FUNGAL CELLULASES

X. FURTHER PURIFICATION OF THE β-GLUCOSIDASE OF STACHYBOTRYS ATRA

By M. A. JERMYN*

[*Manuscript received April* 17, 1962]

Summary

Chromatographic methods for preparing a carbohydrate–protein complex containing an apparently homogeneous single β-glucosidase species are described. This complex may be disrupted to give an enzymically active, but very unstable protein. There seems to be a single β-glucosidase present in *S. atra* culture filtrates, fractionations on the column being due to complexing with polysaccharide. Many of the cases reported in the literature of "multiple enzyme components" in similar systems have never been shown to have components of distinctly different enzyme properties. In the absence of such demonstrations, the present observations cast doubt on the individuality of these reported components in the biochemical sense and they may best be interpreted as a series of physically stable complexes.

I. INTRODUCTION

The partial purification of the β-glucosidase of *Stachybotrys atra* has been reported in Part IV of this series (Jermyn 1955*a*). There was no positive evidence that the enzyme preparations contained more than a single active species. However, even the best preparations did contain a considerable excess of the complex polysaccharide, containing glucose, galactose, mannose, a little xylose, and traces of uronic acid, previously described by Thomas (1956) in Part VII of this series.

There has been much recent interest in the existence of "micro-enzymes" (cf. Haldane 1954), i.e. mixtures of enzymes produced by the same organism which are sufficiently distinct in substrate specificity and protein structure to be regarded as separate entities, but not so distinct in physical properties that they are easily separable. Since the earlier observations had not excluded the possibility that the β-glucosidase of *S. atra* was a mixture of this type, the use of the enzyme in refined kinetic studies (Jermyn 1962*a*) would then depend on either disproving this supposition or isolating a demonstrably homogeneous enzyme species from the mixture.

As the purification of the enzyme by fractional precipitation methods had achieved only limited success, attention was turned to chromatography on ion-exchange celluloses. This paper describes the use of these methods to prepare a protein–carbohydrate complex which appears to meet the test of containing a single enzyme species.

Since it became evident during the course of this purification that the distribution of enzyme activity in the eluates from columns was very like that of carbohydrate, it was suspected that fractionations of the polysaccharide moiety of the

* Division of Protein Chemistry, C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

crude concentrates controlled the apparent fractionation of the enzyme. This hypothesis was therefore investigated in some detail, since it seemed to be of great general interest. There are many papers in the literature claiming "multiple enzyme components" from fractionations carried out in the presence of excess inactive material to which the present results with β-glucosidase–polysaccharide complexes are relevant.

II. Materials and Methods

(*a*) *Ion-exchange Cellulose*

Diethylaminoethyl (DEAE)-cellulose was a commercial Eastman Kodak sample. Pyridiniumacetylhydrazone of oxycellulose (PAHO-cellulose) and *p*-sulphophenylhydrazone of oxycellulose (SPHO-cellulose) were prepared from Whatman chromatographic cellulose powder following methods outlined by Jermyn and Thomas (1953) for filter paper and Thomas (1955) for textiles. They are both stable indefinitely in salt form as light yellow powders.

PAHO-cellulose can be prepared in about the same substitution range as DEAE-cellulose (0·5–1·0 millequivalents exchange capacity per gram) and can be used in a similar way for protein fractionation.

(*b*) *Chromatography*

Chromatography on ion-exchange celluloses followed the general methods of Thompson and O'Donnell (1960). The β-glucosidase of *S. atra* becomes markedly unstable at pH values very little below those most satisfactory for its chromatography and also in the absence of most of its associated carbohydrate (Jermyn 1955*b*). Both kinds of instability increase with increasing temperature. For this reason and also to avoid changes of chromatographic behaviour with changing temperature, all columns were jacketed with circulating water from a bath of melting ice to provide a constant minimum temperature.

Chromatography of test samples and purified fractions was carried out on a small column loaded with 10 g of ion-exchanger, while bulk chromatography was done on a large 200 g column of 4 cm internal diameter. Sodium chloride gradients were provided by a magnetically stirred mixing chamber. The characteristics of the small column and the shape of the standard sodium chloride gradient have already been described (Jermyn 1962*b*).

(*c*) *Partition Experiments*

The dextran used was Glaxo "Intradex" prepared by lyophilizing a commercial solution from which glucose had been removed by dialysis. The polyethylene glycol was "Carbowax 6000" (Union Carbide Corporation). For phase separation at 20°C, a mixture of 5 g of "Carbowax 6000" and 8 g of "Intradex" with 100 ml of water was found to be satisfactory. More "Intradex" was needed than has been described for other dextrans (Albertsson 1958), probably because the chain length of dextran is deliberately reduced during the manufacture of pharmaceutical preparations.

(*d*) *Analytical Methods*

β-Glucosidase activity and kinetic constants were estimated by methods set out elsewhere (Jermyn 1955*c*, 1962*a*) both for routine determinations with *p*-nitrophenyl β-glucoside and occasional experiments with non-chromogenic β-glucosides. Other glucosidase activities were measured when required by using the corresponding *p*-nitrophenyl glycoside.

Carbohydrate was estimated by the anthrone method (Jermyn 1956) with a glucose standard, and expressed as glucose equivalent. Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (B.S.A.) as a reference protein.

Protein end-groups were determined by the fluorodinitrobenzene method (Fraenkel-Conrat, Harris, and Levy 1954).

TABLE 1

ANALYTICAL DATA FOR THE BATCH OF S. ATRA β-GLUCOSIDASE WHOSE PURIFICATION IS ILLUSTRATED IN FIGURES 1, 2, AND 3

Values in brackets are the "best" values obtained with any batch

	Original Sample	Main Peak from Acetate Column	Main Peak from Borate Column
β-Glucosidase activity (units/ml)	1400	31·6	61·5
Protein (mg/ml)	2·94	0·047	0·099
Carbohydrate (mg/ml)	21·63	0·0495	0·0377
Units enzyme per milligram protein	476	672	621 (1400)
Units enzyme per milligram carbohydrate	64·7	638	1630 (3020)
Carbohydrate/protein ratio	7·36	1·05	0·37 (0·11)

(*e*) *Enzyme*

The mould has grown and culture filtrates prepared essentially as described in earlier parts of this series; modifications were made in the growth conditions to give higher enzyme yields (Jermyn and McQuade, unpublished data).

