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THE PURIFICATION AND PROPERTIES OF EXTRACELLULAR β-GLUCOSIDASE FROM *BOTRYODIPLODIA THEOBROMAE* PAT

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SUMMARY

1. A high molecular weight β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) has been purified from culture filtrates of *Botryodiplodia theobromae* Pat. grown on D-cellobiose as sole carbon source.

2. Enzyme activity was obtained in a single zone after gel filtration, polyacrylamide gel electrophoresis and sucrose density gradient centrifugation of the purified enzyme except after prolonged storage.

3. The sedimentation coefficient, $s_{20,w}$, and Stokes radius were estimated to be 11.5 S and 7.0 ± 0.4 nm, respectively; and the diffusion coefficient, molecular weight and frictional ratio, f/f_0, were calculated to be $3.06 \cdot 10^{-7}$ cm²/sec, 332 000 and 1.53, respectively.

4. The enzyme hydrolysed both *p*-nitrophenyl-β-D-glucopyranoside and D-cellobiose, and K_m values for these substrates were 0.33 mM and 1.0 mM, respectively.

5. Treatment with 8 M urea and 0.1 M 2-mercaptoethanol followed by alkylation with iodoacetamide led to irreversible dissociation of the purified enzyme into electrophoretically identical polypeptide subunits.

6. Treatment with *p*-chloromercuribenzoic acid (2.5 mM) or with iodoacetamide (0.05 M) inhibited enzymic activity by 70 and 25%, respectively, and this inhibition was partially reversed by the addition of cysteine·HCl (5 mM).

7. The high molecular weight enzyme is probably an aggregate of about 32 polypeptide subunits.

INTRODUCTION

Botryodiplodia theobromae Pat. is similar to a number of organisms that degrade cellulose in being able to produce cellulase (β-1,4-glucan 4-glucanohydrolase, EC 3.2.1.4) and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) in chemically defined media containing cellulose or D-cellobiose as sole carbon source[1,2]. When the organism is grown in D-cellobiose media for 1–2 weeks the β-glucosidase produced seems to have a higher molecular weight than the components obtained from cellu-

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lose media after a longer incubation period[2] probably because under the latter conditions the enzyme had dissociated into low molecular weight forms. This paper describes the purification and the physical and kinetic properties of the high-molecular-weight β-glucosidase obtained from D-cellobiose cultures.

MATERIALS AND METHODS

Organism

Botryodiplodia theobromae (IMI. 115626) was originally isolated from dead and decaying wood of *Bombax buonopozense*[3].

Chemicals

Sephadex G-200, DEAE-Sephadex A-50 and blue dextran were obtained from Pharmacia (G.B.) Ltd., London. Bovine serum albumin (Cohn fraction V), *p*-nitrophenyl-β-D-glucopyranoside, 2-mercaptoethanol, iodoacetamide, D-cellobiose, *p*-chloromercuribenzoic acid (sodium salt) and cysteine·HCl were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England; and crude *Aspergillus niger* glucose oxidase from Sigma (London) Chemical Co. Ltd., London. All other chemicals were of Analar grade.

Preparation of crude enzyme

The culture filtrate from which the crude enzyme was derived was obtained from 1–2 week old cultures of *Botryodiplodia theobromae* grown at 25° as described previously[1,2] except that the medium contained 1% (w/v) D-cellobiose as sole carbon source. The culture filtrate was dialysed against distilled water at 4° (15–24 h).

Enzyme assays

Unless otherwise stated β-glucosidase activity was determined with *p*-nitrophenyl-β-D-glucopyranoside as substrate[1,2] using 0.1–0.5 ml of the enzyme solution. *p*-Nitrophenol standards were also prepared, and enzyme activity was expressed as μmoles or nmoles of *p*-nitrophenol formed per min at 40°. For experiments where D-cellobiose was used as substrate, the reaction mixture contained 2 ml of D-cellobiose solution in 0.05 M sodium acetate–acetic acid buffer (pH 5.0) (to give a final cellobiose concentration of from 0.05 to 2.0 mM), and 0.1 ml enzyme solution in the same buffer. The mixture was incubated at 40° for 15 min and the glucose formed was estimated with the glucose oxidase colorimetric reagents of C. F. Boehringer and Soehne, G.M.B.H., Mannheim, Germany. Units of enzyme activity were expressed as nmoles of glucose formed per min at 40°.

