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The Subunit Structure of β-Glucosidase from *Botryodiplodia theobromae* Pat

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1. A homologous series of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), which varied in relative amounts in different preparations from cultures of similar and different age, was observed in cultures of *Botryodiplodia theobromae* Pat grown for 4–8 weeks on cotton flock (cellulose) as carbon source. 2. Aging of the purified high-molecular-weight species led to some amount of dissociation into a homologous series of lower-molecular-weight species. 3. Rough molecular-weight estimates, by gel filtration, of the various species derived from the purified high-molecular-weight enzyme were 350000–380000, 170000–180000, 83000–87000 and 45000–47000. 4. Electron micrographs of the negatively stained 350000–380000-molecular-weight enzyme showed that the molecule is an octamer in which each roughly spherical monomer occupies a corner of a cube with each side about 7.14 nm long. 5. Carboxamidomethylation of the reduced form of each molecular-weight species of the enzyme led to irreversible dissociation of the molecules into electrophoretically identical polypeptides with a molecular weight of 10000–12000. 6. These results suggest a slow association–dissociation of the type (8n) ⇌ 2 (4n) ⇌ 4 (2n) ⇌ 8 (n), where n is defined as the monomer. The monomer is in turn made up of four polypeptide subunits which are non-catalytic. 7. The Michaelis constants (K_m) and heat stability of the four enzymically active molecular species derived from the purified enzyme increased with molecular complexity, whereas all four species were inhibited by glycerol (100 mM) at low concentrations of substrate (*o*-nitrophenyl β-D-glucopyranoside) but activated at high substrate concentrations. 8. Only the lowest-molecular-weight species (45000–47000 mol.wt.) showed substrate inhibition.

The enzyme β-glucosidase has been found to be widely distributed among plants, fungi, yeasts, bacteria and animal tissues. In fungi, yeast and bacteria the inducible nature of the enzyme has been demonstrated repeatedly. Partially purified preparations of β-glucosidase from a variety of yeasts have indicated that the enzyme is a high-molecular-weight protein with the molecular weight determined by various methods to range from 300000 to 335000 (Duerksen & Halvorson, 1958; Hu *et al.*, 1960; Fleming & Duerksen, 1967*a*,*b*; Marchin & Duerksen, 1968*a*,*b*). Fleming & Duerksen (1967*b*) and Marchin & Duerksen (1968*b*) have postulated that the enzyme from yeast is composed of three or four monomers with molecular weights of 110000 or 80000 respectively. The monomer was found to be very unstable (Marchin & Duerksen, 1968*a*). These enzyme preparations had very low carbohydrate content.

β-Glucosidase of fungal origin has repeatedly been reported to adopt multiple molecular forms (Jermyn, 1955*a*,*b*, 1962; Pettersson, 1963; Mahadevan & Eberhart, 1964; Horikoshi & Yonosuke, 1965), and Jermyn (1955*a*,*b*, 1962) has attributed the multiplicity of aryl-β-glucosidase from *Stachybotrys atra* to the formation of stable complexes between a single enzyme and a variety of polysaccharides. The quoted molecular weights of the enzymes from fungal sources vary widely according to source and/or method of isolation. Wood (1971) estimated the molecular weight of β-glucosidase from *Fusarium solani* to be 400000. Umezurike (1971) reported a molecular weight of about 321000 for the high-molecular-weight enzyme from *Botryodiplodia theobromae*. This value is in the same range reported for the yeast enzyme. The enzyme from *Aspergillus wentii* has a molecular weight of 170000 (Legler, 1968) and a bacterial β-glucosidase has been reported to have a molecular weight of 120000–160000 (Han & Srinivasan, 1969). The last two enzymes seem therefore to have about half the molecular weight of the yeast and *Botryodiplodia theobromae* (and probably also of the *F. solani*) enzymes. Li *et al.* (1965) have reported a molecular weight of 76000 for the enzyme from *Trichoderma viride*. In contrast with the fungal enzymes, the enzyme isolated from pig kidney has been found to have a molecular weight of 40000–50000 (Abrahams & Robinson, 1969; Robinson *et al.*, 1967*a*,*b*). The former enzyme seems therefore to be about one-quarter and the latter about one-eighth the molecular weight of the yeast and *B. theobromae* enzymes. With this review in mind, it is interesting that the high-molecular-weight enzyme

from *B. theobromae* has been observed to dissociate into catalytically active lower-molecular-weight species which appear to be lower aggregates of similar subunits, and their molecular weights correspond to those given above for the various low-molecular-weight species of β-glucosidase from various sources (Umezurike, 1971).

