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Purification and Characterization of an Intracellular NADH: Quinone Reductase from *Trametes versicolor*

Sang-Soo Lee, Dong-Soo Moon, Hyoung T. Choi, and Hong-Gyu Song*

Division of Life Sciences, and Research Institute of Life Sciences, Kangwon National University, Chuncheon 200-701, Republic of Korea

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Intracellular NADH:quinone reductase involved in degradation of aromatic compounds including lignin was purified and characterized from white rot fungus *Trametes versicolor*. The activity of quinone reductase was maximal after 3 days of incubation in fungal culture, and the enzyme was purified to homogeneity using ion-exchange, hydrophobic interaction, and gel filtration chromatographies. The purified enzyme has a molecular mass of 41 kDa as determined by SDS-PAGE, and exhibits a broad temperature optimum between 20-40°C, with a pH optimum of 6.0. The enzyme preferred FAD as a cofactor and NADH rather than NADPH as an electron donor. Among quinone compounds tested as substrate, menadione showed the highest enzyme activity followed by 1,4-benzoquinone. The enzyme activity was inhibited by $CuSO_4$, $HgCl_2$, $MgSO_4$, $MnSO_4$, $AgNO_3$, dicumarol, KCN, NaN_3, and EDTA. Its K_m and V_{max} with NADH as an electron donor were 23 μM and 101 mM/mg per min, respectively, and showed a high substrate affinity. Purified quinone reductase could reduce 1,4-benzoquinone to hydroquinone, and induction of this enzyme was higher by 1,4-benzoquinone than those of other quinone compounds.

Keywords: quinone, NADH:quinone reductase, *Trametes versicolor*, enzyme purification

White rot fungi can degrade not only lignin, but also various aromatic compounds. The roles of extracellular ligninolytic enzymes of wood rot fungi in degradation of aromatic compounds, such as lignin peroxidase, manganese-dependent peroxidase and laccase have been extensively investigated (Ichinose *et al.*, 2002; Xiao *et al.*, 2003; Shin *et al.*, 2005), but much less is known about intracellular enzymes involved in the further degradation of monoaromatic intermediates (Jensen *et al.*, 2002). Quinone compounds are common metabolites produced during the fungal degradation of aromatic compounds. However, they act as a redox-active toxin and damage fungal biomass. They must be reduced for the protection of fungal cells, and also in order to be further degraded by fungi (Stahl *et al.*, 1995; Cohen *et al.*, 2004). Many wood rotting fungi also use a quinone redox cycle to generate extracellular Fenton reagent, a key component for fungal degradation of aromatic compounds (Jensen *et al.*, 2002). Several quinone reductases have been described from various organisms, and unlike type I NADH:quinone oxidoreductase, also called rotenone-sensitive NADH dehydrogenase in mammalian respiratory chains, type II NADH:quinone oxidoreductase, also called rotenone-insensitive NADH dehydrogenase are usually present in the electron transfer chains of prokaryotes, fungi and plants (Melo *et al.*, 2004). Many microorganisms contain type II NADH:quinone oxidoreductases, however only few have been isolated and characterized, moreover, some of those enzymes studied were extracellular enzymes (Rasmussen *et al.*, 1995; Melo *et al.*, 2004). Intracellular quinone reductase from white rot fungus has been reported only from *Phanerochaete chrysosporium* (Brock *et al.*, 1995; Stahl *et al.*, 1995). Recently, intracellular quinone reductase from brown rot fungus *Gloeophyllum trabeum* has been investigated in details (Jensen *et al.*, 2002; Cohen *et al.*, 2004; Qi and Jellison, 2004a, 2004b).

