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Isolation and characterization of novel pI 4.8 MnP isoenzyme from white-rot fungus *Irpex lacteus*

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ABSTRACT

Growth of *Irpex lacteus* on polyurethane solid support led to secretion of various manganese-dependent peroxidase (MnP) isoenzymes with pI in the range of 3.8–6.7. The highest MnP activity was obtained at 8.9 mmol l^{-1} Mn^{2+} when predominantly a pI 4.8 isoenzyme was produced. Optimization of anion exchange chromatography (Mono Q) allowed the separation of MnP isoenzymes and collection of extensive sequence information by tandem LC–MS. The pI 4.8 isoenzyme was purified 109-fold (6% activity yield) by ion exchange chromatography, its specific activity of Mn^{2+} oxidation was 2800 nkat mg^{-1}, molecular mass 37 kDa and pH optimum 5. The other properties were: respective half-lives (pH 4.5) of 575, 325 and 7 min at 40, 50 and 60 °C, K_m values 46.7; 9.5; 21.4 μmol l^{-1} and k_{cat} values 69.0; 64.2; 15.7 s^{-1} for Mn^{2+}, H_2O_2 and DMP with Mn^{2+}, respectively. The pI 4.8 isoenzyme was shown to be a true MnP affecting secondary substrates via the oxidation of Mn^{2+}. Its biochemical properties and production by *I. lacteus* make the enzyme a candidate for biotechnological applications.

1. Introduction

White-rot fungus *Irpex lacteus* is a potential candidate for use in bioremediation *in situ*, because of its ability to efficiently remove organopollutants during growth, colonize contaminated soil, and grow as a co-culture with the indigenous microflora [1–3]. The main enzyme during biodegradation of synthetic dyes in liquid cultures was manganese peroxidase, Mn(II):hydrogen peroxide oxidoreductase (MnP, EC 1.11.1.13), while lignin peroxidase (LiP, EC 1.11.1.14), laccase (EC 1.10.3.2) and an unclassified enzyme named RBBR oxidase were also detected [4–6].

Generally, white-rot fungi have been shown to effectively degrade a wide range of organopollutants in aqueous cultivation media [7]. Degradation of pollutants has been correlated with the simultaneous secretion of extracellular oxidative enzymes, however, relative contributions of individual enzymes may differ in different fungi [8–10]. In order to provide theoretical background for the use of *I. lacteus* in bioremediation, a detailed characterization of main oxidoreductive enzymes and their isoforms is needed. LiP has already been purified and characterized [11]. Recently, an isoform of MnP having a pI of 3.7 was partially characterized [3,12] and its role in the biodegradation of synthetic dyes specified [6,13,14]. Shin et al. [12] and Baborova et al. [3] isolated the acidic MnP isoenzyme but other isoenzymes were not detected. Another study described the synthesis of six isoforms of MnP in semisolid state cultures of *I. lacteus* growing at 65 μM or 2.9 mM Mn^{2+} without isolating or quantifying any of them [15]. The total amount of all isoforms characterized by pI values of 3.28, 3.50, 3.75, 3.81, 5.88 and 6.04 was rather low to equal to about 1 nkat/ml. The above mentioned purified acidic MnP of *I. lacteus* was proven to decolorize several synthetic dyes and participate in cleavage of PAH aromatic rings [3,12].

The main purpose of our study was to isolate, purify and characterize in detail an unknown pI 4.8 isoenzyme of MnP whose strong production by immobilized *I. lacteus* growing at high Mn^{2+} concentrations was detected at early phases of growth, and investigate its glycosylation. The effect of Mn concentration on the synthesis of various MnP isoenzymes existing in this fungus was also documented and the production of pI 4.8 isoenzyme was optimized. The new primary sequences of all the synthesized MnP isoenzymes were determined and compared with the known ones.

