FUNGAL CELLULASES

IV. PRODUCTION AND PURIFICATION OF AN EXTRACELLULAR β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

By M. A. JERMYN*

[*Manuscript received April* 14, 1955]

Summary

The accumulation of β-glucosidase in shake cultures of *Stachybotrys atra* is dependent on a variety of physiological conditions but there is no evidence that it is induced by the presence of β-glucosides in the medium. One strain of the mould requires a factor present in yeast extract for the production of the enzyme.

The enzyme is concentrated by precipitation with ethanol from the crude culture medium. It is then fractionally precipitated from the dialysed concentrate by increasing the pH in the presence of 0·1M lead acetate. The bulk of the associated polysaccharide can be removed from the most active preparations by adsorbing the enzyme on a magnesium carbonate column and eluting it with sodium sulphate solution. A single peak of activity appears in the eluate fractions indicating that there is probably only one enzyme component.

Some of the relevant chemical and physical properties of the enzyme are discussed. It is not a cellobiase or a cellulase.

I. INTRODUCTION

Attempts at deducing the course of the enzymatic hydrolysis of cellulose have usually proceeded by the synthesis of information from a large number of distinct organisms. We prefer to investigate the nature and activities of the complete range of enzymes capable of splitting β-glucosidic linkages that are produced by a single organism. In other papers of this series, Jermyn (1953*a*) has shown that *Stachybotrys atra* secretes into the culture medium an enzyme that hydrolyses *p*-nitrophenyl-β-D-glucoside when grown in shake culture on a variety of non-cellulosic substrates, and Thomas (1956) has shown that little or none of this enzyme is secreted during growth on cellulose under conditions where considerable amounts of cellulase are produced. This readily-measured activity in which a β-glucosidic linkage is split by an enzyme other than cellulase has therefore been used as an indicator in the purification of a β-glucosidase, the properties of which will be described in subsequent papers.

II. METHODS

(*a*) *Estimation of β-Glucosidase by Liberation of* p-*Nitrophenol*

Three ml of $1{\cdot}67 \times 10^{-3}$M *p*-nitrophenyl-β-D-glucoside, 1 ml of McIlvaine citrate-phosphate buffer (pH 5·0), and 1 ml of enzyme solution are incubated

* Biochemistry Unit, Wool Textile Research Laboratory, C.S.I.R.O., Melbourne.

for 20 min at 28°C. Two ml of 7·5 per cent. (w/v) K_2HPO_4 solution are added (final pH 8·5) and the optical density at 400 mμ measured in a Coleman Universal spectrophotometer, using blanks for the colour due to enzyme and substrate. The plot of enzyme concentration against optical activity is linear to an optical density of 0·45 (0·5 in. light path); alkaline *p*-nitrophenolate solutions are known to show deviations from Beer's law in wide-band spectrophotometers (Goldenberg 1954). It is possible to correct for this by a calibration curve, but it is more accurate in practice to adjust enzyme concentrations so that the final optical density is low.

The unit of β-glucosidase activity is defined as the amount of enzyme which when dissolved in 1 ml gives an optical density of 1·0 under the standard conditions. In practice an enzyme solution that gives an optical density of *D*, has an activity of *D* units per ml.

The same method has been used with *p*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-β-cellobioside and the *p*-nitrophenyl-D-mannosides as substrates; *m*-nitrophenyl-β-D-glucoside can also be used, but *o*-nitrophenyl-β-D-glucoside, theoretically a more attractive substrate (lower pH for colour development), is scarcely attacked by the S. *atra* enzyme.

(*b*) *Estimation of β-Glucosidase by Phenol Liberation*

Jermyn and Thomas (1953) amongst others (Miwa *et al.* 1949; Takano and Miwa 1950; Barker, Bourne, and Stacey 1953; Buston and Jabbar 1954) have shown that the fact that β-glucosidases are also glucotransferases makes it unsafe to use glucose liberation as a measure of the enzymic hydrolysis of phenyl-β-glucosides. A method was therefore devised for measuring phenol liberation based on the A.O.A.C. (1940) method for phosphatase.

Seven ml of a $1{\cdot}43 \times 10^{-3}$M solution of a given phenyl-β-glucoside was incubated with 2 ml of McIlvaine citrate-phosphate buffer and 1 ml of enzyme for 30 min at 28°C, 5 ml of diluted (1 in 3) Folin-Ciocalteau reagent added, followed by 3 ml of 14 per cent. (w/v) Na_2CO_3 solution after 3 min, and the test tube plunged into boiling water for 5 min. After cooling and filtering the contents of the tube, the colour developed was measured in the Coleman Universal spectrophotometer at 700 mμ with appropriate blanks for the substrate and enzyme.

Other glycosides of the phenols may be used as substrates in the same way.

(*c*) *Estimation of Cellobiase, Sucrase, Amylase, and Related Activities*

To 9 ml of a solution of sucrose (10^{-3}M) or cellobiose (5×10^{-4}M) in diluted pH 5·0 McIlvaine buffer (1 in 10) is added 1 ml of enzyme solution and the mixture incubated for 1 hr at 37°C. One ml is then withdrawn and the reducing sugar estimated by the Somogyi-Nelson colorimetric method (Nelson 1944), with a blank for the reducing power of the enzyme. For sucrose, the total reducing sugar present was expressed as μg of invert sugar by comparison with a standard mixture of glucose and fructose; for cellobiose it was possible to calculate the degree of breakdown from the known reducing

power of cellobiose and glucose, but for most purposes it was sufficient to use the ratio of the increase in optical density above that of a blank containing cellobiose alone to the optical density of the blank as a measure of cellobiase activity.

The enzymic hydrolysis of other oligosaccharides was estimated by the same method.

In the later stages of this study it was found that a 0·1 per cent. solution of starch could be used to estimate amylase by this method, although earlier the method of Crewther and Lennox (1953) had been used; the two methods gave results in agreement. The hydrolysis of other polysaccharides such as laminarin, xylan, inulin, and *Pseudomonas tumefaciens* polysaccharide was studied similarly.

(*d*) *Estimation of Protease*

The method employed *p*-dimethylaminobenzeneazocasein (Jermyn 1953*b*) as the substrate. Eight ml of a 1 per cent. solution of the azocasein in sodium phosphate buffer (final pH 7·0) and 1 ml of enzyme solution are incubated for 30 min at 37°C. The protein is precipitated by the addition of 1 ml of N trichloroacetic acid and, after centrifugation, the optical density of the supernatant is measured at 500 mμ. A calibration curve is necessary to convert optical density to enzyme concentration.

(*e*) *Estimation of Esterase*

The chlorophenyl red acetate (Jermyn 1953*a*) method was used.

(*f*) *Estimation of Carbohydrate*

Anthrone (20 mg) is dissolved in 70 ml of conc. H_2SO_4 and 30 ml of water added. Ten ml of the cooled reagent are added to 1 ml of the solution to be tested which has been adjusted to contain 50-150 μg of carbohydrate. The tube and its contents are heated in a boiling water-bath for 7·5 min, cooled, and the optical density at 625 mμ measured against a carbohydrate-free blank. A standard containing 100 μg of a sugar is heated simultaneously.

Outside the concentration ranges indicated there is considerable departure from proportionality between carbohydrate content and optical density. The method is an adaptation of that of Black (1951) and uses the optimal reagent concentration and heating time.

