D-glucopyranoside.

molecule of aryl-β-glucosidase has two sorts of binding site, one for the donor (aryl-β-D-glucosides) and one for the acceptor. It has also been reported[4–6] that in the hydrolysis of aryl-β-glucosides by aryl-β-glucosidase from *S. atra*, water is an acceptor for glucosyl residues in the absence of more efficient acceptors (aliphatic alcohols). From these observations, JERMYN[4] has proposed a ternary mechanism involving an enzyme–donor–acceptor complex to explain the observed kinetics of aryl-β-glucosidase.

These results have prompted an investigation of the effects of a number of substances on the kinetics of purified high molecular weight β-glucosidase from *B. theobromae*, and the data fitted to equations modified from those derived by FRIEDEN[8] to account for the kinetics of allosteric single substrate enzymes.

MATERIALS AND METHODS

Chemicals

All chemicals were as described previously (see ref. 7).

Preparation and purification of enzyme

β-Glucosidase was obtained from culture filtrates of *B. theobromae* grown on D-cellobiose as sole carbon source[9,10]. The high molecular weight component was purified as described previously[7].

Enzyme assay

β-Glucosidase activity was determined with *p*-nitrophenyl-β-D-glucopyranoside (PNPG) as substrate[7,9,10]. For kinetic experiments where various concentrations of the substrate (PNPG) were required, the substrate solutions were adjusted to give a final concentration of PNPG from 0.05 to 2.0 mM in the assay mixture, and the results expressed as Lineweaver–Burk plots[11]. Units of enzymic activity are expressed as nmoles of *p*-nitrophenol formed per min at 40° (UMEZURIKE[7]).

p-Nitrophenol estimation

p-Nitrophenol was estimated by its absorbance at 400 nm as for enzyme assay.

Glucose estimation

Glucose was estimated by the Folin–Wu method[12,13].

Paper chromatography

Samples were concentrated under reduced pressure and spotted on Whatman No. 4 chromatographic papers. The papers were developed with a solvent consisting of isopropanol–water (160:40, v/v) by descending chromatography. The carbohydrate spots were detected with $AgNO_3$–NaOH reagents. The papers were cleared in 2 M ammonia (see ref. 14).

Analysis of kinetic data

FRIEDEN's[8] equation accounts for the effects of modifiers on the kinetic parameters of allosteric single substrate enzymes. This equation has been modified for treatment of the kinetic properties of the more complex glutamate dehydrogenase[15].

In this paper, the equation has also been modified to fit the observed kinetic properties of β-glucosidase.

Our present knowledge of the properties of β-glucosidase (see INTRODUCTION) has to be borne in mind in deriving an equation that fits the observed kinetics of this enzyme. Specifically, the following assumptions have to be made. Consider an enzyme (E) which has a distinct donor site and a distinct acceptor site. If it is assumed that (a) the donor (D) does not compete with the acceptor (A) for the acceptor site and *vice versa*, (b) a ternary complex involving the enzyme, the donor and an acceptor is required for transglucosylation products to be formed, and (c) in aqueous solutions, water (H) takes part in the formation of a ternary complex (HED) which breaks down to products, then the situation can be described by the following mechanism (Mechanism 1):

$$\text{Step 1. } E + \text{D} \rightleftharpoons E\text{D} \qquad K_1 = \frac{[E]\,[\text{D}]}{[E\text{D}]}$$

$$\text{Step 2. } E + \text{A} \rightleftharpoons \text{A}E \qquad K_2 = \frac{[E]\,[\text{A}]}{[\text{A}E]}$$

$$\text{Step 3. } E\text{D} + \text{A} \rightleftharpoons \text{A}E\text{D} \qquad K_3 = \frac{[E\text{D}]\,[\text{A}]}{[\text{A}E\text{D}]}$$

$$\text{Step 4. } \text{A}E + \text{D} \rightleftharpoons \text{A}E\text{D} \qquad K_4 = \frac{[\text{A}E]\,[\text{D}]}{[\text{A}E\text{D}]}$$

and

$$\text{Step 5. } E\text{D} \xrightarrow[+\text{H}_2\text{O}]{k_1} (\text{H}E\text{D}) \rightarrow E + \text{P}_1 + \text{P}_2$$

$$\text{Step 6. } \text{A}E\text{D} \xrightarrow{k_2} E + \text{P}_1 + \text{P}_3$$