Although *Trametes versicolor* in this study has a high degrading capability of recalcitrant aromatic compounds, its removal patterns of some aromatic compounds did not coincide with the activities of its well-known ligninolytic enzymes (Han *et al.*, 2004; Cheong *et al.*, 2006). It has been also reported that various enzymes are involved in biodegradation by white rot fungi (Vyas *et al.*, 1994; Conesa *et al.*, 2002; Han *et al.*, 2005). In this study an intracellular quinone reductase from *T. versicolor* which is important for biodegradation of aromatic compounds and protection of fungal biomass from reactive quinone compounds, but rarely studied was isolated and characterized.

Materials and Methods

Fungal strain and cultivation

T. versicolor 951022 originally isolated in Kangwon-do, Korea was obtained from the Mycology Laboratory of Kangnung National University. Five agar plugs (5×5 mm) of active mycelium on potato dextrose agar medium (Difco Lab., USA) were grown in YMG broth medium (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%) on a rotary shaker (130 rpm) for 5 days at 28°C. The fungal culture was blended with a homogenizer, and used as an inoculum for further fungal cultivation.

* To whom correspondence should be addressed.
(Tel) 82-33-250-8545; (Fax) 82-33-251-3990
(E-mail) hgsong@kangwon.ac.kr

Confirmation and measurement of quinone reductase activity
Fungal biomass grown 3 days in YMG broth was harvested by centrifugation, and washed 3 times with distilled water. Washed mycelium was mixed with 4 volumes of buffer A (50 mM Tri-HCl buffer; pH 7.5+5 μM FMN) and homogenized for 5 min (4°C, 15,000 rpm). After centrifugation (5,000×g, 30 min) crude extract was separated, and loaded on 10% non-enaturing polyacrylamide gel. The staining solution for NAD(P)H quinone reductase activity [50 mM Tris-HCl buffer (pH 7.5) 25 ml, NADH or NADPH 8 mg, menadione (2-ethyl-1,4-naphthoquinone) 8 mg, nitroblue tetrazolium 3.5 mg] was added on the gel. The activity staining was carried for 1 h under dark conditions at 37°C, and the active band of crude enzyme was observed (Manchenko, 1994).

Quinone reductase activity was determined with an assay mixture containing 100 μM menadione, 2 mM NADH and enzyme solution 0.1 ml in a final volume of 1.0 ml 100 mM citrate buffer (pH 6.0). One unit of enzyme activity was defined as the amount of enzyme required oxidizing 1 μmol NADH per minute at room temperature and was measured by following the initial linear decrease in absorbance at 340 nm.

Purification of quinone reductase
The high-speed supernatant was fractionated by sequential addition of ammonium sulfate, with each addition being followed by centrifugation (17,000×g). The quinone reductase activity precipitated between 40 and 65% ammonium sulfate saturation and was redissolved in buffer A and dialyzed for 12 h. The crude enzyme was transferred to a DEAE-Sepharose Fast Flow (Amersham Biosciences, Sweden) column (4.0×25 cm) which was preequilibrated with buffer A. The column was subsequently washed with 4 column volumes of buffer A, and the enzyme fractions eluted with a linear concentration gradient of 0-0.5 M NaCl in the same buffer at a rate of 1 ml/min. The collected fractions were analyzed for quinone reductase activity and the active fractions were pooled. These samples were resuspended in a solution of 20% ammonium sulfate saturation, and then applied to a column (2.8×8.5 cm) of Phenyl Sepharose 6 Fast Flow (high sub) (Amersham Biosciences, Sweden) preequlibrated with buffer A containing 20% saturation of ammonium sulfate and was then eluted with a reverse gradient of saturated ammonium sulfate from 20-0% at a rate of 1 ml/min. The active fractions were pooled and dialyzed against 2 L of buffer A using a dialysis membrane for 12 h. The sample was loaded on to a column (2.8×5.5 cm) of Q-Sepharose (Amersham Biosciences, Sweden) preequlibrated with buffer A. The column was subsequently washed with 4 column volumes of buffer A, and the enzyme fractions eluted with a linear concentration gradient of 0-0.3 M NaCl in the same buffer at a rate of 0.5 ml/min. The active fractions were then pooled, concentrated by ultrafiltration (Centriprep YM-10, Millipore, USA), and loaded onto a column (2.4×18 cm) of Sephadex G-100 (Sigma, USA) preequilibrated with buffer A containing 0.2 M NaCl. The enzyme fractions were eluted with the same buffer at a rate of 0.5 ml/min. The active fractions were then pooled, concentrated by ultrafiltration.