2. Materials and methods

2.1. Strain and culture conditions

I. lacteus Fr. 238 617/93 was obtained from the Culture Collection of Basidiomycetes of the Institute of Microbiology of the ASCR, v.v.i. in Prague (CCBAS). The fungus was maintained at 4 °C on malt extract-glucose agar (malt extract 0.5%, glucose 1%, agar 2%, w/w). The inoculum was prepared by transferring 2–4

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agar plugs (diameter 7 mm) from a fresh culture into 25 ml potato dextrose broth (PDB), pH 4.5, in 250 ml Erlenmeyer flasks, and incubated as static cultures for 3–4 days at 28 °C. The cultures were subsequently homogenized for use as a 10% (v/v) inoculum. Cultivations were carried out in 2–5 l Erlenmeyer flasks containing the medium up to one tenth of their volume. The cultivation medium was a low nitrogen mineral medium (LNMM), pH 4.5 [16], modified to contain 0.1 g l^{-1} ammonium tartrate, 0.105 g l^{-1} nitrilotriacetic acid, 0.107 g l^{-1} $CaCl_2 \times 2H_2O$, 0.035 g l^{-1} $MnSO_4 \times H_2O$ (low Mn^{2+}) or 1.5 g l^{-1} $MnSO_4 \times H_2O$ (high Mn^{2+}), 1 g l^{-1} Tween 80, without $KAl(SO_4)_2 \times 18H_2O$, $Na_2MoO_4 \times 2H_2O$ and without veratryl alcohol. Commercial polyurethane foam was used as the growth support. Before use, the foam was washed three times in a washing machine with tap water at 90 °C, dried at 80 °C, cut into pieces (approx. 5 mm^3), washed with deionized water, dried and added, at 20 g l^{-1}, to the cultivation medium. The total volume of free liquid in a cultivation flask was approximately 1/10 to the foam volume to enable sampling. The medium was autoclaved (120 °C, 20 min) and thiamine was added at 0.001 g l^{-1}. After inoculation, a static cultivation was carried out at 28 °C for 5–10 days, (6 days for pI 4.8 isoenzyme in high Mn^{2+}). In the cultivation in a 1.5-l fermenter (Chemap CMF Mini fermenter, Switzerland) the same conditions as in the flasks were used, however, the solid support was completely submerged in a volume of 1 l of the medium and the aeration at a flow rate of 1 l min^{-1} with a mixture of the air and oxygen (1:1, v/v) was started on the 7th day of cultivation. MnP activity and protein concentration were analyzed daily until the maximum activity was reached. The isoelectric focusing was used to measure MnP isoform content. The culture supernatant was separated from the mycelium and solid support using a juice press and was frozen at −80 °C.

2.2. Analytical methods

Frozen samples (−80 °C) were thawed and centrifuged (10 min, 13,000 × *g*, 4 °C) before analyses. MnP activity was analysed by the oxidation of Mn^{2+} to Mn^{3+} [17]. The enzyme samples were incubated in 50 mmol l^{-1} malonate buffer, pH 4.5, containing 0.2 mmol l^{-1} of $MnSO_4$ and 0.05 mmol l^{-1} of H_2O_2. The absorbance was monitored at 270 nm for the first 5 s of the reaction at 25 °C. The activity was calculated using an extinction coefficient of 11,590 l mol^{-1} cm^{-1}. Laccase and LiP activities were estimated in 50 mmol l^{-1} malonate buffer, pH 4.5, using the oxidation of 5 mmol l^{-1} 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Roche) and 0.02 mmol l^{-1} veratryl alcohol (Aldrich), respectively [18]. The activities were expressed as katals (mol s^{-1}).

The protein amount was determined using the Lowry method (DC Protein Assay Kit, Microplate assay, Bio-Rad), with bovine serum albumin (Sigma) as the standard. Microcon YM-10 centrifugal filter devices (regenerated cellulose membrane, cut-off 10 kDa, Millipore) were used for concentration of samples (20 times) and washing with 50 mmol l^{-1} malonate buffer, pH 4.5.