Glucose oxidase activity was determined by incubating a mixture consisting of 2.48 ml of 0.1 M sodium phosphate buffer containing 0.66 mg of *o*-dianisidine·HCl per ml (pH 7.0), 0.5 ml of D-glucose solution (100 mg/ml), 0.01 ml of peroxidase solution (2 mg/ml) and 0.01 ml of the enzyme solution at 25° for 15 min, and absorbance at 450 nm measured against a blank. A unit of activity was arbitrarily taken as the amount of enzyme that produced an increase in absorbance, $\Delta A_{450\ nm}$, of 0.1.

Preparation of chromatographic columns

Sephadex G-200 and DEAE-Sephadex A-50 columns were prepared as de-

scribed elsewhere[2]. All chromatographic separations were performed in a cold room at 4°.

Protein determination

Protein was determined by the method of LOWRY *et al.*[4] using bovine albumin standards.

Carbohydrate determination

Carbohydrate was determined by the anthrone–H_2SO_4 method using glucose standards[5,6].

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in a cold room at 4° by the standard method of ORNSTEIN AND DAVIS[7]. Enzymatically active bands on the gels were located as described previously[2].

Reduction and alkylation of protein

Reduction of enzyme protein with 8 M urea and 0.1 M 2-mercaptoethanol and alkylation with 0.05 M iodoacetamide were carried out according to the modified technique of CRAVEN *et al.*[8] and SHAPIRO *et al.*[9] as reported elsewhere[2].

Sucrose density gradient centrifugation

Linear sucrose gradients (5.0 ml each) were made from 5% (w/v) and 20% (w/v) sucrose solutions in 0.05 M sodium acetate buffer (pH 5.0) in cellulose acetate tubes. Aliquots of 0.1 ml of the enzyme preparations to be examined were layered on the gradient and centrifugation was carried out at 4° in a Beckman Model L-2 ultracentrifuge at 38 500 rev./min for 12 h, using the SW-39 swing-out-bucket rotor[10]. After centrifugation, the tubes were removed and punctured on the bottom, and the contents collected in 60–68 fractions of about 0.075–0.085 ml each. To each fraction were added 2 ml of 0.05 M sodium acetate buffer (pH 5.0) and aliquots were removed for enzyme assay.

RESULTS

Purification of β-glucosidase

Step 1. $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to the dialysed culture filtrate to give 40% saturation (22.6 g/100 ml of solution). After the solution had stood for 45 min at 4° it was centrifuged at 10 000 × *g* for 30 min. This treatment did not precipitate much β-glucosidase activity. Additional $(NH_4)_2SO_4$ was then added to the supernatant solution to give 75% saturation (22.2 g/100 ml of supernatant), and after standing for 30 min at 4° the precipitate was removed by centrifugation at 10 000 × *g* for 30 min at 4°. The precipitate was dissolved in 0.05 M sodium acetate buffer (pH 5.0) and residual $(NH_4)_2SO_4$ removed by dialysis against 2 l of the same buffer for 24 h at 4°.

Step 2. Fractionation on Sephadex G-200. The sample from Step 1 was dialysed against the elution buffer (0.73 mM Na_2HPO_4, 66.7 mM NaH_2PO_4, 0.1 M NaCl and 5.0 mM NaN_3 (pH 5.0)) and fractionated on a column of Sephadex G-200. β-Gluco-