There exists some uncertainty whether the low-molecular-weight species obtained from yeasts are catalytically active or become so only as a result of re-association into the high-molecular-weight species (Marchin & Duerksen, 1968*a*,*b*; Fleming & Duerksen, 1967*b*). The properties of β-glucosidase have therefore been re-examined. It is a matter of some importance whether the smaller units of β-glucosidase are catalytically active or whether reassociation is required before activity is expressed in the enzyme from *B. theobromae*. Recently (G. M. Umezurike, unpublished results), β-glucosidase preparations richer in the low-molecular-weight enzyme species than previously observed have been obtained. The present paper reports results obtained by using one such preparation to establish the catalytic activity of the low-molecular-weight enzyme species. Results that throw further light on the subunit structure of the β-glucosidase from *B. theobromae* are also reported, and a model of this fungal enzyme, based on these results, is described.

Materials and Methods

Organism

Botryodiplodia theobromae (I.M.I. 115626, A.T.C.C. 26123) was originally isolated from decaying wood (Umezurike, 1969).

Chemicals

o-Nitrophenyl β-D-glucopyranoside and the protein markers used for gel filtration were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. All other chemicals and materials were as described previously (Umezurike, 1970*b*, 1971).

Enzyme assay

β-Glucosidase activity was determined as described previously (Umezurike, 1971) except that *o*-nitrophenyl β-D-glucopyranoside was used as a substrate (donor).

Ion-exchange chromatography

DEAE-Sephadex A-50 columns were prepared as previously described (Umezurike, 1970*b*); 0.1M-Tris–HCl buffer (pH 7.1) was used.

Gel filtration

Columns of Sephadex G-75 and G-200 were prepared as described elsewhere (Umezurike, 1970*b*). All chromatographic separations were performed in a cold-room at 4°C. Estimation of molecular weight by gel filtration, by the method of Andrews (1965), was carried out with Blue Dextran (mol.wt. 2000000), β-galactosidase (mol.wt. 540000), catalase (mol.wt. 250000), bovine serum albumin (mol.wt. 65000), β-lactoglobulin (mol.wt. 35000), cytochrome *c* (mol.wt. 12270) and glucose (mol.wt. 180) as markers.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed in a cold-room at 4°C by the standard method of Ornstein & Davis (1961) and by the double-disc technique of Racusen (1967). The distribution of enzymic activity on 2mm slices of the gels was determined as described previously (Umezurike, 1970*b*).

Enzyme preparation and purification

The culture filtrates from which the crude β-glucosidase was derived was obtained from 4–8-week-old cultures of *B. theobromae* grown at 30°C as described previously (Umezurike, 1970*a*,*b*). The medium contained 0.5% (w/v) cotton flock as carbon source.

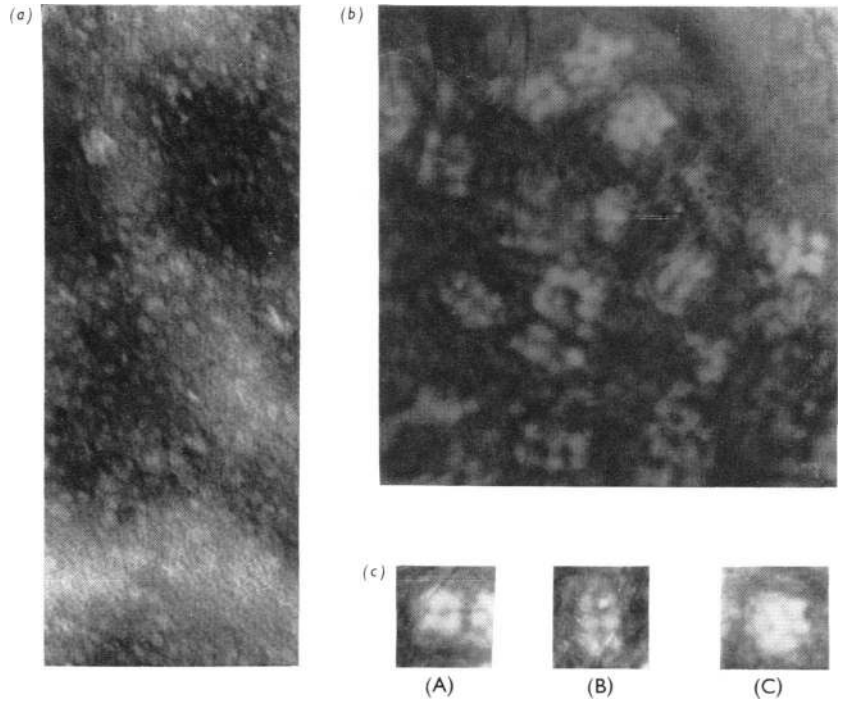
Crude enzyme preparations were obtained from the culture filtrates by precipitation of proteins with cold acetone (twice the volume of the culture filtrate). Where the purified high-molecular-weight species (i.e. the 11.5S species; Umezurike, 1971) was required, the purification from the acetone-dried powder was carried out as previously described (Umezurike, 1971).

Carboxamidomethylation of reduced enzyme protein

Reduction of enzyme protein with 8M-urea and 0.1M-2-mercaptoethanol followed by alkylation with 0.05M-iodoacetamide was carried out as reported previously (Umezurike, 1971).

