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A *Phanerochaete chrysosporium* β-D-glucosidase/β-D-xylosidase with specificity for (1 → 3)-β-D-glucan linkages

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ABSTRACT

Phanerochaete chrysosporium is the best studied organism with respect to lignin degradation, but its degradation of the xylan component of lignocellulose is only now being studied. When grown on oat spelt xylan (mainly arabinoxylan), it produces an enzyme with β-D-xylosidase and β-D-glucosidase activity. This enzyme was purified by ultrafiltration followed by ammonium sulphate precipitation, anion-exchange chromatography using DEAE Biogel and Mono Q, and gel filtration using Superose 12. It is extracellular, with an apparent M_r value of 44500 as determined by SDS-PAGE; the pI is 4.67 and activity is maximal at pH 5 and 60°C. The enzyme is of particular interest because its principal activity is against laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucopyranose and laminarin [(1 → 3)-β-D-glucan with ca. 3% of β-(1 → 6) branches] rather than cellobiose and xylobiose. It was competitively inhibited by D-glucono-1,5-lactone and deoxynojirimycin; with *p*-nitrophenyl β-D-xylopyranoside as substrate, the K_i values were 32 and 87.5 μM, respectively, and with *p*-nitrophenyl β-D-glucopyranoside, they were 35 and 68.7 μM, respectively. The K_m values with *p*-nitrophenyl β-D-xylopyranoside and *p*-nitrophenyl β-D-glucopyranoside as substrates were 3.51 and 5.30 mM, respectively.

INTRODUCTION

Phanerochaete chrysosporium is a white rot fungus that is able to degrade the three main components of lignocellulose[1]. Until now, studies with this fungus have concentrated on its lignin and cellulose degradation abilities[1–6]. However, we have recently demonstrated that it also produces an extracellular xylanolytic system[7]. The characterization of this system is essential to a full understanding of lignocellulose degradation by *P. chrysosporium*.

Xylan-degrading enzymes are of potential biotechnological importance in utilizing xylan-containing waste, and for the production of xylose and other important industrial chemicals. β-Xylosidases [β-D-xylosidase; (1 → 4)-β-D-xylan xylanohydro-

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lase; EC 3.2.1.37] from many fungi and also from some bacteria have been purified and characterized[8,9]. Such enzymes hydrolyse xylo-oligosaccharides from the nonreducing end to yield xylose[10], and are essential to the complete degradation of xylan. β-Xylosidases and other xylan-degrading enzymes interact synergistically to effect the conversion of xylans. For example, when β-xylosidase was included in reaction mixtures, the yields of xylose from xylan were markedly increased[11–13]. β-Xylosidases from a number of fungi have been found to be inducible and to be subject to catabolite repression by D-glucose or D-xylose[14].

In this paper, we describe the properties of an enzyme that was purified using β-xylosidase activity as the assay. It is of particular interest because of its activity against the β-(1 → 3)-linked laminaribiose and laminarin, since a major component of the *P. chrysosporium* sheath is a glucan with β-(1 → 3) and β-(1 → 6) linkages[15].

EXPERIMENTAL

Strain and growth conditions.—Phanerochaete chrysosporium ME446 (ATCC 34541) was maintained on agar medium that contained 20 g L^{-1} malt extract (Oxoid) and 15 g L^{-1} agar, and incubated at 37°C until sporulation occurred (5 days). It was grown in the medium described by Copa-Patiño et al.[7]. For routine liquid cultures, distilled water suspensions of sporulating growth were used to inoculate 1-L flasks containing 100 mL of medium to give a final spore concentration of 2.5×10^5 spores mL^{-1}. Cultures were incubated for 7 days at 37°C without shaking.

Enzyme assay and protein determination.—β-Xylosidase activity was assayed using 4 mM *p*-nitrophenyl β-D-xylopyranoside (PNPhβX) in citric acid–citrate buffer (50 mM, pH 5) as the substrate. Twenty-five μL of enzyme were incubated with 225 μL of PNPhβX for 10 min at 45°C. The reaction was stopped by adding 750 μL of 2 M Na_2CO_3 and the concentration of the product was determined from the A_{410}.

