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Purification by Immunoaffinity Chromatography, Characterization, and Structural Analysis of a Thermostable Pyranose Oxidase from the White Rot Fungus *Phlebiopsis gigantea*

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A moderately thermostable pyranose oxidase (PROD) was purified to apparent homogeneity with a yield of 71% from mycelium extracts of the white rot fungus *Phlebiopsis gigantea* by an efficient three-step procedure that included heat treatment, immunoaffinity chromatography, and gel filtration on Superdex 200. PROD of *P. gigantea* is a glycoprotein with a pI between pH 5.3 and 5.7. The relative molecular weight (M_r) of native PROD is 295,600 ± 5% as determined by four independent methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PROD revealed two distinct but similar stained bands corresponding to polypeptides with M_rs of 77,000 and 70,000, suggesting a heterotetrameric enzyme structure. The tetrameric structure of PROD was confirmed by electron microscopic examinations, which additionally showed the ellipsoidal shape (4.6 by 10 nm) of each subunit. Spectral analyses and direct determinations showed the presence of covalently bound flavin adenine dinucleotide with a stoichiometry of 3.12 mol/mol of enzyme. A broad pH optimum was determined in the range pH 5.0 to 8.0 in 100 mM sodium phosphate, and the activation energy for D-glucose oxidation was 24.7 kJ/mol. The main substrates of PROD are D-glucose, L-sorbose, and D-xylose, for which K_m values 1.2, 16.5, and 22.2 mM were determined, respectively. PROD showed high stability during storage. In 100 mM sodium phosphate (pH 6.0 to 8.0), the half-life of PROD activity was >300 days at 40°C, >110 days at 50°C (pH 7.0), and 1 h at 65°C.

Pyranose oxidase (PROD) (EC 1.1.3.10) is a fungal enzyme that catalyzes the C-2 oxidation of several aldopyranoses, with the preferred substrate being D-glucose. The electrons of substrate oxidation are transferred to molecular oxygen, resulting in the formation of hydrogen peroxide (6). PRODs are widely distributed among white rot fungi (7, 13–15, 22, 37), in which they are supposed to participate in lignin decomposition by supplying peroxidases with hydrogen peroxide (4). Only recently it was shown that in submerged cultures of the white rot fungus *Peniophora gigantea*, PROD is expressed when the activity of an alcohol oxidase declines (5). Because of their selective mode of action, PRODs were used as highly efficient biocatalysts in synthetic carbohydrate chemistry for the production of industrially significant compounds (9, 10, 12, 17, 20, 27, 38). In addition, analytical applications of PROD have been reported in clinical chemistry (35, 39) as well as in industrial-process monitoring (16). Altogether, the biotechnical importance of PRODs is reflected by the existence of ample patent literature on these enzymes (27). Although PROD plays a prominent physiological role in white rot fungi, and many applications for PROD exist, the enzyme itself is relatively poorly investigated, and the biochemical data so far available suggest some inconsistencies among PRODs from different fungal sources with respect to a number of structural properties (6). In this contribution, we describe a rapid and highly efficient method for isolating PROD from mycelium extracts of the white rot fungus *Phlebiopsis gigantea*, with immunoaffinity chromatography as the central step, and suggest its general application to the isolation of immunologically related PRODs from various sources. We also describe for the first time the structure of PROD on the basis of biochemical and electron microscopic data.

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MATERIALS AND METHODS

Organism and growth. The organism used in this study was a basidiomycetous fungus isolated from forest soil (7). It was classified as the white rot fungus *Phlebiopsis gigantea* by the German Culture Collection (DSM), Braunschweig. The fungus was grown in a medium consisting of 1% (wt/vol) malt extract, 0.5% (wt/vol) yeast extract, and 0.5% (wt/vol) (each) polyols sorbitol, mannitol, dulcitol, and xylitol. Solid media were prepared by adding 1.5% (wt/vol) agar. Stock cultures were grown on agar slants or agar plates and stored at 4°C. Liquid cultures were grown as surface cultures in 1-liter Erlenmeyer flasks containing 250 ml of medium. After 14 days of incubation without agitation at 30°C, the mycelia were harvested by filtration. The mycelia were washed twice with 25 mM sodium phosphate (pH 7.0) and then stored frozen at −20°C. The average yield was about 4 g of dry mycelium per liter.

Enzyme assay. PROD activity was determined spectrophotometrically at 420 nm by a coupled chromogenic assay (6). The reaction mixture contained 100 μmol of potassium phosphate (pH 6.5), 1 μmol of ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)], 2 U of peroxidase (EC 1.11.1.7), and 5 to 20 mU of PROD in a final volume of 1 ml at 30°C. The reaction was started by the addition of 100 μmol of sugar substrate (preferably D-glucose). Two molecules of ABTS are oxidized per molecule of hydrogen peroxide reduced. One unit of pyranose oxidase was defined as the amount of enzyme required to oxidize 2 μmol of ABTS per min under standard assay conditions.