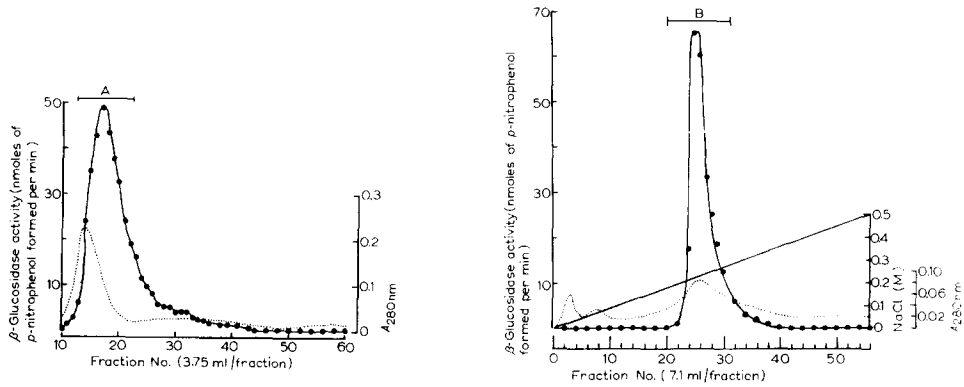


Fig. 1. Elution of β-glucosidase activity from a column of Sephadex G-200 (38.5 cm × 2.5 cm) during the preparation of purified enzyme. The sample used was a 40–75% saturated $(NH_4)_2SO_4$ precipitate, and elution was with buffer consisting of 0.73 mM Na_2HPO_4, 66.7 mM NaH_2PO_4, 0.1 M NaCl and 5.0 mM NaN_3 (pH 5.0). · · · · ·, protein distribution determined from $A_{280\ nm}$; ●—●, β-glucosidase activity expressed as nmoles of *p*-nitrophenol formed per min at 40°. The fractions labelled A were pooled for further purification.

Fig. 2. Elution of β-glucosidase activity from a column of DEAE-Sephadex A-50 (12.0 cm × 1.5 cm) during the purification of the fractions marked A in Fig. 1. Elution was with a linear NaCl gradient (0–0.5 M) in 0.1 M Tris–HCl buffer containing 5.0 mM NaN_3 (pH 7.1). · · · · ·, protein distribution determined from $A_{280\ nm}$; ●—●, β-glucosidase activity expressed as nmoles of *p*-nitrophenol formed per min at 40°. The fractions marked B were pooled for further purification. For details see the text.

sidase activity was eluted mainly in one peak (Fig. 1). The fractions containing β-glucosidase activity (marked A in Fig. 1) were pooled and dialysed overnight (15 h) against 2 l of 0.05 M sodium acetate buffer (pH 5.0) at 4°. By these procedures β-glucosidase was separated from a trace of carboxymethylcellulase components present in the culture filtrate[2].

Step 3. Fractionation on DEAE-Sephadex A-50. The sample from Step 2 was dialysed against 0.1 M Tris–HCl buffer containing 5.0 mM NaN_3 (pH 7.1) and applied on to a column of DEAE-Sephadex A-50. The column was washed with 150 ml of the buffer at pH 7.1, and subsequently eluted with a linear NaCl gradient increasing from 0 to 0.5 M in 400 ml of the same buffer. This procedure separated the non-enzymic proteins in the sample from β-glucosidase. Fig. 2 shows the good correspondence between enzyme activity and the protein peak. The fractions marked B in Fig. 2 were pooled and dialysed against 0.05 M sodium acetate buffer (pH 5.0) as in the preceding purification steps.

Step 4. Second $(NH_4)_2SO_4$ precipitation. The $(NH_4)_2SO_4$ precipitation procedure outlined in Step 1 was repeated on the sample from Step 3. The 40–75% $(NH_4)_2SO_4$ precipitate was dissolved in 0.05 M sodium acetate buffer containing 1.0 mM 2-mercaptoethanol (pH 5.0) and dialysed against the same buffer for 24 h at 4°, and the purified enzyme stored at 4°. About 14-fold purification was achieved. The progress of the purification is shown in Table I.

Physical properties of purified β-glucosidase

Behaviour during gel filtration on Sephadex. The results obtained when the purified enzyme was used for gel filtration on a column of Sephadex G-200 in the

TABLE I

PROGRESS OF β-GLUCOSIDASE PURIFICATION

Units of enzyme activity are expressed as μmoles of *p*-nitrophenol formed per min at 40°.

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Total activity units (μmoles/ min)*	*Specific activity (units/ mg protein)*	*Recovery of activity (%)*	*Total carbohydrates (mg)*	*Carbohydrates/ protein ratio*
Culture filtrate	500	90	50.8	0.56	100	75	0.83
1st 40–75% $(NH_4)_2SO_4$ ppt.	5	14.5	23.1	1.59	45.5	10	0.69
Pooled Sephadex G-200 eluates	60	4.8	25.5	5.3	50.2	4.2	0.88
Pooled DEAE-Sephadex eluates	80	1.6	10.7	6.7	21.1	0.8	0.50
2nd 40–75% $(NH_4)_2SO_4$ ppt.	1.5	0.5	3.8	7.6	7.5	0.12	0.24