Culture filtrates were precipitated three times in the cold with two volumes of ethanol, considerable amounts of insoluble material being centrifuged out after the precipitate had been taken up in water at each stage. The final precipitate was dissolved in water, dialysed exhaustively against running tap water, and centrifuged to give a clear, brown, rather viscous solution. The volume was reduced to 1% of the original and 50–90% of the activity was retained.

These crude concentrates contained 15–20 mg of carbohydrate (glucose equivalent) per millilitre, being too viscous to centrifuge satisfactorily above this concentration. The ratio glucose equivalent/B.S.A. equivalent ranged between 3/1 and 20/1. On the assumption that the enzyme activity per milligram of protein in the best samples recovered at later stages of purification was that of a pure and completely active enzyme, then only about 25% of the protein in the crude

concentrates was enzyme protein. In fact, the enzyme protein was probably an even smaller proportion of the total protein.

Samples and fractions were stored as frozen solutions at −20°C between experiments. It was found that any attempts to recover them in the solid state by lyophilization led to drastic inactivation; where most of the contaminating carbohydrate had been removed this inactivation was normally complete.

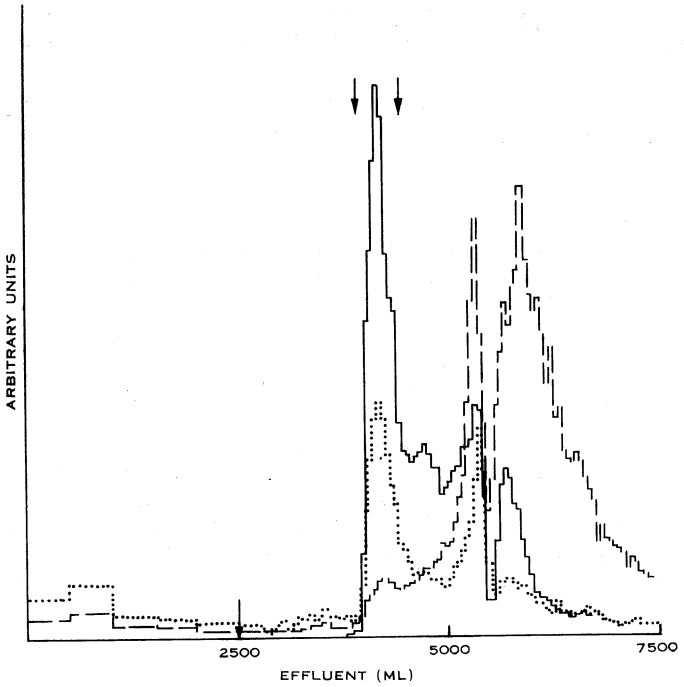

Fig. 1.—Sodium chloride gradient elution of a typical crude *S. atra* β-glucosidase concentrate on the large DEAE-cellulose column in 0·02M sodium acetate buffer, pH 4·1, at 1°C. ———— Enzyme; ..... protein; — — — carbohydrate. The material eluting in the region between the arrows was taken for further chromatography. Column size 50 × 4 cm, load 200 g, gradient 0–0·5M NaCl, 50-ml fractions taken.

III. Results

(a) *Preparation of Purified Samples*

The purification by chromatography of a typical crude concentrate on DEAE-cellulose is shown in Figures 1, 2, and 3, and analytical data are given in Table 1.

The fractionation of the crude concentrate (Fig. 1) in 0·01M sodium acetate at pH 4·1 leads to an obvious major peak of enzyme activity. The fractions under this peak were bulked and dialysed. When a portion of this material was re-run under exactly the same pH and buffer conditions on the small column,

no significant further dissociation of enzyme, protein, and carbohydrate was achieved (Fig. 2), although a small amount of inactive material was removed.

Similar results were obtained when specimens of the main enzyme fraction were eluted by a sodium chloride gradient from DEAE-cellulose columns on which the enzyme had been absorbed from any dilute non-complexing buffer over the pH range 4–9. The enzyme is both absorbable on DEAE-cellulose and stable in this pH range.

The material at this stage appears to consist essentially of a carbohydrate–protein complex where the ratio glucose equivalent/B.S.A. equivalent $= 1 \cdot 5 \pm 0 \cdot 5$. Attempts to disrupt the complex by stepwise elution with fine increments of sodium chloride concentration or frontal analysis with sodium chloride solutions

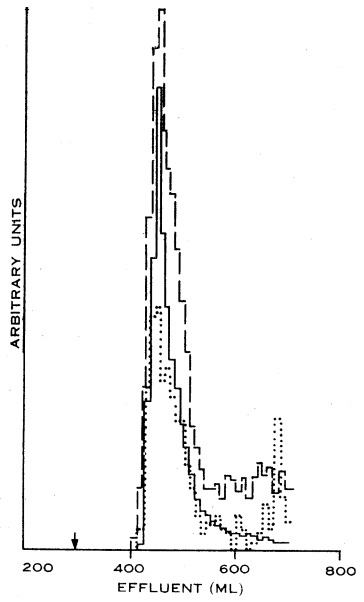

Fig. 2

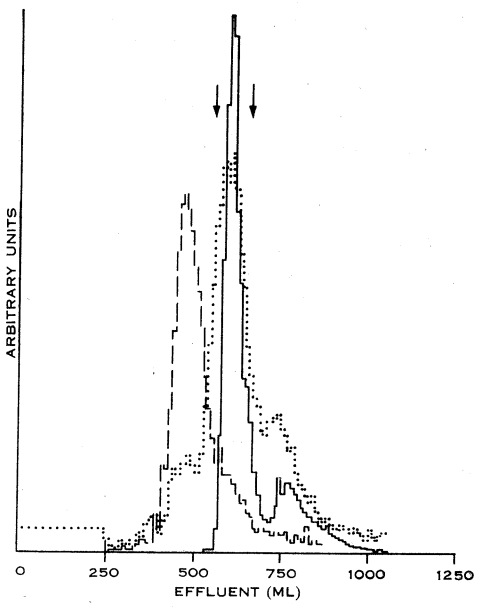

Fig. 3

Fig. 2.—Rechromatography of the material from the "main peak" of Figure 1; DEAE-cellulose, small column, standard conditions, 0·02M sodium acetate buffer, pH 4·1. ———— Enzyme; ..... protein; — — — carbohydrate.

