The octameric structure of β -glucosidase from *Botryodiplodia theobromae* Pat.

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1. Whereas only β -glucosidase A (β -D-glucoside glucohydrolase, EC 3.2.1.21) was produced by the tropical fungus *Botryodiplodia theobromae* Pat. (I.M.I. 115626; A.T.C.C. 26123) in young cultures containing D-cellobiose as carbon source, lower- M_r forms (B, C and D) were found in older cultures when the pH had drifted from the initial value of pH 6.2 to pH 7.9. 2. The Michaelis constants (K_m) of the various molecular forms of the enzyme were 0.30 ± 0.03 mM-, 0.26 ± 0.01 mM-, 0.20 ± 0.02 mM- and 0.16 ± 0.01 mM- *o*-nitrophenyl β -D-glucopyranoside for β -glucosidase forms A (M_r 320 000), B (M_r 160 000), C (M_r 80 000) and D (M_r 40 000) respectively. 3. Only β -glucosidase D showed substrate inhibition. 4. Only L-arginine was found as the N-terminal residue, and β -glucosidase A contained 31.7 ± 0.6 mol of N-terminal L-arginine/mol of the enzyme. 5. Storage of purified β -glucosidase A under mildly alkaline conditions caused its dissociation into the lower- M_r forms, whereas adjustment of the pH of a solution of β -glucosidase A to pH 12.0 with 1 M-NaOH led to complete inactivation on incubation at 40 °C for 1 h and to the release of 25.2 ± 1.5 mol of inorganic phosphate/mol of the enzyme. 6. O-Phospho-L-serine was isolated from the acid-hydrolysis product of β -glucosidase A but not from that of β -glucosidase D. 7. Reduction and carboxamidomethylation of the various forms of β -glucosidase gave only one enzymically inactive protein with an M_r of 10 000–11 000. 8. After partial succinylation (3-carboxypropionylation) of β -glucosidase D at pH 5.0 and removal of the precipitated protein formed, the supernatant solution contained β -glucosidase components similar to the other molecular forms (A, B and C) and an aggregate (β -glucosidase X_s) that gave a positive result in the alkaline hydroxylamine test, whereas N-succinylated β -glucosidase D, an aggregate (form X_p) that behaved like β -glucosidase X_s and traces of forms A, B and C were found by gel filtration of the solution of the precipitate solubilized at neutral pH (7.0–7.7). 9. These observations are discussed in terms of the proposed octameric structure of β -glucosidase A based on the result of electron microscopy [Umezurike (1975) Biochem. J. 145, 361–368].

INTRODUCTION

The tropical fungus *Botryodiplodia theobromae* Pat. produces multiple forms of β -glucosidase in culture in the presence of cellulose or D-cellobiose as sole carbon source, and the four molecular forms of the enzyme produced are enzymically active (Umezurike, 1971, 1975). It appears, on the basis of their M_r values, that the multiple forms are derived from the polymerization of the monomer to give the dimer, tetramer and octamer (Umezurike, 1971, 1975). Electron-microscopic examination of the highest- M_r form (β -glucosidase A with M_r about 320 000) showed that the molecule has a cubic configuration in which eight roughly spherical monomers occupy the corners (vertices) of the cube with each side measuring about 7.14 nm long (Umezurike, 1975).

Four molecular forms of β -glucosidase that have similar M_r values to those of the forms from *B. theobromae* have also been found in the gut contents of the giant African snail *Achatina achatina* (Umezurike, 1976a,b). Incubation of the gut contents of the snail in the presence of ATP and Mg^{2+} causes polymerization of low- M_r forms of β -glucosidase to the high- M_r forms (Umezurike, 1976a). Further studies have shown that the formation of the higher- M_r forms of β -glucosidase from the monomer and vice versa was catalysed by enzymes that are also present in the gut contents of the snails (Umezurike, 1976b). These observations suggested that phosphorylation and dephosphorylation reactions could be responsible for the structural changes.

This possibility has necessitated the further examination of the subunit structure of β -glucosidase from *B. theobromae* and an investigation of the effect of the introduction of negatively charged groups into the monomeric form of the enzyme, by

partial succinylation (3-carboxypropionylation), on the structure of the enzyme. The results obtained are considered in this paper.