Under certain conditions, PROD activity was measured as the rate of oxygen consumption with an oxygen electrode (Rank Brothers Ltd.) in a thermostatically controlled vessel at 30°C (6). The vessel contained 300 μl of sodium acetate (pH 5.0) and 300 mU of PROD in a final volume of 3 ml. The reaction was started by addition of 300 μmol of D-glucose in assay buffer. One unit of enzyme was defined as the amount that consumed 1 μmol of O_2 per min under standard assay conditions. Protein concentrations were determined with bovine serum albumin (BSA) as the standard (21).

Preparation of antibodies and immunoadsorbent. The antiserum was obtained by immunizing a rabbit with PROD of the white rot fungus *Bjercandera adusta* (29). Antibody activity was tested by the double-immunodiffusion assay (25). The immune serum was applied to a protein A-Sepharose CL-4B column (1 by 10 cm; Pharmacia) (8) equilibrated with 25 mM sodium phosphate (pH 7.0) containing

150 mM NaCl and 0.02% NaN_3. The immunoglobulin G was eluted with 100 mM glycine (pH 3.0) and collected in 1-ml fractions in tubes containing 1.25 ml of 1 M sodium phosphate (pH 8.0). The immunoadsorbent was prepared by incubating crude immunoglobulin G (197 mg) with 22 ml of Affi-Gel HZ hydrazide gel (Bio-Rad) according to the manufacturer's instructions. Under these conditions, 122 mg of immunoglobulin G with a high antigen-binding capacity was coupled to the gel (19). After chromatography, the column was prepared for repeated use by washing it with 5 volumes of equilibration buffer (see purification step 2 below). No significant loss of the column capacity was observed during eight cycles of operation.

Purification of pyranose oxidase. After 14 days of growth, the mycelia were harvested by filtration and washed with 25 mM sodium phosphate (pH 7.0). About 100 g (wet weight) of mycelium was suspended in 200 ml of the same buffer. Then 200 g of glass beads (0.1- to 0.25-mm diameter) was added, and the mycelia were homogenized in a Waring blender for 10 min, with interruptions for chilling to about 4°C. Subsequently, the homogenate was centrifuged for 30 min at 54,000 × *g* and 4°C.

(i) Step 1. The mycelium extract was heated to 60°C in a water bath and maintained at that temperature for 10 min with gentle stirring. Then the suspension was chilled on ice, and the precipitate was removed by centrifugation as described above.

(ii) Step 2. The supernatant from step 1 was applied at a rate of 30 ml/h to the immunoaffinity column (1.6 by 20 cm) equilibrated with 20 mM sodium phosphate (pH 7.0) containing 500 mM NaCl and 0.02% NaN_3. Unbound material was eluted with 600 ml of the same buffer until the A_{280} had declined to the baseline. Then PROD was eluted at a rate of 120 ml/h with 100 mM glycine (pH 11.0) containing 10% (vol/vol) ethylene glycol and 0.02% NaN_3. Fractions (2.5 ml) were collected in tubes containing 0.25 ml of 1 M sodium acetate (pH 4.0) to adjust the pH to a neutral value (see Fig. 1).

(iii) Step 3. The fractions with high PROD activities were combined, concentrated by ultrafiltration with a PM-10 membrane (Amicon) to a volume of about 2 ml, and then applied to a Superdex 200 column (1.6 by 60 cm) equilibrated with 25 mM sodium phosphate (pH 7.0). PROD was eluted in 1-ml fractions at a flow rate of 60 ml/h. Fractions 60 to 69 were combined, filter sterilized, and stored at 4°C.

Molecular mass determinations. The Stoke's radius of PROD was determined by gel filtration on Sephacryl S-300 with fast protein liquid chromatography (FPLC), using standards of known Stoke's radius, such as ferritin (6.10 nm), catalase (5.22 nm), aldolase (4.74 nm), and BSA (3.70 nm) (33). The column (1.6 by 50 cm) was equilibrated with 25 mM sodium phosphate (pH 7.0) containing 100 mM NaCl and eluted at a flow rate of 60 ml/h. Sedimentation coefficients were obtained by ultracentrifugation with a Spinco model E analytical ultracentrifuge (Beckman Instruments). After 5 h of centrifugation at 10°C and 140,000 × *g*, the sedimentation coefficient was determined with aldolase ($s_{20,w}$ = 7.35) and catalase ($s_{20,w}$ = 11.3) being used as reference materials (23, 28).