In this mechanism K_1 to K_4 are the dissociation constants of Steps 1 to 4, respectively; k_1 is the rate constant for the hydrolysis of the ED complex; and k_2 is the rate constant for the breakdown of the AED complex. P_1, P_2 and P_3 are products. With PNPG, for example, as donor, P_1 is *p*-nitrophenol, P_2 is glucose and P_3 is a transglucosylation product. If it is assumed that Steps 1 to 4 in the above mechanism attain rapid equilibrium, and that k_1 and k_2 are rate-limiting, the following equation is obtained, which is a modification of that derived by FRIEDEN[8] from the original form of SEGAL *et al.*[16].

$$\frac{v_0}{[E]_0} = \frac{k_1(1 + k_2[\text{A}]/k_1K_3)/(1 + [\text{A}]/K_3)}{1 + \dfrac{K_1}{[\text{D}]}[(1 + [\text{A}]/K_2)/(1 + [\text{A}]/K_3)]} \tag{1}$$

In the absence of added acceptor (glycerol) Eqn. 1 reduces to

$$\frac{v_0}{[E]_0} = \frac{k_1}{1 + K_1/[\text{D}]} = \frac{v_{\max}}{1 + K_m/[\text{D}]} \tag{2}$$

Under this condition only Step 1 and the reaction involving the ED complex and water to form products (*i.e.* Step 6) take place. In the application of these equations to the kinetic data obtained with β-glucosidase, K_1 was taken to be equal to the Michaelis constant (K_m) in the absence of added acceptor; K_2 and K_3 were given arbitrary values based on the assumption that the ratio K_3/K_2 is equal to the ratio of the Michaelis constant in the presence of added acceptor to that in its absence; and

k_1 and k_2 were replaced with the maximal velocities in the absence and presence of added acceptor, respectively[8].

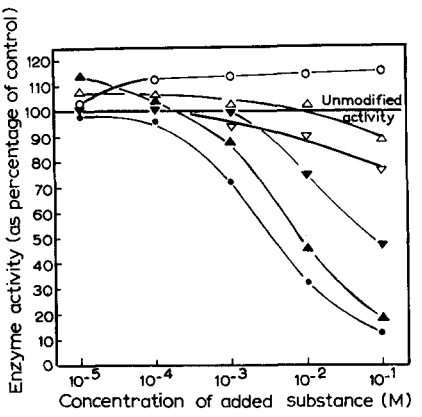
RESULTS

Effect of added substances on enzymic activity

Fig. 1 shows the effect of a number of substances on the activity of purified β-glucosidase. Of particular interest was the activation, by 10–20%, of β-glucosidase activity in the presence of low concentrations of cellobiose, and its inhibition at higher concentrations. This biphasic response of reaction velocity is reminiscent of the effect of substrate analogues on the reaction velocities of allosteric enzymes[17].

Inhibition of hydrolytic activity by glucose

It has been shown[1,2] that the aryl-β-glucosidase from *S. atra* is inhibited by glucose but not by the aglycone produced in the hydrolysis of aryl-β-glucosides.