Fig. 3.—Rechromatography of the "main peak" of Figure 1; DEAE-cellulose, small column, standard conditions, 0·05M sodium borate, pH 8·1. ———— Enzyme; ..... protein; — — — carbohydrate. The material eluting in the region between the arrows was taken for analysis as "purified enzyme".

somewhat stronger than that needed to elute the activity (*c.* 0·05M) failed. A number of ions which give charged complexes with polysaccharides (sulphite, molybdate, tungstate, arsenate, borate) were now tried as eluting or buffering agents. Of them only borate was successful, both in retaining the stability of the enzyme and disrupting the complex. Figure 3 illustrates how the complex is disrupted in 0·05M sodium borate buffer, pH 8·1.

When the material taken from the fractions indicated in Figures 1 and 3 was analysed along with original material, the data set out in Table 1 were obtained. The ratio carbohydrate/protein in the most purified fraction (0·37) was higher than expected, since Figure 3 indicates that much less than a third of the carbohydrate in the original complex (carbohydrate/protein = 1·05) is eluted along with the protein; however, there is always a slow leakage of carbohydrate from the material of the column itself (Jermyn 1962*b*) which sets a limit to the apparent purification. The value quoted as the lowest for the ratio (0·11) was obtained with a particularly active preparation where a much larger amount of protein was put on the column. The amount of protein in the purest fractions was so low that it could not be measured accurately without considerable concentration of the solution, and the carbohydrate/protein ratio was therefore high.

The other striking feature of Table 1 is that the value for the enzyme activity of the protein increases so little during the purification of this particular concentrate. The value quoted as the maximum for any preparation (1400 units/mg protein) was attained or approached a number of times with procedures in which small test samples of culture filtrate (500 ml) were carefully precipitated under conditions that precluded any temperature rise and the fractions put under refrigeration as soon as they left the column. It was also important to dialyse under cold-room conditions. The activity per milligram of protein was then consistently near the limit quoted, and no fraction has ever been found with a higher specific activity.

The appearance of a considerable minor peak of less active material in Figure 3 and the fact that the specific enzymic activity of the protein is much less than in more gently treated samples suggest that the enzyme protein is being inactivated or converted into a less active form by the handling it has received. Re-running of the material from the major peak of Figure 3 shows that it has now split into two peaks (Fig. 4), occupying the positions of the major and minor peaks of Figure 3. Separation of these peaks and two more passages through the column bring about further inactivation and shift in physical properties. The material was deliberately dialysed against running tap water between passages and no attempt made to protect it against the effects of normal room temperatures. The marked instability of the enzyme in the absence of its protecting polysaccharide is in contrast to its robustness as the crude concentrate; in the presence of enough suitable antiseptic this latter can be left for a year or more at refrigerator temperatures without much loss of activity or change in chromatographic pattern.

(*b*) *Homogeneity of the Purified Enzyme*

Gradient-elution chromatography, whether of the salt- or pH-gradient variety, gives no guarantee that the peaks observed represent homogeneous components (Tiselius, Hjerten, and Levin 1956). If the peaks are very sharp and there is a concordance throughout of various properties, both protein and enzymic, there is an indication of homogeneity and no more.

Various conditions of pH, temperature, ionic strength, and urea concentration were used in a large number of attempts to discover systems in which the

enzyme, whether as the carbohydrate complex or not, would exhibit true chromatography, i.e. elution analysis in buffer of constant ionic strength, with DEAE-, PAHO-, and SPHO-celluloses. The sources of ionic strength included chloride, sulphate, phosphate, and borate salts. These attempts have been uniformly unsuccessful and the most powerful single test for homogeneity is therefore not applicable.

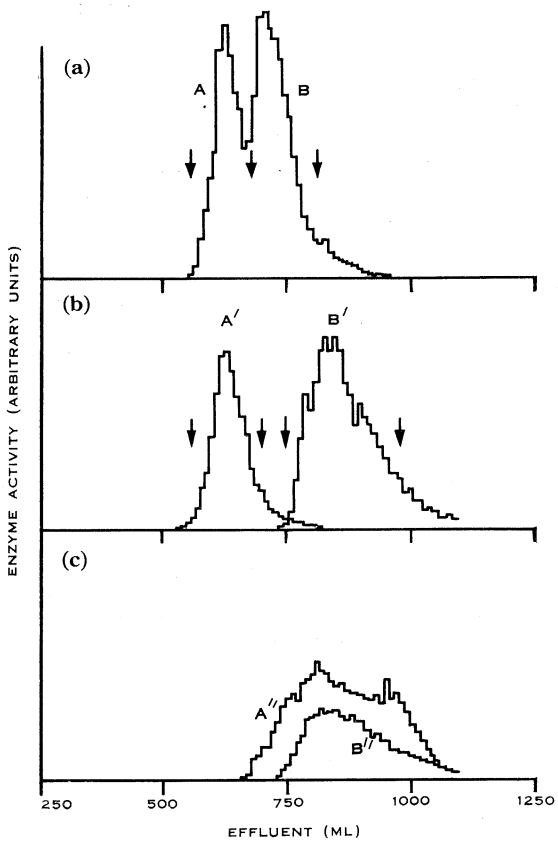

Fig. 4.—Rechromatography of "purified enzyme" (cf. Fig. 3) under conditions identical with those of Figure 3. (*a*) First re-run: material then eluting in the regions indicated was taken as fractions A and B; (*b*) second re-runs: fractions A and B chromatographed in separate experiments but the results combined in a single figure. Indicated material taken for rechromatography (A′ and B′); (*c*) third re-runs: fractions A′ and B′ chromatographed in separate experiments. The scale of enzyme activity is identical for all three sets of curves.

