Properties of the β -Glucosidases of *Talaromyces emersonii*

By ANTHONY MCHALE AND MICHAEL P. COUGHLAN*

Department of Biochemistry, University College, Galway, Ireland

(Received 10 December 1981; revised 5 February 1982)

The thermophilic fungus *Talaromyces emersonii* when grown on cellulose-containing medium produces three extracellular forms of β -glucosidase, I (M_r , 135 000), II (M_r , 100 000), III (M_r , 45 700), and one intracellular form, β -glucosidase IV (M_r , 57 600). With the exception of β -glucosidase II which has yet to be isolated in quantities sufficient for characterization each has been shown to be a glycoprotein and to exist as a single polypeptide. Each of the purified enzyme preparations catalysed cellobiose hydrolysis but only β -glucosidase IV, at the concentrations of substrates used, also catalysed glucosyl transfer to cellobiose.

INTRODUCTION

The extracellular cellulase system of the thermophilic fungus, *Talaromyces emersonii*, is comprised of a number of endocellulases (EC 3.2.1.4), exocellulases (EC 3.2.1.91) and three forms of β -glucosidase (EC 3.2.1.21) (McHale & Coughlan, 1980, 1981*a*). The latter enzymes can be separated from one another by polyacrylamide gradient gel electrophoresis and on the basis of their relative mobilities have been termed β -glucosidases I, II and III (McHale & Coughlan, 1981*a*). Two of these, viz. β -glucosidases I and III, and an intracellular form, β -glucosidase IV, were isolated in sufficient quantity to permit their characterization (McHale & Coughlan, 1981*b*). In this paper we present the results of further investigations on the properties of these enzymes.

METHODS

Chemicals. Chemicals were obtained from the following suppliers: purified ball-milled spruce cellulose (BW.40) was from Brown & Co., Berlin, N.H., U.S.A.; corn steep liquor from Biocon Ltd, Cork, Republic of Ireland; non-ionic detergent (NP-40) from Shell Chemicals (Ireland), Dublin; SDS, Blue dextran, *p*-nitro-phenyl- β -D-glucoside, acrylamide and *N,N'*-methylene-bis-acrylamide from Sigma; dinitrosalicylate, sulphosalicylic acid, PAGE blue 83 and *N,N,N',N'*-tetramethylethylenediamine (TEMED) from BDH; Pharmalytes, high and low molecular weight protein calibration kits, isoelectric focusing apparatus (FEB 3000) and the electrophoresis constant power supply (ECPS 3000/150) from Pharmacia.

Organism. *Talaromyces emersonii* CBS 814.70 obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands was grown at 45 °C as described earlier (Folan & Coughlan, 1978, 1981) on medium containing 2% (w/v) cellulose 0.5% (w/v) corn steep liquor and 1% (w/v) NH_4NO_3 .

Enzyme purification and assay. The various enzymes displaying β -glucosidase (cellobiase) activity were assayed and purified to homogeneity (as judged by polyacrylamide gradient gel electrophoresis by the procedures reported earlier (Folan & Coughlan, 1978; McHale & Coughlan, 1980, 1981*b*).

Protein and carbohydrate determination. Protein concentration was measured by the method of Hartree (1972). The carbohydrate content of purified enzyme samples was determined using a scaled-down version of the method of Dubois *et al.* (1965). Samples (0.2 ml) of each enzyme solution were incubated at 28 °C for 15 min with 10 μ l phenol/water (80%, w/v) and 1 ml H_2SO_4 (sp. gr. 1.84). Absorbance of the mixture at 490 nm was measured and the carbohydrate content as mannose equivalents calculated by reference to a mannose standard curve. Under the conditions used 10 μ g of mannose gave an A_{490} of 0.56.

Electrophoresis and gel filtration. Subunit molecular weights of the various enzymes exhibiting β -glucosidase activity were estimated by subjecting samples of the purified preparations to SDS-polyacrylamide gradient gel electrophoresis (Margolis & Kenrick, 1968) following pretreatment of unknowns and of calibration standards as recommended by Pharmacia (publication no. 11-B-037-01, August 1978). Thus, unknowns and standards were

dissolved in 88.7 mM-Tris/2.5 mM-disodium EDTA/81.5 mM-boric acid, pH 8.3, containing 0.6% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and heated at 100 °C for 5 min. The running buffer was Tris/disodium EDTA/boric acid, pH 8.3, as above but containing 0.1% (w/v) SDS. Gels were fixed in water/methanol (7:3, v/v) containing trichloroacetic acid (114 g l⁻¹) and sulphosalicylic acid (34 g l⁻¹) followed by staining for protein in 0.2% (w/v) PAGE blue 83 in water/methanol/acetic acid (6:6:1, by vol.). Gels were destained in water/methanol/acetic acid (6:6:1, by vol.) until the background was clear. Molecular weights were estimated by gel filtration on a column of Sephadex G-200 (1.2 × 25.5 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 7.5. Standards and unknowns eluted with the same buffer were detected by their absorbance at 280 nm. The void volume was determined using Blue dextran.