MATERIALS AND METHODS

Chemicals

o-Nitrophenyl β -D-glucopyranoside and O-phospho-L-serine were purchased from Sigma Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Sephadex G-200 and Blue Dextran 2000 000 were obtained from Pharmacia (G.B.) Ltd. (London, U.K.). Dansyl chloride, dansylated amino acids and polyamide sheets for t.l.c. were purchased from BDH Biochemicals (Poole, Dorset, U.K.). Ferritin (M_r 540 000), aldolase (rabbit muscle; M_r 158 000), albumin (hen's-egg; M_r 45 000) and cytochrome c (horse heart; M_r 12 500) were obtained from Boehringer Biochemicals (Mannheim, Germany).

Methods

Enzyme purification. Each molecular form of β -glucosidase was obtained from culture filtrates of *B. theobromae* Pat. (I.M.I. 115626; A.T.C.C. 26123) grown at 25 °C with D-cellobiose as carbon source, and purified as described previously (Umezurike, 1970a, 1971).

Enzyme assay. β -Glucosidase activity was assayed with *o*-nitrophenyl β -D-glucopyranoside in 0.05 M-sodium acetate/acetic acid buffer, pH 5.0, as described previously (Umezurike, 1971).

Reduction and alkylation of enzyme. Reduction of enzyme with 8 M-urea and 0.1 M-2-mercaptoethanol and carboxamido-methylation with 0.05 M-iodoacetamide were carried out as described previously (Umezurike, 1971).

N-Terminal determination. N-Terminal residues were identified as dansyl derivatives by the method of Woods & Wang (1967) after t.l.c. on polyamide sheets. The solvents used were water/90% (v/v) formic acid (200:3, v/v) for the first run and benzene/acetic acid (9:1, v/v) for the second run. Quantitative analysis was carried out as described by Sakamoto *et al.* (1972).

Partial succinylation. Solid succinic anhydride was added to a solution of β -glucosidase in 0.05 M-sodium acetate/acetic acid buffer, pH 5.0, to give a final concentration of 1–5% (w/v). The solution was stored at 18–20 °C for 1–2 h, during which the pH was maintained at pH 4.5–5.0 by the addition of 1 M-NaOH. Protein precipitated by this treatment was removed by centrifugation at 10000 *g* for 15 min at 4 °C. Protein in the supernatant solution was precipitated with 2 vol. of cold acetone and dissolved in a minimal amount of buffer (1–3 ml) when necessary.

Gel filtration. Gel filtration on columns of Sephadex G-200 was carried out with McIlvaine's sodium phosphate/citrate buffer (McIlvaine, 1921), pH 5.0, containing 0.1 M-NaCl and 5.0 mM- NaN_3 as elution buffer. M_r values were determined by gel filtration on columns calibrated with Blue Dextran (M_r 2000000), ferritin (M_r 540000), aldolase (rabbit muscle; M_r 158000), albumin (hen's-egg; M_r 45000) and cytochrome *c* (horse heart; M_r 12500) by the method of Andrews (1964, 1965).

PAGE. Electrophoresis on 7.5% (w/v) polyacrylamide gels was carried out by the method of Ornstein & Davis (1961). Protein from gel slices (2 mm thick) was extracted and assayed for enzyme activity as described previously (Umezurike, 1970b, 1971).

Phosphate determination. Free phosphate content of a solution was determined by the method of Watts & Moreland (1970).

Carbohydrate determination. Carbohydrate was estimated by the anthrone/ H_2SO_4 method, with glucose as standard (Morris, 1948).

Alkaline hydroxylamine test. The qualitative detection of *O*-succinylated hydroxy amino acids was carried out by the alkaline hydroxylamine reaction as described by Gounaris & Perlmann (1967).