presence of blue dextran (mol. wt. 2 000 000), which is completely excluded from the gel, and bovine serum albumin (mol. wt. 65 000, Stokes radius 3.5 nm)[11] or *Aspergillus niger* glucose oxidase (mol. wt. 186 000, Stokes radius 5.2 nm which was calculated from the diffusion coefficient, $D_{20,w}$, of $4.12 \cdot 10^{-7}$ cm²/sec (ref. 12); and containing about 16% carbohydrates[13]) are shown in Fig. 3. A Stokes radius of 7.0 ± 0.4 nm was calculated for purified β-glucosidase according to the method of ACKERS[14]. A similar result was also obtained when a dilute enzyme solution (*i.e.* capable of producing only 10 nmoles of *p*-nitrophenol per min at 40°) was used for gel filtration, indicating that the high-molecular-weight enzyme does not undergo a concentration-dependent dissociation under the conditions of enzyme assay.

Sedimentation coefficient. When the purified enzyme was examined by sucrose

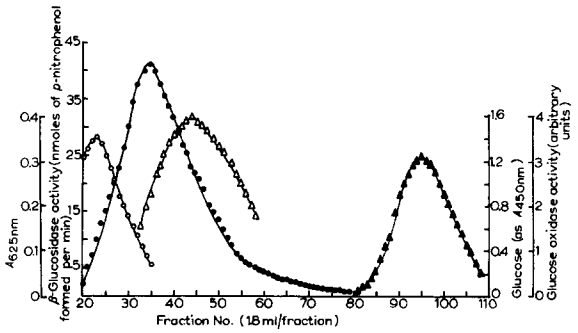


Fig. 3. Elution, from a column of Sephadex G-200 (38.5 cm × 2.5 cm), of a mixture containing blue dextran (5 mg), purified β-glucosidase, purified glucose oxidase from *Aspergillus niger* (purified by a modification of the method of SWOBODA AND MASSEY[12]) and D-glucose (2 mg). Elution was as in Fig. 1. ○—○, blue dextran determined from $A_{625\ nm}$; ●—●, β-glucosidase activity expressed as nmoles of *p*-nitrophenol formed per min at 40°; △—△, glucose oxidase activity determined from $A_{450\ nm}$ (see MATERIALS AND METHODS); ▲—▲, glucose concentration determined colorimetrically with glucose oxidase reagents from $A_{450\ nm}$. For details see the text.

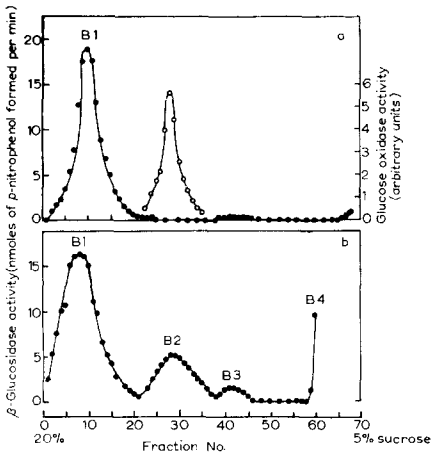


Fig. 4. Sucrose density gradient centrifugation of purified β-glucosidase (a) immediately after purification and (b) after storage at 4° for 3 weeks. Each gradient (5 ml) was prepared from 5–20% (w/v) sucrose solutions in 0.05 M sodium acetate buffer (pH 5.0). Aliquots (0.1 ml) of the purified enzyme solution containing purified *Aspergillus niger* glucose oxidase ($s_{20,w}$ = 8.0 S)[12] were layered on the gradients which were then centrifuged at 38 500 rev./min for 12 h at 4°. Each tube was then removed and punctured at the bottom and the contents collected in 68 fractions of about 0.075 ml each for Gradient a and in 60 fractions of about 0.085 ml each for Gradient b. To each fraction were added 2.0 ml of 0.05 M sodium acetate buffer (pH 5.0) and aliquots removed for enzyme assay. ●—●, β-glucosidase activity expressed as nmoles of *p*-nitrophenol formed per min at 40°; ○—○, glucose oxidase activity expressed as arbitrary units determined from $A_{450\ nm}$.

gradient centrifugation soon after purification, β-glucosidase activity was obtained in a single peak (Fig. 4). An average sedimentation coefficient, $s_{20,w}$, of 11.5 S was calculated for this component according to the method of MARTIN AND AMES[10]. A dilute enzyme solution capable of producing 10 nmoles of *p*-nitrophenol per min at 40° gave a similar result. If the centrifugation was carried out on a sample that had been stored for about 2–4 weeks at 4°, minor β-glucosidase components with average $s_{20,w}$ values of 7.21 and 4.51 s and a fourth component that remained at the top of the gradient were consistently obtained (Fig. 4b). These various components are labelled B1, B2, B3 and B4, respectively, in Fig. 4b and Table II.