Isoelectric focusing. This technique was carried out using Pharmalytes covering the pH range 2.5 to 5.0 and the procedure recommended by Pharmacia (publication no. 01-900-2-1464-02). β -Glucosidase I was also subjected to isofocusing in the presence of urea (9 M) and the non-ionic detergent NP-40 (3%, v/v) as recommended by M. L. O'Connor & D. R. Headon (personal communication). Gels were stained for β -glucosidase activity as described previously (McHale & Coughlan, 1981a). After each run the pH gradient was determined by measurement of pH in samples of gel scraped from various points between the electrode strips from which the Pharmalytes were eluted into 1 ml of degassed distilled water.

RESULTS AND DISCUSSION

Molecular weights, isoelectric points and carbohydrate contents

The native molecular weights of the β -glucosidases were estimated by gel filtration (Fig. 1). The values obtained are in close agreement with the corresponding subunit molecular weight values determined by SDS-polyacrylamide gradient gel electrophoresis of proteins that had been fully reduced with mercaptoethanol (Fig. 1 and Table 1). As these β -glucosidases are glycoproteins (see below), we must treat with caution the molecular weight values obtained by using the above techniques (see e.g. Andrews, 1965). Nevertheless, since all three enzymes migrated as a single component in both analyses it is clear that each is a single polypeptide.

β -Glucosidase III also migrated as a single band, pI 3.6, on isoelectric focusing. In contrast, isofocusing of purified preparations of β -glucosidase I and β -glucosidase IV showed evidence of heterogeneity. With the former staining for activity gave a more or less continuous smear between points corresponding to pH values 3.40 and 4.17. A similar smear was obtained after staining for glycoprotein by the method of Zacharius *et al.* (1969). The formation of different size aggregates of enzyme as a cause of such smearing was ruled out, since the inclusion of urea and the non-ionic detergent NP-40 in the isofocusing process did not lessen the smearing. In the case of β -glucosidase IV three distinct bands, with pI values 4.41, 4.47 and 4.50 were obtained. All three bands stained for glycoprotein.

We expect that this 'microheterogeneity' of β -glucosidase I reflects variation in the content or composition of its carbohydrate rather than differences in primary structure and that the same could well be true for β -glucosidase IV. If this is so, then the carbohydrate contents listed for β -glucosidases I and IV (Table I) must be considered as average values. Differences between the carbohydrate contents of the three enzymes are highlighted when expressed as mannose equivalents per mol enzyme. These are as follows (depending on whether the calculation was based upon the molecular weight values obtained by gel filtration or by electrophoresis, Table 1): β -glucosidase I, 382–391; β -glucosidase III, 59–66; β -glucosidase IV, 35–38. The extracellular enzymes, viz. I and III have greater carbohydrate contents than has β -glucosidase IV, the intracellular form. Hayashida & Yoshioka (1980) have shown that the carbohydrate moieties of the cellulases from *Humicola insolens* contribute significantly to the thermal stability of these enzymes. This situation may also obtain for β -glucosidases I, III and IV which have half lives (70 °C, pH 5) of 410 min, 175 min and 2 min, respectively (McHale & Coughlan, 1981b), although β -glucosidases III and IV have a small difference in carbohydrate content for such a large difference in thermal stability.

Transferase activity

Synthesis of the cellulase system of *T. emersonii* is induced by the presence of cellulosic materials in the growth medium (Folan & Coughlan, 1978). However, the identity of the actual

Fig. 1. Molecular weights of the β -glucosidases of *T. emersonii*. Subunit molecular weights were estimated by polyacrylamide gradient gel electrophoresis in the presence of SDS (a, b). Molecular weights were estimated by gel filtration on Sephadex G-200 (1.2 $\times$ 25.5 cm) equilibrated with 20 mM-Tris/HCl, pH 7.5 (c). Standards and their molecular weights ($M_r \times 10^{-3}$), were as follows: 1, thyroglobulin (330); 2, ferritin (half unit, 220); 3, uricase (150); 4, phosphorylase b (94); 5, bovine serum albumin (67); 6, catalase (60); 7, ovalbumin (43); 8, lactate dehydrogenase (36); 9, carbonic anhydrase (30); 10, trypsin inhibitor (20.1); 11, myoglobin (17.2); 12, α -lactalbumin (14.4). β GI, β GIII and β GIV are defined in the Introduction and in the legend to Table I.