At the beginning of this work a system that produces a powerful but illusory appearance of true chromatography was developed. DEAE-cellulose in the dry –OH form, which is the end-product of purification of the commercial material, is mixed to a slurry with a KH_2PO_4 solution, the slurry run into a small column, and excess liquid allowed to drain off. A little of the same solution containing the positively charged dye methyl green as indicator of the front, together with enzyme purified by the gradient elution in acetate and borate buffer, is run on to

the top of the column, and the enzyme and dye eluted with more of the solution. The results shown in Figure 5 are then obtained. The illusion of chromatography is almost perfect, with an "R_F" depending on phosphate concentration and markedly susceptible to temperature. The effect is in fact due to a pH gradient and disappears if the column is thoroughly equilibrated beforehand. Nevertheless 90% of the enzyme emerges over about half the hold-up volume and a pH change of 0·07–0·15, and the enzyme is thus at least presumptively homogeneous by this test also.

Since the protein–polysaccharide complex was capable of being divided into an indefinite number of subfractions by stepwise elution, this fact could be used to check the homogeneity of the enzyme protein when chromatography of the protein by elution analysis in buffer of constant ionic strength had failed. A sample

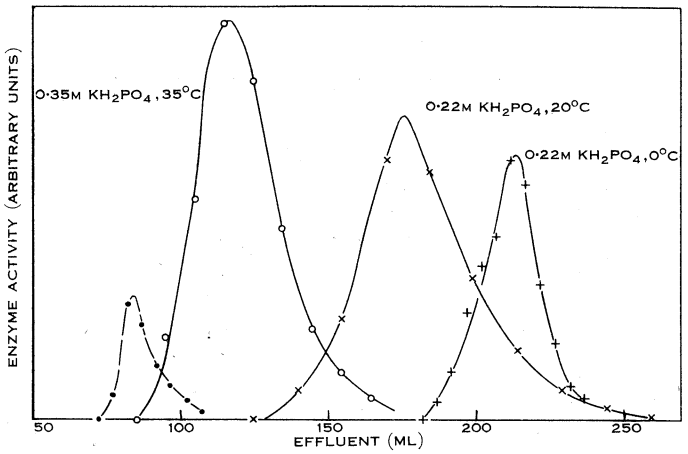

Fig. 5.—Apparent true chromatography using the small column and DEAE-cellulose not in true equilibrium with KH_2PO_4 solutions. ● — — ● Methyl green (front indicator);————β-glucosidase. The observations are fully described in the text.

of protein–carbohydrate complex, purified by a second elution from DEAE-cellulose in acetate buffer (experiment of the type of Fig. 2) and having a carbohydrate/protein ratio of 1·4, was adsorbed on PAHO-cellulose from sodium phosphate buffer (0·15M, pH 6·0). Stepwise elution with increases of 0·005M in buffer strength for each fraction gave the results set down in Table 2. There is not the slightest evidence that the fractions differ in enzymic properties.

When a sample of highly purified enzyme (1260 units/mg; carbohydrate/protein ratio 0·28) was examined in the Spinco analytical ultracentrifuge, it was found that the shape of the boundary obtained was highly concentration-dependent at pH 5·0 with 0·1M NaCl as the supporting electrolyte. The single boundary obtained in all experiments was quite sharp at 10 mg/ml; at 2 mg/ml it was so diffuse that it was only measurable under initial conditions. The simple interpretation of these results is that the sample was polydisperse perhaps even to the point of a continuous gradation of molecular weights, a conclusion that agrees with the chromatographic results.

If the purified preparation contains only one protein, end-group analysis should show most probably a single end-group, or less probably two or three in approximately equal amounts if the enzyme is a multi-chain one. A number of

TABLE 2

ENZYMIC PROPERTIES OF PARTIALLY PURIFIED S. ATRA β-GLUCOSIDASE FRACTIONATED BY STEPWISE ELUTION FROM PAHO-CELLULOSE IN SODIUM PHOSPHATE BUFFERS AT pH 6·0 AND 20°C

About 5000 units, representing 7 mg of protein and 10 mg of carbohydrate, were absorbed on 200 mg resin from 0·15M buffer. About 20 ml eluate collected at each stage as 2×10 ml supernatants from centrifugation

Molarity of Eluting Buffer	Percentage (of total eluted)			Affinity for *p*-Nitrophenyl β-Glucoside ($K_m \times 10^5$) (M)	Affinity for 2-Naphthyl β-Glucoside ($K_i \times 10^5$) (M)
	Enzyme	Carbohydrate	Protein		
0·155	2	2	2	3·8	8·4
0·160	8	7	12	4·4	9·0
0·165	25	18	22	4·1	9·1
0·170	40	34	44	3·8	9·2
0·175	57	51	60	3·8	8·5
0·180	70	68	68	4·0	8·5
0·185	80	83	84	3·7	8·4
0·190	89	93	88	3·6	8·8
0·195	95	100	97	4·3	8·2
0·200	98	104	102	3·8	8·9

end-groups in widely varying amounts would demonstrate the presence of several components. It was difficult to accumulate the enzyme except as the polysaccharide

TABLE 3

COLOUR YIELDS OF DNP-AMINO ACIDS FROM IDENTICALLY TREATED BOOT'S INSULIN, INSULIN PLUS INULIN, AND S. ATRA β-GLUCOSIDASE

A sample of highly purified DNP-insulin was included at the hydrolysis stage for comparison. Values in parentheses represent percentage of yield in absence of inulin

Chromogen	DNP-Insulin (10 mg)	Insulin (10 mg)	Insulin (10 mg) +Inulin (20 mg)	β-Glucosidase (30 mg protein + 45 mg carbohydrate)
DNP-glycine	266	198	153 (65%)	
DNP-phenylalanine	264	238	106 (34%)	
ε-DNP-lysine	142	121	106 (68%)	76
DNP-threonine				84

complex, and the presence of carbohydrate is known to lead to low yields of dinitrophenyl (DNP)-amino acids due to reductions and condensations during the hydrolysis step. A cross-check on the findings with the enzyme was therefore

performed; insulin in the presence of two times as much of the fructose polysaccharide inulin was used as the material for assay. The end-groups of insulin and their yield under the conditions of the assay are well known; the decrease of this yield in the presence of a somewhat greater amount of much more labile ketose sugar should set a limit for the destruction of end-groups in the assay of the enzyme complex.

