

Direct over-expression, characterization and H₂O₂ stability study of active *Pleurotus eryngii* versatile peroxidase in *Escherichia coli*

Xue Bao · Aiqiu Liu · Xuefeng Lu · Jian-Jun Li

Received: 27 February 2012 / Accepted: 17 April 2012 / Published online: 8 May 2012
© Springer Science+Business Media B.V. 2012

Abstract The *vpl2* gene, encoding versatile peroxidase (VP) from *Pleurotus eryngii*, was synthesized with codon optimization and cloned into vector-pET-32a(+) and over-expressed in *Escherichia coli* BL21(DE3). An active peroxidase fused to the thio-redoxin–hexahistidine tag was directly obtained by IPTG induction in the presence of hemin. Most of over-expressed protein was in the soluble form, and was purified on a nickel column with >85 % purity at a yield of 12.5 mg/l. The purified fusion protein, having an Rz value (A_{407}/A_{280} , a measure of hemin content of the peroxidases) of 1.2, oxidized ABTS veratryl alcohol, Mn²⁺, and Reactive Black 5. Activity of the

enzyme increased after removing the tag. It lost only 5 % of its activity in 6.4 mM H₂O₂. This is the first report on direct over-expression of active VP in *E. coli*.

Keywords *Escherichia coli* · Hydrogen peroxide stability · Overexpression · *Pleurotus eryngii* · Peroxidase

Introduction

Lignin-degrading peroxidases can oxidize a variety of aromatic compounds including high-redox-potential compounds such as lignin, industrial dyes, pesticides, etc. (Martínez et al. 2009). Four types of lignin-modifying enzymes from the white-rot fungus have been identified and characterized: lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and laccase (Martínez et al. 2009). Among them, laccases belong to low-redox-potential oxidoreductases, which can only oxidize high-redox-potential substrates in the presence of redox mediators, whereas the other three belong to high-redox-potential peroxidases, which can directly oxidize high-redox-potential substrates and/or Mn²⁺. VP presents special biotechnological interests due to its catalytic versatility (Martínez et al. 2009).

VP has been described in several species from the genera *Pleurotus* and *Bjerkandera* sharing the

X. Bao · A. Liu · X. Lu · J.-J. Li (✉)
Key Laboratory of Biofuels, Qingdao Institute of BioEnergy and Bioprocess Technology, Chinese Academy of Sciences, No. 189 Songling Road, Qingdao 266101, China
e-mail: lij@qibebt.ac.cn

X. Bao
e-mail: baoxue@qibebt.ac.cn

A. Liu
e-mail: liuaq@qibebt.ac.cn

X. Lu
e-mail: lvxf@qibebt.ac.cn

X. Bao · A. Liu
Graduate School of Chinese Academy of Sciences, Beijing 100049, China

catalytic properties of LiP and MnP (Martínez et al. 1996; Mester and Field 1998). The crystal structure of VP from *Pleurotus eryngii*, shows a hybrid molecular architecture that includes different catalytic sites for oxidation of Mn²⁺ and high-redox-potential aromatic compounds in the same protein, which are closely related to those found in the crystal structures of MnP and LiP (Pérez-Boada et al. 2005). The exposed Trp 164 in VP is involved in long-range electron transfer for oxidation of high-redox-potential compounds like recalcitrant lignin (Pérez-Boada et al. 2005). VP follows similar catalytic cycle to other fungal peroxidases, including LiP and MnP, except that VP is unique regarding the substrates that it is able to oxidize (Pérez-Boada et al. 2005).

The first successful example to produce a fungal ligninolytic peroxidase is that of MnP which was expressed in baculovirus, but only giving a low yield of protein (Pease et al. 1991). Expression of MnP in *Phanerochaete chrysosporium* has been achieved (Mayfield et al. 1994); however, the main challenges involve contamination from the wild-type enzyme and extensive screening productive clones. MnP and LiP were also heterologously over-expressed in *Escherichia coli*, but the recombinant proteins usually accumulate as an insoluble form in the cytoplasmic inclusion bodies (Doyle and Smith 1996; Whitwam et al. 1995). It is not easy to obtain an active enzyme at high yield even after optimizing the refolding conditions, only a refolding yield less than 7 % was achieved for *P. eryngii* VP expressed in *E. coli* (Pérez-Boada et al. 2002). VP was also expressed in the ascomycete *Emericella nidulans*, but this approach demanded extensive screening and provided different glycosylation from the original fungus (Lu-Chau et al. 2004). Over-expression of soluble *Bjerkandera adusta* VP has been achieved in *E. coli* under auto-induction conditions in the presence of hemin, but a complicated procedure was required (Mohorčič et al. 2009). A fusion VP from *P. eryngii* has been produced up to 21 mg/l in *Saccharomyces cerevisiae* after six rounds of directed evolution (Garcia-Ruiz et al. 2012).