Detection of *O*-phospho-L-serine in β -glucosidase A. The method used was a slight modification of that described by Engström (1962). β -Glucosidase A or D (about 2.0 mg) in 3.5 ml of 0.05 M-sodium acetate/acetic acid buffer, pH 5.0, was mixed with 0.5 ml of 1 M-HCl, and protein was precipitated by the addition of 8 ml of acetone. After being washed with 5 ml of acetone containing 1% (v/v) 1 M-HCl, the precipitate was dried under reduced pressure at room temperature and then hydrolysed with 2 M-HCl at 100 °C for 21 h in an evacuated and sealed glass tube. The dried hydrolysate was used for chromatography on a column of Dowex 50W (X8), and fractions containing *O*-phospho-L-serine were pooled and dried *in vacuo*. The isolated *O*-phospho-L-serine was compared with the authentic material by paper chromatography (Engström, 1962).

RESULTS

Culture filtrates

The number of the various molecular forms of β -glucosidase found in culture filtrates from cultures of *B. theobromae* grown

in liquid medium containing D-cellobiose as sole carbon source was found to vary with the age of the culture (Fig. 1). Only β -glucosidase A was found by gel filtration on a column of

Fig. 1. Gel filtration of β -glucosidase from culture filtrates of *B. theobromae* on a column (29.5 cm $\times$ 2.5 cm) of Sephadex G-200

The culture filtrates were obtained from (a) a 1-week-old culture, (b) a 2-week-old culture and (c) a 4-week-old culture with D-cellobiose as carbon source. β -Glucosidase activity (●) is expressed as nmol of *o*-nitrophenol liberated/min, and protein (----) was estimated as A_{280} . A, B, C and D refer to β -glucosidase forms A, B, C and D respectively. The elution buffer was McIlvaine's sodium phosphate/citrate buffer, pH 5.0, containing 0.1 M-NaCl. The fraction size was 4.0 ml.

Fig. 2. Distribution of β -glucosidase activity in gel slices (2 mm each) after PAGE

The samples of enzyme were (a) purified β -glucosidase A stored at 4 °C in 0.05 M-sodium acetate/acetic acid buffer, pH 5.0 for 3 weeks, and (b) the same sample of purified enzyme stored for 3 weeks at 4 °C in 0.1 M-Tris/HCl buffer, pH 8.0. The Bromophenol Blue front was in slice 22.

Sephadex G-200 of protein obtained from the culture filtrate obtained from a 1-week-old culture with a final pH value of 6.5 (Fig. 1a). β -Glucosidase A and a small amount of β -glucosidase D were detected in the culture filtrate from a 2-week-old culture with a final pH of 7.0 (Fig. 1b), whereas the four molecular forms of β -glucosidase (A, B, C and D) were detected in a 4-week-old culture filtrate with a pH of 7.9 (Fig. 1c). The proportions of the various forms of β -glucosidase were found to vary with the age of the culture, but the lower- M_r forms of the enzyme (C and D) were found consistently in old cultures in which the pH of the medium had become mildly alkaline.

Properties of the purified enzymes. A determination of the M_r values of the various forms of β -glucosidase has indicated values of about 320 000, 160 000, 80 000 and 40 000 for β -glucosidase forms A, B, C and D respectively (cf. Umezurike, 1971, 1975). The Michaelis constants (K_m) of β -glucosidase forms A, B, C and D obtained from cultures containing cotton flock as carbon source have been reported to be about 0.27 mM-, 0.25 mM-, 0.22 mM- and 0.15 mM-*o*-nitrophenyl β -D-glucopyranoside respectively (Umezurike, 1975). Similar results have been obtained with the purified enzymes prepared from the culture filtrates from cultures containing D-cellobiose as carbon source, and the K_m values are 0.30 ± 0.03 mM-, 0.26 ± 0.01 mM-, 0.20 ± 0.01 mM- and 0.16 ± 0.01 mM-*o*-nitrophenyl β -D-glucopyranoside respectively. The enzymes also behaved like those obtained from cotton-flock medium (Umezurike, 1975) in that only β -glucosidase D showed substrate inhibition, and their activities conformed to the mechanism of enzyme action proposed previously (Umezurike, 1981, 1987, 1988).

