FUNGAL CELLULASES

V. ENZYMIC PROPERTIES OF *STACHYBOTRYS ATRA* β-GLUCOSIDASE

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Summary

The effect of enzyme and substrate concentration, pH, temperature, and the nature and concentration of added salts on the activity of the β-glucosidase from *Stachybotrys atra* have been investigated. The inactivation of the enzyme by heat and acid has also been studied.

Energies of activation and Michaelis constants have been calculated for the enzyme under various conditions.

I. INTRODUCTION

Part IV of this series (Jermyn 1955) described the isolation and partial purification of a β-glucosidase from culture filtrates of *Stachybotrys atra.* The present paper summarizes some of the data necessary for the characterization of this enzyme. Only such information on substrate specificity as is necessary to correlate the other data will be considered here; Part VI in this series will consider the relation of the enzyme to its substrates and competitive inhibitors.

The most recent review devoted to β-glucosidases is that of Veibel (1950) which summarizes and gives references to much of the older literature.

II. METHODS

The methods for estimating β-glucosidase activity, outlined in Part IV of this series (Jermyn 1955), have been modified as required to estimate the effect of different variables. Thus in the *p*-nitrophenol-β-glucosidase technique the volume was held constant at 5 ml while pH and buffer, enzyme, substrate, and salt concentrations were varied by suitable modification of the original solutions. The 2 ml of 7·5 per cent. (w/v) K_2HPO_4 solution added at the end of the chosen period of reaction was occasionally modified to 75 per cent. (w/v) K_2HPO_4 for strongly acid reaction media or even to 10 per cent. (w/v) K_2CO_3 in order to bring the final pH to *c.* 8·5. In some instances insoluble precipitates had to be centrifuged off before making readings in the spectrophotometer. So long as the final pH was in the indicated range, the concentration of salts had no significant effect on the optical density.

Both techniques could be scaled up by any suitable factor so that samples of 5 ml (*p*-nitrophenol method) or 10 ml (Folin-Ciocalteau method) could be removed at intervals over a period of time. Temperatures were controlled throughout by the use of a thermostat bath which could be regulated to ± 0·1°C.

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The enzyme preparations used were lead-precipitated and dialysed fractions prepared by the methods outlined in Part IV. Only material with activity greater than 2000 units per mg N was used; the polysaccharide/protein ratio ranged from 10 to 15. Variations in the amount of polysaccharide present did not seem to have any detectable effect on enzymic properties. The Michaelis constant against *p*-nitrophenyl-β-glucoside (the property which on the available evidence should be most affected by carbohydrate impurities) was tested for

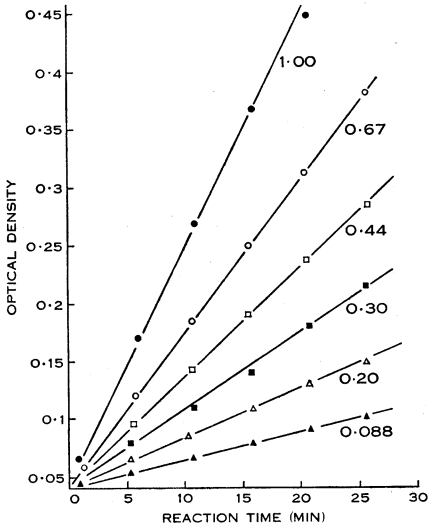

Fig. 1

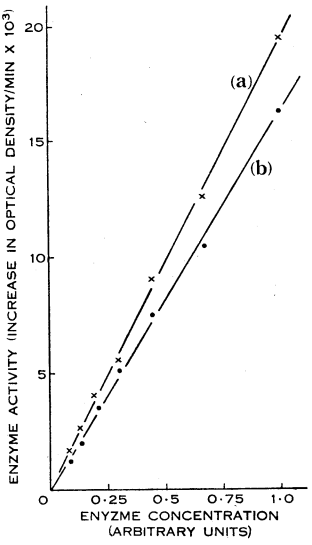

Fig. 2

Fig. 1.—Optical density at 400 mμ of standard enzyme reaction mixture at various times after adding enzyme. 5-ml samples withdrawn and added to 2 ml of 7·5 per cent. K_2HPO_4 solution. Incubation temperature 28°C. The numbers are relative enzyme strengths on an arbitrary scale.

Fig. 2.—Effect of enzyme concentration on rate of decomposition of *p*-nitrophenyl-β-glucoside. Reaction velocities are calculated from the slope of lines similar to those of Figure 1. Incubation temperature 28°C. Under otherwise standard conditions. (*a*) Substrate concentration 3×10^{-3}M. (*b*) Substrate concentration 3×10^{-4}M.

each sample and found to be sensibly constant. Nor was the Michaelis constant affected by the fall of the polysaccharide/protein ratio to 2 in stable column fractions.

