β-Glucosidase from *Penicillium purpurogenum*: Purification and Properties[1]

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β-Glucosidase was purified from the culture supernatant of *Penicillium purpurogenum*. The purified enzyme was homogeneous on both nondenaturing and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The enzyme is a monomeric glycoprotein with M_r of 90,000 as determined by gel filtration on Bio-Gel P-300 and SDS–polyacrylamide gels. Two enzyme forms were resolved by chromatofocusing and isoelectric focusing, and the p*I* values obtained with both methods were 4.2 (major form) and 6.0. The major form was characterised further. Enzyme activity was optimal at pH 3.5 and at 60°C. The enzyme was stable in the pH range 2.5–9.5 for 24 h at 4°C. Kinetic analysis gave K_ms of 0.8 mM for cellobiose and 85 μM for *p*-nitrophenyl-β-D-glucopyranoside. The enzyme hydrolyses a wide range of substrates including aryl-β-glucosides, cellobiose, and amygdalin. Glucose inhibits competitively and glucono-δ-lactone is a mixed inhibitor of the enzyme. © 1992 Academic Press, Inc.

Cellulose degradation is an important natural and biotechnological process. It takes place by the synergistic action of a group of enzymes collectively called "cellulases." The principal components of the cellulase complex are the endoglucanases (E.C. 3.2.1.4.), which randomly hydrolyze internal glycosidic bonds of cellulose, and the exoglucanase (E.C. 3.2.1.91.) enzymes that split cellobiose from the nonreducing end of the cellulose molecule. β-Glucosidase (E.C. 3.2.1.21.) hydrolyzes cellobiose to glucose (1). The action of β-glucosidase is important, since cellobiose is an inhibitor of both exo- and endoglucanases, and must be removed to allow complete saccharification of cellulose (2).

Since cellulose is a renewable resource, there is great interest in its utilisation as a source of fuels and chemicals. Therefore, a considerable amount of work has been done to isolate cellulolytic strains of microbes and to study the enzymes they produce. The most active cellulolytic organisms isolated to date are the fungi of the genus *Trichoderma* (3). These fungi, however, secrete relatively low levels of β-glucosidase (4); this fact has prompted the study of other fungal species, among them those of the genus *Penicillium. Penicillium herquei* Banier and Sartory has been shown to produce large amounts of two extracellular β-glucosidases, along with the components of

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the cellulase complex (5). The present work refers to the purification and characterisation of β-glucosidase from *P. purpurogenum*.

MATERIALS AND METHODS

Chemicals. Bio-Gel P-100, Bio-Gel P-300, and DEAE–Bio-Gel A were supplied by Bio-Rad. CM-cellulose, crystalline cellulose (Sigmacell), oat spelt xylan, pNPG,[3] cellobiose, xylose, pNPX, amygdalin, salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside), MβG, arbutin (hydroquinone-β-glucopyranoside), glucose oxidase, peroxidase, glucono-δ-lactone, and corn steep liquor were obtained from Sigma Chemical Co. PBE 94 and Polybuffer 74 for chromatofocusing were from Pharmacia Fine Chemicals. Carrier ampholytes (Ampholine pH 3.5–10) were obtained from LKB-Produkter. All other chemicals were reagent grade.

Fungal strain. The strain of *P. purpurogenum* used in this work was isolated from a soil sample and grows well on wheat straw as a carbon source (6). The strain can be obtained from the authors.

Culture conditions. P. purpurogenum was grown on potato dextrose agar slants at 28°C and transferred bimonthly. Growth for enzyme production was carried out in submerged cultures. The cultures contained the following components: (a) salt mixture (2 g/liter KH_2PO_4; 0.3 g/liter $MgSO_4 \cdot 7H_2O$; 0.3 g/liter $CaCl_2$; 5 mg/liter $FeSO_4 \cdot 7H_2O$; 1.56 mg/liter $MnSO_4 \cdot H_2O$; 2.49 mg/liter $ZnSO_4 \cdot 7H_2O$; 3.66 mg/liter $CoCl_2 \cdot 6H_2O$); (b) carbon source: 0.5% wheat straw (milled and sieved to a particle size of less than 1.2 mm) and 0.5% wheat bran; and (c) nitrogen source: corn steep liquor (final concentration, 0.1% normal) Conidia (4×10^6) obtained from growth on potato dextrose agar plates were inoculated into 100 ml of medium in 250-ml Erlenmeyer flasks. The cultures were incubated in an orbital shaker (200 rpm) at 28°C for 13 days.