A Superose 6 column (1 by 30 cm) was equilibrated with 25 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. The column was calibrated with the standards ferritin (M_r = 440,000), catalase (M_r = 232,000), aldolase (M_r = 158,000), BSA (M_r = 67,000), and ovalbumin (M_r = 43,000), each at a concentration of 0.4 mg/ml. The flow rate was 24 ml/h.

Gradient polyacrylamide gel electrophoresis (PAGE) (2 to 15% acrylamide) was performed in vertical slab gels (10 by 7 by 0.1 cm) in 25 mM Tris-glycine (pH 8.3) at 100 V for 15 h (1). The standards used were lactate dehydrogenase (M_r = 140,000), catalase (M_r = 232,000), ferritin (M_r = 440,000), and thyroglobulin (M_r = 660,000). Sodium dodecyl sulfate (SDS) gradient PAGE (5 to 20% acrylamide) was performed in vertical slab gels as described above at a current of 20 mA for 3 h (18). The subunit standards trypsin inhibitor (M_r = 20,100), triosephosphate isomerase (M_r = 26,626), aldolase (M_r = 39,212), glutamate dehydrogenase (M_r = 55,562), fructose-6-phosphate kinase (M_r = 85,204), and β-galactosidase (M_r = 116,353) were used. The silver staining (24) and Serva Blue G methods were used for protein staining.

Electron microscopic examinations. Purified PROD was diluted in 20 mM potassium phosphate (pH 7.0) to a concentration of 50 μg of protein per ml. From this solution, the enzyme molecules were adsorbed to an ultrathin carbon film, rinsed with water, and then negatively stained with 4% (wt/vol) uranyl acetate, pH 4.5 (36). The carbon film was mounted on a 400-mesh copper grid and air dried. The samples were examined with a Zeiss CEM 902 transmission electron microscope at calibrated magnifications with an acceleration voltage of 80 kV. Molecular weights were calculated according to the relation 1 Da ≅ 1.37×10^{-3} nm^3 (42).

Analytical PAGE and isoelectric focusing. Analytical PAGE was performed in 7.5 or 10% vertical slab gels (10 by 7 by 0.1 cm) in 25 mM Tris-glycine (pH 8.3) at a current of 20 mA for 1.5 h. For activity staining of PROD, the gel was incubated for 3 min in 40 mM sodium-potassium phosphate containing 5 mM *o*-dianisidine, 100 mM D-glucose, and 200 U of peroxidase. Isoelectric focusing of PROD was performed with Servalyt Precotes 3-10 (12.5 by 12.5 by 0.125 cm) according to the supplier's instructions (Serva). The anode solution contained 3.3 g of aspartic acid and 3.7 g of glutamic acid per liter of water. The cathode solution contained 3 g of arginine, 4 g of lysine, and 120 ml of ethylenediamine per liter of water. The initial voltage was 200 V and the final voltage was adjusted to 1,500 V, with a final power of 4 W after 4 h (power supply 2197; Pharmacia-LKB).

TABLE 1. Purification of PROD from *Phlebiopsis gigantea*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Mycelium extract	97.3	884.5	0.11	1.0	100
Heat treatment	88.5	158.1	0.56	5.1	91
Immunoaffinity chromatography	71.9	9.6	7.46	67.8	74
Superdex 200	69.1	8.5	8.15	74.1	71

Densitometry. Peptide bands in SDS gradient gels were stained with Serva Blue G and scanned with a gel scanner (model 1312; Isco) linked with an integrator (Chromatopac; Shimadzu).

Determination of flavins. PROD (5.92 mg) in 2 ml of 50 mM Tris-HCl (pH 7.5) was digested by incubation with 2 mg of pronase per ml for 20 h at 37°C (40). The digestion was terminated by heating the solution for 5 min at 100°C. After removal of the precipitate by centrifugation (15 min at 4°C and 15,000 × *g*), the supernatant was lyophilized and then resuspended in 0.4 ml of deionized water. The solution was treated with 25 μg of phosphodiesterase for 40 min at room temperature. The reaction was stopped by boiling the solution for 5 min, and the precipitate was removed by centrifugation as described above. The supernatant was used for AMP determination. As a control experiment, 57 nmol of authentic flavin adenine dinucleotide (FAD) was treated in the same manner to evaluate the efficiency of AMP release from FAD. AMP was determined by high-performance liquid chromatography (HPLC) (26) on a Nucleosil 120-3 C_{18} column (Macherey & Nagel), with 10% methanol in 0.1 M sodium phosphate (pH 6.5) being used as the mobile phase, at a flow rate of 0.8 ml/min. AMP was monitored at 254 nm. A calibration curve was established showing a linear range from 1 to 500 μM AMP.

