

Gene cloning and heterologous expression of pyranose 2-oxidase from the brown-rot fungus, *Gloeophyllum trabeum*

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Abstract A pyranose 2-oxidase gene from the brown-rot basidiomycete *Gloeophyllum trabeum* was isolated using homology-based degenerate PCR. The gene structure was determined and compared to that of several pyranose 2-oxidases cloned from white-rot fungi. The *G. trabeum* pyranose 2-oxidase gene consists of 16 coding exons with canonical promoter CAAT and TATA elements in the 5'UTR. The corresponding *G. trabeum* cDNA was cloned and contains an ORF of 1,962 base pairs encoding a 653 amino acid polypeptide with a predicted molecular weight of 72 kDa. A Hisx6 tagged recombinant *G. trabeum* pyranose 2-oxidase was generated and expressed heterologously in *Escherichia coli* yielding 15 U enzyme activity per ml of induced culture. Structural alignment and phylogenetic analysis were performed and are discussed.

Keywords Gene cloning · *Gloeophyllum trabeum* · Glucose oxidase · Pyranose 2-oxidase

Introduction

The enzyme, pyranose 2-oxidase (P2Ox) (EC 1.1.3.10), catalyzes the oxidation of several aldopyranoses and disaccharides at the C-2 position yielding the

corresponding 2-keto sugars (Giffhorn 2000). In addition to O₂ serving as an electron acceptor, P2Ox can also use a number of quinones and metal ions as electron acceptors. The specific activity of P2Ox varies considerably depending on the mono or disaccharide substrate used, with D-glucose as the preferred substrate. Given the range of both potential substrates and electron acceptors, P2Oxs show potential for several biotechnological applications including carbohydrate modification, fine chemical production, antibiotics, and as a bio-element in sensors and biofuel cells (Tamaki et al. 2007). These enzymes are FAD-dependent and harbor a number of highly conserved features including the FAD-binding site consisting of four separate subregions, a flavin attachment loop, and a C-terminal substrate binding domain. Several P2Oxs have been isolated, cloned and characterized from a number of white-rot fungi (de Koker et al. 2004; Leitner et al. 1998; Vecerek et al. 2004; Danneel et al. 1993). While activity in brown-rot fungi, no corresponding gene has yet been isolated and characterized (Volc et al. 1985).

The purpose of this study was to identify a pyranose 2-oxidase gene in the representative brown-rot fungus, *Gloeophyllum trabeum*, compare this to the several known P2Oxs from white-rot fungi, and engineer a cDNA for heterologous expression of this enzyme. We prepared degenerate nucleotide primers from several highly conserved regions of white-rot fungi P2Ox and amplified a small region of the *G. trabeum* P2Ox gene. Starting from this segment genome

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walking was performed, the full gene was isolated, and appropriate primers were used to isolate a full-length cDNA from purified mRNA. Recombinant P2Ox was expressed in *E. coli* and the enzymatic activity was demonstrated.

Materials and methods

Microorganisms

Gloeophyllum trabeum MAD-617 (ATCC 11539) was used for this study. *E. coli* DH5 α and BL21(DE3) (Novagen) were used as hosts for DNA manipulation and recombinant protein expression, respectively.

Genomic DNA purification

A mycelial fragment was inoculated into 50 ml malt extract broth in a 1 l Erlenmeyer flask and incubated at 28°C and 100 rpm for 7 days. Mycelia were harvested by filtration through Miracloth (Calbiochem) snap-frozen in liquid N₂ and ground to a fine powder using a mortar and pestle. The ground mycelia were suspended in extraction buffer [200 mM Tris/HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS] and extracted twice by mixing thoroughly with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), incubated on ice for 15 min, and centrifuged at 4,000 \times g for 45 min. The recovered aqueous phase was supplemented with 50 μ l 10 mg RNase A/ml and incubated at 37°C for 10 min followed by 56° for 15 min. Proteinase K was then added at 0.5 mg/ml and the mixture held at 56°C for 3 h. Nucleic acid was then precipitated by addition of an equal volume of 2-propanol, washed in 70% (v/v) ethanol, and resuspended in an appropriate volume of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Gene cloning of *G. trabeum* P2Ox