Enzyme source. The cultures were centrifuged for 15 min at 27,000*g* and the supernatant was concentrated to one fourth of its volume by ultrafiltration using a Pellicon PTGC membrane with an M_r cutoff at 10,000. The concentrate was used for enzyme purification.

Determination of β-glucosidase activity. When pNPG was used as a substrate, the procedure of Hoffman and Wood (7) was followed; assays were performed at pH 4.0 in 0.2 M acetate buffer incubating for 30 min at 50°C. Activity towards cellobiose and other glycosides was measured under the same conditions, following the appearance of glucose by means of the glucose oxidase method (8), using β-glucosidase-free glucose oxidase and peroxidase. One unit of enzyme activity was defined as the amount of enzyme which hydrolyzes 1 μmol of substrate per minute.

Other enzyme activities. Activities against CM-cellulose and crystalline cellulose (Sigmacell) were estimated following the appearance of reducing power by means of the ferricyanide method, using glucose as a standard (9). Xylanase was measured using oat spelt xylan as a substrate and the reducing sugars were quantified by the method of Somogyi-Nelson using xylose as a standard (10). One unit of activity was

[3] Abbreviations used: pNPG, *p*-nitrophenyl-β-D-glucopyranoside; pNPX, *p*-nitrophenyl-β-D-xyloside; MβG, metyl-β-D-glucopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Con A, concanavalin A; CMCase, carboxymethyl cellulase.

defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute.

Protein determination. The protein concentration was determined according to Lowry as modified by Tan *et al.* (11), using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. SDS–PAGE was performed by the method of Laemmli (12) using 10% acrylamide; protein was stained as described below. Zymograms were performed using nondenaturing gels prepared with 8% acrylamide. After running the gels (with duplicates of each sample), they were cut in half. One half was used for activity determination by incubating for 30 min at 50°C in 0.2 M sodium acetate buffer, pH 4.0, in the presence of pNPG (0.4 mg/ml), followed by incubation in 0.4 M glycine–NaOH, pH 10.7. The other half was stained for protein with Coomassie brilliant blue.

M_r determination. The M_r of the native enzyme was determined by gel filtration in a Bio-Gel P 300 column, using the following standards: rabbit muscle pyruvate kinase (237,000), rabbit muscle aldolase (162,000), and bovine serum albumin (66,000). Subunit M_r was established by means of SDS–PAGE (see above), using as standards: myosin (205,000), β-galactosidase (116,000), phosphorylase b (92,000), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000).

Glycoprotein stain. The presence of glycoprotein was established as follows: the protein from an SDS–PAGE was transferred by electroelution to a nitrocellulose membrane, followed by staining with Con A and horseradish peroxidase according to the procedure of Clegg (13).

Chromatofocusing. A kit supplied by Pharmacia was employed, following the manufacturer's instructions. The pH gradient ranged from pH 4.0 and 7.4, and 0.025 M imidazole–HCl was used as starting buffer. Eluent buffer was Polybuffer 74–HCl, pH 4.0, diluted eightfold with water.

Isoelectric focusing. Isoelectric focusing was carried out in a cooled vertical slab unit (Hoefer SE 600) as described by Giulian *et al.* (14). The gels contained 7.5% polyacrylamide and 2.7% ampholyte and a pH gradient was formed from pH 3.5 to 10.0. The gels were prerun for 10 to 15 min at a constant power of 20 W. Samples were put in the wells, the gels were run for 2 h at constant power (15 W) and were cut in 1 cm slices, and each slice was put in a separate test tube containing 2 ml of distilled water and homogenised by vortexing. The pH was measured with a glass electrode. The β-glucosidase activities using pNPG and cellobiose as substrates were measured in each sample as indicated above.