Estimation of molecular weight. The average molecular weight of the 11.5 S

TABLE II

SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF VARIOUS FORMS OF β-GLUCOSIDASE FROM *Botryodiplodia theobromae*

The sedimentation coefficient, $s_{20,w}$, values were calculated from data obtained after centrifugation of the purified enzyme in a linear sucrose gradient, 5–20% (w/v), for 12 h at 38 500 rev./min at 4° according to the method of MARTIN AND AMES[10] with purified *Aspergillus niger* glucose oxidase ($s_{20,w}$ = 8.0 S)[12] as standard. Molecular weights were calculated according to the method of MARTIN AND AMES[10]. For other details see the legend to Fig. 4.

	Component B1	*Component B2*	*Component B3*
Mean $s_{20,w}$	11.5	7.21	4.51
Mean mol. wt.	320 700	159 200	78 700

β-glucosidase and those of the slow-sedimenting components calculated according to the method of MARTIN AND AMES[10] are shown in Table II. Using the method of SIEGEL AND MONTY[15] a molecular weight of 331 600 was calculated for the 11.5-S β-glucosidase from the $s_{20,w}$ value of 11.5 S and the Stokes radius of 7.0 nm, and assuming a partial specific volume, $\bar{v}$, of 0.725 ml/g (ref. 10). A molecular weight of 331 500 was also calculated for the 11.5-S β-glucosidase according to the method of ROGERS *et al.*[16].

The frictional ratio, f/f_0, calculated from the value of the Stokes radius (7.0 nm) and a molecular weight of 331 500 is 1.53. This value indicates that the molecule of β-glucosidase is not spherical but somewhat asymmetric.

Electrophoretic behaviour. The purified enzyme gave a single protein band with an R_F value of 0.14 relative to the bromophenol blue front, after electrophoresis on 7.5% polyacrylamide gels[7]. The distribution of enzymic activity on one such gel is shown in Fig. 5a. If, however, the enzyme was left in buffer at pH 7.5 for 1–4 weeks before electrophoresis, part of it dissociated into low molecular weight components

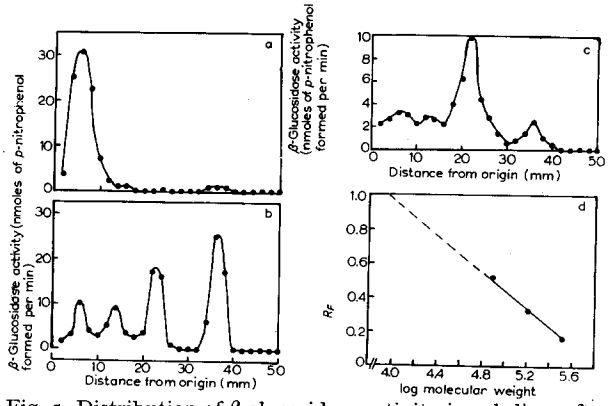


Fig. 5. Distribution of β-glucosidase activity in gel slices after polyacrylamine gel electrophoresis[7] of (a) purified β-glucosidase immediately after purification; (b) purified β-glucosidase after storage for 4 weeks at 4° in 0.01 M Tris–HCl (pH 7.5); and (c) concentrated eluates from gel slices between 20 and 24 mm from the origin on five identical gels similar to the one used in (b); and (d) a plot of the electrophoretic R_F values of the higher-molecular-weight forms against the logarithm of their molecular weights. Electrophoresis was stopped when the bromophenol blue front had migrated to a marked point on the tubes. The gels were then removed and each was cut into slices (2 mm in length) starting from the top of the small pore gel. Each slice was extracted with 0.05 M sodium acetate buffer (pH 5.0) (2 ml), and aliquots of the extracts assayed for enzymic activity. Enzyme activity is expressed as nmoles of *p*-nitrophenol formed per min at 40°.

similar in electrophoretic mobilities to those obtained after electrophoresis of the crude enzyme[2] (Fig. 5b). Re-electrophoresis of the component with an R_F value of 0.52 gave the result shown in Fig. 5c. About 20, 8, 60 and 10% of the total recovered enzymic activity were eluted from gel slices corresponding to the positions of the components with R_F values of 0.14, 0.32, 0.52 and 0.83, respectively. The enzymic activity of the low molecular weight components cannot, therefore, be attributed only to re-association into the high molecular weight form.