Production of large quantities of enzymes with an industrial interest is a key for their industrial use. In the present paper we report direct overexpression of active *P. eryngii* VP in *E. coli* under IPTG induction in the presence of hemin, characterization, and investigation into H₂O₂ stability.

Materials and methods

Materials

Chemicals were from Sigma, Merck or Ameresco. Oligonucleotides and *vpl2* gene encoding VP from *P. eryngii* with codon optimization were synthesized by Shanghai Sangon Biotech Co. Ltd (China). *Taq* DNA polymerases and all restriction endonucleases were from Fermentas or Takara Biotechnology. The kits used for molecular cloning were from Omega Biotech or Takara Biotechnology. Nickel column and expression vectors were from Novagen. Antibodies and chemical reagents used for western blot were from Tiangen (China). Recombinant bovine enterokinase was from Shanghai Sangon Biotech Co. Ltd (China).

Bacterial strains, plasmids, and media

Escherichia coli DH5 α was used for routine DNA transformation and plasmid isolation. *E. coli* BL21(DE3) was utilized for VP over expression. *E. coli* strains were routinely grown in Luria–Bertani broth at 37 °C with aeration or on LB supplemented with 1.5 % (w/v) agar. 50 μ g ampicillin/ml or 15 μ g kanamycin/ml was added when required. Vectors-pET-32a(+) and pET-28a(+) were used for subcloning.

DNA manipulations

General molecular biology techniques were carried out by standard procedures. Restriction and modification enzymes were used following the recommendations of the manufacturers. DNA fragments were purified from agarose gels using the DNA gel extraction kit. Plasmid DNA was isolated using the plasmid miniprep kit.

The *vpl2* gene was amplified with forward primer (CTTCGGAATTCGCCACTTGTGACGATGGTCG AAC, *Eco*RI restriction site underlined) and reverse primer (AATATTGCGGCCGCTTATGATCCGGGA ACTGGAGG, *Not*I restriction site underlined). For PCR, 50 ng plasmid DNA (*vpl* cloned into pBluescript II SK plus) was used as template in 50 μ l reaction mixture containing PCR buffer (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 0.08 % NP-40), 2 mM MgCl₂, 0.5 μ M each of forward and reverse primers and two units of *Taq* DNA polymerase. PCR cycling profiles

were 1 cycle at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s, followed by a final extension step at 72 °C for 10 min. The PCR product was isolated by agarose electrophoresis and extracted from agarose gel using the DNA gel extraction kit, digested with restriction enzymes *EcoRI* and *NotI*, re-cloned into the vectors-pET-32a(+) and pET-28a(+) digested with *EcoRI* and *NotI*, respectively. All constructs were confirmed by DNA sequencing.

Protein over-expression and purification

The expression constructs pET32a-VP and pET28a-VP were transformed into competent *E. coli* BL21(DE3) and expressed under IPTG induction respectively. IPTG induction was carried out following the standard procedure. A single colony was used to inoculate 5 ml LB medium containing 50 µg ampicillin/ml or 15 µg kanamycin/ml and incubated at 225 rpm and 37 °C overnight. The overnight culture was then used for inoculating 300 ml LB medium with 50 µg ampicillin/ml or 15 µg kanamycin/ml. When OD₆₀₀ reached 0.7, 0.1 mM IPTG was added, and incubated in the presence of 0.1 g hemin/l at 16 °C for another 24 h. The cells were harvested by centrifugation at 5,000×g and 4 °C for 15 min.

The cell pellet was suspended in 15 ml lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % NP-40) with 1 mM PMSF as a protease inhibitor. The cell suspension was homogenized by a sonication at 0 °C. The cell lysate was then centrifuged at 20,000×g and 4°C for 25 min. Samples of supernatants and pellets were analyzed by SDS-PAGE and western blotting.