RESULTS AND DISCUSSION

Enzyme Purification

P. purpurogenum cultures were centrifuged and the pooled supernatant (750 ml) was concentrated as indicated under Materials and Methods. This preparation was fractionated with ammonium sulfate (40–85% saturation) and the precipitate was dissolved in 5.7 ml of 20 mM potassium phosphate buffer, pH 6.5 (buffer A). This solution was chromatographed in a Bio-Gel P-300 column (1 × 25 cm) previously equilibrated with the same buffer. Active fractions were pooled and put on a DEAE–Bio-Gel A column (1.5 × 26 cm), equilibrated with buffer A supplemented with 0.1 M NaCl. After washing the column with 30 ml of this buffer, the enzyme was eluted in a

TABLE I

Purification of β-Glucosidase

Fraction	*Total activity (U)*	*Specific activity (U/mg protein)*	*Purification (fold)*
Culture supernatant	1210	3.4	1
$(NH_4)_2SO_4$ fractionation	1030	42	12
Bio-Gel P-300	827	163	47
DEAE–Bio-Gel A	680	354	103
Bio-Gel P-100	610	601	174
Preparative isoelectric focusing			
βG I	375	642	186
βG II	43	619	179

linear gradient from 0.1 to 0.15 M NaCl in 200 ml of buffer A. The active pool was chromatographed in a Bio-Gel P-100 column (1 × 26 cm), using buffer A. Aliquots of the active fractions obtained were added to carrier ampholyte, pH range 3.5–10, up to a concentration of 2% (v/v) and subjected to preparative isoelectric focusing. Two peaks of β-glucosidase activity (βG I and βG II) were detected. The more acidic form (βG I) corresponds to 90% of the activity. The results of the purification are summarized in Table I.

Enzyme Purity and Relative Molecular Mass

Only one band of protein was found when the P-100 column eluate was subjected to nondenaturing PAGE. This band corresponded to a single activity band (data not shown). SDS–PAGE gave a single band with both the P-100 (not shown) and the isoelectrofocusing eluate fractions (Fig. 1). An M_r of 90,000 was estimated for the native enzyme by gel filtration in a Bio-Gel P-300 column; a similar value was obtained under denaturing conditions on SDS–PAGE (data not shown), indicating that the enzyme is a monomer.

This M_r is similar to those found for the β-glucosidase from *Aspergillus niger* (96,000) (15), β-glucosidase I from *Schizophyllum commune* (96,000) (16), and *Trichoderma reesei* (98,000) (17), but it is different from the enzyme from *P. oxalicum* (133,000) (18), *P. herquei* (125,000) (5), *A. aculeatus* No. F-50 (132–136,000) (19), *A. niger* (325,000) (20), and *T. viride* (47,000) (21).

The purified enzyme contained carbohydrates as determined by staining with Con A (not shown). This suggests that α-D-mannopyranosyl and/or α-D-glucopyranosyl units are present in the enzyme. This property can be used to devise an affinity purification method for the enzyme.

Isoelectric Point

Chromatofocusing of the culture supernatant and of the enzyme taken after the Bio-Gel P-100 purification step gave two enzyme forms with isoelectric points of 4.2 and 6.0 (Fig. 2). Similar results were found by means of electrofocusing (not shown). In all cases the predominant form was that with a p*I* of 4.2. If the acidic form was

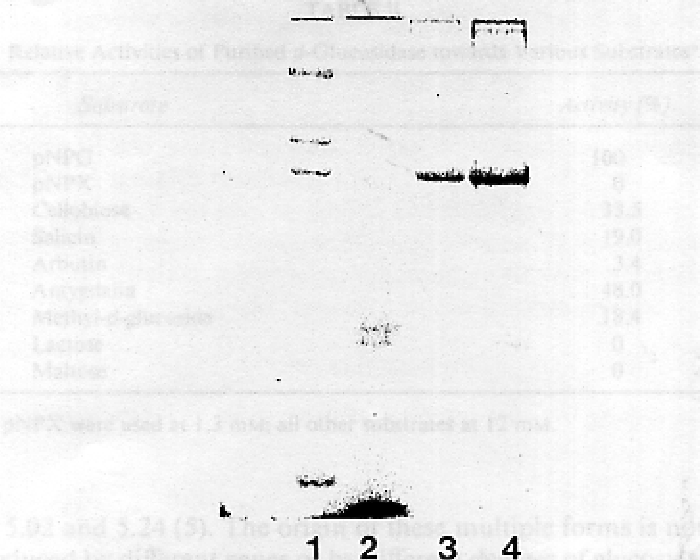

FIG. 1. SDS–polyacrylamide gel electrophoresis of β-glucosidase. Electrophoresis was carried out on 10% acrylamide gels as described under Materials and Methods. Lane 1, M_r standards as indicated under Materials and Methods; lane 2, 30 μg of concentrated supernatant; lanes 3 and 4, 5 and 10 μg of concentrated eluate from isoelectrofocusing gel (p*I* 4.2 band), respectively.

eluted from the electrofocusing gel and subjected again to the same procedure, a single activity peak of p*I* 4.2 was observed (not shown). No interconversion of the two forms could be detected by isoelectrofocusing after incubating them separately (overnight) with an aliquot of a pool of the Bio-Gel P-300 eluate fractions lacking enzyme activity.