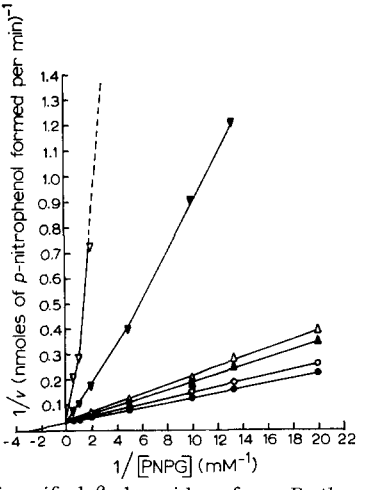


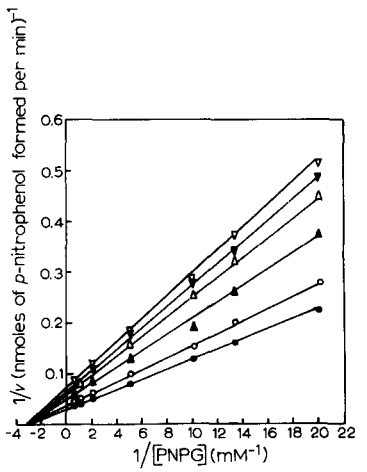
Fig. 1. The effects of various substances on the activity of purified β-glucosidase from *B. theobromae*. Aliquots of the enzyme solution in 0.05 M sodium acetate buffer (pH 5.0), the substrate (1.0 mM PNPG) and the substances at the final concentrations indicated were incubated at 40° for 15 min in the presence of glycerol (○), sucrose (△), glucosamine (▽), maltose (▼), D-cellobiose (▲) and glucose (●).

Fig. 2. Lineweaver–Burk plots showing the effect of glucose on the activity of β-glucosidase. v = nmoles of *p*-nitrophenol formed per min at 40° in the presence of final glucose concentrations of 0 (●), 0.4 mM (○), 0.8 mM (▲), 1.0 mM (△), 10.0 mM (▼) and 100.0 mM (▽).

Similarly, glucose was found to be a competitive inhibitor of the hydrolytic activity of the β-glucosidase from *B. theobromae* (Fig. 2). This inhibition appeared to be of the fully "dead-end" competitive type, for 100 mM glucose inhibited the activity completely at a low PNPG concentration (0.05 mM). The data did not fit Eqn. 1, as there was more inhibition by glucose than is predicted by this equation.

Inhibition of hydrolytic activity by maltose

As shown in Fig. 1, a given concentration of maltose inhibited β-glucosidase



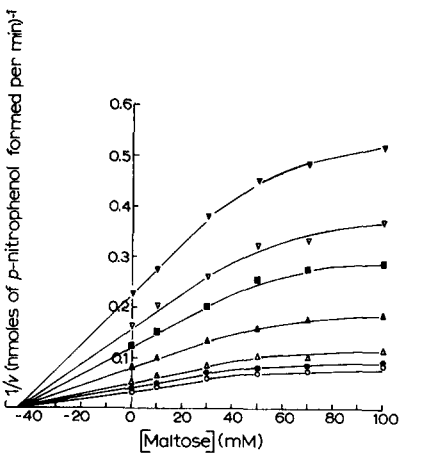


Fig. 3. Lineweaver–Burk plots showing the effect of maltose on the activity of β-glucosidase. The final concentrations of maltose added to the reaction mixtures were: ●, no maltose; ○, 10 mM; ▲, 30 mM; △, 50 mM; ▼, 70 mM; and ▽, 100 mM maltose. The points on the plots were obtained experimentally but the lines were calculated from Eqn. 1 assuming that $K_1 = 0.33$; $K_2 = K_3 = 33.33$ and $k_2 = 0$, 26.67, 20.00, 16.67, 15.38 and 14.29 in the presence of 0, 10, 30, 50, 70 and 100 mM maltose, respectively. The various parameters were calculated as indicated in MATERIALS AND METHODS. v = nmoles of *p*-nitrophenol formed per min at 40°.

Fig. 4. Dixon plots of the data presented in Fig. 3. Final concentrations of *p*-nitrophenyl-β-D-glucopyranoisde were: ▼, 0.05 mM; ▽, 0.075 mM; ■, 0.1 mM; ▲, 0,2 mM; △, 0.5 mM; ●, 1.0 mM; and ○, 2.0 mM. v = nmoles of *p*-nitrophenol formed per min at 40°.