Electron microscopy

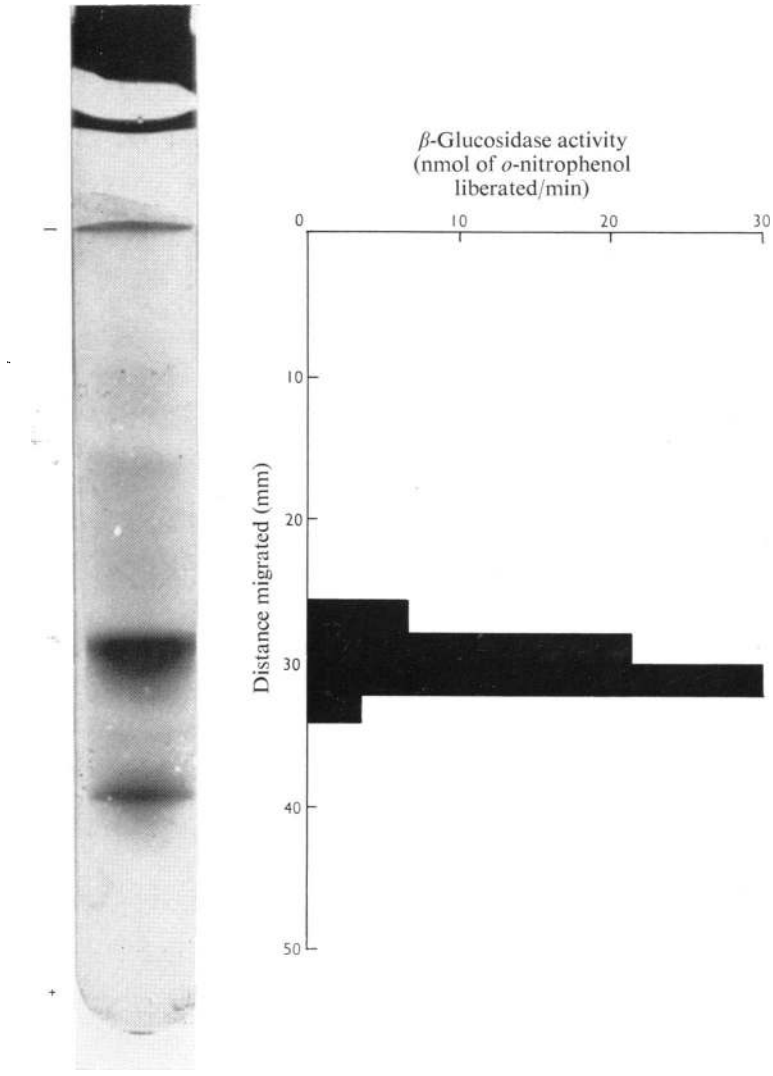
Negative staining of the enzyme preparations for electron microscopy was done at room temperature by the droplet method (van den Broek *et al.*, 1971), by using 0.5% (w/v) phosphotungstic acid solution adjusted to pH 5.0 with 1M-NaOH as contrast agent. The stained protein film was then dried in air and the grids were examined in either a Zeiss E.M.9 or a Hitachi HU-11 E-1 electron microscope.



EXPLANATION OF PLATE 1

Electron micrograph of negatively stained high-molecular-weight β-glucosidase

Staining of a Formvar-coated grid bearing a drop of enzyme solution was done with 0.5% (w/v) phosphotungstic acid adjusted to pH 5.0 with 1 M-NaOH. (*a*) Magnification ×210000; (*b*) magnification ×840000; (*c*) shows the predominant patterns (A, B and C) found in electron micrographs (magnification ×840000).



EXPLANATION OF PLATE 2

Photograph of polyacrylamide gel after electrophoresis in the standard system of Ornstein & Davis (1961) *of the low-molecular-weight β-glucosidase (species D) that had been heated at* 60°*C for* 25 *min*

The histogram on the right shows the distribution of enzymic activity on an identical unstained gel. The gel was cut into 2mm slices, each slice eluted with 2ml of 0.05M-sodium acetate buffer (pH5.0), and samples of the extract were used for enzyme assay.

Concentration of reagents

Unless otherwise stated, the reagent concentrations quoted in this paper are final concentrations in the mixtures.

Results

Consideration of the purified high-molecular-weight β-glucosidase

Physical properties. The high-molecular-weight enzyme purified as previously described (Umezurike, 1971) was found to be homogeneous by gel filtration on Sephadex G-200, by ion-exchange chromatography on DEAE-Sephadex A-50, and by gel electrophoresis on polyacrylamide (cf. Umezurike, 1971). Carboxamidomethylation of the reduced enzyme gave a sample which on electrophoresis by the method of Ornstein & Davis (1961) showed only one fast-moving catalytically inactive protein with a molecular weight of 10000–12000 (cf. Umezurike, 1971). Enzymic activity was not recovered after prolonged dialysis in 0.05M-sodium acetate buffer, pH5.0. 2-Mercaptoethanol (0.1M) had no effect on the activity of the high-molecular-weight enzyme after preincubation at 30°C for up to 3h at pH7.0.