The SI unit of activity is defined as the amount of enzyme that releases 1 mol of *p*-nitrophenol per s under assay condition (katal). The molecular absorption coefficient of *p*-nitrophenol is 18.5×10^3 under the conditions used[16]. Glucose was determined using the D-glucose-oxidase method[17]. Reducing sugars were detected by using the bicinchoninate–Cu^{2+} reagent[18,19]. Protein concentration was determined using the Bradford method[20]. Specific activity is defined as kat mg^{-1} protein.

Concentration of protein, ammonium sulphate precipitation, and DEAE-Biogel chromatography.—These were done as described previously[7].

Mono Q HR 5/5.—A Mono Q column, connected to an FPLC system (Pharmacia), was equilibrated with 20 mM Tris–HCl (pH 8, buffer *A*). The column was washed twice with 1% buffer *B* (0.5 M NaCl in buffer *A*). Protein was then eluted with buffer *B* at a flow rate of 1.0 mL min^{-1} as follows: 2 mL of 1% buffer *B*, 2 mL of a linear gradient (1% to 10%) buffer *B*, 16 mL of 10% to 30% buffer *B*, 1

mL of 30% to 100% buffer *B*, and 4 mL of 100% buffer *B*. Fractions of 0.5 mL were collected. Elution of protein from the column was monitored at 280 nm.

Superose-12 HR 10/30.—A Superose 12 column, connected to the FPLC system, was equilibrated with 50 mM Tris–HCl buffer (pH 8) containing 0.15 M NaCl. Protein was eluted with 30 mL of this buffer at a flow rate of 0.4 mL min^{-1}.

Electrophoresis.—SDS-PAGE was performed according to Laemmli[21] under denaturating conditions in 17% polyacrylamide ($T = 17\%$, $C = 0.085\%$) gel. Protein bands were visualized by staining with Coomassie Blue (Coomassie Brilliant Blue G-250, 1%; methanol, 50%; and acetic acid, 7.5%) for 30 min at 37°C with slow agitation.

Phast-Gel.—The Pharmacia PhastSystem was used for rapid isoelectric focusing (IEF) with minigels. Broad range (pI 3.0 to 9.0) IEF gels, protein calibration kits, isoelectrofocusing, and staining for protein with Coomassie Blue were performed as recommended by the manufacturer. The Dobberstein and Emeis[22] method was used for staining for β-xylosidase activity. After PhastSystem electrophoresis, the gel was equilibrated in 0.1 M acetate buffer (pH 5). A filter paper soaked in solutions of either 4-methylumbelliferyl β-D-xylopyranoside (MeUβX), 4-methylumbelliferyl β-D-glucopyranoside (MeUβG), or 4-methylumbelliferyl α-D-glucopyranoside (MeUαG), each at 10 g L^{-1} in 0.1 M acetate buffer (pH 5), was laid over the gel and incubated at 45°C for 15 min. Activity was visualized as fluorescing bands on the filter paper overlay when illuminated by UV light (366 nm).

Enzyme characterization.—Estimates of optimal temperature and pH as well as thermal and pH stability were made using a temperature range from 30 to 80°C and a pH range from 3.0 to 9.0 (Britton and Robinson Universal Buffer[23]). In the pH stability experiments, the preincubation time was 3 h at room temperature.

To determine half-lives at different temperatures, the enzyme was incubated at a temperature range from 30 to 80°C in a 50 mM Britton and Robinson Universal Buffer at optimum pH. At time intervals, the samples were cooled and enzyme activities were determined under standard conditions.

Michaelis–Menten parameters for the hydrolyses of *p*-nitrophenyl β-D-xylopyranoside and *p*-nitrophenyl β-D-glucopyranoside were estimated from initial rate measurements at substrate concentrations between 0.5 and 10 mM. V_{max} and K_m values were obtained from Eadie–Hofstee plots, and values of V_{max}/K_m from Lineweaver–Burk plots. V_{max} values were converted into k_{cat}, assuming a molecular weight per active site of 46 k.

The effect of D-xylose on activity was tested using 4 mM PNPhβX or PNPhβG in citric acid–citrate buffer (50 mM, pH 5) as substrate. D-Xylose (at 5 to 500 mM) was included in the β-xylosidase assay mixture, with the appropriate controls.