SDS-PAGE was performed in 12 % (v/v) polyacrylamide gels, using low protein molecular weight marker and Coomassie Blue R-250 staining. For western blotting, proteins were transferred from the gel onto a PVDF membrane. The membrane was blocked with 5% (w/v) skimmed milk in TBST (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween 20), incubated with the murine monoclonal anti-polyhistidine immunoglobulin G (IgG), rinsed three times with TBST, incubated with the goat anti-mouse IgG conjugated with alkaline phosphatase, rinsed three times with TBST, and detected with BCIP (5-bromo-4-chloro-3-indolyl phosphate)/nitro blue tetrazolium (NBT) solution.

All purification procedures were carried out at 4 °C. Nickel-chelating resin, 2 ml, was equilibrated with 5 column volumes of equilibration buffer (buffer A: 75 mM Tris/HCl, pH 8.0, 0.5 M NaCl). The crude supernatant was loaded onto the resin, which was sequentially washed with buffer A containing 20–250 mM imidazole. The enzyme purity was analyzed via SDS-PAGE. The fractions containing fusion VP were pooled, first dialyzed against 10 mM sodium tartrate (pH 5.5) supplemented with 1 mM CaCl₂, then against 10 mM sodium tartrate (pH 5.5), finally concentrated with Amicon YM10 membrane (10 kDa cut-off). The protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

The thioredoxin–hexahistidine tag was removed from fusion VP by enterokinase following the standard procedure. 8.07 mg fusion VP was incubated with 60 IU enterokinase in the cleavage buffer (25 mM Tris/HCl, pH 7.6, 50 mM NaCl, 2 mM CaCl₂) at room temperature for 24 h, followed by a second nickel column. The flow-through was collected for further characterization.

Enzyme assay

Mn²⁺-independent oxidation of VA was estimated in 0.1 M sodium tartrate (pH 3.0) at 310 nm (veratraldehyde $\epsilon_{310} = 9,300 \text{ M}^{-1} \text{ cm}^{-1}$), oxidation of RB5 ($\epsilon_{598} = 30,000 \text{ M}^{-1} \text{ cm}^{-1}$) and ABTS (cation radical, $\epsilon_{418} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$) were followed in 0.1 M sodium tartrate (pH 3.5) at 598 and 418 nm, respectively (Ruiz-Dueñas et al. 2008; Garcia-Ruiz et al. 2012). Direct oxidation of Mn²⁺ was followed by the formation of the Mn³⁺-tartrate complex at 238 nm ($\epsilon_{238} = 6,500 \text{ M}^{-1} \text{ cm}^{-1}$) in 0.1 M sodium tartrate (pH 5.0) (Ruiz-Dueñas et al. 2008). All enzymatic assays were measured using initial rates taking linear increase or decrease (RB5) in the presence of 0.1 mM H₂O₂. The reactions were started by the addition of purified fusion VP and followed continuously. For determining specific activities against different substrates, 10 µM ABTS, 1 mM MnSO₄, 10 mM VA, and 10 µM RB5 were used respectively. One unit of enzyme activity (U) is defined as the amount of the enzyme that catalyzes the conversion of 1 µmol substrate per min. K_M and V_{max} were determined using Lineweaver–Burk plot. k_{cat} was calculated from V_{max} on the basis of 52.6 kDa.

H₂O₂ stability assay

H₂O₂ stability was investigated by two approaches: (1) fusion VP activity against ABTS was measured in the presence of different H₂O₂ concentrations (0.01–6.4 mM) at 418 nm in 0.1 M sodium tartrate (pH 3.5). (2) Fusion VP (3 μM) solution was first incubated with 0.5, 1, and 2 mM H₂O₂ at 25 °C for different times (2–45 min) respectively, and then diluted to 0.1 mM H₂O₂. The residual activity was assayed using ABTS as substrate under same conditions as above.