From the relationship between the elution volume of the high-molecular-weight enzyme from a column of Sephadex G-200 and the logarithm of the molecular weights of some spherical protein markers (Andrews, 1965) a rough estimate of the molecular weight was calculated to be 350000–380000. A molecular weight of about 321000 was calculated (Umezurike, 1971) by the technique of Martin & Ames (1961), and a Stokes radius of 7.0–8.3nm was calculated for this enzyme by the method of Ackers (1964).

Electron microscopy. High-magnification electron micrographs of the purified high-molecular-weight enzyme negatively stained with phosphotungstic acid are shown in Plate 1. The ill-defined structures found in these electron micrographs arose from clumped molecules. Fig. 1 shows interpretative diagrams of the predominant well-defined patterns in this and other electron micrographs. The letters (A, B and C) in Fig. 1 indicate the patterns with similar letters in Plate 1. The average dimensions of the various patterns are shown in the legend to Fig. 1. These patterns are consistent with different orientations of an octameric aggregate with each roughly spherical monomer occupying a corner of a cube. The dimensions of the various patterns (x, y and z in Figs. 1A, 1B and 1C) agree with corresponding dimensions (shown in the legend to Fig. 1D) calculated from a cube with each side 7.14nm long.

Effect of aging. When the purified high-molecular-weight enzyme, stored for 12 weeks in 0.1M-Tris–HCl buffer (pH7.1) at 10°C, was used for ion-exchange chromatography on DEAE-Sephadex A-50 and for electrophoresis on polyacrylamide gel the results presented in Figs. 2 and 3 were obtained. Apparently, some of the high-molecular-weight enzyme had dissociated into lower-molecular-weight species. The relative amounts of the various molecular-weight species varied in different preparations. The fractional reassociation observed when a low-molecular-weight β-glucosidase was used for electrophoresis has been reported (Umezurike, 1971).

The order of elution of these forms of the enzyme on Sephadex G-200 was found to be identical with that on DEAE-Sephadex A-50 (see below). The molecular weights of the various species (A–D in Fig. 2) estimated by the method of Andrews (1965) when the aged sample, or each of the pooled fractions marked A–D in Fig. 2, was used for chromatography on Sephadex G-200 and Sephadex G-75 were 350000–380000 (A), 170000–180000 (B), 83000–87000 (C) and 45000–47000 (D).

Carboxamidomethylation of the reduced form of each enzyme species gave a protein which on electrophoresis showed only one enzymically inactive fast-moving protein band with a molecular weight of 10000–12000 estimated from its behaviour during

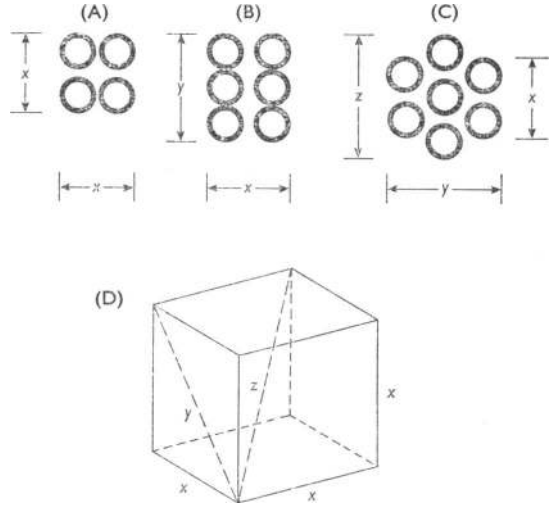


Fig. 1. *Interpretative diagrams of the predominant patterns observed in electron micrographs of the purified high-molecular-weight β-glucosidase*

The patterns labelled (A), (B) and (C) correspond to those labelled similarly in Plate 1. The average dimensions of sides x, y and z in (A), (B) and (C) are 7.14, 10.72 and 12.00nm respectively. Those for the corresponding sides in a cube with each side 7.14nm long (i.e. in D) were calculated to be 7.14nm (x), 10.10nm (y) and 12.37nm (z).