Characterization of purified quinone reductase
Homogeneity of the quinone reductase was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed on 12.5% polyacrylamide gel. Proteins were stained with coomassie G, and the molecular mass of denatured enzyme was estimated with molecular weight markers (Sigma, USA). The optimum pH for the quinone reductase was determined using 100 mM citrate buffer (pH 4.0-6.0) and 100 mM phosphate buffer (pH 6.0-8.0) ontaining 100 μM 2-methyl-1,4-naphthoquinone (menadione). The optimum temperature of the purified enzyme was determined between 10°C and 50°C. The effects of pH and temperature were expressed as the relative enzyme activity measured by the method described above. Preference of electron donor for enzymatic reduction was compared between NADPH and NADH. Substrate specificity of the purified enzyme among quinone compounds was examined with tetramethyl-1,4-benzoquinone (duroquinone), 1,4-benzoquinone, 2-hydroxy-1,4-naphthoquinone, menadione, and hydroquinone. Preference of prosthetic group of the quinone reductase was examined with apoenzyme and FMN, FAD and riboflavin by a method of Watanabe *et al.* (1998). Kinetic studies were performed by measuring the initial velocity in 1 ml glass cuvette with 1 cm path length, and the velocities of enzyme-catalyzed reactions were measured at 420 nm for NADH oxidation. Effects of metals and inhibitors on the activity of the purified enzyme were examined with 1 mM metal ions ($MgSO_4$, $MnSO_4$, $CuSO_4$, $HgCl_2$, $AgNO_3$), 1 mM each of EDTA, KCN, NaN_3, and 5 μM dicumarol.

Results and Discussion

Quinone reductase activity of T. versicolor
Presence of quinone reductase in *T. versicolor* strain 951022 which had shown a high degrading capability of some aromatic compounds (Han *et al.*, 2004; Cheong *et al.*, 2006) was examined by activity staining with crude extract of fungal culture. A band that indicated activity of NADH:quinone reductase was shown in non-denaturing PAGE, but NADPH: quinone reductase activity was not observed in the activity staining (data not shown). Quinone reductase is important in degradation of aromatic compounds, because it is involved in the Fenton reaction that is another extracellular degradation mechanism by wood rot fungi besides ligninolytic enzymes (Jensen *et al.*, 2002). Since catechol, a key intermediate in degradation of aromatic ring, is formed during reduction of quinone compounds, quinone reductase is also necessary for the further degradation of quinone compounds. Moreover, quinone reductase removes quinone compounds which act as a redox-active toxin to fungus (Stahl *et al.*, 1995). The removal patterns of some aromatic compounds of *T. versicolor* in this study did not coincide with the activities of manganese-dependent peroxidase (MnP) and laccase excreted by this fungus (Han *et al.*, 2004; Cheong *et al.*, 2006). The activity of MnP was negligible and laccase showed 441 U/ml in the 3 days culture of this fungus in YMG medium. However, MnP may be induced by Mn in the nitrogen-limited conditions. It has been reported that quinone compounds are produced in the second metabolic event of lignin metabolism and degradation of some other aromatic compounds by MnP and laccase (Wesenberg *et al.*, 2003). Therefore, quinone reductase may have an important role in degrada-

tion of aromatics by *T. versicolor*. The activity of quinone reductase was maximal in 3-day culture of *T. versicolor* (Fig. 1), and it seemed to be linked to fungal growth. The pattern of enzyme activity during fungal growth was similar to quinone reductase of brown rot fungus *Gloeophyllum trabeum* (Jensen *et al.*, 2002).