Enzyme modifications

Incubation of purified β -glucosidase A at 40 °C for up to 5 h in McIlvaine's sodium phosphate/citrate buffer (McIlvaine, 1921), pH 6.5, containing 0.05 M-iodoacetic acid or -iodoacetamide had no effect on its activity. Reduction of the various molecular forms of the enzyme with 8 M-urea and 0.1 M-2-mercaptoethanol followed by alkylation with 0.05 M-iodoacetamide at 45 °C for 3 h in 0.01 M-Tris/HCl buffer, pH 7.5, led to complete inactivation. Dialysis against ten changes of distilled water (for 5 days) at 4 °C did not lead to recovery of enzymic activity. Gel electrophoresis of the reduced and alkylated enzyme gave only one protein band with an R_f value (relative to Bromophenol Blue front) of 0.95 (cf. Umezurike, 1971, 1975). An M_r of 10 000–11 000 was calculated from its behaviour during electrophoresis, as reported previously (Umezurike, 1971, 1975).

Gel electrophoresis of purified β -glucosidase A that had been stored for 3 weeks at 4 °C in 0.05 M-sodium acetate/acetic acid buffer, pH 5.0 did not reveal any dissociation of the enzyme (Fig. 2a). However, when a sample of the purified enzyme stored for 3 weeks in 0.1 M-Tris/HCl buffer, pH 8.0, at 4 °C was used for gel electrophoresis, dissociation of β -glucosidase A into lower- M_r forms was detected, with β -glucosidase D predominating (Fig. 2b). Addition of a solution of 1 M-NaOH to a solution of β -glucosidase A in 0.05 M-sodium acetate/acetic acid buffer, pH 5.0, to give pH 12.0 and incubation at 40 °C for 1 h caused complete inactivation, as neither prolonged dialysis against distilled water at 4 °C nor the adjustment of the pH to 5.0 with 1 M-HCl led to recovery of activity. This treatment at pH 12.0 caused the release of 25.2 ± 1.5 mol of inorganic phosphate/mol of β -glucosidase A (assuming an M_r of 320 000). No carbohydrates were detected in the solution after acetone precipitation of protein and removal of the protein by centrifugation.

A fraction that behaved like *O*-phospho-L-serine was obtained by chromatography of acid-hydrolysed β -glucosidase A on a column of Dowex 50W (X8). This fraction was not detected in

Fig. 3. Gel filtration of purified β -glucosidase D (a) before and (b) after partial succinylation at pH 5.0

A, B, C, D and X_s refer to the activities of β -glucosidase forms A, B, C and D and a large aggregate X_s respectively found in the supernatant solution after centrifugation of the succinylated sample (●). X_p and D_p refer to those of an aggregated form of the enzyme and succinylated β -glucosidase D respectively found in the precipitated protein that had been solubilized by adjustment of the pH of the suspension to pH 7.7 with 1 M-NaOH (○). S_1 , S_2 , S_3 , S_4 and S_5 indicate the elution positions of Blue Dextran, ferritin, rabbit muscle aldolase, hen's-egg albumin and cytochrome *c* respectively. β -Glucosidase activity was expressed as nmol of *o*-nitrophenol liberated/min, and protein (---) was estimated as A_{280} . The fraction size was 4.0 ml.

the sample obtained from acid-hydrolysed β -glucosidase D. The isolated *O*-phospho-L-serine behaved like authentic *O*-phospho-L-serine on paper chromatography, and inorganic phosphate was released on treatment with alkaline hydroxylamine. Quantitative determination was not attempted, as *O*-phospho-L-serine is partially hydrolysed during protein hydrolysis with HCl (Brand & Söling, 1975).

N-Terminal analysis

In order to investigate further the identity and number of subunits present in β -glucosidase A, N-terminal analysis was carried out by the dansyl chloride method. Only dansylarginine was detected by two-dimensional t.l.c. of the hydrolysed dansylated β -glucosidase A on polyamine sheets. In two determinations it was found that 31.7 ± 0.6 nmol of dansyl-arginine was released from 1.0 nmol of β -glucosidase A.