III. Enzyme Kinetics

(*a*) *Effect of Time and Enzyme Concentrations*

Under conditions where the β-glucosidase was stable (in the pH range 4-10 and at temperatures below 45°C) the rate of hydrolysis was found to be linear with time in the presence of excess substrate. When the slope of these lines was plotted against enzyme concentration, another straight line resulted (see Figs. 1 and 2).

The demonstration of first order kinetics with regard to enzyme concentration is a justification of the method described in Part IV of this series for enzyme estimation, since the standard concentration of *p*-nitrophenyl-β-glucoside (10^{-3}M) is well above K_m for this substrate.

TABLE 1

EFFECT OF pH AND BUFFER STRENGTH ON THE MICHAELIS CONSTANT FOR THE HYDROLYSIS OF *p*-NITROPHENYL-β-GLUCOSIDE BY THE β-GLUCOSIDASE OF *S. ATRA*

Buffer	Michaelis Constant ($\times 10^5$)			
	pH 4	pH 5	pH 6	pH 7
Sodium phosphate-citric acid*	3·4	2·9	6·8	1·7
Sodium phosphate-citric acid†		4·4	3·8	3·0
Sodium acetate, 0·2M	2·4	4·9		
Sodium acetate, 0·1M	2·5	2·7		

* Four times McIlvaine concentration. † One-tenth McIlvaine concentration.

(*b*) *Course of Total Hydrolysis*

When the course of hydrolysis of the *p*-nitrophenyl-β-glucoside is followed by withdrawing samples over a period of time, the curve relating percentage substrate decomposed to incubation time falls considerably below the asymptotic

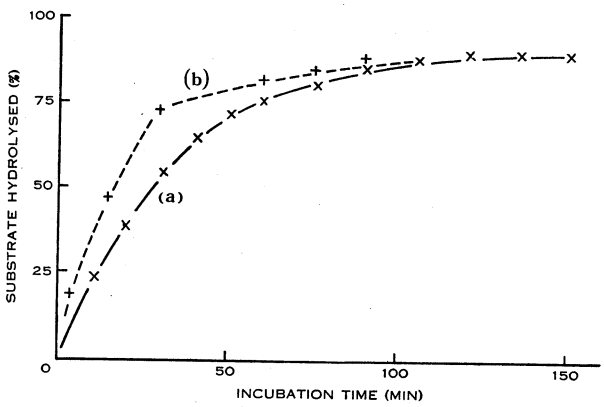

Fig. 3.—Time course of the hydrolysis of *p*-nitrophenyl-β-glucoside by the *S. atra* β-glucosidase under standard conditions. (*a*) Substrate concentration 3×10^{-5}M. (*b*) Substrate concentration 5×10^{-5}M.

approach to 100 per cent. decomposition expected from normal first order kinetics (Fig. 3). This behaviour led to the observation that the glucose liber-

ated by hydrolysis is a powerful competitive inhibitor of the enzyme. No phenol tested was found to exert a comparable effect.

(*c*) *Effect of pH*

In Figure 4 are set out pH-activity curves for the S. *atra* β-glucosidase against a number of substrates. McIlvaine (citric acid-sodium phosphate) buffers were used throughout, since they covered the whole range of enzyme activity. Almond emulsin measured under exactly identical conditions (Fig. 5) gives a single symmetrical peak and the secondary peak given by the S. *atra* enzyme at around pH 6 appears to be characteristic and not due to interactions