(*g*) *Filter Paper Electrophoresis and Chromatography*

The conditions used have been fully set out by Jermyn (1953*b*) and Jermyn and Thomas (1954). The position of β-glucosidase on the paper was shown by spraying with a solution of 6-bromo-2-naphthyl-β-D-glucoside and tetrazotized di-*o*-anisidine (the reagents of Cohen *et al.* 1952) in a buffer solution which would bring the final pH of the paper to about 5. Any active β-glucosidase showed up as a purple area after a short incubation at 37°C; washing the paper in water to remove the reagents left a permanent record of the position of the enzyme.

(*h*) *Shake Culture*

The reciprocating shaker and conditions of spore production used in the earlier work (Jermyn 1953*a*) were used without change.

III. Enzyme Production

The object of this study was to examine the properties of the S. *atra* β-glucosidase rather than to track down all the physiological factors concerned in its secretion. Hence it was found convenient not to alter the general con-

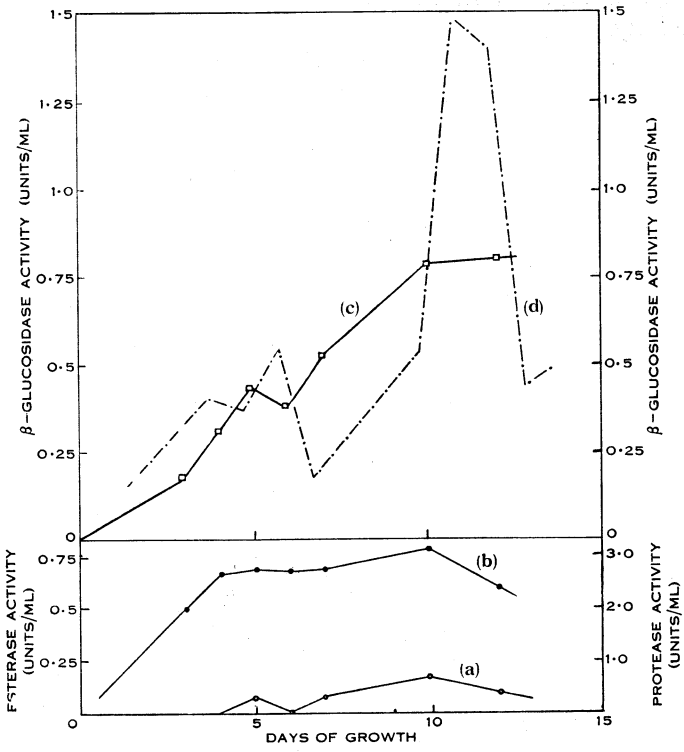


Fig. 1.—Enzyme production by S. *atra* grown on Waksman-Carey medium containing 1 per cent. salicin at 28°C in the continuous recording apparatus. Only those activities relevant to the argument are displayed in this and the following figure. (*a*) Protease activity. (*b*) Esterase activity. (*c*) β-Glucosidase activity. (*d*) β-Glucosidase activity for a parallel experiment with 1·2 per cent. starch (Jermyn 1953*a*).

ditions of growth (shake culture in medium-sized flasks, mineral medium (Waksman-Carey), and temperature of operation (28°C)) that were already employed. Only the effects of variations within this framework have been investigated.

(*a*) *Effects of the Carbon Source*

S. atra will grow readily on a wide variety of carbon sources (Jermyn 1953*a*) with a distinct preference for sugars and polysaccharides. When grown on any of the sugars and their polymers so far tested it secretes β-glucosidase. Growth with enzyme secretion has been found to occur on D- and L-arabinose, D-galactose, D-xylose, and maltose as well as the substrates studied in the earlier paper. None of these carbon sources appeared to be as effective as starch in promoting enzyme secretion.

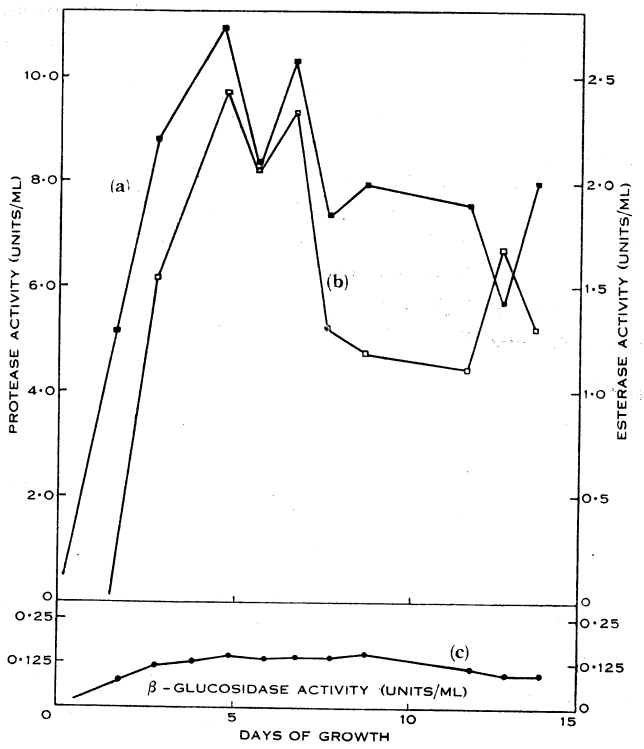


Fig. 2.—Enzyme production by *S. atra* grown on Waksman-Carey medium containing 2 per cent. ground wool toppings at 28°C in the continuous recording apparatus. The scale is identical with that of Figure 1. (*a*) Protease activity. (*b*) Esterase activity. (*c*) β-Glucosidase activity.

Since it appeared possible that there might be an "adaptive" effect in the production of the enzyme, growth on carbon sources containing β-glucosidic linkages was studied. The continuous recording apparatus described earlier (Jermyn 1953*a*) was employed to study growth on a number of such carbon sources.

Figures 1 and 2 show growth curves obtained for growth on salicin (a stable aryl-β-glucoside) and ground wool. In the latter case an apparent adaptive response occurs and protease is secreted into the medium in measur-

able quantities, a result which does not occur with any other carbon source so far tested. There is also a marked increase in esterase activity. Comparison of the salicin curves with those in Jermyn (1953*a*) for glucose, sucrose, and starch show no demonstrable adaptive response. Similar results were obtained for cellobiose, *p*-nitrophenyl-β-glucoside, and carboxymethylcellulose. Thomas (1956) has demonstrated little or no β-glucosidase production by S. *atra* in the presence of the insoluble polymeric β-glucoside cellulose, even though the mould is at the same time producing a cellulase adaptively.

Since the β-glucosides are rapidly hydrolysed in the culture medium, small quantities in the presence of another carbon source as the major substrate might be effective in inducing increased enzyme production. Salicin and cellobiose (1 per cent. on added glucose) in a 2 per cent. glucose medium did not increase the secretion of β-glucosidase over that on the glucose alone.

It thus appears that the secretion of the β-glucosidase has not been shown to be an adaptive response (no experiments bearing on the intracellular production of the enzyme have yet been carried out). Further work on the production of β-glucosidase was therefore carried out on media containing starch as the carbon source. There appears to be no reason to suppose that the occasional β-glucosidic linkages postulated in native starch by Peat, Thomas, and Whelan (1952) play any part in stimulating β-glucosidase production.