Glycoprotein staining. Following PAGE of PROD, the enzyme was treated with periodic acid and Schiff's reagent (30).

Determination of starting materials and reaction products. Sugars and keto sugars were determined by comparison with authentic compounds by the use of HPLC and thin-layer chromatography (TLC) as described recently (12).

Chemicals and enzymes. Enzymes, coenzymes, and SDS-protein standards were from Boehringer GmbH. All other protein standards were purchased from Pharmacia. The chemicals were of the highest grade of purity and were purchased from Fluka.

RESULTS

Growth of *Phlebiopsis gigantea* and production of PROD. The white rot fungus *Phlebiopsis gigantea* produced PROD when grown as a surface culture but not under submerged conditions in a fermentor or in an Erlenmeyer flask with shaking. In surface cultures, PROD activity increased with increasing mycelium mass, reaching a maximum level of 100 U/liter after 14 days, when the culture reached stationary phase. In stationary phase, the appearance of PROD activity correlated with dry-mycelium and protein concentrations of 4 and 0.8 g/liter, respectively. During growth, the pH of the culture medium decreased steadily from 6.3 to 5.0 at stationary phase. Mycelia from early stationary phase were used for PROD purification.

Purification of PROD. The results of a representative purification of PROD (see Materials and Methods) are summarized in Table 1. PROD was purified 74-fold to a specific activity of 8.2 U/mg with a yield of 71%. The most efficient step of the procedure was immunoaffinity chromatography (Fig. 1), which resulted in a 13-fold purification of PROD from the preceding step. With the final gel filtration on Superdex 200 (data not shown), traces of contaminating protein were removed to yield an apparently homogeneous PROD preparation as shown by analytical PAGE (Fig. 2A).

Properties of PROD. The M_r of the native PROD was determined to be 295,600 ± 14,700 by four independent methods (Table 2). On the basis of the estimated values of the Stoke's radius (5.5 nm) and sedimentation coefficient ($s_{20,w}$ = 11.2), an M_r of 273,700 was calculated (33). Two distinct peptide bands were detected by SDS gradient PAGE, correlating with M_rs of

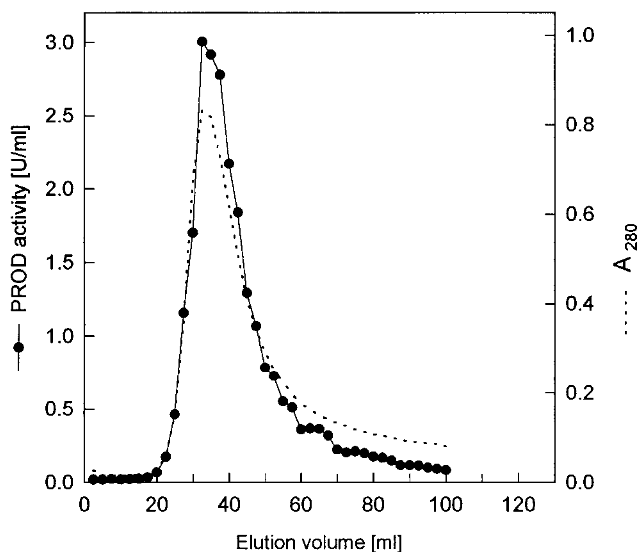


FIG. 1. Elution profile of *Phlebiopsis gigantea* PROD obtained by immunoaffinity chromatography. Experimental details are described in Materials and Methods.

77,000 and 70,000 (Fig. 2B). Densitometric scans of the gels revealed that the two peptides were present in similar mass ratios. On the basis of these findings and the M_r of native PROD, we suggest that the *Phlebiopsis gigantea* PROD is a heterotetramer composed of two subunits of M_r 70,000 and two subunits of M_r 77,000. The tetrameric structure of PROD was confirmed by electron microscopic examinations, which showed that the enzyme is composed of four ellipsoidal subunits (Fig. 3A). The tetrameric structure was clearly depicted in the top views of the enzyme, and the elongated morphology of the subunits was evident in the front views. If the subunits were assumed to be rotational ellipsoids with half-axes of 2.3, 2.3, and 5.0 nm, their calculated M_r was 80,900, which coincided well with the biochemical data. A similar structure was also found for the PRODs of *Peniophora gigantea* and the basidiomycete strain 167Z (Fig. 3B and C).