Inhibition by heavy metal ions was investigated by incorporating appropriate salts in the assay mixture. The substrate used was PNPhβX or PNPhβG (4 mM) in 50 mM acetate buffer (pH 5).

The effects of D-glucono-1,5-lactone and of deoxynojirimycin were determined by incorporating different concentrations of these compounds (50 μM to 1 mM) in

the assay mixture. PNPhβX or PNPhβG at either 4 or 10 mM in 50 mM citrate–citrate buffer (pH 5) was used as substrate.

The *N*-terminal sequence of the enzyme was determined using a Protein Sequencer, model 473A, from Applied Biosystems.

RESULTS

Phanerochaete chrysosporium produced 0.9×10^{-8} kat of β-xylosidase per mg of protein in the culture broth. After concentration by ultrafiltration and precipitation with ammonium sulphate at 70% saturation, the specific activity was 1.50×10^{-8} kat mg^{-1} protein.

Purification of β-xylosidase to apparent homogeneity was achieved in three further steps that involved anion-exchange and gel permeation chromatography. In the first step, the enzyme was bound to DEAE-Biogel at pH 8, as were other xylanolytic enzymes[7], but was separated from the latter using a gradient of NaCl (Fig. 1A). This column also permitted separation of β-xylosidase activity from the contaminating brown polyphenols. The latter was not eluted from the column until the NaCl concentration reached 1 M. The fractions (127–131) showing greatest β-xylosidase activity were pooled and applied to a Mono Q anion-exchange column. The enzyme bound to the column and, when a gradient of NaCl was applied, only a single peak of β-xylosidase activity was obtained (Fig. 1B). In the final step, the appropriate pooled fractions were applied to a Superose 12 column. Again, there was only one peak of activity (Fig. 1C). Compared with the activity of the culture filtrate, the yield of apparently homogeneous β-xylosidase was 11% and the purification factor was 66.7 (Table I).

The final preparation showed a single band on SDS-PAGE (Fig. 2); the M_r value under these conditions was estimated to be 46 000 compared with a value of 14 000 by gel filtration on Superose 12 (Fig. 1C). The preparation also gave a single band on PhastSystem isoelectrophoresis and staining for protein. The pI value was calculated to be 4.64 (Fig. 3). When the gel was stained for activity, only one band, coincident with that staining for protein, was visible. This band had activity against both 4-methylumbelliferyl β-D-xylopyranoside (MeUβX) and 4-methylumbelliferyl β-D-glucopyranoside (MeUβG), but not against 4-methylumbelliferyl α-D-glucopyranoside (MeUαG) (Fig. 4).

The pH and temperature optima of the purified enzyme with PNPhβX as substrate were 5.0 and 60°C, respectively. The same values were obtained for this activity using the crude extract. The PNPhβX-hydrolyzing activity of the crude extract was fully stable on incubation for 3 h at pH values between 4 and 9. By contrast, the purified enzyme was fully stable only at pH 7. After 3 h at pH 4 or 9, activity had dropped to ca. 50 and 80%, respectively, of the original value.

The second-order rate constant for the hydrolysis of PNPhβG (2.1×10^4 M^{-1} s^{-1}) is some 5-fold greater than that for hydrolysis of PNPhβX (4.0×10^3 M^{-1} s^{-1}), and the k_{cat} value some 9-fold greater (118 s^{-1} compared with 13 s^{-1}). The