(*b*) *A Factor in Yeast Extract Affecting β-Glucosidase Production by* S. atra

For two to three years, in the initial stages of these investigations, it was possible to carry out physiological experiments and obtain reliable amounts of enzyme and mycelium from one batch to the next. A change then occurred without apparent reason and only a few flasks in each batch produced enzyme. Finally, enzyme production ceased although growth of the mould was unaffected. It was found that the "degenerated" strain could be induced to produce enzyme when the medium was supplemented with yeast extract (2 g/l).

The properties of the "β-glucosidase factor" which is in no way a "growth factor" will be treated in forthcoming papers by Jermyn and McQuade.

Subsequent to these observations yeast extract was added to all growth media when maximal β-glucosidase production as such was desired.

(*c*) *Physiology of Enzyme Production*

The following factors have been shown to exert a consistent influence on the secretion of β-glucosidase by S. *atra* in shake culture:

(i) *Carbon Source.* Rapid growth on a carbohydrate or related substance (glycerol is as satisfactory as glucose) is necessary for the secretion of workable quantities of enzyme. Insoluble substrates such as cellulose and wool (Fig. 2) are unsatisfactory, nor was appreciable activity developed in acetate media.

(ii) *Aeration.* No enzyme secretion is observed during surface culture of S. *atra* on liquid media with glucose as carbon source. A few runs with a small stainless steel fermenter showed that under conditions of relatively vigorous

aeration it was difficult to apply any conclusions drawn from shake-culture experiments; on one occasion an S. *atra* inoculum which, in the absence of added yeast extract, uniformly gave cultures showing no enzyme secretion on the shaker gave a culture with good activity in the fermenter.

To test the effect of aeration in shake culture a factorial experiment was run in which the influences of size of Erlenmeyer flask and volume of medium (Waksman-Carey containing 1 per cent. starch) were compared. The interaction flask-size × liquid volume had a significant effect on enzyme yield, 100 ml of medium in a 500-ml flask giving optimum results. Such combinations as 50 ml in a 1-l. flask and 150 ml in a 250-ml flask gave little or no enzyme.

(iii) *Mycelial Transfer.* When some of the mycelium from the sporadic flasks containing active enzyme appearing during growth of the degenerated strain on media not containing yeast extract was transferred to a fresh flask, growth was vigorous but no enzyme was secreted. On the other hand, mycelial transfer from an enzymically active culture on a starch medium supplemented with yeast extract to fresh medium of the same type was associated with a heightened production of enzyme. Culture filtrates from S. *atra* grown on starch-yeast extract media (50 ml in 250-ml flasks) for 4-5 days had an enzyme content of 1-2 units/ml. If such cultures were tipped into 700 ml of the same medium in 2-l. flasks, a further 3 days growth gave an activity of 8-10 units/ml. This method was adopted for bulk enzyme production.

(iv) *Duration of Culture.* Jermyn (1953*a*) showed that the main changes during growth of S. *atra* on sugar solutions in shake culture at 28°C (increase in mycelial weight, fall in pH of the medium) took place within a period of 24 hr, 3-5 days after inoculation with mould conidia. Secretion of β-glucosidase into the medium takes place with similar abruptness when the mould is grown under optimal conditions. In cultures derived from conidial inoculations into starch media this liberation of enzyme usually lags 1-2 days behind the mycelial growth. A number of curves illustrating the time relationship of enzyme secretion and the scatter of results between successive flasks are shown in Figures 3(*a*) and 3(*b*). Mycelial growth is more rapid and enzyme secretion occurs earlier in these Erlenmeyer-flask cultures than in the continuous recording apparatus used to obtain the curves figured by Jermyn (1953*a*).

After the maximum level of enzyme activity is reached the subsequent fall takes a variable course in different cultures. This is at least partly due to small differences in the final pH of the medium, since the stability of the enzyme falls sharply in the pH range 4·0-3·0

On yeast-extract medium the degenerated strain shows less variability than did the original strain on the basal medium. Figures 3(*a*) and 3(*b*) are derived from the latter type of experiment.

Cultures initiated by mycelial transfer from an enzymically active culture to a medium supplemented with yeast extract normally show simultaneous growth and enzyme production.

In a series of experiments certain other factors have been eliminated from consideration as factors influencing enzyme production. These include:

(1) Variations in the procedure for making up the medium such as dissolving the starch before autoclaving and altering the brand of soluble starch employed.

(2) Number of conidia in the inoculum. A certain minimum number of spores (10^2-10^3) seems necessary to initiate growth in shake culture (50 ml in 250-ml flasks), but increasing the number of spores has no effect on the final enzyme activity, although it leads to an earlier maximum.

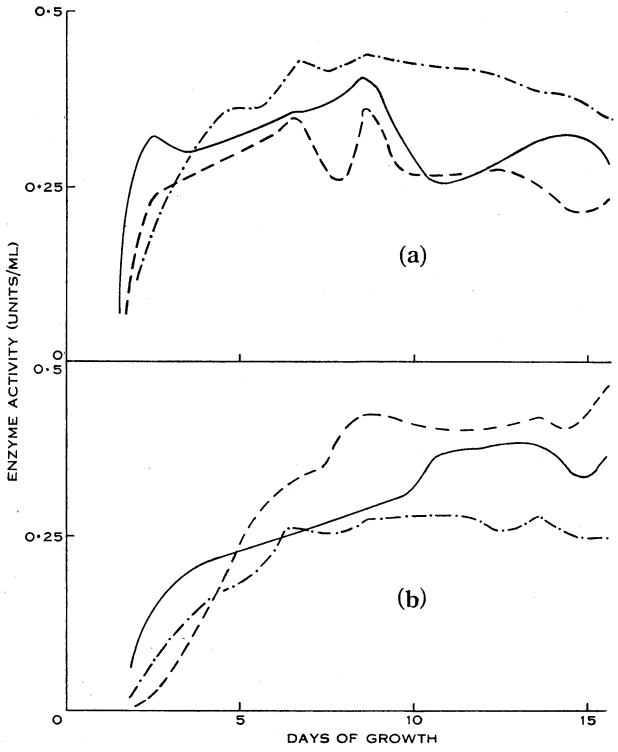


Fig. 3.—Variation in β-glucosidase secretion by *S. atra* grown in shake-culture flasks under identical conditions (yeast extract absent, 100 ml of 1 per cent. starch medium in 500-ml Erlenmeyer flasks at 28°C). Pairs of flasks were inoculated on 3 successive days (solid, dot-dash, and dashed lines) with fresh batches of conidia (*c.* 10^8 per flask). The curve for one member of each pair is given in (*a*) and (*b*).

(3) Batch to batch variations associated with different lots of inoculum. It could not be shown that different conidial inocula from the "undegenerated" strain gave significantly different yields of enzyme. Nor does the degenerated strain show significant variation from batch to batch in the presence of yeast extract. Only during the period of transition did successive inoculations give conflicting results.

IV. Preparation of Culture Filtrates

All experiments on the large-scale purification of *S. atra* β-glucosidase have been done with cultures initiated by mycelial transfer between media supplemented by yeast extract. A 250-ml flask containing 50 ml of medium (Waksman-Carey, 1 per cent. starch by weight, 0·2 per cent. yeast extract by weight) was inoculated with about 10^6 conidia and shaken for 4 days at 28°C. The contents were then tipped into 700 ml of the same medium in a 2-l. flask and shaken until β-glucosidase activity reached a maximum (about 3 days).