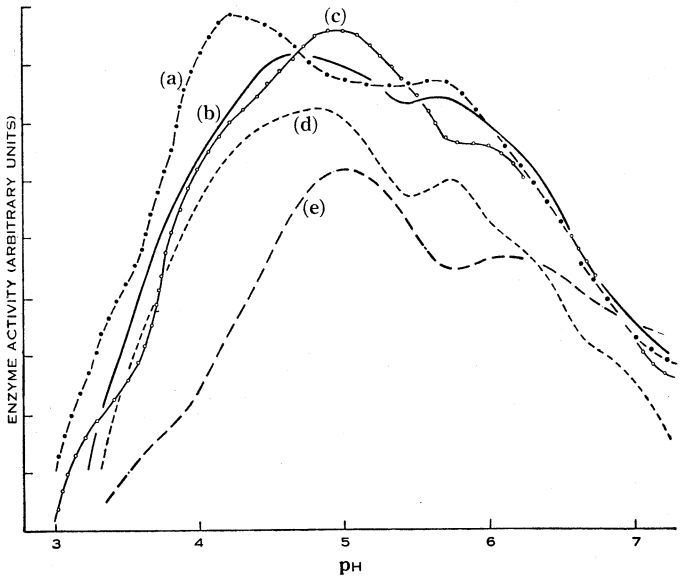

Fig. 4.—pH-activity curves for the S. *atra* β-glucosidase against a number of β-glucosides. Citrate-phosphate buffers at 28°C under identical conditions (10^{-3}M substrates). (*a*) 2-Naphthyl-β-glucoside. (*b*) Phenyl-β-glucoside. (*c*) *m*-Cresyl-β-glucoside. (*d*) *p*-Chlorophenyl-β-glucoside. (*e*) *o*-Hydroxymethylphenyl-β-glucoside (salicin).

which take place between the buffering salts and β-glucosidases in general.

Since it was later found that enzyme activity was affected by the nature of the buffering ion and salt concentration, and that K_m varied with pH and buffer, an attempt was made to construct a pH-activity curve in which these disturbing factors were eliminated.

The points of the curves in Figure 6 were obtained by a process of double extrapolation. A single stock solution of suitably diluted enzyme (shown to be stable over the course of the experiments) was employed throughout. Using this enzyme solution and a range of substrate concentrations where the

Michaelis-Menten relationship was known to hold, V_{max} at infinite substrate concentration was deduced for any given pH and buffer concentration by the extrapolation of Lineweaver-Burk plots. These calculated values of V_{max} were then plotted against buffer concentration and the curve extrapolated (no way was found of obtaining a linear plot) to give a value for V_{max} at zero buffer concentration.

The curves of Figure 6 show that the secondary peak in the curve for citrate-phosphate buffers has been substantially reduced by this procedure, and that the curve now has a single flat maximum and a slight shoulder. The non-equivalence of the citrate-phosphate and acetate curves shows that specific ion effects have not been eliminated.

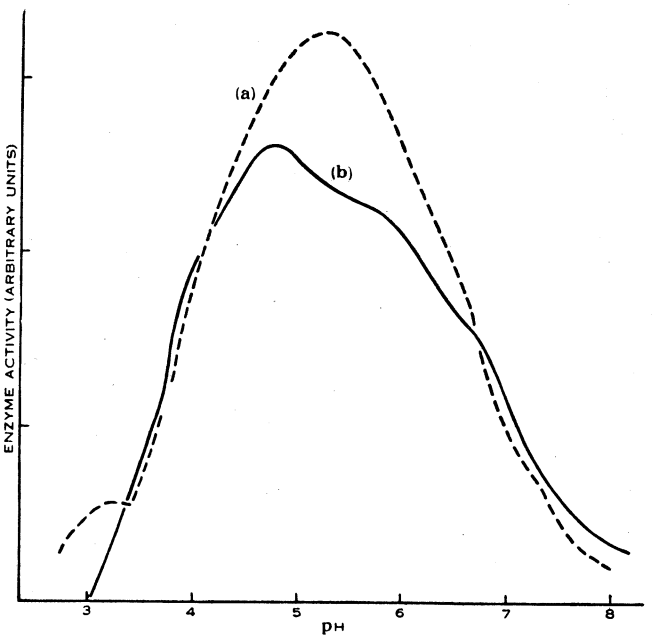

Fig. 5.—Comparison under identical conditions of the pH-activity curves of (*b*) *S. atra* β-glucosidase and (*a*) almond emulsin against *p*-nitrophenyl-β-glucoside. Citrate-phosphate buffers at 28°C.

(*d*) *Effect of Substrate Concentration*

Figure 7 illustrates the relationship between substrate concentration and rate of hydrolysis for two substrates. At high substrate concentrations the results do not accord with the Michaelis-Menten equation. This is one aspect of the general inhibition by excess of polyhydroxy compounds which will be discussed in Part VI of this series. However, at low substrate concentrations, the experimental results lead to rectilinear plots according to the method of Lineweaver and Burk (1934) and K_m can be calculated.