The P2Ox gene from *G. trabeum* was isolated by redundant PCR using 200 ng genomic template, 200 pmol each of redundant primers 5'-GGZGGZATGKCZACNCA $\underline{\text{YTTGGACN}}$ -3' (forward, 64-fold complexity) and 5'-CIARIC $\underline{\text{CIGGITCCATRAAYTGN}}$ -3' (reverse, 32-fold complexity) corresponding to highly conserved regions in the substrate

binding domain of known P2Ox as indicated (Fig. 1). Thermal cycling was at 94°C for 2 min followed by 40 cycles of 94°C for 45 s, 53° for 45 s, 72° for 2 min with a final 7 min extension at 72°C. Products were gel purified, recovered using solid phase extraction (Qiagen), A-tailed and cloned into pGemTeasy (Promega) for DNA sequencing according to the manufacturer's instructions. Based on this P2Ox gene fragment, appropriate specific primers were generated iteratively and used with the Universal Genome Walker kit (Clontech) to recover the full gene. Subsequence genomic DNA purification for overlapping sequence confirmation was performed using the ZR Genomic DNA Kit (Zymo Research). All sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems) according to the manufacturer's instructions and analyzed at the University of Wisconsin Biotechnology Center.

cDNA purification and cloning

Mycelia were prepared as described above and RNA was recovered using a Dynabead extraction kit (Invitrogen) and further purified using the Oligotex mRNA kit (Qiagen). First strand synthesis was performed using an Accuscript Kit (Stratagene) according to the manufacturer's instructions. *Gloeophyllum trabeum* P2Ox was amplified from this cDNA using *Pfu* proofreading DNA polymerase (Stratagene) with primers 5'-ATGTCCCTCAGCCC TGATGAC-3' (forward) and 5'-CCGTAAATCTCA GGTCCCTTCTGACA-3' (reverse). The product was gel purified and cloned into pCR-Blunt (Invitrogen) and sequenced as described above.

Heterologous expression of *G. trabeum* P2Ox

The *G. trabeum* P2Ox cDNA was amplified using primers 5'-CGCGGATCCATGTCCCTCAGCCCT-3' (forward) and 5'-CAGAAGCTTTGCGGCTCCT CG AATCCAAGC-3' (reverse) which introduce *Bam*HI and *Hind*III restriction sites, respectively as indicated by underlining. This fragment was introduced into the *Bam*HI-*Hind*III interval in pET21a(+) (Novagen) resulting in an in-frame fusion with the N-terminal T7-tag and the C-terminal His₆tag. The resulting plasmid was transformed into *E. coli* BL21(DE3) for expression. A fresh colony was inoculated into 25 ml Terrific Broth (TB) supplemented with 100 μ g