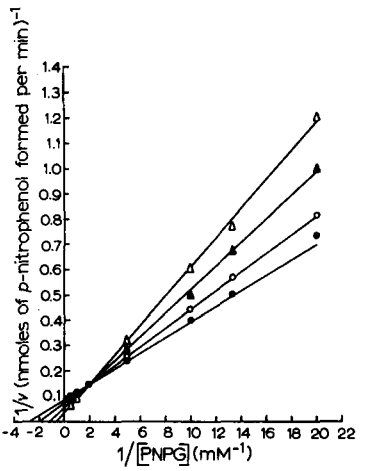
activity less than the same concentration of glucose. A Lineweaver–Burk plot[11] of maltose inhibition (Fig. 3) shows that maltose is a non-competitive inhibitor of the hydrolytic activity of β-glucosidase. Theoretical lines calculated from Eqn. 1 fitted the experimental results satisfactorily (Fig. 3). From Fig. 3 the K_m for PNPG was calculated to be 0.33 mM. Fig. 4 shows the Dixon plots of the same data plotted in Fig. 3. Extrapolation of the linear part of the lines (*i.e.* at lower maltose concentrations) to the x-axis gave a K_i value of 45 mM for maltose.

Effect of glycerol on enzymic activity

At the PNPG concentration (1.0 mM) routinely used for the assay of hydrolytic activity, the addition of glycerol (up to 100 mM) activated β-glucosidase activity (see Fig. 1). Lineweaver–Burk plots[11] showing the effect of varying the concentration of glycerol in the presence of increasing PNPG concentrations are shown in Fig. 5. All the lines intersect at a point corresponding to 0.5 mM PNPG. Theoretical lines calculated from Eqn. 1 fitted the experimental results satisfactorily (Fig. 5). The apparent K_m values for PNPG increased from 0.36 to 1.67 mM as the glycerol concentration was increased from 0 to 166.7 mM; and the apparent v_{max} increased from 11.76 to 28.57 mM *p*-nitrophenol formed per min as the glycerol concentration was increased from 0 to 166.7 mM.

Antagonism of maltose and glucose inhibition by cellobiose

The effect was studied of increasing concentrations of cellobiose (which stimu-



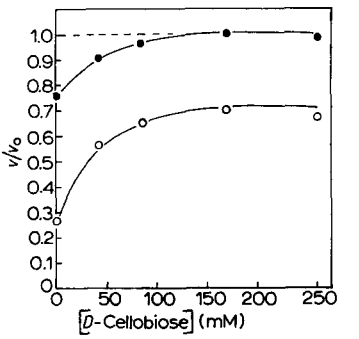


Fig. 5. Lineweaver–Burk plots showing the effect of glycerol on the activity of β-glucosidase. Final glycerol concentrations in the assay mixtures were: ●, no glycerol; ○, 66.7 mM; ▲, 133.4 mM; and △, 166.7 mM glycerol. The points on the plots were obtained experimentally but the lines were calculated from Eqn. 1 assuming that $K_1 = 0.36$; $K_2 = 1.0$; $K_3 = 0$, 1.39, 2.31 and 4.64; and $k_2 = 0$, 13.33, 16.67 and 28.57 in the presence of 0, 66.7 mM, 133.4 mM and 166.7 mM glycerol respectively; and $k_1 = 11.76$ in all cases. The various values were calculated as outlined in MATERIALS AND METHODS. v = nmoles of *p*-nitrophenol formed per min at 40°.

Fig. 6. Antagonism by D-cellobiose of the inhibition of β-glucosidase activity by 14 mM maltose (●) or by 14 mM glucose (○). v_0 and v = nmoles of *p*-nitrophenol formed per min at 40° in the absence and presence of inhibitors, respectively.

lates at low concentrations (Fig. 1) but inhibits competitively at higher concentrations[7]) on the inhibition of β-glucosidase activity by maltose (14 mM) or glucose (14 mM). Fig. 6 shows that up to about 250 mM cellobiose completely abolished the inhibition by maltose. The inhibition by glucose was reduced from about 75% to about 30%. Higher concentrations of cellobiose (*i.e.* more than 250 mM) inhibited enzymic activity with either sugar.