Figure 8 sets out Lineweaver-Burk plots for a number of substrates. For *p*-nitrophenyl-β-glucoside it has been found that this apparent K_m under the

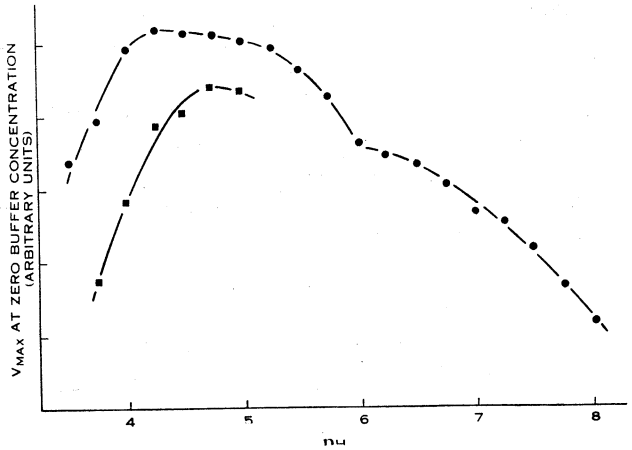

Fig. 6.—Enzyme activity of *S. atra* β-glucosidase extrapolated to zero buffer concentration and infinite substrate concentration (against *p*-nitrophenyl-β-glucoside at 28°C). ● Citrate-phosphate buffers. ■ Sodium acetate buffers. The same enzyme solution was used to determine both curves.

standard conditions (28°C and pH 5 McIlvaine buffer), is not affected by enzyme concentration or the use of different samples of enzyme.

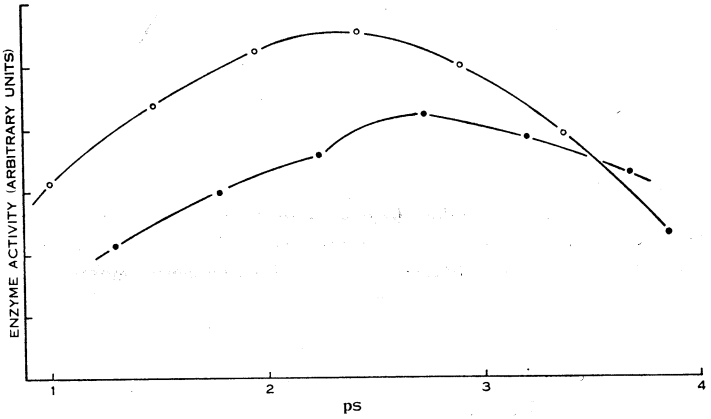

Fig. 7.—Effect of substrate concentration on the activity of *S. atra* β-glucosidase under standard conditions. ○ Phenyl-β-glucoside. ● *p*-Nitrophenyl-β-glucoside.

The values of V_{max} calculated from data obtained at substrate concentrations below 10^{-3}M although fictitious, are extremely useful in comparing the

activity of the enzyme against various substrates with the disturbing effects of differing K_m values eliminated.

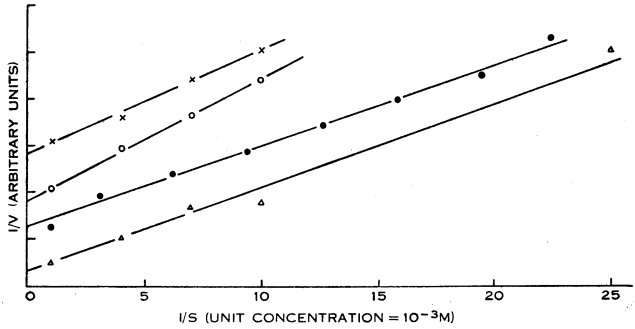

Fig. 8.—Lineweaver-Burk plots of the effect of low substrate concentrations on the velocity of hydrolysis of various β-glucosides by *S. atra* β-glucosidase. Standard conditions, 28°C. × 2-Naphthyl-β-glucoside. ○ Phenyl-β-glucoside. ● *p*-Nitrophenyl-β-glucoside. △ *p*-Cresyl-β glucoside.

(*e*) *Effect of pH, Buffer, and Salt Concentration on the Michaelis Constant*

In Table 1 are set out changes in the K_m values obtained for *p*-nitrophenyl-β-glucoside using acetate and citrate-phosphate buffers at different pH and two different levels of concentration; linear Lineweaver-Burk plots were obtained under all the conditions used. The differences obtained are significant (K_m at

TABLE 2

EFFECT OF 0·01M BUFFERS ON THE ACTIVITY OF *S. ATRA* β-GLUCOSIDASE AGAINST 10^{-3}M *p*-NITROPHENYL-β-GLUCOSIDE AT 28°C

The same dialysed enzyme sample was used throughout

Buffer (pH 5)	Enzyme Activity (relative to acetate buffer)
Sodium phosphate	0·85
Sodium succinate	0·71
Sodium citrate	0·94
Sodium phthalate	0·99
Sodium acetate	1·00
Sodium phenylacetate	0·93
Anilinium hydrochloride	1·07
Pyridinium hydrochloride	0·91

pH 5·0 under standard conditions is reproducibly $4{\cdot}5\text{-}5{\cdot}0 \times 10^{-5}$M) but not profound. It is impossible on this limited evidence to offer a rational explanation of the observed effects.