Partial succinylation

The precipitate formed when β -glucosidase D was subjected to partial succinylation at pH 5.0 was removed by centrifugation and dissolved in 0.05 M-potassium phosphate/NaOH buffer, pH 7.0, or in 0.1 M-Tris/HCl buffer, pH 7.5, or by adjusting the pH of the suspension of the precipitate in 0.05 M-sodium acetate/acetic acid buffer, pH 5.0, in which it was not soluble, to pH 7.7 with 1 M-NaOH solution. Fig. 3 shows the results of gel filtration of both the supernatant solution and the dissolved precipitate after storage at 4 °C for 24 h. Aggregates corresponding to β -glucosidase forms A, B and C and a larger aggregate (X_s) that was virtually excluded from the column were detected in the supernatant solution obtained after partial

succinylation. The dissolved precipitate contained an aggregated form of the enzyme (X_p) that was eluted at the same position as aggregate X_s (Fig. 3) and a component that behaved like β -glucosidase D (i.e. D_p in Fig. 3) as major components. Traces of components A, B and C appeared to be present (Fig. 3). When aggregates X_s and X_p and component D_p were subjected to the alkaline hydroxylamine test, only the aggregated forms X_s and X_p gave a positive result for hydroxamic acid.

DISCUSSION

Electron micrographs of β -glucosidase A taken after negative staining with phosphotungstic acid showed that the molecule is an octamer in which each roughly spherical monomer occupies the corner of a cube with each side measuring about 7.14 nm long (Umezurike, 1975). Furthermore, the estimated M_r values indicate that β -glucosidase forms A, B and C are multimers of β -glucosidase D, molecular species A being an octamer. Thus the detection of 32 mol of N -terminal arginine residues/mol of β -glucosidase A indicates that each monomer (β -glucosidase D) contains four identical polypeptides (protomers), in consonance with the detection of only one electrophoretically homogeneous and enzymically inactive protein with an M_r of 10000–11000 on reduction and alkylation of each molecular form of the enzyme.

The adjustment of the pH of a solution of β -glucosidase A to pH 12.0 with 1 M-NaOH and incubation for 1 h at 40 °C caused the complete loss of enzymic activity and the release of 25.2 ± 1.5 mol of inorganic phosphate/mol of the enzyme (assuming an M_r of 320000), and no carbohydrates were released. This result is consistent with the participation of alkali-catalysed β -elimination of inorganic phosphate from substituted aliphatic hydroxy amino acids in phosphoproteins (cf. Mecham & Olcott, 1949; Anderson & Kelley, 1959; Simpson *et al.*, 1972; Taborsky,

1974). The peptide bond in which the resulting dehydroalanine residue is involved is labile, and the selective cleavage of this bond has been demonstrated (cf. Taborsky, 1974). Such a process may have contributed to the irreversible inactivation of β -glucosidase. The depolymerization of β -glucosidase A to give active lower- M_r forms when the enzyme is stored at 4 °C under mildly alkaline conditions could therefore be attributed to dephosphorylation. The complete dephosphorylation of 1 mol of β -glucosidase A would release 24 mol of inorganic phosphate, if only three out of the four polypeptides (protomers) in the monomer were phosphorylated. This is consistent with the value of 25.2 ± 1.5 mol/mol obtained. The identification of O -phospho-L-serine in the acid-hydrolysis product of β -glucosidase A, and not in that of β -glucosidase D, implicates serine residues as the phosphorylated amino acid residues.

Partial succinylation of the monomeric form of the enzyme at pH 5.0 gave a component (D_p) that behaved like β -glucosidase D and that gave a negative result in the alkaline hydroxylamine test. It is therefore apparently an N -succinylated β -glucosidase D. The other component (the aggregated form X_p) gave a positive result in the alkaline hydroxylamine test, and therefore probably contains O -succinylated aliphatic hydroxy amino acid residues that induce its aggregation and N -succinylated residues and/or other succinylated residues (e.g. histidine or tyrosine) that cause its precipitation at pH 5.0. Histidine derivatives and O -succinyltyrosine are labile at the pH (7.0–7.7) of solubilization of the precipitated protein (cf. Means & Feeney, 1971; Glazer *et al.*, 1975). Succinylation of BSA has been shown to give rise to two components, one of which shows an increased tendency to aggregate, presumably as a result of a change in its conformation (Habeeb, 1967). That aggregate X_p remained aggregated after solubilization at pH 7.0–7.7 indicates that the succinylation of an aliphatic hydroxy amino acid (probably the serine hydroxy group, which has been reported to be more reactive than the threonine hydroxy group during succinylation; Gounaris & Perlmann, 1967) with an exceptionally reactive hydroxy group is involved. The increased nucleophilic reactivity of the hydroxy amino acid residue at the pH (5.0) of succinylation can be achieved by the participation of an imidazole base of a neighbouring histidine residue that acts as a general base catalyst in a 'charge-relay system', particularly in an environment of low polarity as may be found in a hydrophobic centre (cf. Blow *et al.*, 1969).