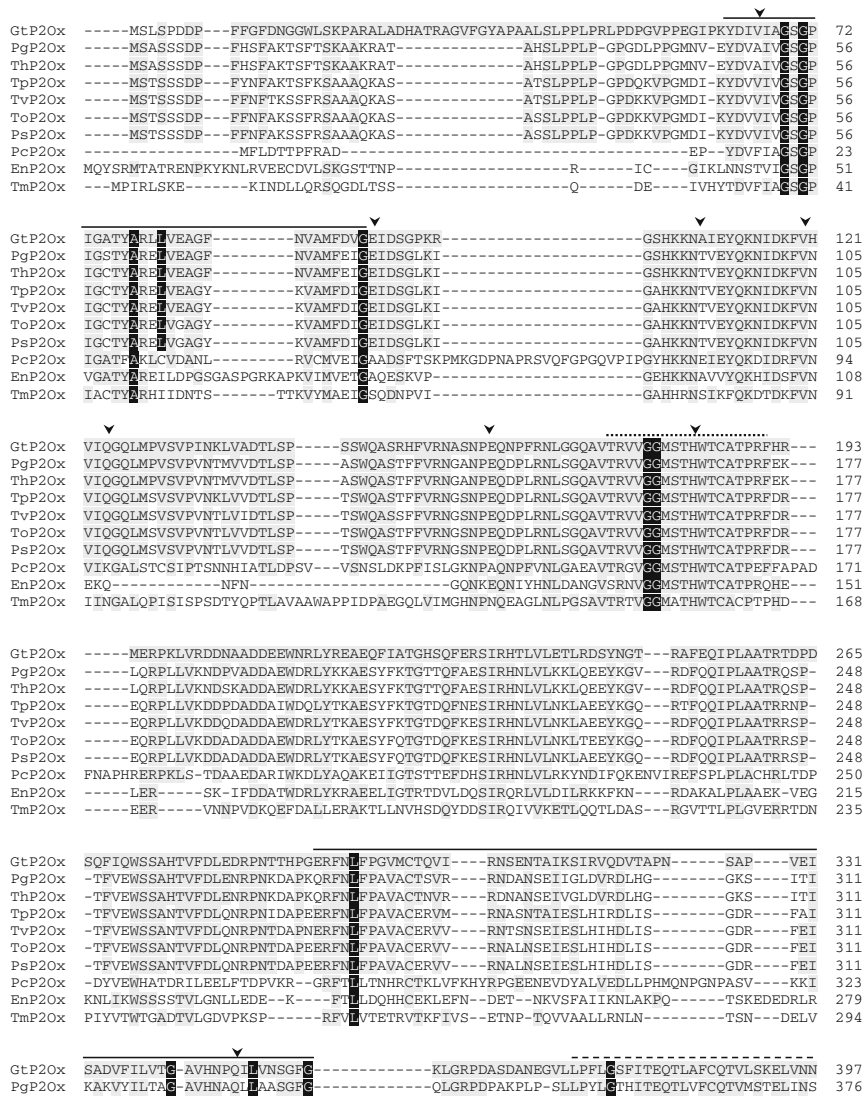


Fig. 1 Alignment of *G. trabeum* MAD-617 P20x (GtP20x; accession ACJ54278) translated from cDNA with P20x from *Peniophora gigantea* (PgP20x; accession GI:34452037), *Trametes hirsuta* (ThP20x; accession GI:25091016; Christensen et al. 1999), *Trametes pubescens* (TpP20x; accession GI: 57867849), *Trametes versicolor* (TvP20x; accession GI:25091018; Nishimura et al. 1996), *Trametes ochracea* (ToP20x; accession GI:31044224) *Peniophora sp.* (PsP20x; accession GI:274364221), *Phanerochaete chrysosporium* (PcP20x; accession AY522922; de Koker et al. 2004) and

Trametes multicolor (TmP20x; accession GI: 215794594; Leitner et al. 2001). Grey shaded amino acids represent identity to GtP20x. Solid lines above sequence indicate FAD-binding regions. Dotted line above sequence indicates flavin attachment loop, dashed line above sequence indicated substrate binding domain, underlined GtP20x sequence indicates regions successfully used for degenerate PCR primers, down arrow head; indicates intron locations and black shaded amino acids with white typeface indicates GMC-OXRED consensus residues (Albrecht and Lengauer 2003)

ampicillin/ml and incubated at 37°C and 150 rpm for 6 h. This culture was then used to inoculate 225 ml TB/ampicillin at 37°C and 150 rpm for an additional 2 h. These cultures were then supplemented with 5 g lactose/l and incubated at 25°C and 150 rpm for 16 h. Bacteria were harvested by centrifugation at 10,000×g

for 10 min at 4°C. The pellet was resuspended in 25 ml lysis buffer (50 mM KPO₄, 1 M NaCl, 5 mM imidazole, pH 6.5) and disrupted by two passages through a French press at 100 mPa. Lysates were clarified by centrifugation at 30,000×g for 30 min at 4°C and filtered through 0.45 μM PVDF membranes (Millipore).