(*f*) *Effect of the Ionic Environment on the Activity of the Enzyme*

The observations which are incorporated in Figure 6 and Table 1 would lead to the expectation that all changes in the ionic environment would profoundly affect the activity of the enzyme. This was found to be the case and any statement of enzyme activity is accordingly meaningless unless this environment is rigidly specified.

Table 2 shows the variation in enzyme activity with variation in the nature of buffering ions at constant pH; and Figure 9 shows the variation with

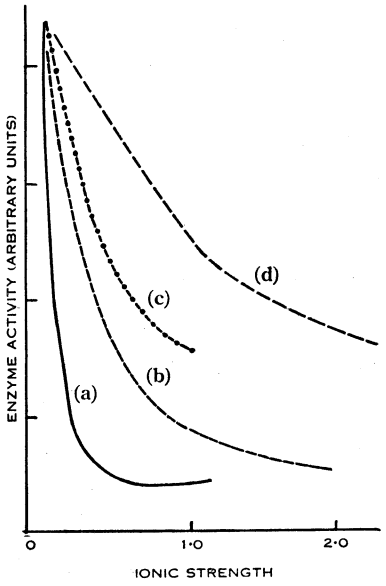

Fig. 9

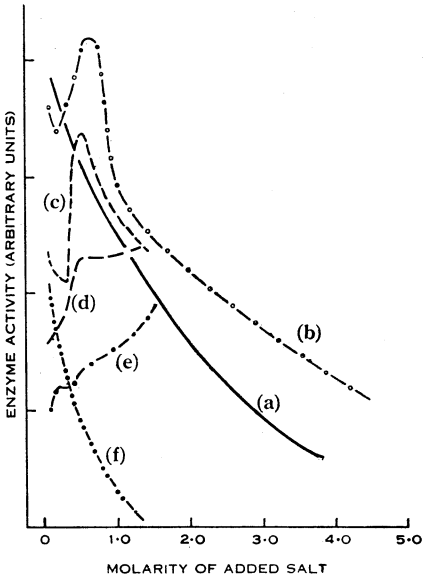

Fig. 10

Fig. 9.—Specific effects of the concentration of various buffering ions on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. (*a*) Pyridinium-HCl, pH 5·0. (*b*) Sodium acetate, pH 5·0. (*c*) NaH_2PO_4. (*d*) Sodium phosphate-citric acid, pH 5·0. In this and the next figure the buffer strengths given are those finally attained in the incubation mixture.

Fig. 10.—Effect of neutral salts on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase in the presence of various buffers. All buffers readjusted to pH 5·0 after the addition of the salts. (*a*) McIlvaine buffer with added NaCl. (*b*) 0·02M Pyridinium-HCl with added NaCl. (*c*) 0·02M Sodium acetate with added NaCl. (*d*) 0·02M Sodium acetate with added Na_2SO_4. (*e*) 0·02M Sodium acetate with added $MgCl_2$. (*f*) 0·02M Sodium acetate with added $MgSO_4$.

buffering ion concentration. These effects are not limited to buffering ions alone; the influence of neutral salts is shown in Figure 10. It is apparent that various salts influence the activity in a way which cannot be explained by greater or lesser ionic strength. The significance of the secondary peak of activity as the concentration of sodium chloride is increased in dilute buffers is quite uncertain.

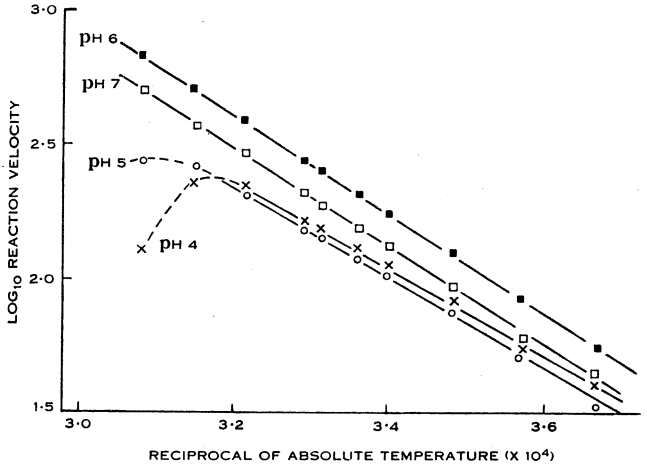

Fig. 11.—Effect of temperature on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase at four pH's. Reaction time 20 min; substrate concentration 10^{-3}M. Sodium phosphate-citric acid (McIlvaine) buffers.