Homogeneous PROD solutions had a yellow color and exhibited the characteristic spectral properties of flavoproteins, with absorption maxima at 275, 350, and 455 nm (Fig. 4). In the presence of substrate or after treatment of the enzyme with reducing agents, the absorption maximum at 455 nm was absent. Treatment with 5% trichloroacetic acid and heat did not release the flavin moiety from the enzyme, suggesting that it is covalently linked to PROD (34). Therefore, 5.9 mg (20 nmol) of PROD was proteolytically digested and then incubated with phosphodiesterase to release AMP from FAD (3, 40). AMP was determined by HPLC (26) through comparison with authentic AMP (see Materials and Methods). It was calculated that 62.4 nmol of AMP was produced per 20 nmol of PROD, corresponding to 3.12 nmol of FAD per nmol of PROD.

TABLE 2. Determination of the M_r and subunit composition of PROD from *Phlebiopsis gigantea*

Method of determination	M_r of: Enzyme	M_r of: Subunit	Number of subunits
Calculation[a]	273,700	ND[b]	ND
Gel filtration on Superose 6	280,000	ND	ND
Native gradient gel electrophoresis	305,000	ND	ND
Electron microscopy	323,600	80,900	4
SDS gradient gel electrophoresis	ND	77,000	2
		70,000	2

[a] On the basis of the Stoke's radius and sedimentation coefficient.
[b] ND, not determined.

The isoelectric point of PROD was determined to be between pH 5.3 and 5.7 by isoelectric focusing.

In order to assess whether PROD is a glycoprotein, the enzyme was treated with periodate and Schiff's reagent following PAGE (30) and then incubated in double-diffusion assays (25) with a lectin from *Momordica charantia* which reacts specifically with D-galactosyl and D-galactosylamine residues (2) and concanavalin A, which is specific for D-mannosyl and D-glucosyl residues (11). PROD gave clear staining reactions in gels when >250 μg of protein was present. In double-diffusion assays, the lectins did not precipitate PROD (150 μg of protein) while reference glycoproteins such as thyroglobulin and glucose oxidase from *Aspergillus niger* were readily precipitated by concanavalin A. These findings indicate that PROD may have little or no glycosylation.

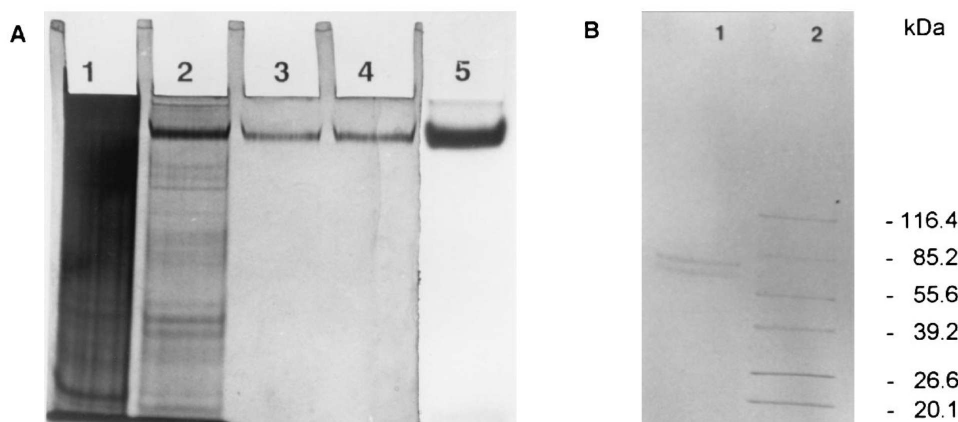
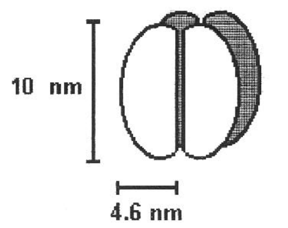
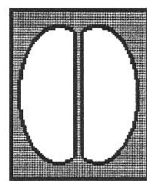
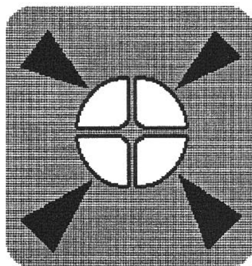
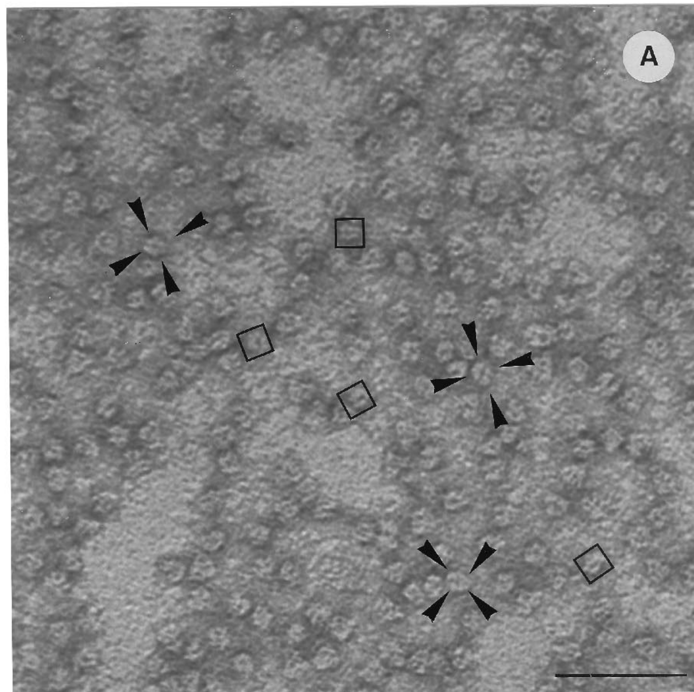