Results and discussion

Cloning, expression and purification of fusion VP

The codon optimized gene *vpl2* encoding peroxidase VPL2 without its signal peptide was synthesized. The gene was cloned into vectors-pET-32a(+) and pET-28a(+) at restriction sites *EcoRI* and *NotI*, respectively (Fig. 1). The resulting constructs were named as pET32a-VP and pET28a-VP, respectively (Fig. 1). The pET32a-VP, including the VP nucleotide sequence fused after the coding sequences for the thioredoxin and hexa-histidine tags, was constructed since it has been suggested that thioredoxin could enhance the solubility of proteins normally produced

in an insoluble form and stabilize the protein (Mhorčič et al. 2009). The pET28a-VP was constructed for expression of VP with the *N*-terminal His-tag.

pET32a-VP was over-expressed in *E. coli* BL21(DE3) under IPTG induction in the presence of hemin. The optimized conditions for higher yield of soluble and active protein were 0.1 mM IPTG, 0.1 g hemin/L, and 16 °C for 24 h. SDS-PAGE (Fig. 2) and western blot (data not shown) analysis confirmed the presence of soluble fusion protein-THHVP (VP with the thioredoxin–hexahistidine tag). The predicted molecular weight for THHVP is 52.6 kDa, which corresponded to the SDS-PAGE value (Fig. 2). No major band corresponding to 52.6 kDa was observed in the vector-pET-32a(+) transformant (lane 11 in Fig. 2), neither detected by western blot (data not shown).

Under the same induction conditions pET28a-VP gave very low yield of protein based on SDS-PAGE and western blot (data not shown), which might be due to the absence of the thioredoxin tag in the expression vector-pET28a-VP (Fig. 1).

The over-expressed protein was purified by Ni-chelating affinity chromatography. The target protein was eluted with buffers containing 60 and 100 mM imidazole, with >85 % purity based on SDS-PAGE (Fig. 2). The collected fractions containing the target protein were combined, dialyzed, and concentrated. The yield of THHVP was about 12.5 mg/l.

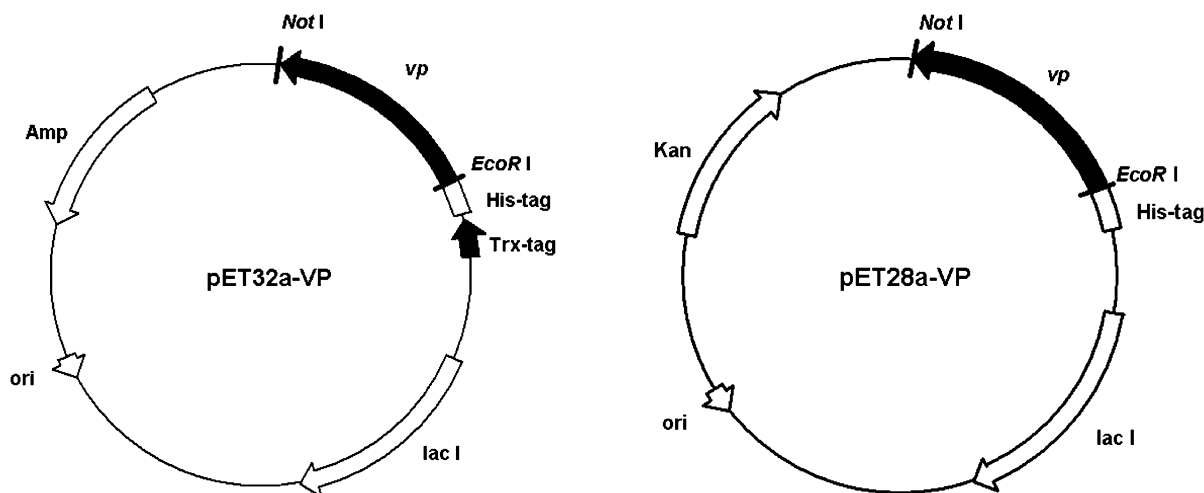


Fig. 1 Schematic structures of the expression vectors-pET28a-VP and pET32a-VP: *ori* the origin of replication, *Kan* kanamycin-resistance gene sequence, *Amp* ampicillin-resistance gene

sequence, *vp* gene sequence encoding VP VPL2, *lacI lacI* repressor gene sequence, *His-tag* hexa-histidine tag, *Trx-tag* thioredoxin tag