Purification and characterization of quinone reductase

Quinone reductase from *T. versicolor* was purified to homogeneity from crude extracts by sequential chromatographies (Table 1). Single band of quinone reductase was confirmed by SDS-PAGE, and its molecular mass was estimated to be 41 kDa, and this enzyme seemed to be active as a monomer (Fig. 2). Well-known white rot fungus *Phanerochaete chrysosporium* has a similar sized benzoquinone reductase (44 kDa), but it was composed of two identical 22 kDa subunits (Brock *et al.*, 1995), which was same with NADH: quinone reductase of brown rot fungus *Gloeophyllum trabeum* (Jensen *et al.*, 2002). *P. chrysosporium* has been reported to have a 69 kDa intracellular NADH:quinone reductase (Constam *et al.*, 1991). Purified enzyme showed a typical absorption spectrum of flavin protein with the absorption maxima at 375 nm and 450 nm (Fig. 3), which was quite similar to NADH: benzoquinone reductase of *G. trabeum* (Jensen *et al.*, 2002).

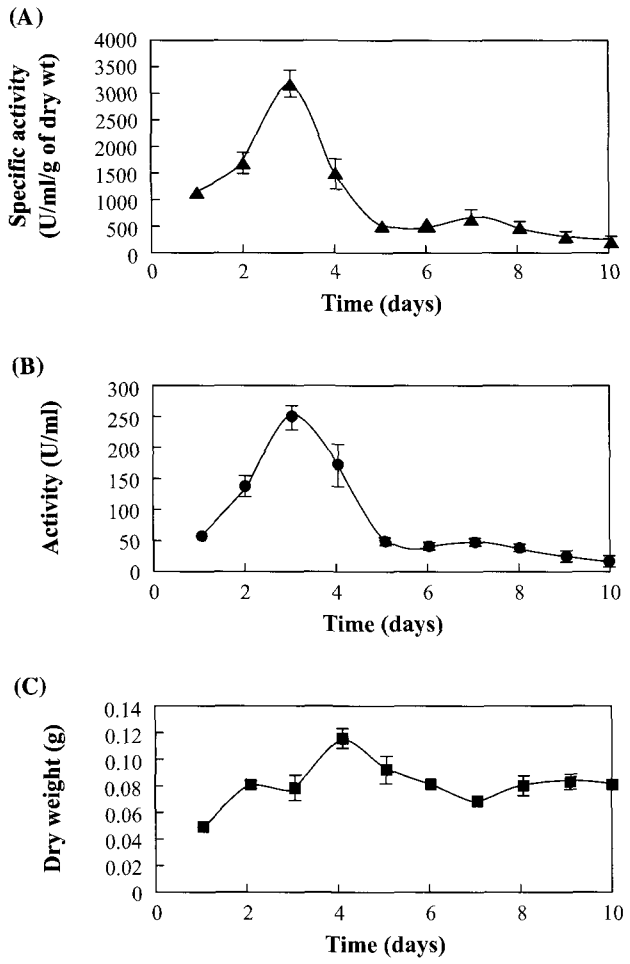

Fig. 1. Time course of specific activity (A) and total activity (B) of quinone reductase and fungal biomass (C) in the crude extract of *T. versicolor* 951022 from fungal culture in YMG medium.

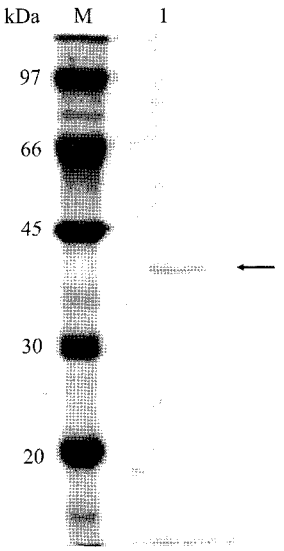

Fig. 2. Denaturing polyacrylamide gel electrophoresis of purified quinone reductase from *T. versicolor* 951022. Lane M; protein marker, lane 1; shows the purified quinone reductase.