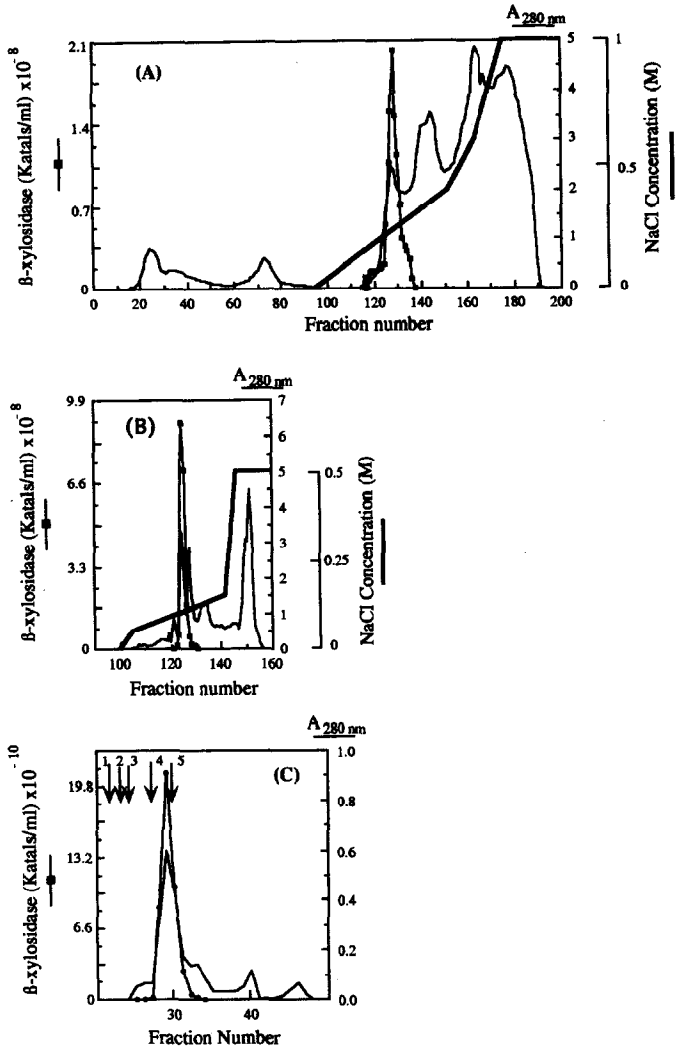

Fig. 1. DEAE-Biogel chromatography (A), Mono Q chromatography (B), and gel filtration chromatography in a Superose 12 column (C) of *Phanerochaete chrysosporium* glucosidase. The M_r standards used in the gel filtration were: 1, beta-amylase (200000); 2, alcohol dehydrogenase (150000); 3, bovine serum albumin (67000); 4, carbonic anhydrase (29000); 5, cytochrome C (12400). For details, see Experimental section.

lower K_m value of the xyloside (3.5 vs. 5.3 mM for the glucoside) may reflect different relative rates of the two chemical steps of this retaining enzyme[24], rather than different affinities.

Apart from activity against PNPhβG and PNPhβX, the purified enzyme hydrolyzed laminaribiose, laminarin, and *o*-nitrophenyl β-D-xylopyranoside (ONPhβX) (Table II). Only low levels of activity were observed on *p*-nitrophenyl α-L-arabinofuranoside (PNPHαAra), methyl β-D-glucopyranoside (MeβG), cel-

TABLE I

Summary of the purification and yield of the enzyme. The values were obtained using PNPX as substrate

Step	Total protein (mg)	Total activity (kat) ($\times 10^{-7}$)	Specific activity (kat/mg of protein) ($\times 10^{-8}$)	Yield (%)	Purification
Culture medium	59	5.5	0.9		1
Ultrafiltration (Bio-2000)	40	5.4	1.3	98	1.4
Ammonium sulfate (70%)	21	3.2	1.5	58	1.6
DEAE-Biogel	2.3	1.8	7.8	33	8.6
Mono-Q	0.4	0.8	20.0	14.5	22.2
Superose 12	0.1	0.6	60.0	11	66.7

lobiose, xylobiose, and xylan. Such low levels could be explained either by low levels of activity of this enzyme or by residual amounts of other activities being present in the preparation. Thus, the primary activity is on β-(1 → 3) rather than β-(1 → 4) linkages. MeUβX and MeUβG were also hydrolyzed (Fig. 4). However, *o*-nitrophenyl α-D-galactopyranoside (ONPhαGa), methyl β-D-xylopyranoside (MeβX), carboxymethylcellulose, arabinogalactan, and MeUαG were not substrates.