ThP2Ox	KAKVYVLTAC-AVHNAQLDAASGFC-----QLGRPDPKAKLP-SLLPYLGHITHEQTLVFCQVMSTELINS	376
TpP2Ox	QADVYVLTAC-AVHNTQLDVSNGFC-----KLGPRDPANP-P-ELLPLGCSYITEQSLVFCQVMSTELIDS	375
TvP2Ox	KADVYVLTAC-AVHNAQLDVSNGFC-----QLGRPDPANP-P-QLLPLGCSYITEQSLVFCQVMSTELIDS	375
ToP2Ox	KADVYVLTAC-AVHNTQLDVSNGFC-----QLGRPNPANP-P-ELLPLGCSYITEQSLVFCQVMSTELIDS	375
PsP2Ox	KADVYVLTAC-AVHNTQLDVSNGFC-----QLGRPNPTNP-P-ELLPLGCSYITEQSLVFCQVMSTELIDS	375
PcP2Ox	YARSYVVIAC-AVATAQVIANSHIPDDVVI PFGGKSGSGGGERDATIPTPLMPLGCSYITEQPMTCQVVLDSLMEV	402
EnP2Ox	IKAKYVIVC-GPILTPQLFKSGFRVD-----EDAEDSEGNKSSLYIPALRNLTQTMCCPCQIVLKKDKWVEE	348
TmP2Ox	VAQSFVIAC-AVCTPQIIMNSNIRP-----HALGRYLSEQSMTCQIVLKRISIVDS	345
▼		
GtP2Ox	VKADMRVSGTGGQPDYKVEWTPGDPANKHPDWWNEKVKHMMHQ--EDLPLIPLHDPPEQVTTLFEDSHPHWTQIHRDA	475
PgP2Ox	VTADMTIVGKPGDPDYSVTYTSGSPNNKHPDWWNEKVKHMMHQ--EDLPLIPFEDPEPQVTTLFKASHPHWTQIHRDA	454
ThP2Ox	VTADMTIVGKPGHPDYSVTYTPGNPNKHPDWWNEKVKHMMHQ--EDLPLIPFEDPEPQVTTLFQATHPHWTQIHRDA	454
TpP2Ox	VKSDMTIIIGNPGELGYSVYMPGASTNKHPDWWNEKVNHHMQH--EDLPLIPFEDPEPQVTTLFQSPHPHTQIHRDA	453
TvP2Ox	VKSDMIIRGNPGDLGYSVYTPGAETNKHPDWWNEKVNHHMQH--EDLPLIPFEDPEPQVTTLFQSPHPHTQIHRDA	453
ToP2Ox	VKSDMTIRGTPEGLTYSVYTPGASTNKHPDWWNEKVNHHMQH--EDLPLIPFEDPEPQVTTLFQSPHPHTQIHRDA	453
PsP2Ox	VKSDMTIRGTPEGLTYSVYTPGASTNKHPDWWNEKVNHHMQH--EDLPLIPFEDPEPQVTTLFQSPHPHTQIHRDA	453
PcP2Ox	VR-----NPP-----WPG-----LDWMEKVARHVEAFP--NDPLIPFEDPEPQVTKFTEHPHWTQIHRDA	459
EnP2Ox	LQKN-----NWGPECEEHRKRYDEEDDLRIPFEDLDPQVTLPEFTENTQIHRDA	401
TmP2Ox	IATDP-----RFAAKVEAHKKHP--DDVLIPIFHEPEPQVMIPTSDFPWHVQVHR--	395
▼		
GtP2Ox	FSYGAVAESIDTRLVDWRFFGRTEPEKEENKLFWSKQ-----ITDQYGMQPPTDFDRFPDGTTSQDADRMTDMCE	546
PgP2Ox	FSYGAVQQSIDSRLIVDWRFFGRTEPEKEENKLFWSKQ-----ITDAYNLQPPTDFDRFPGGR--EAEDMTDMCV	522
ThP2Ox	FSYGAVQQSIDSRLIVDWRFFGRTEPEKEENKLFWSKQ-----ITDAYNLQPPTDFDRFPGGR--EAEDMTDMCV	522
TpP2Ox	FSYGAVQQSIDSRLIVDWRFFGRTEPEKEENKLFWSKQ-----ITDAYNMPQPTDFDRFPAGRTSKEAEDMTDMCV	524
TvP2Ox	FSYGAVQQSIDSRLIVDWRFFGRTEPEKEENKLFWSKQ-----ITDTYNMQPPTDFDRFPAGRTSKEAEDMTDMCV	524
ToP2Ox	FSYGAVQQSIDSRLIVDWRFFGRTEPEKEENKLFWSKQ-----ITDAYNMPQPTDFDRFPAGRTSKEAEDMTDMCV	524
PsP2Ox	FSYGAVQQSIDSRLIVDWRFFGRTEPEKEENKLFWSKQ-----ITDAYNMPQPTDFDRFPAGRTSKEAEDMTDMCV	524
PcP2Ox	FSYGAVENMDTRIVDVRFFGYTEPEQEANLVFQQH-----YRDAYDMPQPTFKFMSQDDR-ARARRMDDMCN	529
EnP2Ox	FSYGAVPPADKRTIVDLRFGRAETQWRNRVTFSKK-----LTDAYGMPQPTDFKLSKDR-LESHRMMQDMEK	471
TmP2Ox	YAFGDGPKADPRVVDLRFPGKSDIVEENRVTFGNPKLRDWEAGVTDYGMQPPTFHVKRTNADG-DRDQRMMDMTN	474
▼		
GtP2Ox	MSSKIGGFLPGSNPQWMEPGLVILGGTH--RMG--FDEQED-KCCVDTDSRVFGFKNPLGGCGNIG--TAYASNPTL	618
PgP2Ox	MSAKIGGFLPGSYPPQFMEPGLVILGGTH--RMG--FDEKAD-KCCVDTDSRVFGFKNPLGGCGNIG--TAYASNPTL	594
ThP2Ox	MSAKIGGFLPGSYPPQFMEPGLVILGGTH--RMG--FDEKAD-KCCVDTDSRVFGFKNPLGGCGNIG--TAYASNPTL	594
TpP2Ox	MSAKIGGFLPGSLPQFMEPGLVILGGTH--RMG--FDEQED-NCCVDTDSRVFGFKNPLGGCGNIG--TAYGANPTL	596
TvP2Ox	MSAKIGGFLPGSLPQFMEPGLVILGGTH--RMG--FDEQED-KCCVNTDSRVFGFKNPLGGCGNIG--TAYGANPTL	596
ToP2Ox	MSAKIGGFLPGSLPQFMEPGLVILGGTH--RMG--FDEKED-NCCVNTDSRVFGFKNPLGGCGNIG--TAYGANPTL	596
PsP2Ox	MSAKIGGFLPGSLPQFMEPGLVILGGTH--RMG--FDEKED-NCCVNTDSRVFGFKNPLGGCGNIG--TAYGANPTL	596
PcP2Ox	IALKIGGFLPGSEPPQFMTPLGLALAGATT--RCG--LDTQ--KTVGNTHCKVHNFNPLVGGGVNIE--TGFAANPTL	599
EnP2Ox	VAGLGGVLPQSEPPQFAPLGLALFVCGTTAALRKGCRSEDEMKRISVCDENSKVGVENHLGGLNIVDPGRSNASNPTL	551
TmP2Ox	VANILGGVLPQSYPPQFMAPGLACHTTGT--RIG--TDDQ--TSVADPTSKVHNFNPLVGGGNCIP--DATACNPTL	544
▼		
GtP2Ox	TAMAMAIKSCSEYIKKNFKPSEIGSSDNRAWIRGAA	653
PgP2Ox	TAMSLAIKSCSEYIKKNFEPSPNPVKHHN	622
ThP2Ox	TAMSLAIKSCSEYIKKNFEPSPNPVKHHN	622
TpP2Ox	TAMSLAIKSCSEYIKKNFTSPPTPAQ	622
TvP2Ox	TAMSLAIKSCSEYIKKNFTSPPTDQAE	623
ToP2Ox	TAMSLAIKSCSEYIKQNTFTSPPTSEAQ	623
PsP2Ox	TAMSLAIKSCSEYIKQNTFTSPPTSEAQ	623
PcP2Ox	TSICYAIRASNDIIAKFGRHRG	621
EnP2Ox	TAMCFAIKGAEIRRLKGGKSHSGNRDDGDVDTDDDDA	591
TmP2Ox	TSVAYALKGAEAVSYSLGVS	564