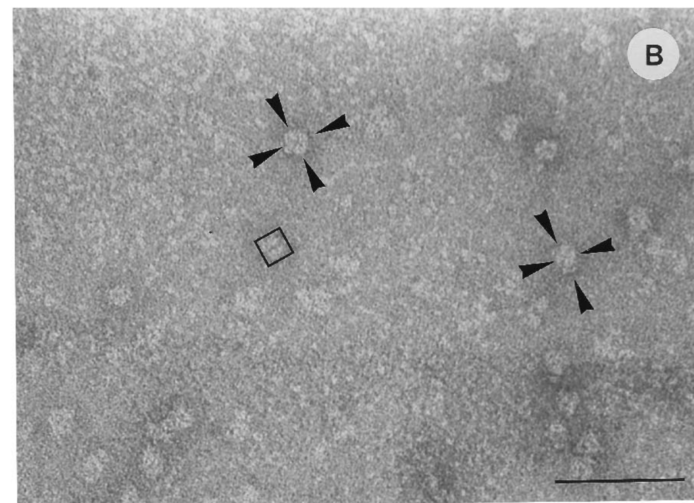
FIG. 2. PAGE of *Phlebiopsis gigantea* PROD fractions. (A) Native PAGE, with a 25-μl sample of each of the purification fractions being applied to lanes 1 to 5 of a 10% polyacrylamide slab gel. Lanes: 1, mycelium extract, 30 μg of protein; 2, heat-treated extract, 20 μg of protein; 3 and 5, immunoaffinity chromatography, 3 μg of protein each; 4, Superdex 200, 3 μg of protein. Lanes 1 to 4 were silver stained, and lane 5 was activity stained. (B) SDS gradient PAGE on a linear-gradient slab gel of 5 to 20% acrylamide. Lane 1, 2 μg of PROD; lane 2, peptide standards (with sizes at right). For experimental details, see Materials and Methods.











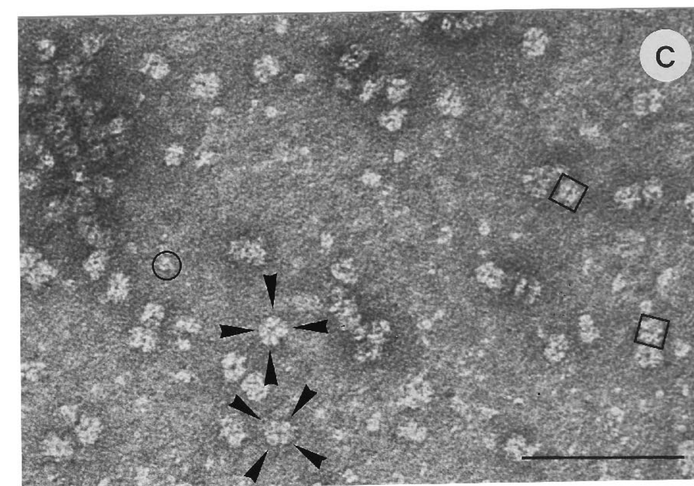


FIG. 3. Electron micrographs of negatively stained PRODs from *Phlebiopsis gigantea* (A), *Peniophora gigantea* (6) (B), and the basidiomycete strain 167Z (C). Arrowheads indicate the top views and boxes show the front views of the tetrameric structures. Bars, 50 nm.