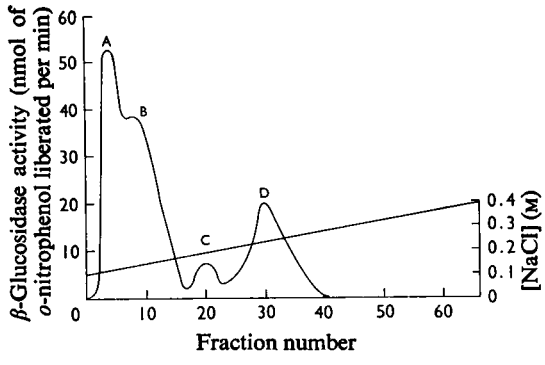


Fig. 2. *Elution of β-glucosidase activity from a column (8.0 cm × 1.5 cm) of DEAE-Sephadex A-50*

The gel was equilibrated with 0.1 mM-Tris–HCl buffer (pH 7.1) and washed with 300 ml of the same buffer after application of sample. The enzyme components were then eluted from the column with a linear gradient of NaCl made from 500 ml of 0.1 M-Tris–HCl buffer (pH 7.1) containing 0.1 M-NaCl and 500 ml of 0.1 M-Tris–HCl buffer (pH 7.1) containing 0.4 M-NaCl. The volume of each fraction was 15 ml. The sample was a purified high-molecular-weight enzyme that had been stored at 10°C for 12 weeks in 0.1 M-Tris–HCl buffer (pH 7.1).

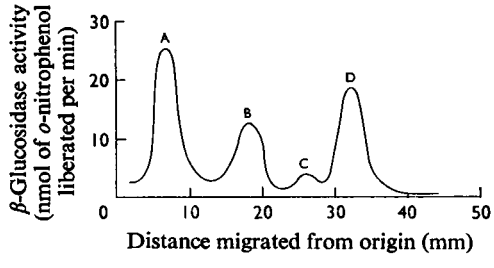


Fig. 3. *Distribution of β-glucosidase activity in gel slices after polyacrylamide-gel electrophoresis of a purified high-molecular-weight β-glucosidase that had been stored in 0.1 M-Tris–HCl buffer (pH 7.1) for 12 weeks at 10°C*

After electrophoresis, the gel was sliced into 2 mm pieces starting from the top of the small-pore gel. Each slice was extracted with 2 ml of 0.05 M-sodium acetate buffer (pH 5.0), and portions of the extracts were assayed for enzymic activity.

electrophoresis, in comparison with those of the higher-molecular-weight forms (cf. Umezurike, 1971).

Kinetic properties of the various molecular species. Fractions 3 (i.e. species A), 12 (species B), 20 (species C) and 30 (species D) from similar separations to that shown in Fig. 2 were dialysed against appropriate buffers and used for kinetic studies.

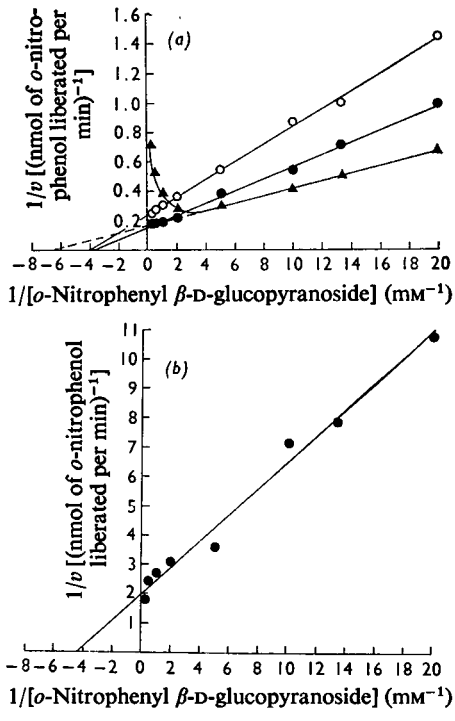


Fig. 4. *Lineweaver–Burk plots of results obtained when various enzyme species were used*

(*a*) Species A (●), B (○) and D (▲); (*b*) species C. Species A, B, C and D of β-glucosidase were obtained from fractions 3, 12, 20 and 30 respectively, of a similar separation on a DEAE-Sephadex A-50 column as shown in Fig. 2. Straight lines were fitted to the points by the least-squares method.

(i) Michaelis constants. Lineweaver–Burk plots (Lineweaver & Burk, 1934) prepared from the results obtained when the various enzyme species were used to determine the relationship between enzymic activity and substrate concentration are shown in Fig. 4. Only the lowest-molecular-weight species (i.e. D, with mol.wt. 45000–47000) showed substrate inhibition. The K_m values calculated from straight lines fitted to the experimental points by the least-squares method were 0.27 mM-, 0.25 mM-, 0.22 mM- and 0.15 mM-*o*-nitrophenyl β-D-glucopyranoside for species A, B, C and D respectively. The affinity of the various β-glucosidase species thus increased with decreasing molecular complexity.

(ii) Effect of glycerol. The effect of glycerol (100 mM) on the activity of species A (highest-molecular-weight form) and D (lowest-molecular-weight form) are shown in Fig. 5. The effect of glycerol on both forms of the enzyme is qualitatively similar (i.e. inhibition by glycerol at low substrate

concentrations and stimulation at high substrate concentrations). The intermediate forms of the enzyme showed similar results.