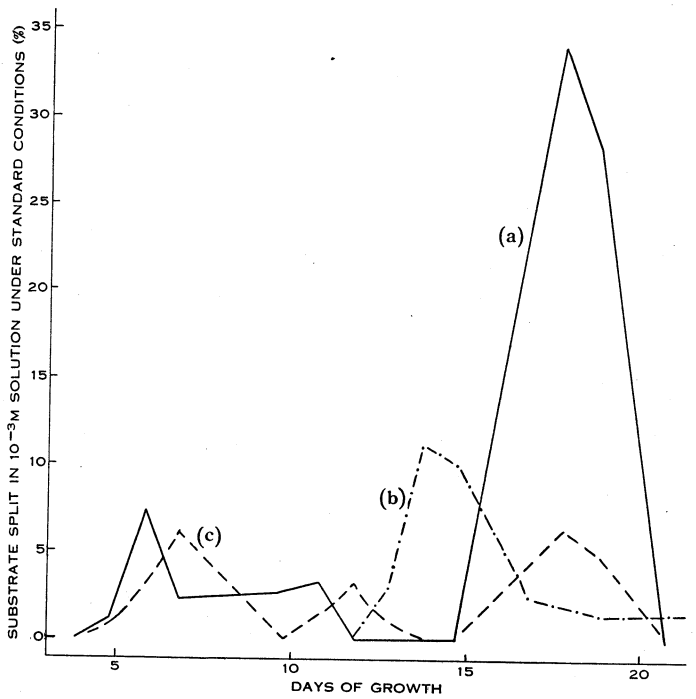


Fig. 4.—Secretion of various enzymes by *S. atra* grown on 2 per cent. glycerol medium in the continuous recorder. (*a*) Cellobiase. (*b*) *p*-Nitrophenyl-β-glucosidase. (*c*) Sucrase.

The contents of the flasks were filtered off through cheese cloth and the compacted mycelium washed with about 1/5 volume of water. The pooled filtrates could be stored at 1°C for an indefinite period without loss of activity.

The bulked mycelium was washed with water till the washings were devoid of β-glucosidase activity and then stirred into 10 volumes of acetone at room temperature. The acetone-extracted mycelium was filtered off, beaten up with further acetone in a Waring Blendor for a short period, centrifuged, and washed with several changes of acetone. The final air-dried powder was stable over a long period and various enzymic activities could be extracted from it after storage for a year at room temperature.

TABLE 1

ENZYMIC ACTIVITIES OF *S. ATRA* GROWN ON STARCH MEDIA

The mycelial extract of Batch B was prepared by ball-milling the acetone-dried mycelium with two parts of silica flour and extracting the resultant powder with water equivalent to the original volume of medium. The activities of the glycosidases are expressed as percentage of enzymically susceptible bonds split in a 10^{-3}M solution of the substrate under the standard conditions

Enzymes and Substrates	Enzyme Activity		
	Batch A Culture Filtrate	Batch B	
		Culture Filtrate	Mycelial Extract
Phosphatase			
Acid	Nil		
Alkaline	Nil		
Protease	Nil		
Esterase	Trace		
Catalase	Negligible		
Laminarinase	Nil	Nil	0·25
Xylanase	Nil		
Amylase	Nil	0·16	0·10
Glycosidase substrates			
Maltose	12·4		
Cellobiose	Nil	6·9	34·9
Lactose	Nil		
Turanose	Nil		
Mellibiose	Nil		
Sucrose	213·0	74·8	80·8
Trehalose	1·0		
Phenyl-α-galactoside	0·59		
2-Naphthyl-β-galactoside	0·48	31·9	46·7
Menthyl-β-glucuronide	1·0		
p-Nitrophenyl-β-xyloside	0·36		
m-Cresyl-β-glucoside	24·3		
o-Cresyl-β-glucoside	5·8		
Thymyl-β-glucoside	3·6	2·0	9·6
Phenyl-β-glucoside	16·8	23·5	41·5
p-Chlorophenyl-β-glucoside	25·1		
2-Naphthyl-β-glucoside	28·3		
6-Bromo-2-naphthyl-β-glucoside	43·7		
Salicin	4·9	5·2	21·0
p-Nitrophenyl-β-glucoside	17·7	25·6	28·1
m-Nitrophenyl-β-glucoside	11·9		
o-Nitrophenyl-β-glucoside	Nil		
Methyl-β-glucoside	2·0		
p-Nitrophenyl-α-glucoside	0·17		
Methyl-α-glucoside	2·3		
Viscometric cellulase (Thomas 1956)	Nil*		

* After concentration of the culture filtrate 35 times by vacuum evaporation, the enzyme could just be detected.

V. Enzymic Activities of the Culture Filtrate and Mycelium

Although the culture filtrate was looked on primarily as a source of β-glucosidase, the other enzymic activities present were investigated since their presence or absence at later stages in the concentration of the β-glucosidase was one index of the purification of the enzyme. In Table 1 are set out results for two batches of culture filtrate and an extract of the mycelium of one of them.

The sporadic appearance of cellobiase activity in the culture medium and its consistent presence in the mycelium are features of all batches investigated. The secretion of a β-glucosidase into the medium by *S. atra* which does not hydrolyse cellobiose is illustrated in Figure 4 where the peak of *p*-nitrophenyl-β-glucosidase activity comes at a point where there is no cellobiase in the medium and the peak activity of the latter comes some days afterwards.

There is evidence from a number of experiments (cf. Table 1) that preparations with strong cellobiase activity show a much higher salicinase to *p*-nitrophenyl-β-glucosidase ratio than preparations without cellobiase. Hence it is probable that "cellobiase" is in fact one or more β-glucosidases with different specificities from the one investigated in this work. In the absence of cellobiase the ease of hydrolysis of various β-glucosides always follows a fixed order which is the same as that deduced from the specificity properties of the enzyme.

Other activities which appear from time to time in the culture medium are laminarinase (always in association with cellobiase), xylanase, and amylase. Preparations hydrolysing laminarin (poly-1,3-β-D-glucose) do not affect *Pseudomonas tumefaciens* polysaccharide (poly-1,2-β-D-glucose). β-Xylosidase and β-cellobiosidase will be discussed in Part VI of this series in connection with the specificity of the β-glucosidase.

VI. Purification of the β-Glucosidase

(*a*) *Concentration of the Enzyme*

Below 40°C and at neutral or slightly alkaline pH, the β-glucosidase is stable for long periods. Culture filtrates can therefore be concentrated readily in a climbing film evaporator to about 1/20 volume with little loss of activity. Such concentrates are rather viscous and cannot be dialysed since insoluble, brown, gel-like material is precipitated on which the enzyme appears to be irreversibly absorbed. This material can be removed by three successive precipitations of the enzyme with two volumes of ethanol, either in highly-coloured supernatants or as a precipitate which does not redissolve in water. The final concentrate (*c.* 1/100 volume) can be dialysed without loss of activity.

The above procedure involves high salt concentrations causing slow crystallization of salts from ethanol-water mixtures and viscous solutions from which it is difficult to centrifuge or filter insoluble material. It was found much easier to use ethanol precipitation without prior concentration in three stages giving volume diminutions of about 1/10, 1/3, 1/3.

Two volumes of ethanol were added to the crude culture filtrate and it was allowed to stand overnight. The supernatant was siphoned off and the loose precipitate centrifuged to give a compact pellet. A certain amount of mycelial debris was useful in obtaining a coherent precipitate; if the culture filtrate was completely cleared by centrifuging, there was a much lower yield of enzyme. Since the enzyme is not precipitated by ethanol below a certain salt concentration, the aqueous solution was made up to 1 per cent. (w/v) NaCl before the addition of ethanol in the second and third steps.