Gel electrophoresis of the four components obtained after sucrose gradient centrifugation of an enzyme preparation that had been stored for 4 weeks at 4° showed

that these components corresponded with the four obtained after gel electrophoresis of the original sample, though traces of some other forms were also observed (*cf.* Figs. 4b and 5b).

A plot of the R_F values obtained after gel electrophoresis of the 11.5-,7.21-and 4.51-S components against the logarithm of their molecular weights (*cf.* ref. 2) is linear (Fig. 5d). By extrapolating this line to the left of the diagram, the molecular weight of Component B4 was roughly estimated from its electrophoretic R_F value to be 18 000–20 000. A 7.5% polyacrylamide gel is suitable for the separation of proteins in the molecular weight range of 10 000–400 000.

Dissociation of enzyme into polypeptide subunits. Dissociation of the 11.5-S enzyme with 8 M urea and 0.1 M 2-mercaptoethanol followed by alkylation with iodoacetamide (0.05 M) resulted in complete inactivation of the enzyme. Prolonged dialysis (4 days) did not lead to recovery of enzymic activity. When the reduced and alkylated derivative was subjected to gel electrophoresis, only one protein band with

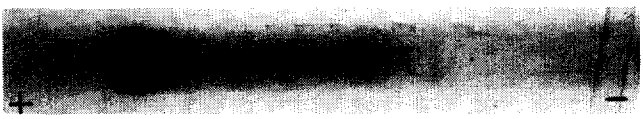


Fig. 6. Photograph of polyacrylamide gel after electrophoresis in the standard system of ORNSTEIN AND DAVIS[7]. The gel was loaded with the purified β-glucosidase from *Botryodiplodia theobromae* after reduction in 8 M urea and 0.1 M 2-mercaptoethanol followed by alkylation with 0.05 M iodoacetamide. The gel was stained in Amido-Schwartz after electrophoresis. The R_F value of the band relative to the bromophenol blue front was 0.95.

an R_F value of 0.95 was obtained (Fig. 6). A molecular weight of 10 000–11 000 was estimated from its behaviour during electrophoresis.

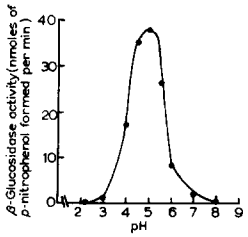
Kinetic properties of purified β-glucosidase

Effect of pH. In McIlvaine buffers the optimum pH for β-glucosidase activity was 5.0 (Fig. 7).

Michaelis constants. The relationship between β-glucosidase activity and the concentration of D-cellobiose or of *p*-nitrophenyl-β-D-glucopyranoside as substrate is shown as Lineweaver–Burk plots in Fig. 8. From these plots the K_m value for D-cellobiose was calculated to be 1.0 mM and that for *p*-nitrophenyl-β-D-glucopyranoside as 0.33 mM.

β-Glucosidase activity was inhibited competitively by D-cellobiose (10 mM) when *p*-nitrophenyl-β-D-glucopyranoside was the substrate (Fig. 8).

Inhibition by thiol reagents. A solution of the purified enzyme in 0.025 M sodium phosphate buffer (pH 7.0) was mixed with an equal volume of a solution of iodoacetamide or of *p*-chloromercuribenzoic acid in 0.025 M sodium phosphate buffer at pH 7.0 so that the final concentration of iodoacetamide was 0.05 M and that for *p*-chloromercuribenzoic acid was 2.5 mM. After incubation for 4 h at 30° the reaction mixtures were assayed for enzymic activity and the results were compared with a control which contained no thiol reagent. Aliquots of the reaction mixtures (2 ml) were treated with an equal volume of a solution of cysteine·HCl in 0.025 M sodium phosphate buffer at pH 7.0 to give a final concentration of 5 mM of cysteine·HCl and left at 30° for 15 min before enzyme assay.