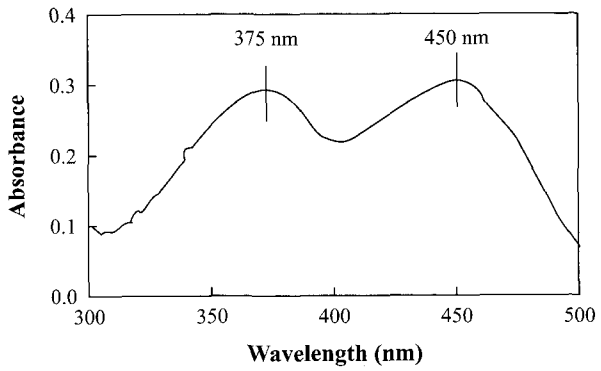

Fig. 3. Absorbance spectrum of purified quinone reductase from *T. versicolor* 951022.

Table 1. Purification of quinine reductase from *T. versicolor* 951022

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	63.90	12,733	199	1.0	100.0
DEAE-Sepharose fast flow	5.24	10,965	2,093	10.5	86.1
Phenyl-Sepharose 6 fast flow	1.51	4,373	2,896	14.6	34.3
Q-Sepharose high performance	0.05	695	13,880	70.7	5.5

When the apoenzyme of quinone reductase was treated with FMN, FAD and riboflavin and enzyme activity was measured, FAD addition could recover enzyme activity over 70% compared to that of control (data not shown). The recovery of enzyme activity by FMN and riboflavin could not reach to 30%, and it showed that the prosthetic group of quinone reductase of *T. versicolor* is FAD. Many extracellular cellobiose quinone reductases of various wood- degrading fungi have two prosthetic groups, FAD and a heme (Henriksson *et al.*, 2000). On the contrary, benzoquinone reductase of *P. chrysosporium* and quinone reductase of *G. trabeum* contained FMN (Brock *et al.*, 1995; Jensen *et al.*, 2002).

Purified quinone reductase of *T. versicolor* exhibited a high relative activity over a broad temperature range between 10-40°C and showed the maximal activity at 30°C (Fig. 4A). The enzyme activity was relatively high under weak acidic conditions and rapidly decreased below pH 5.0 and over pH 7.0, and optimal pH was 6.0 (Fig. 4B). Effects of pH and temperature on enzyme activity were similar to those of intracellular benzoquinone reductase of *P. chrysosporium* (Brock *et al.*, 1995), however, they were quite different from those of extracellular cellobiose:quinone reductase of *T. versicolor*, which had temperature optimum of 50-60°C and pH optimum of 4.5-5.0 (Roy *et al.*, 1996).