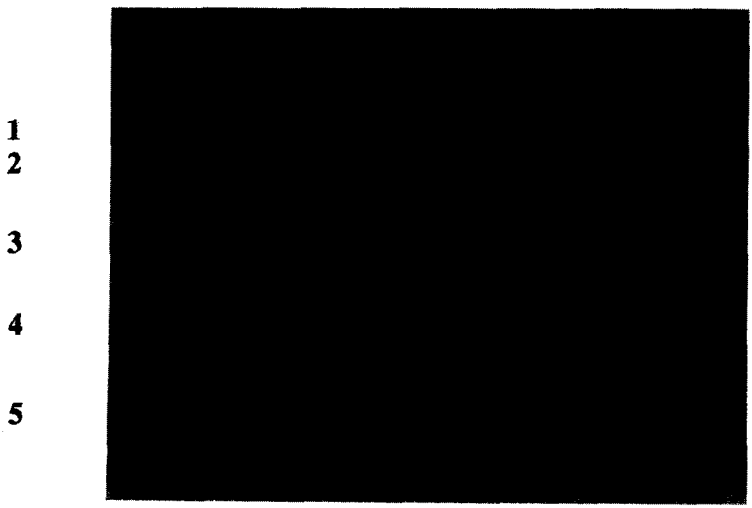

Fig. 2. Molecular weight of *P. chrysosporium* glucosidase. SDS-PAGE was performed as described in the Experimental section. The M_r standards were: 1, phosphorylase B (94000); 2, bovine serum albumin (67000); 3, ovalbumin (43000); 4, carbonic anhydrase (29000); 5, trypsin inhibitor (20100); 6, α-lactalbumin (14400). Lanes A and C: standards (12 μg). Lane B: purified enzyme (0.9 μg).

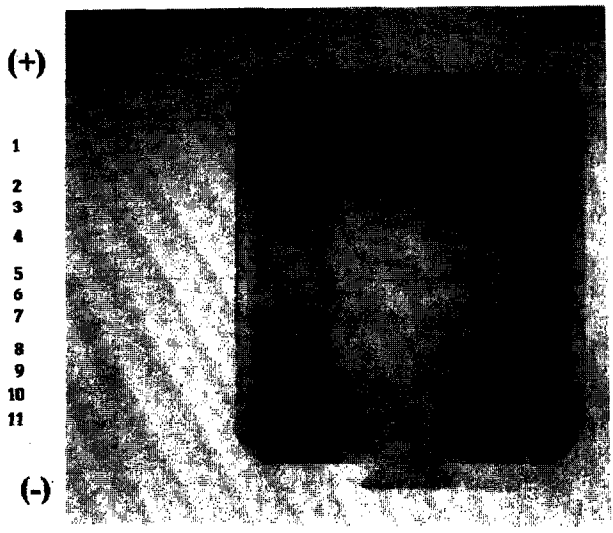

Fig. 3. pI of *P. chrysosporium* glucosidase by PhastSystem electrofocusing. The pI standards were: 1, amyloglucosidase (3.50); 2, soybean trypsin inhibitor (4.55); 3, β-Lactoglobulin A (5.20); 4, bovine carbonic anhydrase B (5.85); 5, human carbonic anhydrase B (6.55); 6, horse myoglobin-acidic band (6.85); 7, horse myoglobin-basic band (7.35); 8, lentil lectin-acidic band (8.15); 9, lentil lectin-middle band (8.45); 10, lentil lectin-basic band (8.65); 11, trypsinogen (9.30). Lanes A and C: standards (200 ng). Lane B: purified enzyme (200 ng).

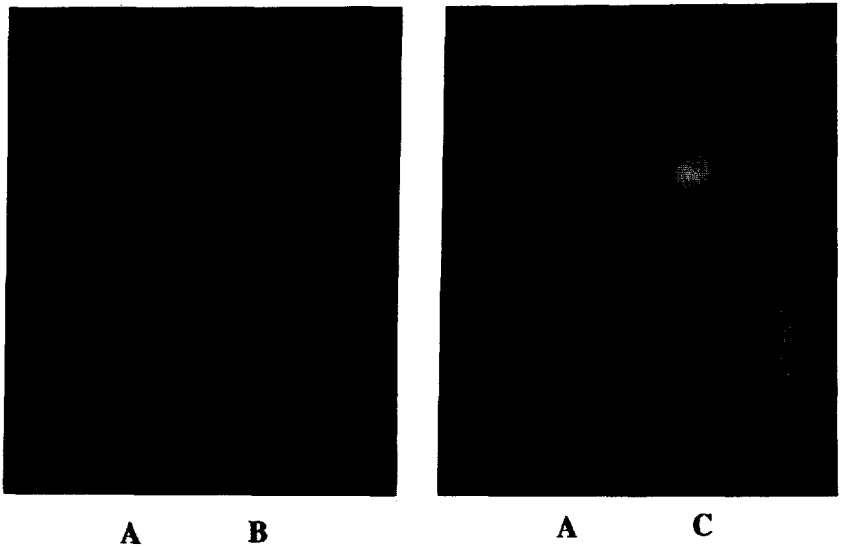

Fig. 4. Electrofocusing gel of *P. chrysosporium* glucosidase stained by activity. For details, see Experimental section. A, MeUβX; B, MeUαG; C, MeUβG. In all cases, 200 ng of purified enzyme were applied in the gels.