(g) *Energy of Activation*

In spite of the profound effect of various alterations of the environment on the activity of the β-glucosidase, it was found that the apparent energy of activation was little altered by changes in pH and substrate. Figure 11 shows

TABLE 3

ENERGY OF ACTIVATION* FOR THE HYDROLYSIS OF VARIOUS β-GLUCOSIDES BY THE β-GLUCOSIDASE OF *S. ATRA* IN SODIUM PHOSPHATE-CIRTIC ACID (McILVAINE) BUFFERS

Substrate ($\times 10^{-3}$M)	pH	Temperature Range (°C)	Energy of Activation ± S.D. (calories/mol.)
p-Nitrophenyl-β-glucoside	7·0	0-50	8390±110
p-Nitrophenyl-β-glucoside	6·0	0-50	8540±140
p-Nitrophenyl-β-glucoside	5·0	0-44	7800±200
p-Nitrophenyl-β-glucoside	4·0	0-38	7440±220
Phenyl-β-glucoside	5·0	0-28	9300±300
o-Methylphenyl-β-glucoside	5·0	0-28	8600±150
o-Hydroxymethyl-phenyl-β-glucoside (salicin)	5·0	0-28	8020±240

* Calculated from the regression of log k on $1/T_{abs}$.

that the hydrolysis of *p*-nitrophenyl-β-glucoside follows the Arrhenius relationship in the pH range 4-7; the departure of the plot of log k against $1/T_{abs}$ from a straight line at the lower pH values occurs only at temperatures where the enzyme can be shown to be inactivated. Table 3 shows some derived values for the energy of activation; there is not sufficient evidence to decide whether the apparently significant difference between the energy of activation for the hydrolysis of *p*-nitrophenyl-β-glucoside at pH 4·0 and 6·0 is genuine or due to slight inactivation of the enzyme at the higher temperatures during incubation.

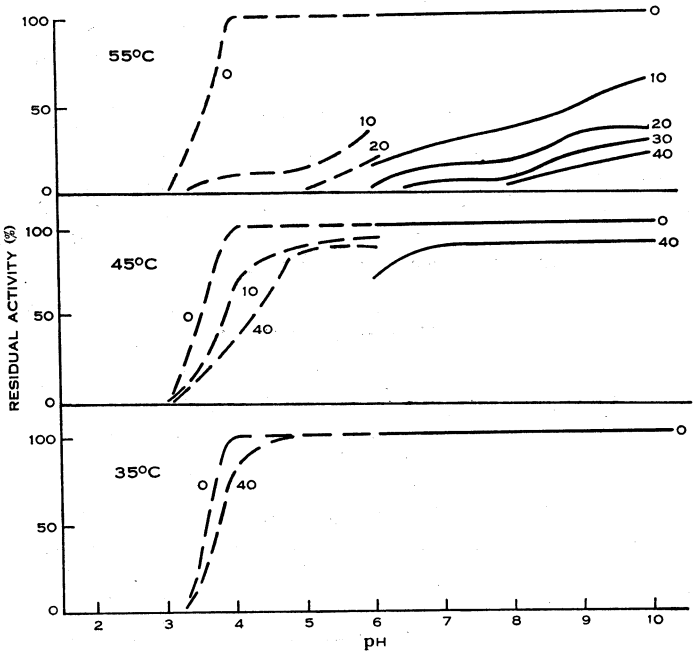

Fig. 12.—Stability of *S. atra* β-glucosidase at different pH's and temperatures. Broken lines, ACA buffers; full lines, ETE buffers. The numbers represent minutes of incubation.

IV. Inactivation of the Enzyme

(*a*) *Influence of pH and Temperature*

A solution of dialysed *S. atra* β-glucosidase (about 4 units per ml) in a buffer was incubated at the required temperature, samples withdrawn from time to time, and the residual activity estimated after dilution with cold McIlvaine buffer (pH 5). In order to cover the maximum range with simple ionic species, the buffers used were 0·05M ethanolamine-triethanolamine hydrochloride adjusted with NaOH (Thies and Kallinich 1953) and 0·05M sodium acetate-chloroacetate (equimolar proportions) adjusted with HCl. These buffers will be referred to as ETE and ACA buffers respectively; besides the

positively or negatively charged buffering species, they contain only varying amounts of sodium and chloride ions (Fig. 12).