(iii) Heat-stability. The semi-logarithmic plots presented in Fig. 6 show the effect on enzymic activity of preincubating the various molecular species of β-glucosidase in 0.1 M-Tris–HCl buffer (pH 7.1) at 60°C for various lengths of time. The results suggest that the instability of the various molecular species of the enzyme during heat treatment increased with decreasing molecular complexity. The inactivation rate profiles of the higher-molecular-weight species are non-linear when the results are plotted as the logarithm of the percentage of initial activity against time, whereas the plot for the lowest-molecular-weight species (D) is linear. The non-linear plots for the higher-molecular-weight forms of β-glucosidase may result from the conversion of a fraction of the active high-molecular-weight enzyme into modified active species (probably by dissociation into active lower-molecular-weight species with different kinetic parameters), which then undergo inactivation. The linearity of the plot for the lowest-molecular-weight form (D) would therefore suggest that during heat treatment it does not give rise to enzymically active species.

Ion-exchange chromatography of the high-molecular-weight enzyme (A) before and after heat treatment for 1 h at 60°C gave the results plotted in Fig. 7. Small amounts of two active lower-molecular-weight species were obtained after heat treatment. Gel electrophoresis of the lowest-molecular-weight form of β-glucosidase (species D) after heating for 25 min at 60°C gave the result shown in Plate 2. An

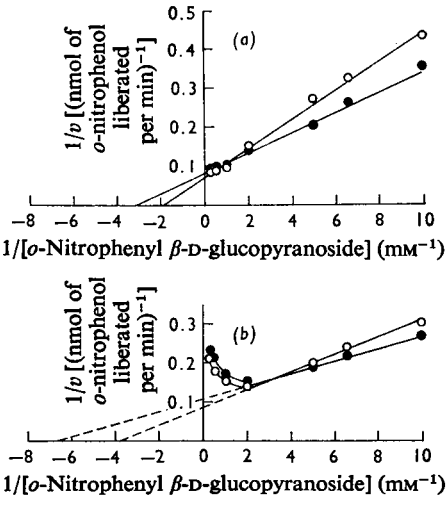


Fig. 5. *Lineweaver–Burk plots showing the effects of glycerol (100 mM) on the activity of (a) β-glucosidase species A and (b) β-glucosidase species D*

The plots are for results obtained in the absence (●) and presence (○) of 100 mM-glycerol. The various enzyme species were obtained as shown in the legend to Fig. 4. Straight lines were fitted to the points in the linear segments by the least-squares method

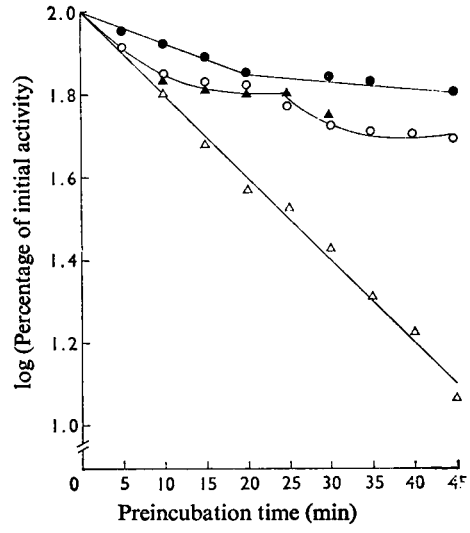


Fig. 6. *Semilogarithmic plots showing the heat-stability of the various β-glucosidase species*

Solutions of the various species, obtained as shown in the legend to Fig. 4, were dialysed against 0.1 M-Tris–HCl buffer (pH 7.1) and preincubated at 60°C. At zero time and at regular time-intervals samples were removed for enzyme assay. The plots are for species A (●), B (○), C (▲) and D (△).

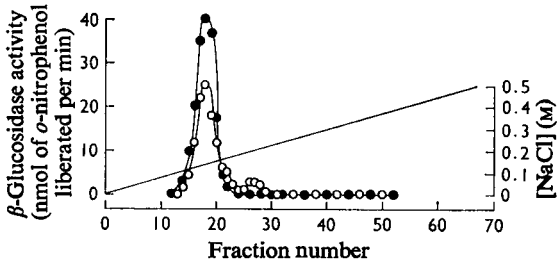


Fig. 7. *Chromatography of the purified high-molecular-weight β-glucosidase on columns (9.0 cm × 1.5 cm) of DEAE-Sephadex A-50 before (●) and after (○) preincubation at 60°C for 1 h in 0.1 M-Tris-HCl buffer (pH 7.1)*

After application of the samples the columns were washed with 200 ml of 0.1 M-Tris–HCl buffer (pH 7.1) and elution was then effected with a linear NaCl gradient prepared from 500 ml of 0.1 M-Tris–HCl buffer (pH 7.1) and 500 ml of 0.1 M-Tris–HCl buffer (pH 7.1) containing 0.5 M-NaCl.

identical unstained gel was cut into 2mm slices and each slice was eluted with 2ml of 0.05M-sodium acetate buffer (pH5.0). Enzyme assay on the eluates showed that the faster-moving protein band had no enzymic activity, whereas the slow-moving protein band was enzymically active. These results seem to support the idea that the non-linear inactivation rate profiles for the higher-molecular-weight species of β-glucosidase may be due to fractional dissociation into enzymically active species with different kinetic properties.