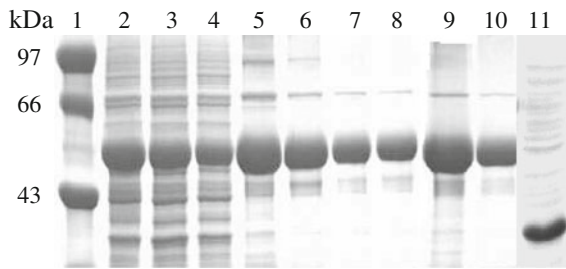


Fig. 2 SDS-PAGE analysis of THHVP. Lane 1 low protein molecular weight marker, lane 2 crude supernatant for over-expressed THHVP, lane 3 flow-through when loaded, lane 4 eluent of buffer A, lanes 5–7 eluents of buffer A + 60 mM imidazole, lanes 8–10 eluents of buffer A + 100 mM imidazole, lane 11 crude supernatant for *E. coli* harboring the empty vector-pET-32a(+)

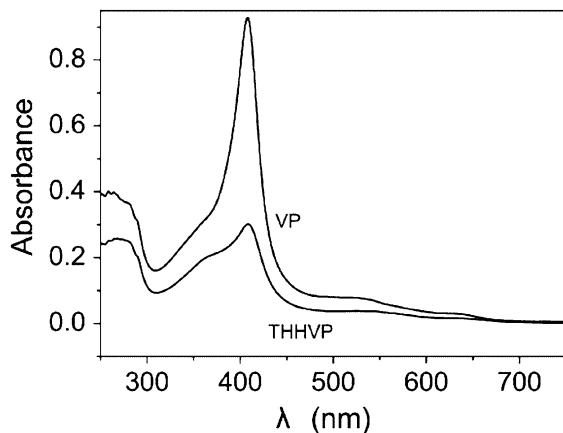


Fig. 3 UV-Vis spectra of THHVP and VP (the thioredoxin-hexahistidine tag was removed using enterokinase). The enzymes were in 10 mM sodium tartrate (pH 5.5)

The UV-Vis spectrum of THHVP showed a characteristic Soret peak at 407 nm (Fig. 3) demonstrating that hemin was incorporated into the heterologously over-expressed THHVP. The R_z value (A_{407}/A_{280} , a measure of hemin content of the peroxidases) for THHVP is 1.2, which is close to the values for fusion VPs from *B. adusta* expressed under auto-induction conditions (Mohorčič et al. 2009), but is much lower than 3.9 for the refolded non-fusion VP from the inclusion body (Pérez-Boada et al. 2002). The R_z value for VP (the thioredoxin-hexahistidine tag was removed from THHVP using enterokinase, there are nine amino acids left in the N-terminal.) is 2.5, indicating that the presence of the thioredoxin-

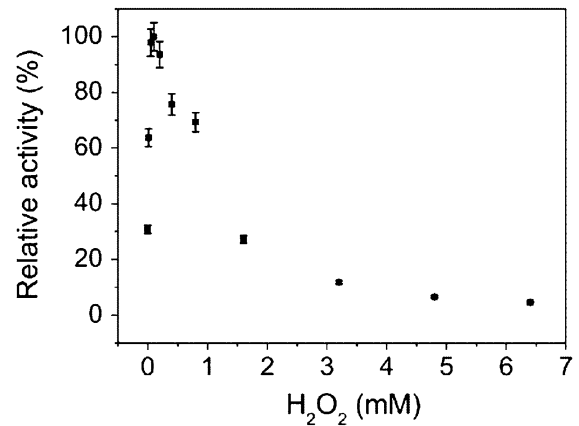


Fig. 4 H₂O₂ dependence assay for THHVP. Enzyme activity against ABTS was assayed in the presence of different H₂O₂ concentrations (0.01–6.4 mM) at 418 nm in 0.1 M sodium tartrate (pH 3.5). The absolute value of 100 % relative activity is 0.17 ± 0.004 ($\Delta A_{418}/\text{min}$)

hexahistidine tag has had some effects on the R_z value of THHVP. Between 400 and 750 nm, THHVP and VP showed three similar bands at 407 (Soret), 532, and 634 nm (Fig. 3), which means that the thioredoxin-hexahistidine tag had not affected the heme environment.