From one-half to two-thirds of the activity was recovered by this process, but the ease of manipulation far outweighed the increased losses.

(*b*) *Properties of the Concentrate*

The β-glucosidase activity of the concentrate was not stable to lyophilization, about one-half the activity being lost. A second lyophilization halved the residual activity and this could be repeated indefinitely. The lyophilized preparation was a fluffy, white powder, in a yield of 1-2 g/l. The ratio of carbohydrate to protein was from 10 : 1 to 20 : 1 in different preparations. An essentially similar product could be obtained from culture filtrates devoid of enzymic activities.

Hydrolysis of the concentrate with boiling 0·5N H_2SO_4 for 2 hr followed by paper chromatography showed the presence of galactose, glucose, and mannose in the estimated ratios 3 : 2 : 1 together with a little uronic acid. Amino sugars were shown to be absent (Thomas (1956) details the tests employed). Galactose was used as the standard sugar in estimating carbohydrate content at the later stages of purification. Hydrolysis with 6N HCl at 100°C for 12 hr followed by paper chromatography gave a chromatogram that could not be readily interpreted owing to humin formation and interference from sugars, but revealed the presence of ninhydrin-reacting spots. In the absence of any evidence for the existence of other ninhydrin-positive substances these have been assumed to be distorted amino acid spots, and the nitrogen of dialysed enzyme preparations has been assumed to be protein nitrogen.

Batch B (Table 1), which was concentrated by evaporation before ethanol precipitation, retained 88 per cent. of the β-glucosidase activity in the dialysed concentrate, but 40 per cent. of the amylase, 65 per cent. of the cellobiase, and 99 per cent. of the sucrase had been eliminated. The β-glucosidase was stable to ethanol and methanol up to 30°C, but sucrase was rapidly destroyed even at 0°C. Use of the higher alcohols to precipitate the β-glucosidase led to inactivation of the enzyme at all temperatures; this enzyme was stable to acetone at low temperatures and salt concentrations, but was rapidly destroyed at high salt concentrations.

The enzyme could not be precipitated by ethanol at low ionic strength nor by saturation with ammonium sulphate. It remained completely in the water phase whatever the pH of an ammonium sulphate-water-ethanol mixture, the only two-phase system in which it was stable. It was not precipitated by specific protein precipitants, even such a general one as the zinc-mercury reagent of Schmid (1953) being ineffective. The only precipitants found were

basic lead acetate and uranyl salts. The only effective adsorbents were activated charcoal and light magnesium carbonate, but no method for eluting the enzyme from charcoal could be discovered.

After precipitation from lead solutions all enzyme activities but the β-glucosidase were eliminated from the most active fraction. Adsorption on magnesium carbonate, as shown in Figure 5, did not lead to differential separation of sucrase (the most persistent enzymic contaminant) from β-glucosidase. Fractionation by lead precipitation was therefore used as the next step in the purification procedure.

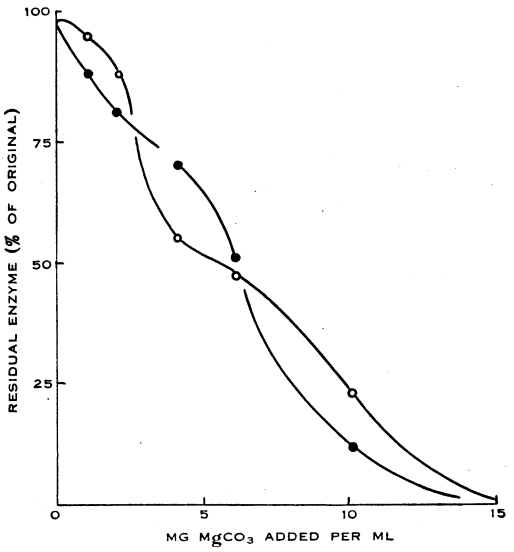


Fig. 5.—Adsorption of enzymes from a dialysed concentrate of *S. atra* culture filtrate on light $MgCO_3$. ○ Sucrase. ● *p*-Nitrophenyl-β-glucosidase.

(*c*) *Fractionation by Lead Precipitation*

When the dialysed concentrate was fractionated by the addition of successive quantities of basic lead acetate solution and centrifuging off the precipitate after each addition, there was no significant increase in the purity of the most active fractions (assessed on the units of enzyme per mg N) over the original material. If the lead was added as the normal acetate to a concentration of 0·1M, no precipitation took place in general, and a series of fractions could be obtained by successive small additions of sodium hydroxide. The initial pH for precipitation varied from experiment to experiment; in one case it was as low as 4·0 and acetic acid had to be added to the normal lead acetate solution. The most active fractions were not the same in different experiments, but in general the bulk of the enzyme activity was precipitated by amounts of NaOH solution sufficient to convert 5-15 per cent. of the normal lead acetate to the basic compound (pH range 5-6).

The most effective reagent for redissolving the lead-precipitated enzyme was found to be a 5 per cent. (w/v) solution of glycine. Any solid matter in the final solution was removed and the latter was then dialysed in a rotary dialyser (16 hr against running tap water). In general all the lead was removed by this dialysis, but occasionally some of it was retained tenaciously. The cause of this anomalous behaviour has not been investigated since the retained lead is removed during the next stage of purification.

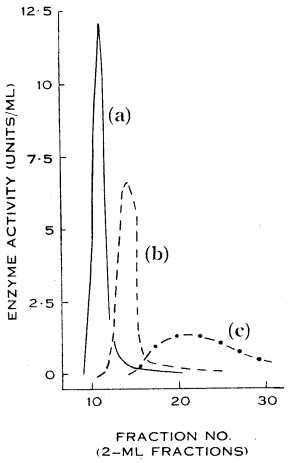


Fig. 6

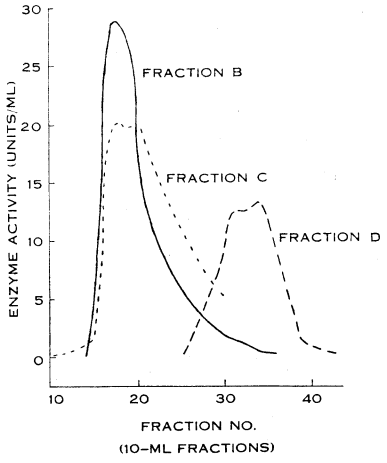


Fig. 7

Fig. 6.—Variation in the elution of 250 units of *S. atra* β-glucosidase from a light $MgCO_3$-cellulose column (5 g of 1 : 1) with concentration of the $(NH_4)_2SO_4$ eluting solution. (*a*) 4 per cent. $(NH_4)_2SO_4$; (*b*) 3·5 per cent. $(NH_4)_2SO_4$; (*c*) 3 per cent. $(NH_4)_2SO_4$. Same sample of lead-fractionated enzyme used throughout.

Fig. 7.—Elution of lead-precipitated fractions B, C, D of batch 8 from a light $MgCO_3$-cellulose column (40 g of 1 : 1) with 2·7 per cent. Na_2SO_4. Curve D is magnified to five times its true height.

The dialysed lead-precipitated enzyme is stable for long periods in the refrigerator. No amylase or cellobiase activity remains, but a little sucrase has occasionally been detected in the least active fractions, which were discarded at this stage.