Several fungi have been found which produce multiple forms of β-glucosidases (22). Two β-glucosidases have been described from *P. herquei* and their p*I*s were

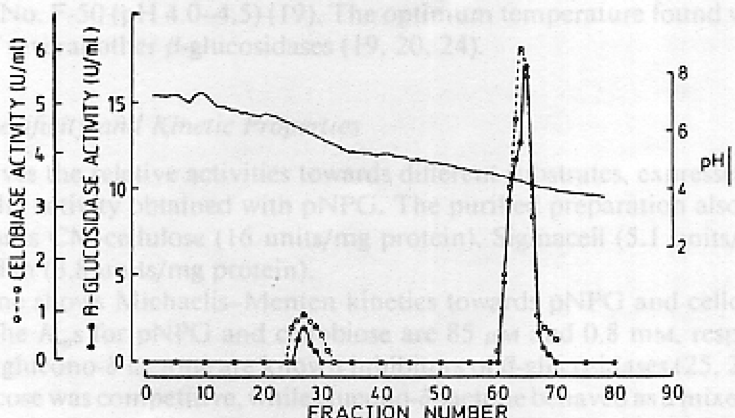

FIG. 2. Determination of the isoelectric point of the β-glucosidase forms in the culture supernatant from *P. purpurogenum* by chromatofocusing. The experiment was performed as described under Materials and Methods.

TABLE II

Relative Activities of Purified β-Glucosidase towards Various Substrates[a]

Substrate	*Activity (%)*
pNPG	100
pNPX	0
Cellobiose	35.5
Salicin	19.0
Arbutin	3.4
Amygdalin	48.0
Methyl-β-glucoside	18.4
Lactose	0
Maltose	0

[a] pNPG and pNPX were used at 1.3 mM; all other substrates at 12 mM.

found to be 5.02 and 5.24 (5). The origin of these multiple forms is not clear. They could be produced by different genes or by different degrees of glycosylation, or they can be the result of proteolysis in the culture supernatant. This last cause is not likely for the enzymes discussed here, since both forms show the same M_r, although terminal removal of a few charged residues may cause large changes in p*I* without significant effects on the M_r.

Effects of pH and Temperature on Activity and Stability

The optimum pH for both pNPG- and cellobiose-hydrolyzing activities was found to be 3.5. The "optimum temperature" with both substrates was 60°C. The enzyme is stable when preincubated up to 60°C for 10 min and shows a broad range of stability to pH (2.5 to 9.5) when incubated at 4°C for 24 h.

The optimum pH for activity on pNPG was similar to β-glucosidase-3 from *A. aculeatus* No. F-50 (19), but is lower than the values found for the enzymes from *P. herquei* (pH 4.0–4.5) (5), *A. japonicus* (pH 5.0) (23), and β-glucosidase-1 and -2 from *A. aculeatus* No. F-50 (pH 4.0–4.5) (19). The optimum temperature found was similar to that of several other β-glucosidases (19, 20, 24).

Substrate Specificity and Kinetic Properties

Table II gives the relative activities towards different substrates, expressed as percentages of the activity obtained with pNPG. The purified preparation also showed activity towards CM-cellulose (16 units/mg protein), Sigmacell (5.1 units/mg protein), and xylan (3.8 units/mg protein).

The enzyme shows Michaelis–Menten kinetics towards pNPG and cellobiose as substrates. The K_ms for pNPG and cellobiose are 85 μM and 0.8 mM, respectively. Glucose and glucono-δ-lactone are known inhibitors of β-glucosidases (25, 26). Inhibition by glucose was competitive, while glucono-δ-lactone behaved as a mixed inhibitor (27), as determined with a Hanes plot (not shown). The K_i values obtained for these inhibitors with pNPG as a substrate were 1.4 mM for glucose and 0.067 mM for glucono-δ-lactone.