Enzyme-catalysed polymerization and depolymerization have been observed in the β -glucosidase found in the gut contents of the giant African snail *Achatina achatina* (Umezurike, 1976a,b). A model illustrating the process of polymerization (or depolymerization) of β -glucosidase, following the introduction of negatively charged groups into the various molecular forms of the enzyme (or the elimination of such groups), based on the results presented in this paper is shown in Fig. 4. The diagonal arrows indicate the process of depolymerization following the elimination of negatively charged groups from the enzyme, the horizontal arrows illustrate the introduction of negatively charged substituent groups into the appropriate amino acid residues of the enzyme, and the vertical arrows indicate the polymerization of modified enzyme forms. Details of this process are shown in Fig. 5. The modification of one of the four protomers, arranged in a tetrahedral configuration in the monomer, by the introduction of a negatively charged group (e.g. by enzymic phosphorylation or by chemical modification or succinylation of the hydroxy group of serine) would cause the polymerization of two modified monomers (n) to form a dimer ($2n$). If two protomers of the monomer are modified, four such monomers polymerize to form a square planar tetramer ($4n$) in which the apical protomers of the four monomers are located on

Fig. 4. Proposed model of interconversion of the various forms of β -glucosidase from *B. theobromae*

Horizontal arrows indicate the introduction of negatively charged groups (x^-) into the protomers by phosphorylation or succinylation; vertical arrows indicate polymerization into dimer ($2n$), tetramer ($4n$) and octamer ($8n$); diagonal arrows indicate the elimination of negatively charged substituent groups (x^-) and depolymerization. The peripheral arrows probably represent the predominant sequence in cultures of *B. theobromae*. See the text for further details.

Fig. 5. Details of the proposed structure of the monomeric (n), dimeric ($2n$) and tetrameric ($4n$) forms of β -glucosidase from *B. theobromae*

The monomer is proposed to consist of four protomers (polypeptides) arranged in a tetrahedral fashion, and only three of these (a, b and c) are capable of being modified by phosphorylation under physiological conditions to induce polymerization. See the text for further details.

one face of the molecule. If these four apical protomers of the tetramer (marked a in Fig. 5) are modified also, two such tetramers will polymerize face-to-face to give the cubic configuration observed by electron microscopy. As the process is enzyme-catalysed in nature, the unmodified protomer of each monomer (marked d in Fig. 5) apparently participates in the interaction between the modifying enzyme (kinase) and β -glucosidase, and is thus not free and properly oriented for phosphorylation.

The detection of β -glucosidase forms B, C and D in culture filtrates from old cultures of *B. theobromae* when the pH of the medium has become mildly alkaline is in consonance with the elimination of negatively charged substituent groups on aliphatic hydroxy amino acid residues. However, until the establishment of a direct phosphorylation of a specific serine residue of *B. theobromae* β -glucosidase by a specific enzyme (kinase) in the presence of [γ - 32 P]ATP, the scheme of Figs. 4 and 5 will remain a highly plausible working model. The scheme, however, explains the effect of partial succinylation and all the other results presented in this paper.

The results presented in this paper thus show that the β -glucosidase of *B. theobromae* is different in its organization from β -glucosidase A_3 from *Aspergillus wentii*, which has been reported to have an M_r of 170 000 and to consist of a single polypeptide

chain with an *N*-terminal aspartic acid residue (Legler *et al.*, 1972; Bause & Legler, 1974).

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