Fig. 1 continued

Enzyme assay

Pyranose 2-oxidase activity was determined using the chromogen ABTS [2,2-azinobis(3-ethylbenzthiazolinesulfonic acid)] ($\epsilon_{420} = 43.2 \text{ mM}^{-1} \text{ cm}^{-1}$) (Danneel et al. 1993). The standard 1 ml assay mixture contained 10 μmol ABTS, 20 U horseradish peroxidase, 100 μmol D-glucose, in 50 mM potassium phosphate buffer (pH 6.5). The reaction was started with the addition of 20 μl diluted enzyme. Absorbance was measured at 420 nm and 25°C for 3 min. One unit of activity was defined as the amount of enzyme necessary for the oxidation of 2 μmol ABTS per min (equivalent to the oxidation of 1 μmol D-glucose) at 25°C.

Bioinformatics

Multiple protein alignment was performed using the ClustalW algorithm with default settings supplied with Lasergene v8.0 software (DNASar). Cladogram construction was performed using Treeview v1.6.6. (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

Results and discussion

Redundant PCR using a number of PCR primer pairs generated against conserved regions of an alignment of known pyranose 2-oxidases (de Koker et al. 2004)

resulted in the successful amplification of a homologous DNA from *G. trabeum* using primers derived from the regions underlined in Fig. 1. Genome walking using an adapter library yielded the full-length gene. The *G. trabeum* P2Ox gene is predicted to be comprised of 16 coding exons with a predicted cDNA of 1,962 nucleotides coding for a polypeptide of 653 amino acids and a predicted molecular weight of 72 kDa. The P2Ox protein is not predicted to have a cleavable *N*-terminal secretory signal sequence.