Isoelectric focusing (IEF) was performed according to the manufacturer's instructions on Ampholine PAG plates, pH 3.5–9.5 (Amersham Biosciences). The enzyme samples were dialysed against water using floating filters (Millipore, "V" Series Membranes, i.d. 25 mm, pore size 0.025 μm). A surface electrode (Ingold) was used to measure the pH gradient of the gel. The samples contained 0.3 nkat per lane. The active bands were visualised with 0.2 mmol l^{-1} ABTS in 50 mmol l^{-1} malonate buffer, pH 4.5, containing 0.05 mmol l^{-1} H_2O_2 and 0.2 mmol l^{-1} $MnSO_4$. The electrophoresis on polyacrylamide gel in denatured conditions (SDS-PAGE) was carried out on 12% Tris/HCl Ready Gels (Bio-Rad). The reference proteins were Prestained SDS-PAGE Standards 6-198 kDa (Bio-Rad). *I. lacteus* proteins were stained with Coomassie Brilliant Blue G-250 (Serva). The groups of isoenzymes are marked. All the fractions seemed to be electrophoretically pure; the second step of separation was employed for the separation of isoenzymes.

2.3. Purification of MnP isoenzymes

Frozen culture filtrates were thawed and centrifuged (20 min, 13,000 × *g* at 4 °C) to remove abundant polysaccharides and concentrated by ultrafiltration. The filtrate obtained in a cultivation with 0.2 mmol l^{-1} Mn^{2+} was concentrated using a cassette-style tangential flow filtration device (Pellicon 2 with a 10 kDa cut-off polyethersulphone membrane, Millipore). The concentrate was fractionated by anion exchange chromatography in a Q Sepharose Fast Flow column (Pharmacia, 1.5 × 8.8 cm) and equilibrated with 20 mmol l^{-1} Bis–Tris buffer, pH 6.7. The proteins were eluted with a linear gradient of 0–1.0 mol l^{-1} NaCl in 150 ml of buffer. The active fractions were pooled, ultrafiltrated (Amicon 8400 ultrafiltration unit, polyethersulphone membrane, cut-off 10 kDa, Millipore) and further purified in a Mono Q HR 5/5 column (Amersham-Pharmacia). The column was equilibrated with a 20 mmol l^{-1} piperazine buffer, pH 5.3, and eluted with a linear gradient of 0.03–0.17 mol l^{-1} NaCl in a volume of 35 ml of buffer. The active fractions were concentrated by ultrafiltration (Microcon YM-10, Millipore).

2.4. Purification and catalytic properties of pI 4.8 MnP isoenzyme

The filtrate from a cultivation with 8.9 mmol l^{-1} of Mn^{2+} was concentrated in an ice bath using an Amicon 8400 ultrafiltration unit. The concentrate was purified at 10 °C in a Q Sepharose column as described above, except that the column size was 3 × 23 cm and a linear NaCl gradient of 0.01–0.5 mol l^{-1} in 1500 ml of buffer was used. The active fractions were concentrated by ultrafiltration (Microcon YM-10, Millipore).

The pH optimum for Mn^{2+} oxidation was determined at 25 °C in the universal McIlvaine buffer within a pH range of 2.6–7.6. The temperature stability was determined by measuring the residual activity after incubation in 50 mmol l^{-1} malonate buffer, pH 4.5, at 40, 50 and 60 °C for 15–210 min.

The Michaelis–Menten curves for determining the K_m and V_{max} values of the purified MnP isoenzyme with a pI of 4.8 were obtained with the GraphPad Prism 3.02 programme (GraphPad Software). k_{cat} values were calculated using a molecular mass of 37 kDa and a protein concentration of 0.12 mg ml^{-1}. Substrate concentrations of 5–200 μmol l^{-1} were used for determination of the K_m value for Mn^{2+} in 50 mmol l^{-1} malonate buffer, pH 4.5, in the presence of 0.05 mmol l^{-1} H_2O_2. When determining the K_m value for H_2O_2, hydrogen peroxide concentrations of 5–200 μmol l^{-1} were used in the presence of 0.2 mmol l^{-1} Mn^{2+}. The oxidation of 2,6-dimethoxyphenol (DMP, Aldrich) was measured in 50 mmol l^{-1} malonate buffer, pH 4.5, in the presence of 0.05 mmol l^{-1} H_2O_2 and 0.2 mmol l^{-1} Mn^{2+} using a DMP concentration of 1–200 μmol l^{-1}. The absorbance was followed at 469 nm and the extinction coefficient was 49,600 l mol^{-1} cm^{-1} [17]. The activities were also measured in the absence of Mn^{2+}. The experiments were carried out at 25 °C in triplicate using 2 nkat of enzyme per assay.