Table 1. *Properties of the β -glucosidases of Talaromyces emersonii*

Three extracellular forms of β -glucosidase, β GI, β GII and β GIII and an intracellular form, β GIV, are produced by *T. emersonii*. Molecular weight was determined by gel filtration of purified preparations on Sephadex G-200. Subunit molecular weight was determined by electrophoresis under denaturing conditions in polyacrylamide gradient gels.

Property	β GI	β GII	β GIII	β GIV
Molecular weight ($\times 10^{-3}$)	138	—	40.7	52.5
Subunit molecular weight ($\times 10^{-3}$)	135	100*	45.7	57.6
pI	3.40–4.17	—	3.60	4.41 4.47 4.50
Carbohydrate content (%)	51	—	26	12

* The value for (subunit) molecular weight of β GIII is a tentative one from electrophoretic analysis as this enzyme has not been purified.

Fig. 2. TLC analysis of the products of action of β -glucosidase on a mixture of cellobiose and glucose. β -glucosidases I (3 μ g) or IV (30 μ g) were incubated at 37 °C with 4 mM-cellobiose plus 4 mM-glucose in 50 mM-sodium acetate buffer, pH 5. Samples of reaction mixture were obtained at 0.5, 1 and 2 h after the start of the incubation, boiled for 2 min and subjected to TLC as described by Wood & McCrae (1978) except that chromatograms were stained with iodine vapour (Dawson *et al.*, 1969). G₁, G₂, G₃ and G₄ refer to the glucose, cellobiose, cellotriose and cellotetraose standards. G₃ and G₄ were prepared as described before (McHale & Coughlan, 1981*b*). S indicates standard mixtures of sugars.

inducer(s) of this or any other cellulase system is not yet known. It is generally believed that low constitutive levels of cellulases cleave cellulose and provide a small molecule which on being transported into the cell brings about an increase in the rate of synthesis of the enzymes in question. Mandels & Reese (1960) reported that during growth of *Trichoderma reesei* on cellobiose, products of glucosyl transfer were found in the growth medium and that these were better than cellobiose as inducers of cellulase synthesis. Sophorose, a β -1,2-linked dimer of glucose, is said to be the most potent inducer of cellulase synthesis by *Trichoderma reesei* (Mandels *et al.*, 1962) although it may not induce all of the components obtained in the presence of cellulose (Sternberg & Mandels, 1979). While sophorose and other β -linked di- and trisaccharides have yet to be tested as possible inducers of cellulase synthesis by *T. emersonii*, it was of interest to determine whether any of the β -glucosidases produced by this organism exhibited transferase activity. Accordingly, purified samples of β -glucosidases I, III and IV were incubated with glucose plus cellobiose and the products analysed at intervals by TLC. The results illustrated in Fig. 2 show that cellobiose is gradually converted to glucose by β -glucosidase I, albeit slowly because of inhibition by glucose (McHale & Coughlan, 1981*b*), with no spot other than those corresponding to cellobiose or glucose being observed. Similar results were obtained with β -glucosidase III. In contrast, when β -glucosidase IV was incubated with cellobiose plus glucose a sugar (arrowed) migrating faster than cellotriose but slower than cellobiose accumulated within half an hour (Fig. 2). The decrease over the next 1.5 h in the size of the spot corresponding to this unidentified sugar clearly indicated that it was acted on further. The unidentified sugar may be a disaccharide that is converted to glucose in the later stages of incubation or a trisaccharide that is converted to glucose via a disaccharide. Further work will be required to identify the compound(s) and to see whether it (they) induces the synthesis of cellulase by *T. emersonii*. It is interesting that of the β -glucosidases of this organism which have been purified, only the intracellular form, β -glucosidase IV, exhibits transferase activity (at the substrate concentrations used). Thus, in keeping with the general scheme proposed by Gong & Tsao (1979), the intracellular β -glucosidase I may regulate cellulase synthesis both by converting incoming

cellobiose to active inducer (transferase action) and cleaving cellobiose to glucose (cellobiase action) repressing cellulase synthesis.

A. McH. thanks the Department of Education, Republic of Ireland, for a maintenance grant. We thank Lynn O'Connor and Dr Denis Heaton of this department for their assistance with electrophoretic and isofocusing techniques.

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