It is evident that the resistance of the enzyme to temperature inactivation rises with increasing pH as far as the maximum (pH 10) used in this study and that the enzyme is more stable in ACA than in ETE at pH 6. The change from relative stability to complete inactivation with decreasing pH is very sharp; the enzyme is stable to above 30°C at pH 4 in ACA but inactivated immediately on mixing at pH 3·6, even at 0°C. Small differences in experimental conditions gave apparently inconsistent results and it was impossible to determine the

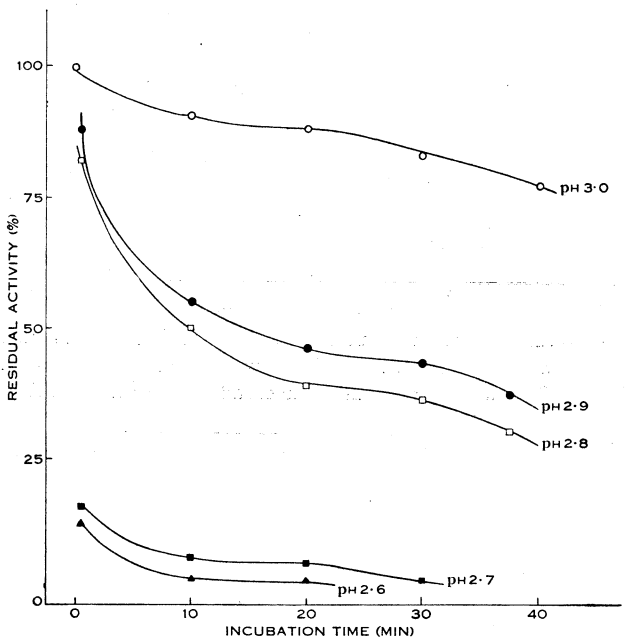

Fig. 13.—Inactivation of *S. atra* β-glucosidase in sodium phosphate-citric acid (McIlvaine) buffers. Enzyme concentration 2 units/ml; incubation temperature 20°C. The pH's at which the inactivation curves were obtained are shown on the diagram.

boundary more accurately than the range 3·6-4·0. In sodium phosphate-citric acid buffers it was possible to demonstrate the transition over a narrow range of pH values (Fig. 13). The enzyme is considerably more resistant to inactivation by acid in phosphate-citrate buffer than in ACA.

The ionic environment thus apparently affects the stability as well as the activity of the enzyme. That these effects extend to neutral salts for the stability may be seen from Figure 14. The maximum stabilization by an intermediate concentration of added sodium chloride is perhaps parallel to the similar effect of sodium chloride on the activity of the enzyme in certain buffers (Fig. 10).

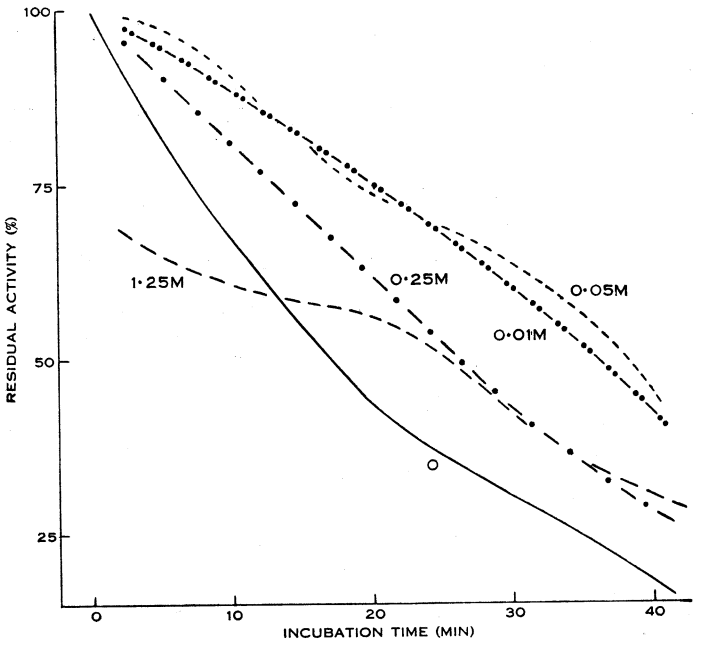

Fig. 14.—Effect of various concentrations of NaCl on the inactivation of *S. atra* β-glucosidase in ACA buffer (0·05M, pH 4·0) at 45°C. The figures give the concentration of added NaCl.