When the purified pI 4.8 isoenzyme activities were measured in the presence and absence of Mn^{2+} ions for comparison, the following conditions were used: 50 mmol l^{-1} malonate buffer, pH 4.5, 0.05 mmol l^{-1} H_2O_2, 0.2 mmol l^{-1} Mn^{2+}, 0.02 mmol l^{-1} veratryl alcohol, 1 mmol l^{-1} DMP, and 5 mmol l^{-1} ABTS. An activity of 370 nkat ml^{-1} in the oxidation of Mn^{2+} was defined as 100%.

2.5. Mass spectrometry and sequencing

The MnP isoenzymes purified from the culture filtrates were analysed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, protein bands were excised from the gel and cut into small pieces. Then they were destained, the proteins reduced with 20 mmol l^{-1} DTT, carbamidomethylated with 100 mmol l^{-1} of iodoacetamide and *in-gel* digested with porcine trypsin (Promega) (*cf.* [19]). The peptide mixture was directly analysed by microcapillary HPLC–nESI-MS/MS (high performance liquid chromatography–nanoelectrospray ionization-tandem mass spectrometry) with a quadrupole-ion-trap, mass spectrometer LCQ^{Deca} (Thermo Finnigan) [19,20]. Tandem mass spectrometric data were searched against a non-redundant protein database using Sequest software (Thermo Finnigan). The derived peptide sequences with XCorr > 3 were validated manually.

2.6. Glycosylation analysis

Enzymatic deglycosylation of MnP isoenzymes was performed with N-glycosidase (Endo F, EC 3.5.1.52, New England BioLabs) in 50 mmol l^{-1} bicarbonate buffer, pH 8, at 37 °C overnight. The digestion was analysed by MALDI MS (matrix-assisted laser desorption/ionization mass spectrometry) [21] and SDS-PAGE. The monosaccharide analysis of the MnP isoenzymes was carried out after further purification. Possible saccharide contaminants were eliminated in conditions where only proteins from a mixture of purified MnP isoenzymes with pI values of 3.8, 4.8 and 6.1 (0.1 mg) were adsorbed by a C4 reverse-phase column (Dionex-VYDAC, 4 × 250 mm). Gradient elution was performed with solvents containing 0.1% trifluoroacetic acid (TFA, Fluka) in water (eluent A) and in acetonitrile (eluent B) (Merck). Proteins were eluted at 175 μl min^{-1} in a 10.5-ml linear gradient of 1–70% B followed by a 1-ml plateau of 70% B. The elution was monitored by absorbance at 280 and 406 nm. Two heme protein fractions were collected and hydrolysed in 2 mol l^{-1} TFA for 4 h at 100 °C. The hydrolysates were evaporated, redissolved in water, and the monosaccharides were analysed by HPAEC-PAD (high performance anion exchange chromatography-pulsed amperometric detection) (Dionex) on a Carbopac PA 10 column [22].

3. Results and discussion

3.1. Promotion of MnP synthesis

Under nitrogen-limited culture conditions *I. lacteus* produced a MnP activity of *ca.* 1 nkat ml^{-1} in submerged and immobilized, 7–10 days cultivations. The MnP synthesis was enhanced when the inoculum was cultivated in the rich PDB medium resulting in a high production of fungal biomass. MnP synthesis was improved by adding Tween 80 to the growth medium (*cf.* [6]). An observation of a rapid MnP activity increase in a 1-l fermentor initiated by aeration after 1 week of growth without any measurable MnP activity led us to a reduction of liquid phase in shallow liquid cultures, originally performed according to Rothschild et al. [11]. A decrease of the volume of liquid medium used in combination with polyurethane foam as the solid support to one tenth of the foam volume ensur-