(*d*) *Chromatography on Magnesium Carbonate Columns*

It could be shown (Table 2) that the enzyme in the dialysed solutions from the lead fractionation was reversibly eluted by dilute ammonium sulphate solutions after adsorption on light magnesium carbonate (B.D.H. laboratory reagent). The necessary condition for the use of magnesium carbonate as the adsorbing agent in a chromatographic column was thus fulfilled. The column packing used was equal parts of light magnesium carbonate and Whatman's standard chromatographic cellulose powder, the latter being added merely to

improve flow through the column. Figure 6 shows results obtained by the elution of the β-glucosidase from cellulose-magnesium carbonate columns with

TABLE 2

REVERSIBLE ADSORPTION OF *S. ATRA* β-GLUCOSIDASE ON MAGNESIUM CARBONATE

All adsorptions carried out on 50 mg of light magnesium carbonate from 5 ml of enzyme solution. All apparent activities corrected for the presence of ammonium sulphate

(*a*) Effect of Enzyme Concentration on Adsorption

Enzyme Concentration (units/ml)		Difference (units/ml)	Units of Enzyme Adsorbed
Before Adsorption	After Adsorption		
1·820	1·360	0·460	2·30
0·920	0·436	0·484	2·42
0·455	0·036	0·419	2·10
0·239	0·016	0·223	1·12
0·117	0·006	0·113	0·57

(*b*) Effect of Ammonium Sulphate Concentration on Desorption

Desorption Process	Enzyme Concentration (units/ml)	Volume (ml)	Total Units
Enzyme adsorbed	0·298	5	1·49
Enzyme desorbed by 2 per cent. ammonium sulphate	0·097	10	0·97
Enzyme desorbed by 5 per cent. ammonium sulphate	0·143	10	1·43
Enzyme desorbed by 10 per cent. ammonium sulphate	0·153	10	1·53

(*c*) Effect of Eluting Volume of 2·5 per cent. Ammonium Sulphate

Elution Process	Enzyme Concentration (units/ml)	Volume (ml)	Total Units
Enzyme adsorbed	0·484	5	2·42
Enzyme desorbed by 2·5 per cent. ammonium sulphate	0·493	2	0·98
	0·318	5	1·59
	0·190	10	1·90
	0·152	15	2·28

ammonium sulphate solutions of varying strengths. Similar results were obtained with other enzyme samples, but usually at slightly lower concentrations of the salt (2·5 per cent. ammonium sulphate was the usual optimum);

some samples could be eluted satisfactorily with as little as 1·5 per cent. ammonium sulphate.

Since the ammonia could only be removed from the enzyme fractions by dialysis, which is very tedious when a large number of samples have to be handled, all quantitative studies were carried out by using sodium sulphate as the eluant. The eluting effect of the two sulphates was identical at identical molarities. The results of various experiments using elution with sodium sulphate solutions are set out in Figures 7, 8, and 9. When the salt concentration of the eluant is reduced far enough, there is almost complete separation of the peak of enzyme activity from that of carbohydrate. The enzyme is therefore not firmly associated with most of the carbohydrate present. None the less it was found that the enzyme became progressively less stable as less carbohydrate was associated with it. The enzyme could be recovered quantitatively from

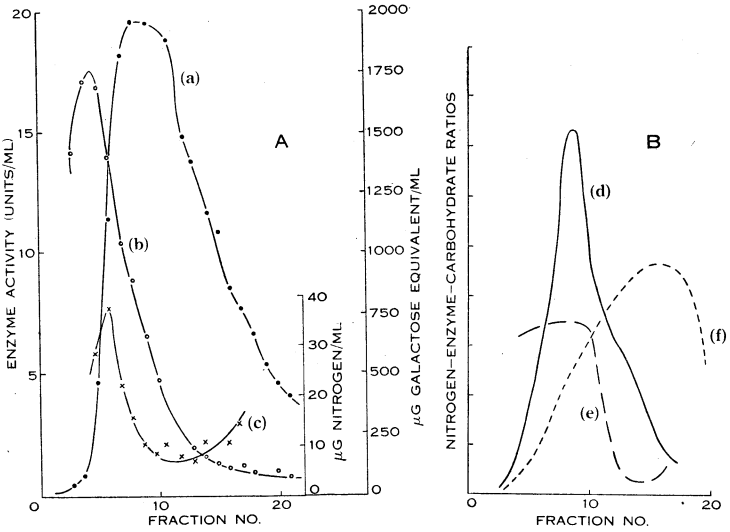


Fig. 8.—*A*, Analysis of the column fractions from the chromatography of lead-precipitated fraction B of batch 8 (see Fig. 7). (*a*) Enzyme activity. (*b*) Carbohydrate. (*c*) Nitrogen. *B*, (*d*) Ratio of enzyme activity to nitrogen. (*e*) Ratio of carbohydrate to nitrogen. (*f*) Ratio of enzyme activity to carbohydrate.

adsorption on magnesium carbonate in test tube experiments and 75-80 per cent. could be recovered from columns on elution with strong ammonium sulphate solutions so that the carbohydrate and enzyme peaks were simultaneous. As the enzyme peak lagged behind the carbohydrate peak, progressively less and less enzyme was recovered although nitrogen and carbohydrate were almost quantitatively eluted. Finally, under conditions where little or no carbohydrate was associated with the enzyme (e.g. fractions after the 30th in Fig. 9), very little active enzyme reached the bottom of the column and this little rapidly disappeared on keeping. The later fractions in Figure 9 were inactive after a further 24 hr in the refrigerator. An exceptional sample from lead fractiona-

tion which had a carbohydrate-protein ratio of only 0·3, although perfectly stable in solution at refrigerator temperatures, lost all activity on chromatographing although carbohydrate and nitrogen emerged normally from the column.

It would appear that some at least of the associated carbohydrate is essential for the stability of the enzyme. Helferich and Pigman (1939) have shown that 3-4 per cent. of carbohydrate is tenaciously retained by almond β-glucosidase although there is no direct correlation between activity and carbohydrate content. Helferich, Richter, and Grünler (1937) showed this carbohydrate to be built up from mannose, arabinose, and possibly glucose.

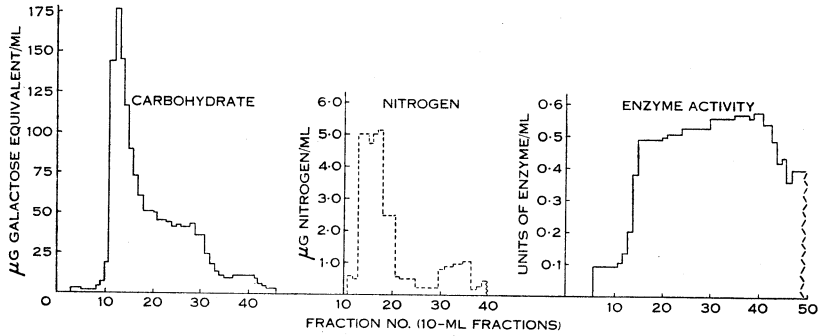


Fig. 9.—Elution of 2000 units of lead-fractionated β-glucosidase from a light $MgCO_3$-cellulose column (25 g of 1 : 1) by 1·5 per cent. Na_2SO_4. The enzyme has been considerably inactivated (see text).