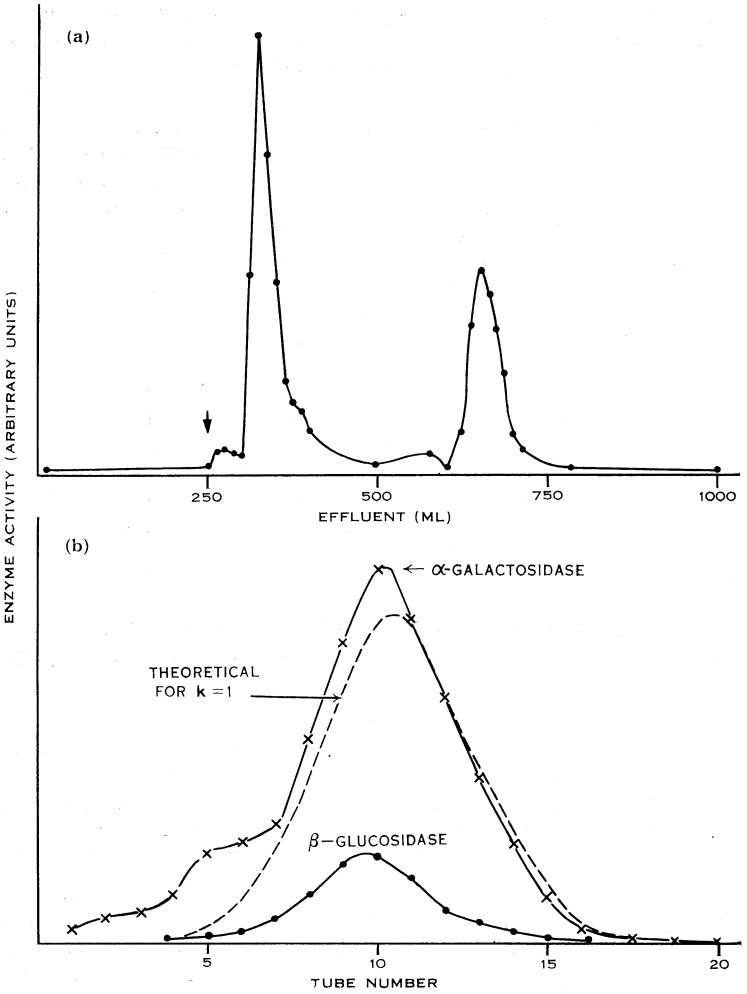

Fig. 6.—Heterogeneity of fenugreek enzymes by two methods: (*a*) sodium chloride gradient elution of fenugreek α-galactosidase from the small column of DEAE-cellulose under standard conditions in 0·02M sodium acetate buffer, pH 5·0; (*b*) distribution of fenugreek α-galactosidase and the accompanying minor β-glucosidase activity in a dextran–carbowax two-phase system (see text) at pH 5·0 (0·02M sodium acetate).

The results (Table 3) show that the yield of DNP-amino acids from the two end-groups of insulin, although reduced in the presence of a double amount of

inulin, is still quite sufficient to allow the end-groups to be identified with certainty; DNP-glycine and DNP-phenylalanine are also present in roughly equal amounts. It is therefore reasonable to claim that threonine, the only end-group detected in the enzyme preparations, must be the terminal amino acid of the great bulk of the protein chains present. It is unlikely, although not impossible, that there are a number of proteins present with terminal threonine; taken in conjunction with the other results, the data of Table 3 strengthen the presumption that the preparation contains a single homogeneous enzyme protein.

The very small amount of ϵ-DNP-lysine (the ratio ϵ-DNP-lysine/DNP-threonine was approximately 1) recovered from the enzyme preparation is in conformity with the observation from paper chromatographic experiments (Jermyn

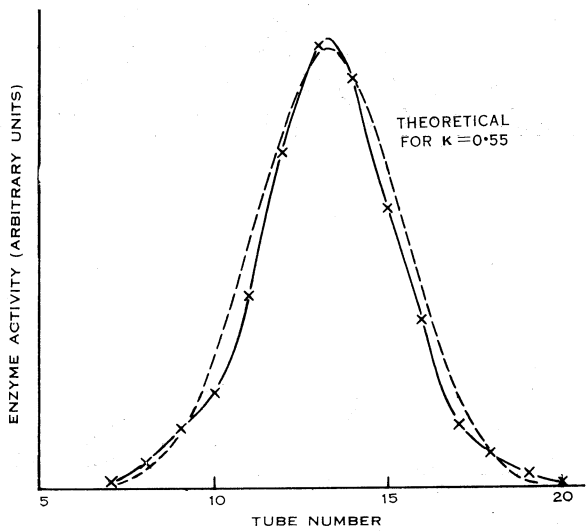

Fig. 7.—Distribution of *S. atra* β-glucosidase in a dextran–carbowax two-phase system at pH 3·5 (standard McIlvaine citric acid–sodium phosphate buffer diluted to one-fifth).

1955*b*) that the isoelectric point of the enzyme is very low ($\ll$pH 4) and it can contain few basic amino acids. The minimum molecular weight for the enzyme protein, assuming a single chain, will be 30,000–40,000, but this estimate might easily be in error by a factor of two.

A further powerful test for inhomogeneity in a protein preparation is to follow the course of distribution experiments, if a two-phase system can be discovered in which the protein is partitioned between two phases. The β-glucosidase could not be partitioned in any of the numerous water–organic solvent–inorganic salt systems mentioned in the literature (cf. Porter 1955) nor in any other that I could devise, the enzyme always passing wholly into the more aqueous phase. An alternative possibility is to use the two aqueous phases formed by "polymer exclusion", using such pairs of incompatibles as dextran and polyethyleneglycol

(Albertsson 1958). Although such systems give little or no hope of recovering the distributed material, they can afford a useful test for purity, and as such they have been used here.