These and the preceding results lead to the conclusion that the β-glucosidase from *B. theobromae*, like the aryl-β-glucosidase from *S. atra*[2], has at least two sorts of binding site: a donor site and an acceptor site. The results in Fig. 6 can be taken as indicating that cellobiose, at low concentrations, first binds at the acceptor site to abolish the inhibition by maltose but subsequently, at higher concentrations, also binds at the donor site to inhibit. The result for glucose can be interpreted similarly. However, the 30% residual inhibition, when glucose was the inhibitor, in the presence of from 100 to 250 mM cellobiose, is probably due to the binding of glucose to the donor site since glucose is a competitive inhibitor.

Transglucosylation reaction

When the liberation of glucose and *p*-nitrophenol from PNPG (2 mM final concentration) by β-glucosidase, in the presence and absence of glycerol, was followed, it was found that within a short period (90 min) equal amounts of glucose and *p*-nitrophenol were liberated in the absence of glycerol (Fig. 7). That is, the ratio μmoles glucose liberated per μmoles *p*-nitrophenol liberated was 1. When the reaction mixture also contained 100 mM glycerol the amount of *p*-nitrophenol liberated increased by

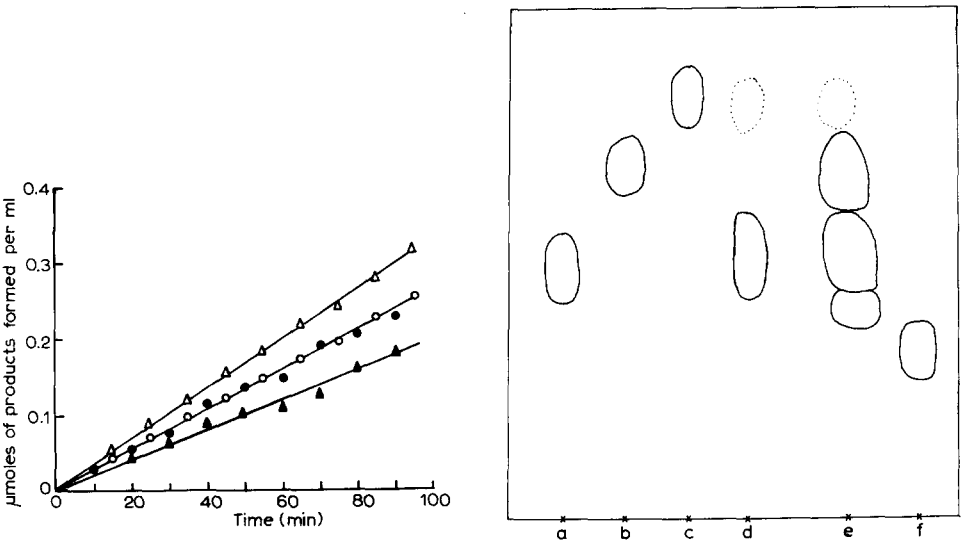


Fig. 7. Time course of the liberation of: ○, *p*-nitrophenol, and ●, glucose (as reducing sugar) in the absence of added glycerol; and △, *p*-nitrophenol, and ▲, glucose in the presence of glycerol (100 mM final concentration) from *p*-nitrophenyl-β-D-glucopyranoside (2 mM final concentration).

Fig. 8. Diagram of paper chromatogram on which was run: (a) glucose; (b) glycerol; (c) *p*-nitrophenyl-β-D-glucopyranoside; (d) concentrated reaction mixture containing β-glucosidase and PNPG (2 mM final concentration) after incubation for 2 h at 40°; (e) same as in (d) but the reaction mixture also contained glycerol (100 mM final concentration); and (f) cellobiose. The solvent was isopropanol–water (160:40, v/v). For further details see MATERIALS AND METHODS. *p*-Nitrophenol moved with the solvent front.