(*e*) *Purification of a Typical Batch of Enzyme*

In Table 3 is set out the flow sheet for the purification of a typical batch of enzyme. Table 4 gives the basic data for the estimation of the purity of this enzyme at various stages in the process. Figures 7 and 8 have been chosen both to illustrate the stages in the purification of this batch and the general chromatographic behaviour of the enzyme.

VII. Physicochemical Properties of the Enzyme

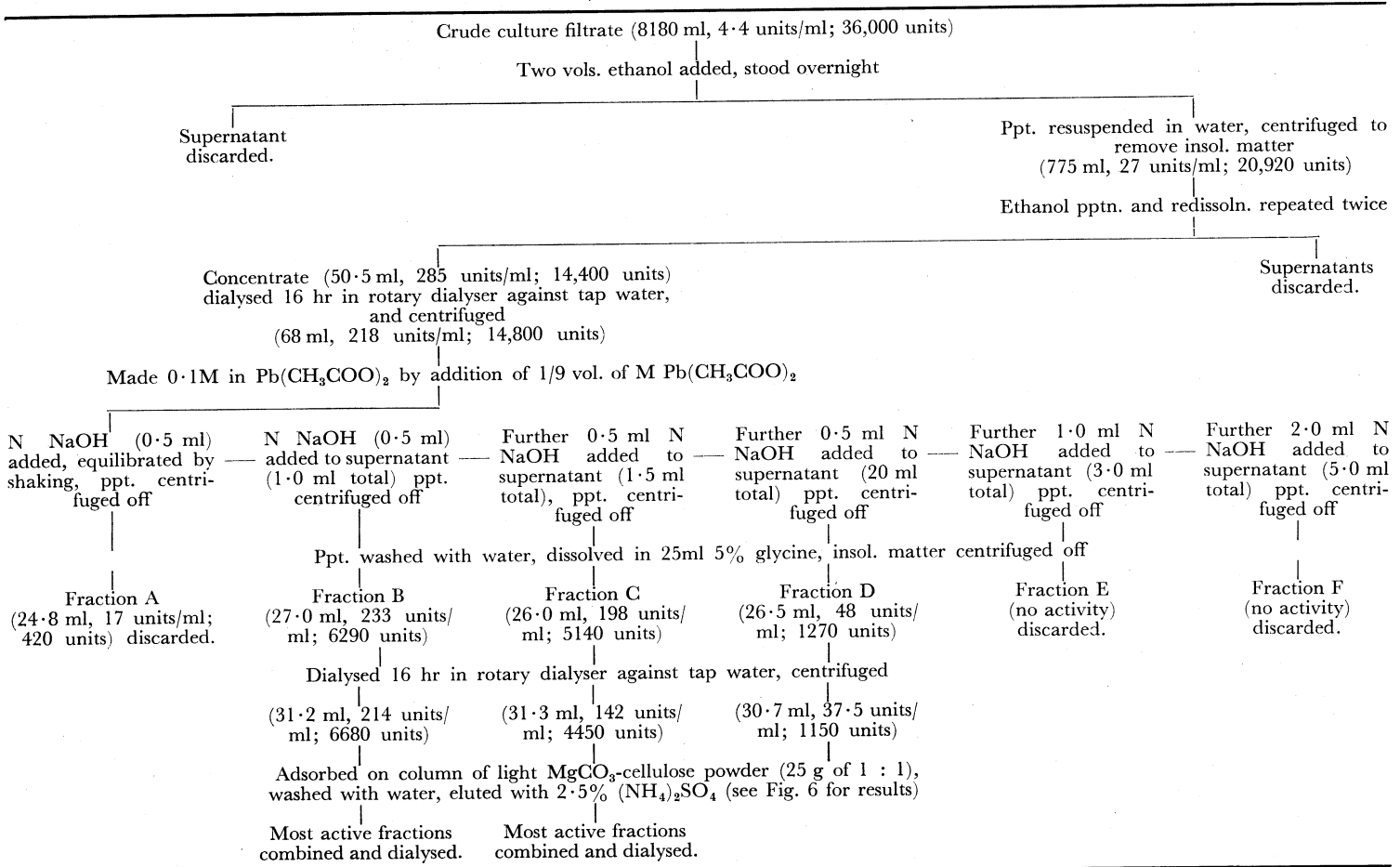
Not more than a few milligrams in dilute solution of highly purified *S. atra* β-glucosidase have yet been prepared. Since this is not sufficient for detailed examination and the behaviour of less pure preparations is dominated by the carbohydrate/protein ratio, no attempt has been made to establish any absolute physicochemical properties. Some incidental observations appear, however, to be of interest in showing how this association modifies the observed properties.

(*a*) *Filter Paper Electrophoresis and Chromatography*

When the crude culture filtrate was used the enzyme travelled as a single fairly well-defined spot and the isoelectric point (inaccessible because of inactivation of the enzyme) appeared to be below pH 3. The enzyme in the ethanol-precipitated concentrate was heavily adsorbed at lower pH, but still

TABLE 3

FLOW SHEET FOR PURIFICATION OF THE β-GLUCOSIDASE FROM BATCH 8 OF *S. ATRA* CULTURE FILTRATES



moved as a well-defined spot at pH 8-10; extrapolation resulted in a similar value for the isoelectric point. The lead-precipitated enzyme and, still more, column fractions with carbohydrate/protein ratios of 2-3 were so heavily adsorbed on the paper, no matter how it had been treated, that no deductions were possible.

Similar behaviour was observed with paper chromatography, the crude enzyme moving freely in methanol-buffer mixture and semi-purified preparations being tightly held at the point of application.

TABLE 4

ANALYTICAL DATA FOR BATCH 8 AT VARIOUS STAGES OF PURIFICATION

Stage	Units of Enzyme Activity per mg N	Units of Enzyme Activity per mg Galactose Equivalent	Polysaccharide/ Protein Ratio	Purification Factor
Ethanol-precipitated and dialysed	525	5·95	12·7	1
Lead-precipitated:				
Fraction B	2280	25·7	12·8	4·3
Fraction C	1580	23·1	9·9	3·0
Fraction D	770	18·6	6·0	1·45
Column fractions:				
B 21-22	2660	66	5·8	5·1
B 23-26	2410	100	3·5	4·6
C 17-21	2280	132	2·5	4·3

(*b*) *Adsorption*

In keeping with the increased adsorption on filter paper of small amounts of enzyme during its progressive purification, the same behaviour was observed with a variety of other adsorbents. The adsorbability of the enzyme on magnesium carbonate rises rapidly as carbohydrate is removed from enzyme preparations and much smaller columns can be used than would be predicted from experiments on the adsorption of the crude enzyme. A slight adsorption on calcium phosphate gel and calcium carbonate is sometimes evident.

It was not possible to find conditions in which the enzyme was retained by columns of ion-exchange resins. The activity disappeared on contact with strongly acidic cation exchangers, alone or in mixed-bed resins, but this could be shown to be due to the normal acid inactivation of the enzyme.

(*c*) *Optical Properties*

Dilute solutions of the enzyme showed no absorption in the visible range after lead fractionation (carbohydrate/protein ratio about 12 : 1), but an intense end-adsorption in the ultraviolet. Only when the carbohydrate/protein

ratio fell to about 2 : 1 after passage through a column was the characteristic maximum at 2800 Å in the protein ultraviolet adsorption curve visible as a hump on the enzyme absorption curve. Figure 10 illustrates this point.