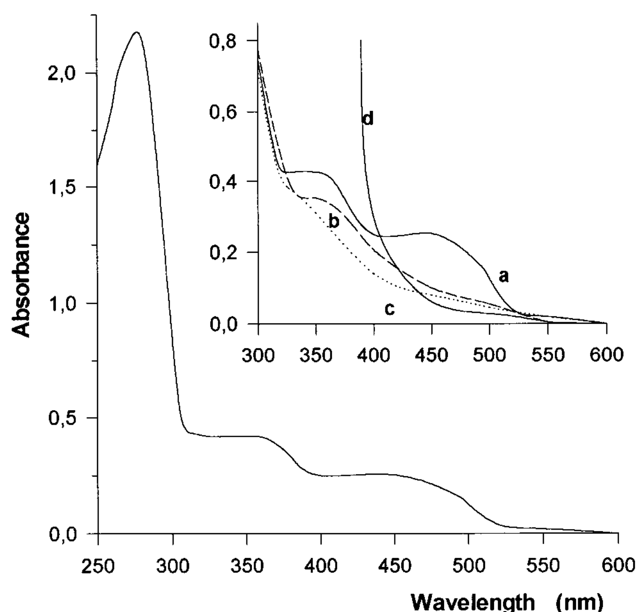


FIG. 4. Absorption spectrum of pyranose oxidase from *Phlebiopsis gigantea*. The absorption spectrum of purified PROD was analyzed with a Hitachi U 2000 spectrophotometer and quartz cuvettes. The protein concentration was 1.6 mg/ml in 25 mM sodium phosphate (pH 7.0). The large representation shows the absorption spectrum of the oxidized enzyme. The inset shows the spectrum of the untreated enzyme (a) and the spectra of the enzyme in the presence of 100 mM D-glucose (b), 100 mM $NaHSO_3$ (c), and 100 mM Na_2SO_4 (d).

The reaction velocity of PROD increased with increasing temperature, showing a maximum at 65°C. Appropriate controls showed that peroxidase present in the coupled assay (see Materials and Methods) did not influence the reaction at elevated temperatures. From the linear range of an Arrhenius plot, an activation energy of 24.7 kJ mol^{-1} for glucose oxidation was calculated.

Storage stability of PROD was studied in 100 mM sodium citrate (pH 3 to 5), sodium phosphate (pH 6 to 7), and Tris-HCl (pH 8 to 10). Irrespective of the pH, the enzyme was inactivated by freezing and thawing. The enzyme was relatively unstable at 4°C in the pH range 3.0 to 6.0 but exhibited a half-life of >300 days at pH 7.0 to 10.0. At room temperature and 30°C, a half-life of >300 days was determined within the pH range 5.0 to 10.0 at 30°C and within the pH range 5.0 to 8.0 at 40°C. At 50°C, the half-lives at pH 6.0, 7.0, and 8.0 were 90, 110, and 70 days, respectively. At 60, 65, and 70°C (at pH 7.0), the half-lives were 180, 50, and 5 min, respectively.

The pH optimum of PROD, as determined in 100 mM potassium phosphate, was relatively broad, ranging from pH 5.0 to 7.5.

The substrate specificity of PROD is shown in Table 3. D-Glucose, L-sorbose, and D-xylose are the preferred substrates of PROD. The saturation curves for substrate oxidation were hyperbolic, and the double-reciprocal plots were linear. The apparent K_m values determined for D-glucose, L-sorbose, and D-xylose were 1.2, 16.5, and 22.2 mM, respectively. The products of substrate oxidation were identified to be 2-keto-D-glucose (D-arabino-hexos-2-ulose), 5-keto-D-fructose (D-threo-hexos-2,5-diulose), and 2-keto-D-xylose (D-threo-pentos-2-ulose) by comparison with authentic keto sugars by HPLC and TLC (6, 12).

In order to determine the direct influence of various metal salts, thiol reagents, reducing reagents, and chelators on PROD activity, an assay was performed (see Materials and Methods) in which the oxygen consumption was measured with an oxygen electrode. This assay excludes possible interactions of the compound tested with peroxidase present in the chromogenic assay mixture. At concentrations of 1 mM, the metal salts $MgSO_4$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, and $ZnSO_4$ had no significant effects on the activity of PROD. The effect of $FeSO_4$ was tested in the chromogenic assay because it is oxidized by oxygen. No interfering effect of $FeSO_4$ in concentrations up to 1 mM could be observed in this assay. Of the chelating reagents, each at a concentration of 10 mM, EDTA, α,α'-dipyridyl, *o*-phenanthroline, Tiron, and NaN_3 had no inhibitory effects on PROD activity. Of the thiol reagents, each at a concentration of 1 mM, *p*-chloromercuribenzoate and bromosuccinimide were completely inhibitory but iodoacetic acid had no effect.