25% but the amount of glucose liberated decreased by 25% in relation to the values obtained in the absence of glycerol (Fig. 7). These results suggest that some of the glucose which would have been liberated in the presence of glycerol, had only hydrolysis taken place, must have been transferred to glycerol to form a non-reducing glucoside[3–6]. Examination of these reaction mixtures by paper chromatography revealed the presence of a transglucosylation product only when glycerol was present (Fig. 8).

Effect of varying glycerol concentration

Fig. 9 shows double reciprocal plots illustrating the effects of fixed PNPG concentrations on overall enzymic activity as a function of glycerol concentration. Again, the data fitted Eqn. 1. In the presence of less than 0.5 mM PNPG the non-linear lines seem to be parallel at low glycerol concentrations, but at higher glycerol concentrations the lines are markedly curved. At PNPG concentrations more than 0.5 mM the lines are also parallel but seem to be mirror images of those in the presence of lower PNPG concentrations. By extrapolating the linear portions of these lines (*i.e.* at low glycerol concentrations) to the *y*-axis and plotting the intercepts against the reciprocal of PNPG concentrations (see ref. 18) a straight line was obtained (Fig. 10). From the point of intersection of the line with the *x*-axis a K_m^{PNPG} of 0.5 mM was calculated, and from the intercept of the line with the *y*-axis a v_{max} value of 13.3 mM *p*-nitrophenol formed per min was calculated for the reaction in the presence of

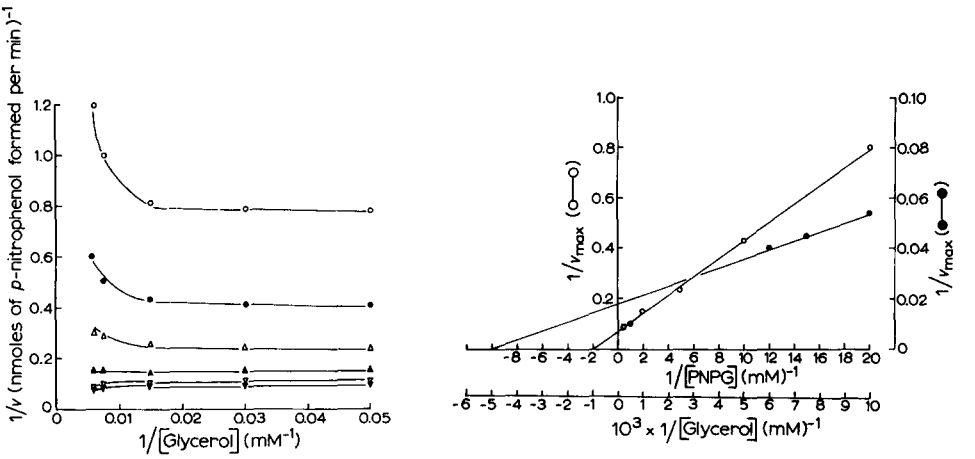


Fig. 9. Plot of reciprocal of reaction velocity (v) against reciprocal of glycerol concentration in the presence of ○, 0.05 mM; ●, 0.1 mM; △, 0.2 mM; ▲, 0.5 mM; ▽, 1.0 mM; and ▼, 2.0 mM p-nitrophenyl-β-D-glucopyranoside. The points were obtained experimentally and the lines were calculated from Eqn. 1.

Fig. 10. Secondary plots of (○) y-axis intercepts in Fig. 9 against reciprocals of p-nitrophenyl-β-D-glucopyranoside concentrations, and (●) y-axis intercepts against reciprocals of glycerol concentrations of results obtained from an experiment similar to that presented in Fig. 5.

glycerol (*i.e.* transglucosylation). Similarly, a plot of the results obtained in an experiment similar to that shown in Fig. 5 gave a K_m^{glycerol} of 200 mM and a v_{max} value of 55.6 mM for the transglucosylation reaction (Fig. 10).