A cDNA for *G. trabeum* P2Ox was isolated from polyA-selected RNA and found to correspond to the cDNA predicted from genomic material. A multiple sequence alignment shows that *G. trabeum* P2Ox conserves essential features of other known P2Ox including four regions of a predicted FAD-binding domain, predicted flavin attachment loops and a predicted substrate binding domain. Moreover, as described in a structural similarity analysis of *Trametes versicolor* (*Coriolus versicolor*) and *T. hirsuta*, *G. trabeum* P2Ox is conserved at 14 of the 15 identified strictly conserved residues for the GMC-OXRED family (Albrecht and Lengauer 2003) (Fig. 1). *Gloeophyllum trabeum* represents a phylogenetic intermediate between a cluster of closely interrelated P2Oxs including PgP2ox (70% identity to GtP2Ox as determined by blastp, <http://www.ncbi.nlm.nih.gov>, see Fig. 1 for abbreviations) ThP2Ox (69% identity), TpP2Ox (70% identity), TvP2Ox (70% identity), ToP2Ox (70% identity), PsP2Ox (70% identity), and the comparatively less related (PcP2Ox 46% identity), and TmP2Ox (37% identity) (Fig. 2).

Gloeophyllum trabeum P2Ox was introduced into an *E. coli* expression system and analyzed in crude lysates and IMAC column purified fractions. A typical experiment resulted in 15 U pyranose-oxidizing activity per ml of induced heterologous expression culture. The recombinant P2Ox was recovered by IMAC chromatography and shown by SDS-PAGE to consist of a polypeptide of 72 kDa under reducing conditions as predicted from the translated cDNA (Fig. 3).

Given the considerable promise that pyranose 2-oxidase enzymes have in the production of fine chemicals, antibiotics, carbohydrate modification and biofuel sensors, the identification of novel P2Ox contributes to the pool of candidates for further characterization and improvement (Giffhorn 2000; Tamaki et al. 2007). As the first P2Ox isolated from a

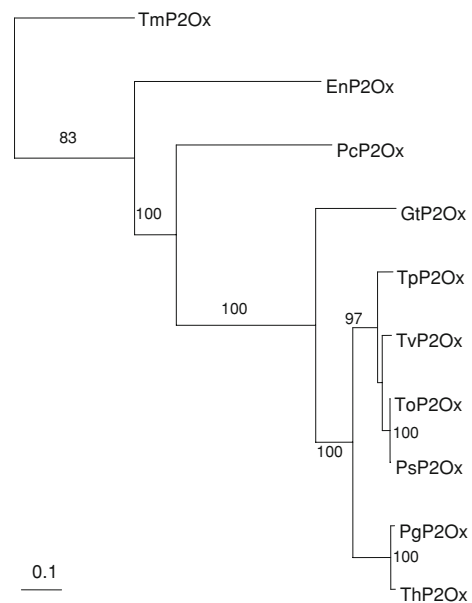


Fig. 2 Cladogram of P2Ox phylogeny. Distance analysis and tree construction was performed using the neighbor joining method of Megalign (DNASTar). Numbers within the tree indicate the percent support after 1,000 replications of bootstrap analysis. The bar represents 10% estimated sequence difference

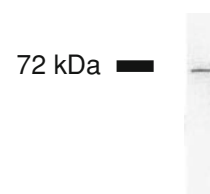


Fig. 3 Analysis of recombinant GtP2Ox. Coomassie blue stain of approximately 0.5 µg of GtP2Ox in a reducing 10% SDS-PAGE gel showing the recombinant protein comigrating with a 72 kDa marker using EZ-Run Pre-Stained Rec protein ladder (Fisher) as indicated

brown-rot fungus, this enzyme appears to represent, from an identity standpoint, a midpoint between a tight cluster of described white-rot fungi P2Ox and a group of comparatively less related members. Further understanding of the unique characteristics of this P2Ox versus those described for a variety of white-rot fungi is a matter of ongoing investigation. As efforts to amend the characteristics of known P2Ox through rational design and directed evolution continue, the recovery of novel P2Ox provide another avenue to the identification of candidates with characteristics best suited to biotechnological utilization (Spadiut et al. 2008).

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