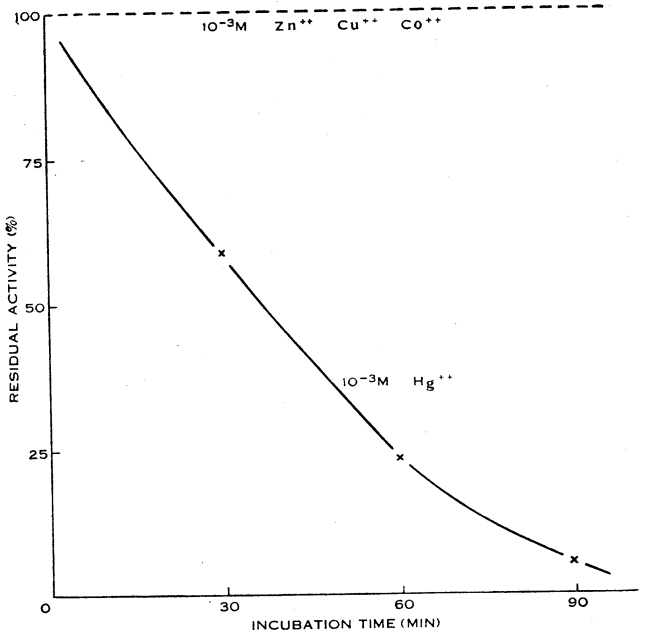

Fig. 15.—Progressive inactivation of *S. atra* β-glucosidase (2 units/ml) in sodium acetate buffer (0·1M, pH 5·0) by 10^{-3}M $HgCl_2$ at 28°C.

(*b*) *Inactivation by Heavy Metals*

A large number of anions and cations were examined to see whether they had any specific effect on the inactivation of the enzyme (in McIlvaine buffer at pH 5·0). The only effective ions were found to be the heavy metal cations mercury, silver, and thallium. This inactivation was irreversible and, since lead has no irreversible effects on the activity (see Part IV of this series), it does not appear to be due to reaction with sulphydryl groups. Thomas (1956) has shown that the cellulase of *S. atra* is inactivated by mercuric salts, but is insensitive to sulphydryl reagents.

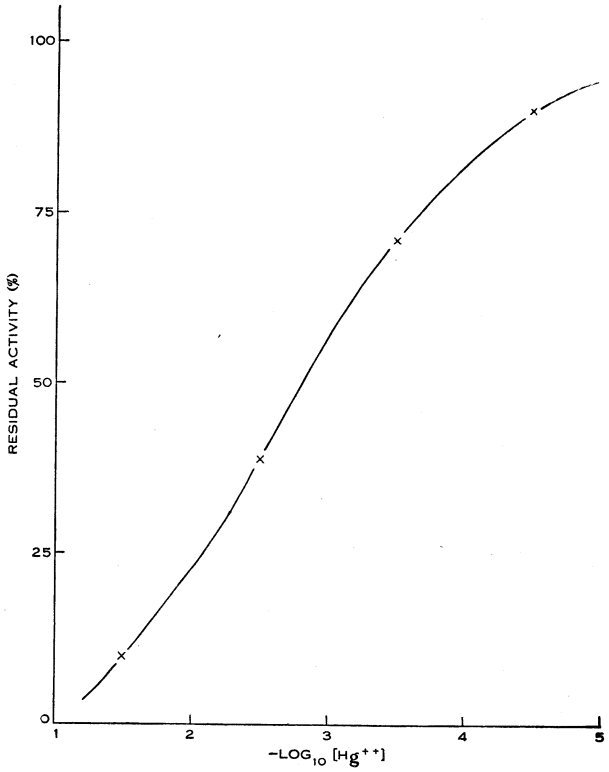

Fig. 16.—Dependence of the rate of inactivation of *S. atra* β-glucosidase on $HgCl_2$ concentration. Residual activity after 30 min incubation at 28°C; 2 units/ml of enzyme in sodium acetate buffer (pH 5·0, 0·1M).

Since thallium and silver slowly gave precipitates in the incubation medium used, all further work was done using mercury. The progressive inactivation of the enzyme with time and the dependence of the rate on the concentration of mercuric ion (see Figs. 15 and 16) suggest that the inactivation is due to some relatively slow chemical reaction.

IV. Discussion

Most of the data presented here for the β-glucosidase of *S. atra* are more suggestive of lines for future research than informative in themselves. The main purpose of this study was to determine whether the enzyme possessed unusual properties that would have to be taken into account in any scheme for interrelating the activities of the various β-glucoside-splitting enzymes of *S. atra*. Judged by this criterion, the β-glucosidase here studied is in no way a remarkable enzyme and all the data presented can be paralleled by other workers' results as summarized in Veibel (1950).

V. Acknowledgment

I am indebted to Miss S. Plaisted for carrying out the greater part of the experimental work.

VI. References

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