Independence of binding sites

By using the results obtained with β-glucosidase in the absence or presence of glycerol, glucose or maltose for Hill plots[19–21], it was found that the slope, n, of the plot of log $[v/v_{max} - v]$ against log (PNPG) was 1 in the absence of glycerol, glucose or maltose. In the presence of increasing glucose concentrations K_m^{PNPG} increased, but the value of the slope, n, remained at 1, except in the presence of 10 to 100 mM glucose and at low donor concentrations when the value of n increased to 1.3 presumably because glucose was binding to both the donor and acceptor sites. Maltose (up to 100 mM) did not affect the slope of the Hill plots, and, as expected, all the lines were superimposed. Up to 100 mM, glycerol did not alter the value of n.

DISCUSSION

It is noteworthy that the conditions under which the kinetic data obtained in the presence of maltose or glycerol fitted Eqn. 1 were such that the parameter K_2/K_3 was less than k_1/k_2 (see ref. 8). In the presence of maltose K_2 equalled K_3, and k_1 was greater than k_2. In the presence of glycerol K_2 was much less than K_3, and k_1 was less than k_2. The results obtained in the presence of glucose (Fig. 2) did not fit Eqn. 1 because the equation does not account for the effects of substances that compete with the donor. These results and the total or partial antagonism by cellobiose of the inhibitory effects of maltose or glucose, respectively (Fig. 6), show that the

β-glucosidase of *B. theobromae*, like the aryl-β-glucosidase of *Stachybotrys atra*[2], possesses two sorts of binding site: one that binds the donor (PNPG) and the other that binds the acceptor (glycerol or water). Evidence presented in this paper indicates that glucose or cellobiose (which is also a substrate[7]) can bind to both the donor and acceptor sites whereas maltose apparently binds only to the acceptor site.

That the results obtained in the presence of glycerol fitted Eqn. 1 shows that products are formed *via* two pathways: hydrolysis and transglucosylation. Water is the acceptor in the hydrolytic process whereas glycerol is the glucosyl acceptor in the transglucosylation reaction. Under the conditions of the experiment reported in Fig. 7 only the hydrolytic process took place in the absence of glycerol. When glycerol (100 mM) was present, the transglycosylation process represented 40% of the overall cleavage of *p*-nitrophenyl-β-D-glucopyranoside, but both hydrolysis and transglucosylation activities were parallel. Water and glycerol are thus competitive acceptors for glucosyl residues from the donor.

The effect of glycerol on the kinetics of β-glucosidase (Fig. 5) is similar to the condition postulated by FRIEDEN[8] for a single-substrate allosteric enzyme when the rate of breakdown of the enzyme–substrate–modifier complex is greater than that for the enzyme–substrate complex (*i.e.* k_2 is greater than k_1). Similarly, it can be argued that, in the presence of glycerol, a ternary complex involving the enzyme, the donor and glycerol (as glucosyl acceptor) liberates the products at a faster rate than does the hydrolytic process where water is the glucosyl acceptor. This leads to inhibition at low donor concentrations and to stimulation at higher donor concentrations when the reaction velocity is measured by the release of *p*-nitrophenol. Thus glycerol is a better glucosyl acceptor than water (see ref. 4). The stimulatory effect of low concentrations of cellobiose (Fig. 1), when it does not compete with *p*-nitrophenyl-β-D-glucopyranoside for the donor site, indicates that cellobiose is also a better glucosyl acceptor than water.

The Hill plots of the results obtained in the presence of glycerol and of maltose or glucose show that the donor and the acceptor or inhibitors are bound independently to the enzyme.

Since both hydrolysis and transglucosylation reactions take place in the presence of glycerol, and *p*-nitrophenol is a product of both processes, experiments in which reaction velocities are measured by the amount of the transglucosylation product, rather than of *p*-nitrophenol, formed should throw some light on the effects of maltose or glucose on the binding of glycerol to the acceptor site.

Although, by definition, the β-glucosidase of *B. theobromae* is a cellobiase (see ref. 7) its kinetic properties are basically similar to those of the aryl-β-glucosidase of *S. atra* which is not a cellobiase.

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