The nature of the material responsible for this intense ultraviolet absorption is not known. The sum of total carbohydrate estimated by the anthrone method and protein ($6{\cdot}25 \times N$) usually came to about 90-95 per cent. of the dry weight. Analytical figures were a little high in carbon and hydrogen and much too high in oxygen for a mixture of carbohydrate and protein, and a component with the type of analytical figures expected for an oxygenated organic acid is suggested.

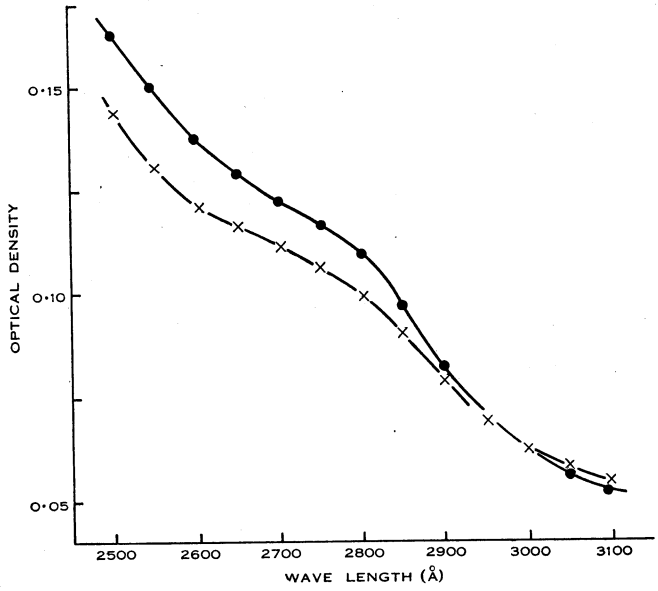


Fig. 10.—Ultraviolet absorption spectrum of two samples of S. *atra* β-glucosidase eluted from a $MgCO_3$ column. ● Carbohydrate/protein ratio 2·5. × Carbohydrate/protein ratio 3·5.

(*d*) *Electrophoresis*

A sample of dialysed, lead-fractionated enzyme, free of ash and with an enzyme activity of about 2000 units per mg N and a carbohydrate/protein ratio of 12 : 1 was submitted to electrophoresis in the Tiselius apparatus by Mr. J. M. Gillespie. The electrophoresis pattern showed rapid flattening of the boundary with re-sharpening on reversal of the current. This behaviour is consistent with the existence of a considerable number of molecular species, of not very dissimilar mobilities.

VIII. Discussion

Thomas (1956) found that his cellulase preparations from S. *atra* contained a polysaccharide very similar in properties to that encountered in the β-glucosi-

dase preparations. These polysaccharides seem to be a characteristic product of *S. atra,* but in the case of the cellulase preparations they were not present in such a large excess as to dominate the behaviour of the enzyme. The dependence of the stability of the *p*-nitrophenyl-β-glucosidase on the presence of an associated but separable polysaccharide resembles the experience of Fischer (1954) with yeast invertase.

Although the variation in the physicochemical properties of the enzyme with the amount of associated polysaccharide is such as to make its complete purification beyond the scope of the present study, it will be shown in Parts V and VI of this series that this variation is not incompatible with constancy of enzymic properties. The constant value of K_m against various substrates and the reproducibility of the peculiar features of the pH-activity curve make it probable that a single enzymic species is being studied. This is supported by the single peak of enzyme activity obtained on elution from magnesium carbonate columns.

By correlating the present observations with those of Thomas (1956) (Part VII of this series) it can be shown that *S. atra* produces at least the following four enzymes concerned in the hydrolysis of β-glucosidic linkages:

(i) The β-glucosidase here studied; incapable of splitting cellobiose.

(ii) A cellobiase, probably capable also of splitting aryl-β-glucosides. The upper limiting size of substrate for this enzyme is not known but the enzyme producing reducing groups from carboxymethylcellulose studied by Jermyn (1953*a*) and cellobiase activity always seem to occur together.

(iii) The adaptive "viscometric cellulase" of Thomas (1956). This enzyme attacks soluble cellulose derivatives with cellotriose as the smallest molecule attacked; it has no action on cellobiose. Preparations containing this activity also attack many insoluble celluloses (the inseparability of the two activities has not yet been rigorously proved) but not unscoured cotton duck. The ability of the fungus to grow on this substrate may be provisionally attributed to the presence of (iv).

(iv) A "swelling factor." This enzyme opens up highly crystalline celluloses to attack by the cellulase (Thomas 1955).

The full biological significance of the observations recorded in Parts IV, V, and VI of this series cannot be discussed until the mode of action of yet unstudied enzymes has been elucidated and the physiology of their production understood.

IX. Acknowledgments

The author wishes to thank Miss B. Lyons and Mr. A. B. McQuade for maintaining and growing the fungus, and Mr. W. B. Hall for helpful discussion of statistical problems. He is also indebted to Mrs. M. Wilkinson and Miss S. Plaisted for technical assistance.

X. References

Association of Official Agricultural Chemists (1940).—"Official and Tentative Methods of Analysis." 5th Ed. (Washington D.C.)

Barker, S. A., Bourne, E. J., and Stacey, M. (1953).—*Chem. Ind.* 31: 1287.

BLACK, H. C. (1951).—*Anal. Chem.* **23**: 1792.
BUSTON, H. W., and JABBAR, A. (1954).—*Biochem. Biophys. Acta* **15**: 543.
COHEN, R. B., RUTENBERG, S. H., TSOU, K. C., WOODBURY, M. A., and SELIGMAN, A. M. (1952).—*J. Biol. Chem.* **195**: 607.
CREWTHER, W. G., and LENNOX, F. G. (1953).—*Aust. J. Biol. Sci.* **6**: 410.
FISCHER, E. H. (1954).—*Arch. Sci. Genève* **7**: 131.
GOLDENBERG, H. (1954).—*Analyt. Chem.* **26**: 690.
HELFERICH, B., and PIGMAN, W. W. (1939).—*Hoppe-Seyl. Z.* **259**: 253.
HELFERICH, B., RICHTER, W., and GRÜNLER, S. (1937).—*Ber. sächs Ges. (Akad.) Wiss.* **89**: 385.
JERMYN, M. A. (1953*a*).—*Aust. J. Biol. Sci.* **6**: 48.
JERMYN, M. A. (1953*b*).—*Aust. J. Biol. Sci.* **6**: 77.
JERMYN, M. A., and THOMAS, R. (1953).—*Aust. J. Biol. Sci.* **6**: 70.
JERMYN, M. A., and THOMAS, R. (1954).—*Biochem. J.* **56**: 631.
MIWA, T., TAKANO, K., MAFUNE, K., and FURUTANI, S. (1949).—*Proc. Imp. Acad. Japan* **25**: 111.
NELSON, N. (1944).—*J. Biol. Chem.* **153**: 375.
PEAT, S., THOMAS, G. J., and WHELAN, W. J. (1952).—*J. Chem. Soc.* **1952**: 722.
SCHMID, K. (1953).—Quoted by Cohn, W. J., in "Blood Cells and Plasma Proteins." No. 2. (Ed. J. L. Tullis.) (Academic Press Inc.: New York.)
TAKANO, K., and MIWA, T. (1950).—*J. Biochem. Tokyo* **37**: 435.
THOMAS, R. (1955).—*J. Text. Res.* **25**: 559.
THOMAS, R. (1956).—*Aust. J. Biol. Sci.* **9**: (in press).