Enzymatic activity assay

Proteins expressed in *E. coli* lack adequate glycosylation and, although lack of glycosylation may not interfere with enzyme activity, its thermal stability can be lower than that of corresponding native enzyme (Nie et al. 1999). Thus, activity of VP over-expressed in *E. coli* should not be affected by lack of glycosylation.

Fusion protein THHVP oxidized all substrates tested: ABTS, Mn²⁺, VA, and RB5 (Table 1). Specific activities and kinetic parameters of non-fusion VP are also listed in Table 1. Specific activities against ABTS, Mn²⁺, VA, and RB5 were improved 9-, 13-, 18-, and 11-fold, respectively, by removing the thioredoxin-hexahistidine tag. K_m were not affected by the tag whereas the turnover numbers were. The catalytic efficiencies (k_{cat}/K_m) against ABTS, Mn²⁺, VA, and RB5 increased 12-, 11-, 6-, and 12-times, respectively, after the tag was removed. These data have demonstrated that the tag has greatly affected enzyme activity.

Table 1 Specific activities and kinetic parameters for THHV and VP

Substrate	THHV			VP		
	Specific activity (U/mg)	K_m (μM)	k_{cat} (s^{-1})	Specific activity (U/mg)	K_m (μM)	k_{cat} (s^{-1})
ABTS	0.9 ± 0.02	1.2 ± 0.07	0.7 ± 0.01	8.8 ± 0.03	0.7 ± 0.04	5.4 ± 0.1
Mn^{2+}	14.5 ± 0.2	100 ± 6.1	15.1 ± 0.3	194 ± 3.1	78 ± 7.9	138 ± 5.2
VA	0.4 ± 0.002	$3,900 \pm 296$	0.9 ± 0.02	7.2 ± 0.6	$4,090 \pm 523$	6.4 ± 0.3
RB5	0.2 ± 0.01	8.8 ± 0.3	0.4 ± 0.006	2.2 ± 0.2	4.8 ± 0.3	2.7 ± 0.08

All enzymatic assays were done at 1 ml scale at 25 °C in duplicate. Oxidations of ABTS and RB5 were followed in 0.1 M sodium tartrate (pH 3.5) at 418 and 598 nm, respectively, oxidation of Mn^{2+} was followed by the formation of the Mn^{3+} -tartrate complex at 238 nm in 0.1 M sodium tartrate (pH 5.0), oxidation of VA was carried out in 0.1 M sodium tartrate (pH 3.0) at 310 nm. For determining specific activities, 10 μM ABTS, 1 mM MnSO_4 , 10 mM VA, and 10 μM RB5 were used respectively

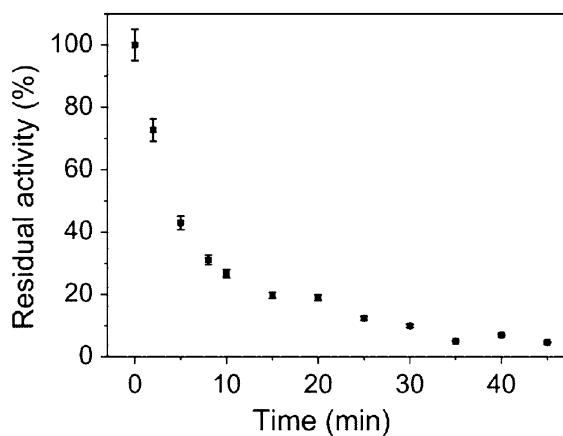


Fig. 5 H_2O_2 stability assay for THHV. THHV (3 μM) solution was first incubated with 1 mM H_2O_2 at 25 °C for different times (2–45 min), then diluted to 0.1 mM H_2O_2 . The residual enzyme activity was measured using ABTS as substrate under standard conditions. The absolute value of 100 % residual activity is 0.1 ± 0.005 ($\Delta A_{418}/\text{min}$)

H_2O_2 stability study

Peroxidases tend to lose activity in the presence of H_2O_2 through a mechanism-based process known as a suicide inactivation (Valderrama et al. 2002). The optimum H_2O_2 concentration for THHV is 0.1 mM, above which enzyme gradually lost activity, it retained only about 4.6 % of original activity when H_2O_2 reached 6.4 mM (Fig. 4). THHV lost 50 % of original activity after being incubated with 0.5, 1, and 2 mM H_2O_2 for 25, 6, and 1 min, respectively (Fig. 5) and completely lost activity with 2 mM H_2O_2 for 30 min (data not shown). In addition, inactivation of VP by H_2O_2 is dependent on pH (Böckle et al. 1999). Thus, protein engineering has to be carried out

for VP in order to improve its H_2O_2 stability (Garcia-Ruiz et al. 2012).