The purified enzyme has greater affinity towards pNPG than to the natural substrate cellobiose as shown by the respective K_m values. Therefore, it should be considered an aryl-β-glucosidase. In this respect, it is similar to the enzyme produced by *T. viride* (21), and β-glucosidase-1 from *P. herquei* (5), but it is different from the enzyme of the thermophilic fungus *Humicola lanuginosa* (24). The latter has similar affinities towards pNPG and cellobiose. Some β-glucosidases, on the other hand, show greater affinity towards cellobiose (28).

The enzyme was active not only on pNPG and cellobiose but also on other aryl-β-glucosides such as amygdalin, salicin, MβG, and arbutin. It is not clear whether the low but measurable activities towards carboxymethylcellulose, Sigmacell, and xylan are due to the same protein or correspond to minor contaminants.

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REFERENCES

1. KHAN, A. W., MEE, E., AND HENSCHEL, J. R. (1985) *Enzyme Microb. Technol.* 4, 73–79.
2. WOOD, T. M., AND MCCRAE, S. I. (1978) *Biochem. J.* 171, 61–72.
3. MANDELS, M. (1985) *Biochem. Soc. Trans.* 13, 414–416.
4. STERNBERG, D., VIJAYAKUMAR, P., AND REESE, E. T. (1982) *Exp. Mycol.* 6, 115–124.
5. FUNAGUMA, T., AND HARA, A. (1988) *Agric. Biol. Chem.* 52, 749–755.
6. MUSALEM, M., STEINER, J., AND CONTRERAS, I. (1984) *Bol. Micol.* 2, 17–26.
7. HOFFMAN, R. M., AND WOOD, T. M. (1985) *Biotechnol. Bioeng.* 27, 81–85.
8. DAY, D. F., AND WORKMAN, W. E. (1982) *Anal. Biochem.* 126, 205–207.
9. WOOD, T. M., AND MCCRAE, S. I. (1972) *Biochem. J.* 128, 1183–1192.
10. NELSON, N. (1944) *J. Biol. Chem.* 153, 375–381.
11. TAN, L. U. L., CHAN, M. K.-H., AND SADDLER, J. N. (1984) *Biotechnol. Lett.* 6, 199–204.
12. LAEMMLI, U. K. (1970) *Nature* 227, 680–685.
13. CLEGG, J. C. S. (1982) *Anal. Biochem.* 127, 387–394.
14. GIULIAN, G. G., MOSS, R. L., AND GREASER, M. (1984) *Anal. Biochem.* 142, 421–436.
15. WITTE, K., AND WATENBERG, A. (1989) *Acta Biotechnol.* 9, 179–190.
16. LO, A. C., BARBIER, J.-C., AND WILLICK, G. E. (1990) *Eur. J. Biochem.* 192, 175–181.
17. INGLIN, M., FEINBERG, B. A., AND LOEWENBERG, J. R. (1980) *Biochem. J.* 185, 515–519.
18. COPA-PATIÑO, J. L., RODRÍGUEZ, J., AND PÉREZ-LEBLIC, M. I. (1990) *FEMS Microbiol. Lett.* 67, 191–196.
19. SAKAMOTO, R., ARAI, M., AND MURAO, S. (1985) *Agric. Biol. Chem.* 49, 1283–1290.
20. YEOH, H. H., TAN, T. K., CHUA, S. L., AND LIM, G. (1988) *MIRCEN J.* 4, 425–430.
21. BERGHEM, L. E. R., AND PETTERSSON, L. G. (1974) *Eur. J. Biochem.* 46, 295–305.
22. SCHMID, G., AND WANDREY, C. H. (1987) *Biotechnol Bioeng.* 30, 571–585.
23. RUDICK, M. J., AND ELBEIN, A. D. (1973) *J. Biol. Chem.* 248, 6506–6513.
24. ANAND, L., AND VITHAYATHIL, P. J. (1989) *J. Ferment. Bioeng.* 67, 380–386.
25. CHIRICO, W. J., AND BROWN, R. D. JR. (1987) *Eur. J. Biochem.* 165, 333–341.
26. REESE, E. T., PARRISH, F. W., AND ETTLINGER, M. (1971) *Carbohydr. Res.* 18, 381–388.
27. CORNISH-BOWDEN, A. (1979) Fundamentals of Enzyme Kinetics, p. 76, Butterworths, London.
28. LEGLER, G. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* 348, 1359–1366.