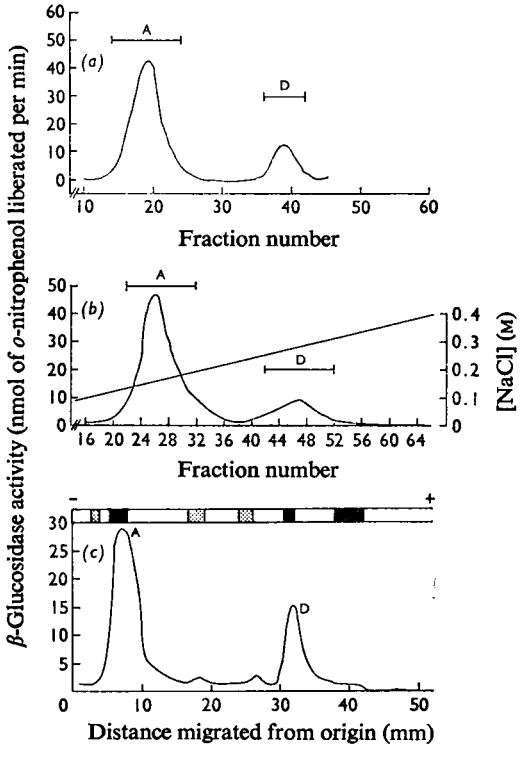


Fig. 8. *Separation of the β-glucosidase components in a crude preparation from a 4-week-old culture filtrate*

(*a*) Elution of β-glucosidase activity from a column (38.5cm×2.5cm) of Sephadex G-200 with a buffer consisting of 0.73mM-Na_2HPO_4, 66.7mM-NaH_2PO_4, 0.1mM-NaCl and 5.0mM-NaN_3 (pH5.0). (*b*) Elution of β-glucosidase activity from a column (11cm×1.5cm) of DEAE-Sephadex A-50 with a linear NaCl gradient prepared from 500ml of 0.1M-Tris–HCl buffer (pH7.1) and 500ml of 0.1M-Tris–HCl buffer (pH7.1) containing 0.4M-NaCl. (*c*) Electrophoresis on 7.5% polyacrylamide gel of the same crude enzyme preparation. Slices (2mm) of the gel were extracted with 2ml of 0.05M-sodium acetate buffer (pH5.0) and samples of the extracts were used for enzyme assay. The diagram on the top of the plot shows the distribution of proteins on an identical gel after staining with 1% Amido Schwartz in 7% (v/v) acetic acid.

Consideration of the crude β-glucosidase

The crude enzyme preparation from which the high-molecular-weight β-glucosidase used in the preceding studies was purified was obtained by precipitating the proteins in a 4-week-old culture of *B. theobromae* by treatment with cold acetone (twice the volume of the culture filtrate). Fig. 8(*a*) shows the elution profile of the two predominant species of β-glucosidase in the acetone-dried powder from this culture from a column of Sephadex G-200. Ion-exchange chromatography on DEAE-Sephadex A-50 by using the same crude enzyme preparation gave the results presented in Fig. 8(*b*). Fig. 8(*c*) shows the separation of the two enzyme species in the same crude sample by electrophoresis on polyacrylamide gels. That the order of elution of the components marked A and D in Figs. 8(*a*) and 8(*b*) corresponded was confirmed when each of the pooled fractions containing the two forms of the enzyme in Fig. 8(*a*) were separately used for ion-exchange chromatography on DEAE-Sephadex. Similarly, the electrophoretic mobility of each of these separated components agreed with that shown in Fig. 8(*c*). However, small amounts of the other enzyme species were sometimes observed after electrophoresis of separated components (cf. Umezurike, 1971).

The relative amounts of the various molecular species of β-glucosidase, observed after separation by

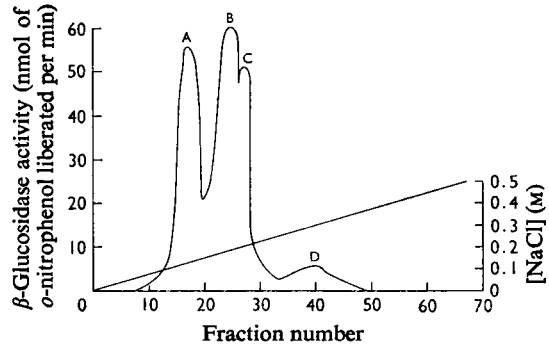


Fig. 9. *Chromatography of a crude enzyme preparation from an 8-week-old culture filtrate on a column (8.0cm×1.5cm) of DEAE-Sephadex A-50*

After application of the sample, the column was washed with 250ml of 0.1M-Tris–HCl buffer (pH7.1) and elution effected with a linear gradient of NaCl prepared from 500ml of 0.1M-Tris–HCl buffer (pH7.1) and 500ml of 0.1M-Tris–HCl buffer (pH7.1) containing 0.5M-NaCl.

the various techniques, in preparations of the crude enzyme from different cultures of the same age, were found to differ. Similarly, wide differences in the relative amounts of the various enzyme species were observed in cultures of different age. Fig. 9 shows the elution profile obtained when a crude enzyme preparation from an 8-week-old culture was used for ion-exchange chromatography on a column of DEAE-Sephadex A-50. Generally, only preparations from old cultures contained appreciable amounts of the lower-molecular-weight species of β-glucosidase (compare Figs. 8*b* and 9).