Conclusion

Active VP from *P. eryngii* was directly produced in *E. coli* under IPTG induction in the presence of hemin at moderate yields, thus the labor-intensive, time-consuming, and low-efficient refolding was avoided. This will be useful for production of active VP for structure–function investigation.

Acknowledgments We are grateful to Knowledge Innovation Program of the Chinese Academy of Sciences (Grant KSCX1-YW-11A3) and Natural Science Foundation of Shandong Province (China) (Grant ZR2010CM058) for the financial support.

References

- Böckle B, Martínez MJ, Guillén F, Martínez AT (1999) Mechanism of peroxidase inactivation in liquid cultures of the ligninolytic fungus *Pleurotus pulmonarius*. Appl Environ Microbiol 65:923–928
- Doyle WA, Smith AT (1996) Expression of lignin peroxidase H8 in *Escherichia coli*: folding and activation of the recombinant enzyme with Ca^{2+} and haem. Biochem J 315: 15–19
- Garcia-Ruiz E, Gonzalez-Perez D, Ruiz-Dueñas FJ, Martínez ÁT, Alcalde M (2012) Directed evolution of a temperature-, peroxide- and alkaline pH tolerant versatile peroxidase. Biochem J 441:487–498
- Lu-Chau TA, Ruiz-Duenas FJ, Camarero S, Feijoo G, Martinez MJ, Lema JM, Martinez AT (2004) Effect of pH on the stability of *Pleurotus eryngii* versatile peroxidase during heterologous production in *Emricella nidulans*. Bioprocess Biosyst Eng 26:287–293

- Martínez MJ, Ruiz-Dueñas FJ, Guillén F, Martínez ÁT (1996) Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. Eur J Biochem 237:424–432
- Martínez ÁT, Ruiz-Duenas FJ, Martínez MJ, Río JC, Gutiérrez A (2009) Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotechnol 20:348–357
- Mayfield MB, Kishi K, Alic M, Gold MH (1994) Homologous expression of recombinant manganese peroxidase in *Phanerochaete chrysosporium*. Appl Environ Microb 60: 4303–4309
- Mester T, Field JA (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J Biol Chem 273:15412–15417
- Mohorčič M, Benčina M, Friedrich J, Jerala R (2009) Expression of soluble versatile peroxidase of *Bjerkandera adusta* in *Escherichia coli*. Bioresour Technol 100:851–858
- Nie GJ, Reading NS, Aust SD (1999) Relative stability of recombinant versus native peroxidases from *Phanerochaete chrysosporium*. Arch Biochem Biophys 365:328–334
- Pease EA, Aust SD, Tien M (1991) Heterologous expression of active manganese peroxidase from *Phanerochaete chrysosporium* using the baculovirus expression system. Biochem Biophys Res Commun 179:897–903
- Pérez-Boada M, Doyle WA, Ruiz-Dueñas FJ, Martínez MJ, Martínez AT, Smith ÁT (2002) Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimisation of in vitro folding. Enzyme Microb Tech 30: 518–524
- Pérez-Boada M, Ruiz-Dueñas FJ, Pogni R, Basosi R, Choinowski T, Martínez MJ, Piontek K, Martínez ÁT (2005) Versatile peroxidase oxidation of high potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. J Mol Biol 345:385–402
- Ruiz-Dueñas FJ, Morales M, Mate MJ, Romero A, Martínez MJ, Smith AT, Martínez ÁT (2008) Site-directed mutagenesis of the catalytic tryptophan environment in *Pleurotus eryngii* versatile peroxidase. Biochemistry 47:1685–1695
- Valderrama B, Ayala M, Vazquez-Duhalt R (2002) Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes. Chem Biol 9:555–565
- Whitwam RE, Gazarian IG, Tien M (1995) Expression of fungal Mn peroxidase in *E. coli* and refolding to yield active enzyme. Biochem Biophys Res Commun 216:1013–1017