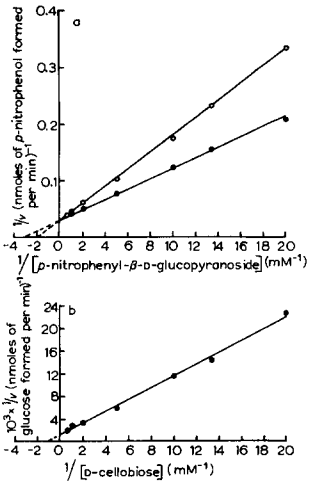


Fig. 7. Effect of pH on the activity of purified β-glucosidase in McIlvaine buffers. The assay was carried out with *p*-nitrophenyl-β-D-glucopyranoside as substrate. Enzyme activity is expressed as nmoles of *p*-nitrophenol formed per min at 40°.

Fig. 8. Lineweaver–Burk plots showing the effects of (a) *p*-nitrophenyl-β-D-glucopyranoside concentration in the presence (○) and absence (●) of 1.0 mM D-cellobiose, and (b) D-cellobiose concentration on the activity of purified β-glucosidase from *Botryodiplodia theobromae*. The initial rate, *v*, is expressed as nmoles of *p*-nitrophenol or glucose formed per min at 40° from *p*-nitrophenyl-β-D-glucopyranoside or D-cellobiose, respectively.

Treatment with iodoacetamide or *p*-chloromercuribenzoic acid resulted in 25 and 70% inhibition of β-glucosidase activity ,respectively. After treatment with cysteine·HCl the inhibition by iodoacetamide or by *p*-chloromercuribenzoic acid was partially reversed; the percentage activity of the enzyme relative to the control was 92.4% for the sample originally treated with iodoacetamide and 62.8% for the sample originally treated with *p*-chloromercuribenzoic acid.

DISCUSSION

The behaviour of purified β-glucosidase from *Botryodiplodia theobromae* during gel filtration, gel electrophoresis and sucrose density gradient centrifugation, and the dissociation of the high molecular weight enzyme after storage indicate that the enzyme is polymeric. A comparison of the molecular weights of the various components suggests that the 11.5-S component is a multiple of the low molecular weight forms. It is possible that the polypeptide subunits obtained after reduction of the 11.5-S component may differ in nature but have approximately equal molecular weight and charge, and would therefore appear as a single band after electrophoresis on polyacrylamide gels. However, the 11.5-S component appears to consist of about 32 polypeptide subunits, the 7.21-S component of about 16, the 4.51-S component of about 8 and Component B4 of about 2 such units.

The effects of thiol reagents suggest that sulphydryl groups are involved in the catalytic activity of the enzyme. Similar results have been reported for the β-glucosidase from other organisms[17–19].

The molecular weight of 320 000–332 000 calculated for the 11.5-S β-glucosidase is in agreement with the values of 300 000 (ref. 17) and 325 000 (ref. 20) reported for the β-glucosidase from yeast.

The existence of multiple forms of β-glucosidase from some organisms has been reported[21–23]. The various forms of the enzyme usually showed different thermal inactivation properties and different specificities[19]. However, MAHADEVAN AND EBERHART[19] have shown that the two forms of *Neurospora crassa* β-glucosidase they obtained attacked both D-cellobiose and *p*-nitrophenyl-β-D-glucopyranoside though the two enzyme forms differed in that one was more active on D-cellobiose and the other on *p*-nitrophenyl-β-D-glucopyranoside. The enzyme from *Botryodiplodia theobromae* is similar to those from *Neurospora crassa* in that it is able to attack both substrates, although it has about 3 times more affinity for *p*-nitrophenyl-β-D-glucopyranoside than for D-cellobiose. In this respect, it differs from that of *Stachybotrys atra* which lacked any activity on D-cellobiose[6,24]. The multiplicity of β-glucosidase from *Stachybotrys atra* has been attributed to the formation of stable complexes between a single enzyme and a variety of polysaccharides[6,25]. The results presented in this paper agree with those of JERMYN[6,25] only in so far as they indicate that the β-glucosidase from *Botryodiplodia theobromae* seems to be a single enzyme which can partially dissociate into lower molecular weight forms under certain conditions.

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