The slow separation of the two phases under standard gravity and the consequent necessary centrifugation at each distribution limits the number of tubes

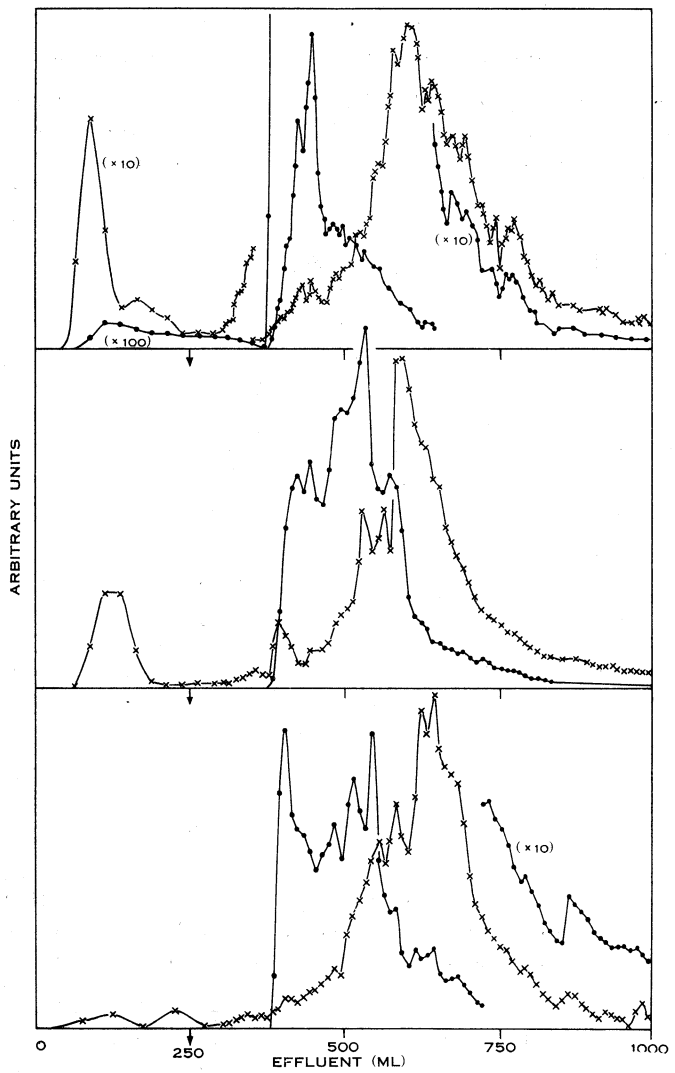

Fig. 8.—Sodium chloride gradient elution chromatography of test samples from three successive batches of crude *S. atra* β-glucosidase. DEAE-cellulose, small column, standard conditions, 0·02M sodium acetate buffer at pH 4·1. ●——● Enzyme; ×——× carbohydrate.

to what can be achieved in a working day (20–30). However, Figure 6 shows that this number of tubes is enough to demonstrate marked heterogeneity in an enzyme

system (fenugreek α-galactosidase) which can be readily shown to be multicomponent by gradient-elution chromatography. The weak β-glucosidase activity in the same preparation is, in contrast, apparently homogeneous.

At high pH values, the partition of *S. atra* β-glucosidase is strongly in favour of the lower (dextran) phase. Only at pH 3·5, where the enzyme is at best metastable, does the partition coefficient approach a value (2 : 1 in favour of the dextran phase) where efficient partition is possible. Within the limitations imposed by this diminished stability, the results set out in Figure 7 were obtained. There is no evidence that the β-glucosidase is not homogeneous.

The fact that the distribution curve is narrower than the theoretical distribution curve fitted to the observed maximum implies that the partition coefficient in favour of the moving (dextran) phase should be higher at high enzyme concentrations than at low enzyme concentrations. This hypothesis was tested and found to be correct; between the highest and lowest measurable enzyme concentration present in any tube in the experiment of Figure 7, the partition coefficient fell from about 4 to about 1.

(*c*) *Heterogeneity of Crude Preparations*

Section III(*b*) has concerned itself with material from the main peak of enzyme activity that can be seen on Figure 1. At least three other peaks are visible in that figure. Do these peaks contain enzymes different in any way from that present in the main peak? An approach to this question may be made by considering the data for a number of crude concentrates shown in Figure 8.

Test samples of crude concentrate from three successive batches were run under conditions made as nearly identical as possible on the small column. There is very little likeness between the three elution curves; numerous enzyme "components" can be seen in each curve. Some of these components seem to be identical in position in the three curves but are never identical in relative amounts. Having regard to the conclusion demonstrated elsewhere (Jermyn 1962*b*) that acidic polysaccharides show normal chromatographic behaviour in systems like the one studied and the general (though by no means perfect) correlation between peaks and shoulders in the enzyme and carbohydrate elution curves, the following hypotheses were formed:

(1) The main bulk of the polysaccharides present chromatograph as separate entities having, at the most, only very slight interaction with the enzyme.

(2) Some of the polysaccharides interact specifically with the enzyme protein; these complexes chromatograph as entities.

(3) The absolute and relative amounts of the specific polysaccharides varies from experiment to experiment with slight variations in the growth conditions of the mould.

(4) There is only a single enzyme protein present.

It may be remarked that the first three of these hypotheses would have been even more strongly suggested by the data if "protein" had been substituted for

"enzyme". Protein-elution curves (not included in Fig. 8 for reasons of clarity) show almost one-to-one correspondence between protein peaks and carbohydrate peaks.

To test these hypotheses, a run was made with a batch of enzyme that resulted from growing *S. atra* on a starch medium devoid of specific inducers (Jermyn 1955*a*). This had an extremely low activity (*c.* 1 unit per milligram of carbohydrate) and a reduced amount of protein (carbohydrate/protein ratio about 22/1) and it was felt that chromatography of the polysaccharide should dominate the observed elution curves.