Kinetic studies using fractions 16(A), 24(B), 28(C) and 39(D) of the separation shown in Fig. 9 showed that these components have similar properties [e.g. K_m, effect of glycerol, heat inactivation and substrate inhibition of the lowest-molecular-weight species (D)] to the corresponding components derived from the purified enzyme (cf. Figs. 4–6).

Discussion

Evidence is presented in this paper for what appears to indicate an association–dissociation phenomenon in the β-glucosidase of *B. theobromae*. The rate of the association–dissociation processes must be slow, making it possible for the different components in the system to be readily separable by gel filtration and gel electrophoresis.

The purified high-molecular-weight β-glucosidase from *B. theobromae* has been shown to have a carbohydrate/protein ratio of about 0.24 (Umezurike, 1971). Calculations using those results indicated a carbohydrate content of about 19.4%. Indeed, recent results indicate that the aryl-β-glucosidase from *S. atra* is a glycoprotein (M. A. Jermyn, personal communication). Glycoproteins behave anomalously in gel filtration apparently because of their greater hydration than globular proteins, when the column is calibrated with globular proteins (Andrews, 1965). The rough estimates of molecular weights reported in the present paper were calculated from the behaviour of the enzyme species relative to those of globular protein markers, and are therefore expected to be higher than the true values. The molecular-weight values previously reported (Umezurike, 1971) were obtained from experiments in which both the columns for gel filtration and the gradient centrifugation media were calibrated with the glycoprotein *Aspergillus niger* glucose oxidase, with a molecular weight of 186000, a sedimentation coefficient, $s_{20,w}$ of 8.0S and a diffusion coefficient, $D_{20,w}$ of 4.12×10^{-7}cm^2/s (Swoboda & Massey, 1965) and which contained about 16% carbohydrate (Pazur *et al.*, 1963). These values are therefore considered more accurate. However, assuming that a given glycoprotein and its aggregated species have similar hydration, the results in the present paper indicate that in the β-glucosidase of *B. theobromae* the following association–dissociation exists: $(8n) \rightleftharpoons 2(4n) \rightleftharpoons 4(2n) \rightleftharpoons 8(n)$, where n is defined as the enzymically active monomer. On the basis of the preceding discussion, the average molecular weights of the octamer (8n), tetramer (4n), dimer (2n) and monomer (n) are therefore more likely to be about 320000, 160000, 80000 and 40000 respectively (cf. Umezurike, 1971). One cannot escape the conclusion that, at least in the β-glucosidase of *B. theobromae*, all the various molecular species are catalytically active. Evidence in support of this conclusion include the behaviour of the various species on rechromatography and re-electrophoresis, (cf. Umezurike, 1971). Evidence presented indicates that the affinity of the various molecular-weight species of the enzyme for the substrate (*o*-nitrophenyl β-D-glucopyranoside) decreases with increasing molecular complexity, and the rate of heat inactivation decreases with increasing molecular complexity.

Electron micrographs of the negatively stained high-molecular-weight β-glucosidase provide strong evidence in support of the octameric structure proposed where each of the eight monomers occupies a corner of a cube with each side about 7.14nm long. The dimensions of the octameric structure deduced from electron micrographs were consistent with those from molecular-sieve chromatography. There is evidence which suggests that the monomeric (n) species of the enzyme is itself made up of about four catalytically inactive subunits.

The subunit structure of β-glucosidase from *B. theobromae* is of some importance, since the structure described above permits some amount of speculation about the nature of the enzymes from other sources. One would guess that, as with the β-glucosidase of *B. theobromae*, the enzymes from other microbial sources are a homologous series of macromolecules. The different molecular species of β-glucosidase obtained by various workers, as reviewed in the introduction, may be partly reflecting differences in the history of the cultures from which the enzymes were derived, the isolation and/or purification methods employed, conditions of storage and the nature of the enzyme source. All the published molecular weights of β-glucosidase from various sources fall neatly into one or the other range of molecular weights for the monomer or the various multimeric species, if allowance is made for some experimental errors. Protein structure studied in isolated systems may be affected by the various factors enumerated above.

However, by combining the results presented in this paper and those presented previously (Umezurike, 1971) one cannot help concluding that the native β-glucosidase from *B. theobromae* is an aggregate of eight roughly spherical active monomers, and has a molecular weight of about 320000.

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