TABLE II

Substrate specificity of β-xylosidase

Substrate	Concentration	Relative activity (%)
PNPhβX	10 mM	23
PNPhβG	10 mM	100
ONPhβX	10 mM	13
ONPhαGa	10 mM	0.5
PNPhαAra	10 mM	2.5
MeβX	10 mM	0
MeβG	10 mM	1.63
Cellobiose	0.5 mM	0.79 [a]
	10 mM	2.0 (2.6 [a])
Laminaribiose	10 mM	57.2 (63.0 [a])
Xylobiose	0.5 mM	1
	10 mM	2.7
Arabinogalactan	5 g L^{-1}	0
CMC	5 g L^{-1}	0
Xylan	0.05 g L^{-1}	0.5
	5 g L^{-1}	1
Laminarin	0.005 g L^{-1}	1.62
	0.05 g L^{-1}	10.4 (4.3 [a])
	5 g L^{-1}	92 (66 [a])

[a] Measured by the D-glucose-oxidase method. See Experimental section for details.

Hydrolysis of PNPhβX was lowered slightly (11%) with a high concentration of D-xylose (0.5 M), while action against PNPhβG was unaffected under these conditions. Inhibition of hydrolysis by D-glucono-1,5-lactone and deoxynojirimycin, using either PNPhβX or PNPhβG, was competitive. The K_i values were the same with the two substrates, and were lower with D-glucono-1,5-lactone (32 and 35 μM with PNPhβX and PNPhβG) than with deoxynojirimycin (87 and 69 μM). The effects of different metal ions on enzyme activity were tested. The strongest inhibition (92%) was obtained when Cu^{2+} was present at 3.2 mM. The enzyme was also inhibited by Ag^{+} (41% at 1 mM), by Fe^{2+} (40% at 2.7 mM), and by Hg^{2+} (20% at 5 mM). It was not affected by Mg^{2+} (2.4 mM) or Ca^{2+} (3.8 mM). The extent of inhibition by heavy metals was the same using either PNPhβX or PNPhβG as substrate. This is consistent with the hypothesis that a single protein acts on both substrates.

The *N*-terminal amino acid sequence of this enzyme was found to be: Arg-Asn-Pro-Ile-Asn-Ala-Gly-Phe-. No similar sequence was found on searching the Protein Identification Research database of The National Biomedical Research Foundation.

DISCUSSION

In previous work, we showed that the yield of extracellular β-xylosidase by *Phanerochaete chrysosporium* was enhanced by the inclusion of yeast extract in

medium containing xylan as growth/inducing substrate[7]. During fractionation of culture filtrates, only one major peak of β-xylosidase activity was detected. An apparently single enzyme exhibiting this activity was purified and characterized.

The low M_r value obtained by Superose 12 size-exclusion chromotography contrasts markedly with that obtained using SDS-PAGE. This might be due to adsorption of the enzyme to the Superose 12 matrix, thereby causing it to elute as if it were a smaller molecule. Alternatively, as suggested by Grabski and Jeffries[25], the protein might have an elongated or tapered shape and for this reason be able to diffuse through small pores. This would be an advantageous characteristic since it would permit diffusion of the enzyme in the lignin–cellulose–hemicellulose matrix of wood[25]. Similar differences between M_r values calculated from gel filtration and SDS-PAGE have been observed with other enzymes from the xylanolytic system of *Phanerochaete chrysosporium*[27].

The reported pI values of β-xylosidases range from 3.0 for the enzyme from *Aerobasidium pullulans*[22] to 7.4 for that from *Trichoderma lignorum*[26]. The apparent pI, 4.64, of the *P. chrysosporium* enzyme is within this range and close to the value (4.7) reported for a β-xylosidase from *Trichoderma reesei*[13]. The observed pH optimum, using PNPhβX as substrate, also falls within the range of values reported for other fungal β-xylosidases[13,22,27].