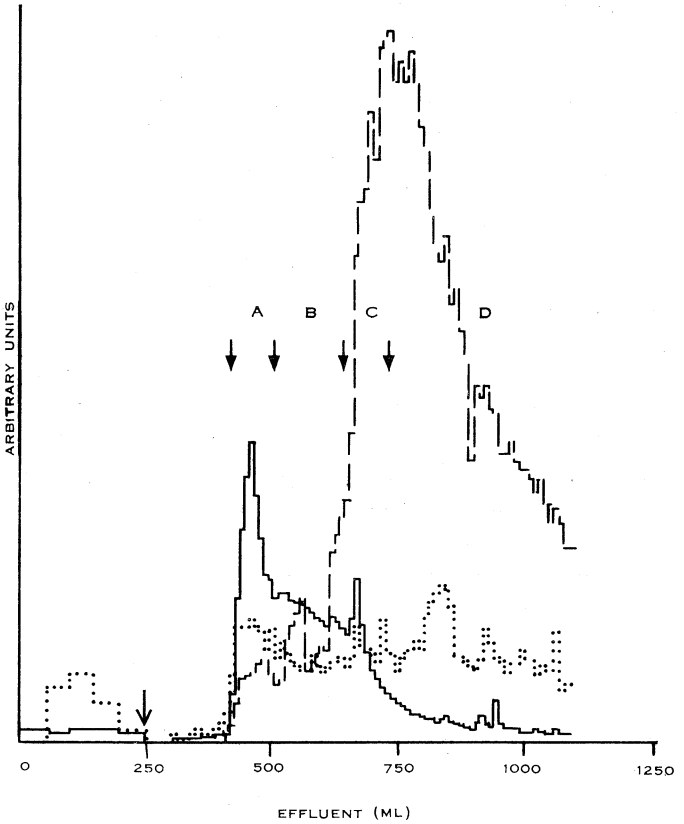

Fig. 9.—Sodium chloride gradient elution chromatography of a batch of *S. atra* β-glucosidase with a higher carbohydrate content and a very low enzyme activity. DEAE-cellulose, small column, standard conditions, 0·02M sodium acetate buffer at pH 4·1. ———— Enzyme; ..... protein; — — — carbohydrate. The arrows indicate the limits of the various fractions into which the eluate was divided.

The result of running this material in the crude state is shown in Figure 9. Re-running of the indicated fractions gave the results shown in Figure 10. The indicated fractions A′, B′, C′, D′, which constitute well-spaced components, were tested for their enzymic properties. The results are shown in Table 4; there is no significant difference in enzymic properties either among the eluted fractions

or as compared with the original. In addition, these observed properties agree with those obtained for the most highly purified material as indicated earlier. The components therefore appear to be illusory in the enzymatic sense.

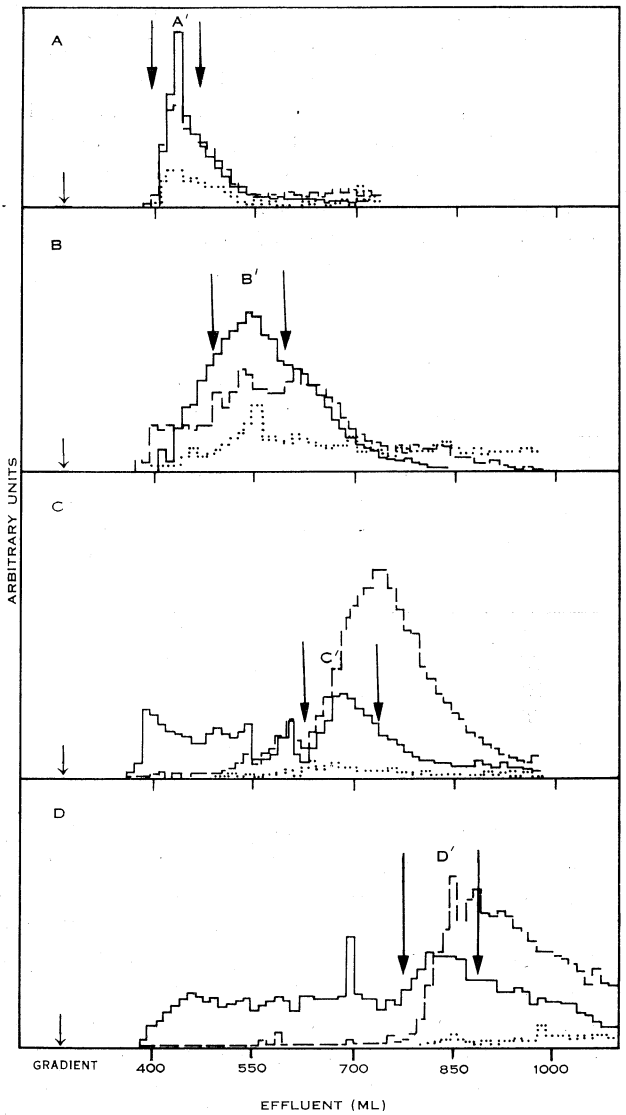

Fig. 10.—Rechromatography of the fractions A–D of Figure 9, under the identical conditions. Fractions A′, B′, C′, and D′ were taken from the material eluted between the positions marked by the arrows.

(*d*) *Properties of the β-Glucosidase*

The last section has shown that the properties of the purified enzyme fractions may be taken as typical of the β-glucosidase of *S. atra* as a whole so that certain conclusions may now be stated with reasonable firmness.

The enzyme is devoid of the following activities: α-D-glucosidase, α- and β-D-galactosidases, α- and β-D-mannosidases, β-D-xylosidase, β-L-arabinosidase, β-cellobiosidase, β-lactosidase, β-maltosidase, cellobiase, cellulase. In the most purified preparations sucrase, amylase, protease, and esterase cannot be demonstrated. The sole activity of the enzyme appears to be that of a transferring β-glucosidase with specificity directed towards aryl β-glucosides. None of the attempts to demonstrate that the total activity present in partially purified material can be split into fractions of differing enzymic properties has been successful. This negative evidence can be taken as the current validation of earlier work with the enzyme and more recent kinetic studies (Jermyn 1962*a*). On the basis of the known behaviour of the enzyme, the attempt to provide positive evidence by isolating a crystalline protein of high and constant specific activity does not appear to have any chance of being fruitful.

TABLE 4
ENZYMIC PROPERTIES OF "COMPONENTS" ISOLATED AS SHOWN IN FIGURES 9 AND 10
All of data at pH 5 and 28°C

Enzyme Sample	Michaelis Constant ($K_m \times 10^5$) for *p*-Nitrophenyl β-Glucoside (M)	Inhibitor Constant ($K_i \times 10^5$) for 2-Naphthyl β-Glucoside (M)
Original	3·8	10·0
A′	3·7	8·7
B′	4·3	8·7
C′	4·3	9·5
D′	4·1	8·9

One further point that will require elucidation is whether the properties of the enzyme are themselves constant. The organism has now been in continuous subculture for 12 years in this laboratory and it is likely that within this period there has been some degree of unconscious selection. The K_m for *p*-nitrophenyl β-glucoside was approximately 6×10^{-5}M in the first experiments on the mould; in later experiments over a long period (Jermyn 1955*c*) it averaged $5{\cdot}0 \times 10^{-5}$M. Some years later it averaged $3{\cdot}8 \times 10^{-5}$M in the present experiments (carried out in 1960). Two years later the K_m is now (1962) averaging $3{\cdot}2 \times 10^{-5}$M. The slight activity against phenyl β-thioglucoside detected earlier (Jermyn 1955*c*) has now disappeared (Professor G. Wagner, private communication; and tests in this laboratory).