DISCUSSION

In a previous study on PROD from the white rot fungus *Peniophora gigantea*, we pointed out structural differences among PRODs from various fungal sources with respect to size, subunit composition, and glycosylation (6). References were made to three homogeneous PRODs from *Coriolus versicolor* (22), the basidiomycetous fungus no. 52 (14), and *Phanerochaete chrysosporium* (37). In view of the important physiological role of PROD in white rot fungi (4) and its biotechnical importance (9, 12, 27), we wanted to clarify some unanswered questions by characterizing another PROD from a newly isolated white rot fungus, *Phlebiopsis gigantea*. A very efficient purification procedure involving heat treatment and immunoaffinity chromatography was used. This procedure generally can be applied to the isolation of immunologically related PRODs from other fungal sources. The final PROD preparation was homogeneous, as shown by native PAGE, in which a single protein band correlated with a single activity stain band. On the other hand, SDS-PAGE yielded two distinct peptide bands, indicating the presence of two types of subunit in the enzyme complex. Since the difference in sizes of the two polypeptides is relatively small and all other PRODs are composed of uniform types of subunits, it cannot be ruled out that the different mobilities of the two peptides were due to unequal glycosylation of the PROD subunits, which is known to influence molecular weight determinations in SDS gels (30). To date, data on six homogeneous PRODs from different fungal sources are available (6, 14, 22, 32, 37), including an enzyme from *Pleurotus ostreatus* designated as a glucose oxidase (32). This enzyme is very similar to PRODs from other white rot fungi but is different from glucose oxidases from ascomycetes with respect to size, subunit composition, and substrate specificity

TABLE 3. Substrate specificity of PROD from *Phlebiopsis gigantea*[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}[b] (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	Relative activity (%)
D-Glucose	8.2	40.5	1.2	33.8×10^3	100.0
L-Sorbose	7.9	38.9	16.5	2.4×10^3	96.3
D-Xylose	3.6	17.7	22.2	0.8×10^3	43.7
D-Galactose	0.7	ND[c]	ND	ND	8.5
Methyl-β-D-glucoside	2.2	ND	ND	ND	26.3
Methyl-α-D-glucoside	0.25	ND	ND	ND	3.0

[a] Initial reaction velocities were determined in the chromogenic assay (see Materials and Methods).
[b] k_{cat}, turnover number of the enzyme.
[c] ND, not determined.

(34, 41). PRODs are high-molecular-weight flavoenzymes (M_r, ~300,000) consisting of four subunits (M_r, ~70,000) of identical size. The only exception is the PROD of *Coriolus versicolor*, which has an M_r of 220,000 and presumably consists of three subunits (22). FAD is the cofactor of all PRODs investigated. It has been demonstrated to be covalently linked to PROD, and the stoichiometric data suggest the presence of one FAD molecule per subunit (6, 32). From our results we conclude that FAD is covalently attached to PROD of *Phlebiopsis gigantea*, but the stoichiometry only roughly corresponds to the theoretical value. Investigated PRODs have been shown to be glycoproteins (6, 14), although the sugar moiety seems to be relatively small. A carbohydrate content of 0.7% has been determined for PROD from the basiodiomycetous fungus no. 52 (14). Whether the *Phlebiopsis gigantea* PROD is a glycoprotein cannot be definitively answered. While Schiff's reagent gave a positive reaction, the two lectins tested did not precipitate the enzyme. It is plausible that Schiff's reagent reacted nonspecifically with the protein, but it is rather unlikely that concanavalin A does not react with its target sugars D-mannose and D-glucose which generally represent the largest proportion of carbohydrates in glycoproteins (11). Most PRODs have a relatively broad pH optimum ranging from weakly acidic to weakly alkaline. This allows the use of the enzyme at neutral pH for bioconversions of a broad range of sugars into the corresponding relatively unstable keto sugars (9, 12, 20). Interestingly, the temperature optima of most of the PRODs are relatively high, with a maximum of 65°C for the *Phlebiopsis gigantea* enzyme. Also, the thermal stability is relatively high. At 50°C, the half-life of the *Phlebiopsis gigantea* PROD is 100 days, and even at 70°C, the *Pleurotus ostreatus* enzyme is stable for 2 h. However, there are significant differences among PRODs with respect to operational stability, a most important criterion for biotechnological use of an enzyme. While both products of D-glucose oxidation, 2-keto-D-glucose and the byproduct H_2O_2, caused rapid inactivation of the PROD from *Polyporus obtusus* (31), this was not the case for the *Peniophora gigantea* PROD, which has an economically useful half-life of 9 days under the operating conditions found in an enzyme reactor (12) and in an enzyme cartridge for process monitoring (16). Other differences among PRODs may be attributable to their primary structures and are reflected by the differences in their isoelectric points, activation energies, and immunologic specificities (13).

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