The fact that the enzyme was inhibited by Cu^{2+}, Ag^{+}, and Fe^{2+} might indicate that thiol groups at the active site are involved in binding or catalysis, or that such groups are essential for maintenance of the three-dimensional structure of the active protein.

Specific β-xylosidases i.e., free from β-glucosidase activity, are produced by *Emericella nidulans*[28], *Penicillium wortmanni* IFO 7237[29], and *Sclerotium rolfsii*[16]. However, enzymes with activity against both substrates are commonly found. A β-glucosidase from *Aspergillus niger* hydrolyzed PNPhβX at 2–3% the rate at which PNPhβG was cleaved[30]. An enzyme described as a β-xylosidase/β-glucosidase was isolated from *Chaetomium trilaterale*[31]; the calculated V_{max} of this enzyme against PNPhβX was 3.5% of that against PNPhβG. Various other papers describe β-xylosidases with β-glucosidase activity[22,32].

Fractionation of the 30–70% ammonium sulphate precipitate of *P. chrysosporium* culture filtrate showed that a single enzyme exhibited β-xylosidase activity. Greatest activity was shown against PNPhβG. However, it is clear that the enzyme is not a typical β-glucosidase (EC 3.2.1.21) since activity against cellobiose was relatively low. Similarly, the low activity against xylobiose indicates that it is not primarily a β-xylosidase (EC 3.2.1.37). Indeed, the greatest activities against "natural" substrates were those against laminaribiose and laminarin. Such activity could be due to a laminaribiosidase [which would formerly have been termed a (1 → 3)-β-D-glucoside glucohydrolase], an exo-laminarinase [(1 → 3)-β-D-glucan glucohydrolase; EC 3.2.1.58], or an endo-laminarinase [(1 → 3)-β-D-glucan glucanohydrolase; EC 3.2.1.39]. However, the fact that glucose accounts for the bulk (> 70%) of the products produced from laminarin would indicate that the purified

enzyme is either a laminaribiosidase [(1 → 3)-β-D-glucan glucohydrolase] or an exo-laminarinase. The results of the physicochemical and biochemical studies reported here indicate that a single enzyme is responsible for all of the observed activities. Thus, on the basis of the specificity for natural substrates, plus the stereochemistry of the reaction catalyzed, one would term the enzyme a non-specific laminaribiosidase.

An exo-(1 → 3)-β-D-glucanase from the basidiomycete QM806, later called *Sporotrichum dimorphosporum* ATCC 24562, has been described[33–36]. The M_r value, acidic pI value, the inability to use cellulose or (1 → 4)-β-D-xylan as substrates, plus the capacity to hydrolyze laminarin and laminaribiose (the V_{max} against the latter is 0.3% of that against the former) resemble the observed characteristics of our enzyme. However, the *S. dimorphosporum* enzyme cannot cleave PNPhβX or xylobiose. *P. chrysosporium* is the perfect state of *Sporotrichum pulverulentum*, so it was interesting to compare the mechanisms of action of these two enzymes. We have shown that their hydrolyses of β-D-glucopyranosyl fluoride have opposite stereochemistries, being respectively inverting and retaining for the *S. dimorphorosporum* and *P. chrysosporium* enzymes[24].

No homology between the *N*-terminal 8-residue sequence of our enzyme and those of other enzymes was uncovered on a search of a database. Thus, although large variations in the *N*-terminal sequences of otherwise related proteins have been reported before, the unusual substrate specificity and the lack of any relatedness of the *N*-terminal sequence to those of other proteins suggest that we have isolated a novel enzyme.

Ruel et al.[15] reported that the sheath surrounding *P. chrysosporium* is a (1 → 3)-(1 → 6)-β-D-glucan. These authors suggested that the sheath might function in adsorbing the enzymes that catalyze the degradation of wood and, following its hydrolysis by (1 → 3)-β-D-glucanases, that it could allow the immobilized proteins access to the site of wood degradation. It is possible that the enzyme we describe could effect sheath hydrolysis in vivo as well as participating directly in the conversion of cellulose and hemicellulose when suitable conditions obtain.

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