It has been shown (Jermyn 1959) that a variety of moulds, some of which are closely related to *S. atra*, all produce a β-glucosidase of basically similar type, but of varying specificity towards substrates. *Stachybotrys* is not a particularly stable genus in culture and Zuck (1948) has claimed that isolates of morphology similar not only to other named species of the genus but also to *Memnoniella* can be recovered from subcultures of an apparently homogeneous isolate of *S. atra*.

Although an attempt has been made to keep such variation to a minimum by subculturing by mycelial transfer from an original culture derived from a single spore, it is likely that these precautions have not been altogether successful. Drift in the properties of an enzyme on continued subculture could be explained on the basis of selection, perhaps for quite other characters of the mould, that led to just sufficient alterations in the relevant protein-synthesizing systems to replace some amino acids in the enzyme by new ones. Kinetic properties of an enzyme from a "species" of microorganism seem to depend not only on the isolate but also its cultural history.

This effect will be discussed more fully in a forthcoming paper on adaptive enzyme systems in the mould, where its operation is much more obvious.

IV. Discussion

It was shown by Gillespie, Jermyn, and Woods (1952) that many enzymes from microorganisms can be split into components by paper electrophoresis; and subsequently enzymes from many sources have been split into components by various physical techniques. Sometimes these components are so different in properties as to be obviously distinct enzymes: in others they are so numerous and so poorly defined as to justify Haldane's (1954) concept of "micro-enzymes". The evidence of different workers is sometimes in direct conflict; a case in point is the cellulase of *Myrothecium verrucaria* where Whitaker (who reviewed his work in 1960) has always found a single component and other groups (Reese and Gilligan 1953; Hash and King 1958) have found multiple components. It can be deduced from the observations on the β-glucosidase of *S. atra* that both conclusions may be right. In the *S. atra* case, variations in the cultural conditions giving rise to different amounts of the complexing polysaccharides give all apparent states of affairs from one component to many. In the absence of proof that the enzymic properties of components in any given sample are markedly different, the presumption ought still to be that they all contain the same enzyme. Miller and Birgzalis (1961) have, in fact, not been able to show that the enzymic properties of nine separate components of a sample of *M. verrucaria* cellulase are more distinct than would be expected for a series of complexes of a single enzyme species.

The present work bears out the conclusion drawn earlier (Jermyn 1955*b*) that the β-glucosidase of *S. atra* is less stable the purer it becomes. In particular, when the complexing polysaccharide is almost completely removed, the enzyme only remains active for a short while in solution and is immediately inactivated on reduction to the solid state by lyophilization. This dependence on a complexing polysaccharide for stability resembles the relationship between yeast sucrase and yeast mannan described by Fischer, Kohtès, and Fellig (1951). Where a single enzyme can complex with a variety of macromolecules, it is to be expected that the complexes will show an equal variety of stability against heat, organic solvents, etc. Any evidence that tends to show components as distinct enzymes on the basis of stability differences must therefore be treated with extreme caution. It is possible that some of the differences seen in Figure 8 between the spectrum of components in different batches of enzyme may depend on small differences in the thermal history of the batches.

V. Acknowledgments

I wish to acknowledge the technical assistance of Messrs. B. W. Wilson and P. Drew. I am indebted to Dr. E. O. P. Thompson for a sample of pure DNP-insulin and to Professor G. Wagner for his continued interest in the thioglucosidase activity of the *S. atra* β-glucosidase.

VI. References

Albertsson, P. (1958).—*Biochim. Biophys. Acta* **27**: 378.

Fischer, E. H., Kohtès, L., and Fellig, J. (1951).—*Helv. Chim. Acta* **34**: 1132.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1954).—In "Methods of Biochemical Analysis". (Ed. D. Glick.) (Interscience Publishers, Inc.: New York.)

Gillespie, J. M., Jermyn, M. A., and Woods, E. F. (1952).—*Nature* **169**: 487.

Haldane, J. B. S. (1954).—"The Biochemistry of Genetics." (Allen and Unwin: London.)

Hash, J. H., and King, K. W. (1958).—*J. Biol. Chem.* **232**: 381.

Jermyn, M. A. (1955*a*).—*Aust. J. Biol. Sci.* **8**: 541.

Jermyn, M. A. (1955*b*).—*Aust. J. Biol. Sci.* **8**: 563.

Jermyn, M. A. (1955*c*).—*Aust. J. Biol. Sci.* **8**: 577.

Jermyn, M. A. (1956).—*Nature* **177**: 38.

Jermyn, M. A. (1959).—*Aust. J. Biol. Sci.* **12**: 213.

Jermyn, M. A. (1962*a*).—*Aust. J. Biol. Sci.* **15**: 248.

Jermyn, M. A. (1962*b*).—*Aust. J. Biol. Sci.* **15**: 787.

Jermyn, M. A., and Thomas, R. (1953).—*Nature* **172**: 728.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951).—*J. Biol. Chem.* **193**: 265.

Miller, G. L., and Birgzalis, R. (1961).—*Arch. Biochem. Biophys.* **95**: 19.

Porter, R. R. (1955).—In "Methods in Enzymology". Vol. 1. (Eds. S. P. Colowick and N. O. Kaplan.) (Academic Press Inc.: New York.)

Reese, E. T., and Gilligan, W. (1953).—*Arch. Biochem. Biophys.* **45**: 74.

Thomas, R. (1955).—*Text. Res. J.* **25**: 559.

Thomas, R. (1956).—*Aust. J. Biol. Sci.* **9**: 159.

Thompson, E. O. P., and O'Donnell, I. J. (1960).—*Aust. J. Biol. Sci.* **13**: 393.

Tiselius, A., Hjerten, S., and Levin, Ö. (1956).—*Arch. Biochem. Biophys.* **65**: 132.

Whitaker, D. R. (1960).—*Bull. Soc. Chim. Biol.* **42**: 1701.

Zuck, R. K. (1948).—*Mycologia* **38**: 69.