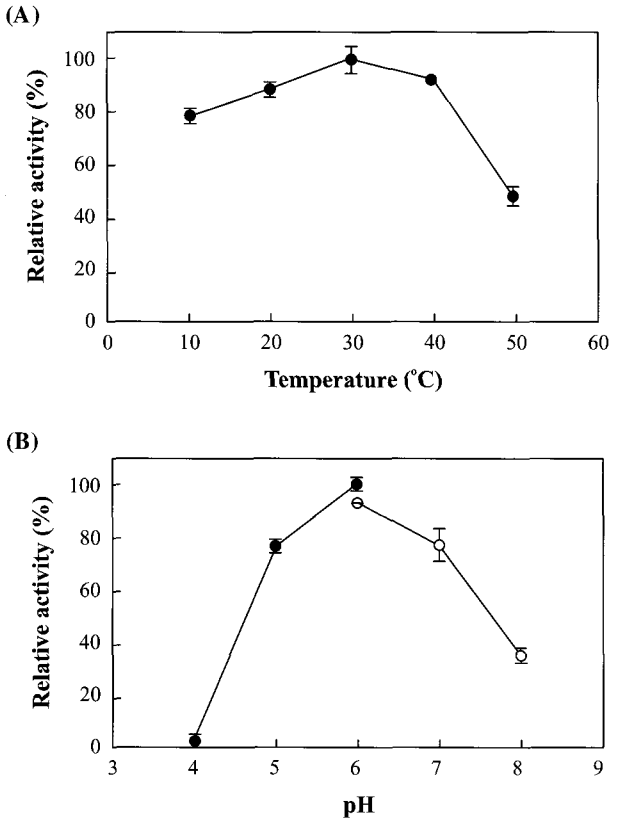

Fig. 4. Effects of temperature (A) and pH (B) on activity of purified quinone reductase from *T. versicolor* 951022.

Table 2. Enzyme activity of purified quinone reductase from *T. versicolor* 951022 with different quinone compounds as a substrate

Quinone compounds	Activity (U/ml)
Menadione	197.5±10.8
1,4-Benzoquinone	169.0±3.4
2-Hydroxy-1,4-naphthoquinone	16.1±5.0
Duroquinone	39.9±0.0
Hydroquinone	5.6±2.2

When NADPH and NADH were added as an electron donor into crude extract of *T. versicolor*, enzyme activities were 28 U/ml and 305 U/ml, respectively. The purified enzyme was active with NADH (80 U/ml), but did not show any activity with NADPH. These results indicated that the electron donor for quinone reductase of *T. versicolor* is NADH, and it was already shown in activity staining of crude extract of fungal culture, which did not exhibit NADPH: quinone reductase. Quinone reductase from *G. trabeum* also showed the same preference of electron donor (Jensen *et al.*, 2002). However, an intracellular benzoquinone reductase of *P. chrysosporium* showed similar enzyme activity with both NADH and NADPH (Brock *et al.*, 1995), and many other quinone reductases of other organisms also use NADPH as an electron donor in addition to NADH (Melo *et al.*, 2004). The quinone reductase of *T. versicolor* showed the highest enzyme activity (197 U/ml) with 2-methyl-1,4-naphthoquinone (menadione) as a substrate among several quinone compounds, and hydroquinone had the lowest activity as expected (Table 2). K_m and V_{max} of the purified enzyme were 23 μM and 101 mM/mg per min with menadione as a substrate and NADH as an electron donor. Its K_m was higher than that of *Neurospora crassa* (11 μM), but lower than those of *E. coli* (50 μM), *Saccharomyces cerevisiae* (31 μM), and *P. chrysosporium* (32 μM) (Melo *et al.*, 2004), which indicated a high substrate affinity of the quinone reductase of *T. versicolor*.

Purified quinone reductase showed about 10% inhibition of enzyme activity compared to control by 1 mM of $MgSO_4$, $MnSO_4$, $CuSO_4$, EDTA, KCN, and NaN_3. Among the metal tested, $AgNO_3$ exhibited the highest enzyme inhibition (72% by 0.05 mM $AgNO_3$), and Ag^+ has been reported to lower enzyme activity by binding to sulfhydryl group in protein (Haynes *et al.*, 2002). Dicumarol known as an inhibitor of mammalian NAD(P)H:quinone oxidoreductase also showed a high inhibition of the enzyme (70%) by 5 μM concentration. Low inhibition by several metals and EDTA suggested that this enzyme does not require a transition metal for activity, and inhibition by dicumarol indicated that the mechanism of this quinone reductase may be similar to that of DT diaphorase [NAD(P)H(quinone-acceptor) oxidoreductase] (Brock *et al.*, 1995).

Enzymatic transformation and enzyme induction

When 1,4-benzoquinone was reacted with the purified enzyme, Optical Density at 277 nm which is an absorption wavelength of 1,4-benzoquinone decreased, and Optical Density at 288 nm which is an absorption peak of hydroquinone increased (Fig. 5). This change of absorption spectrum suggested that 1,4-benzoquinone was reduced to hydroquinone by the purified quinone reductase. It has been reported that enzymatically reduced hydroquinone produces Fenton

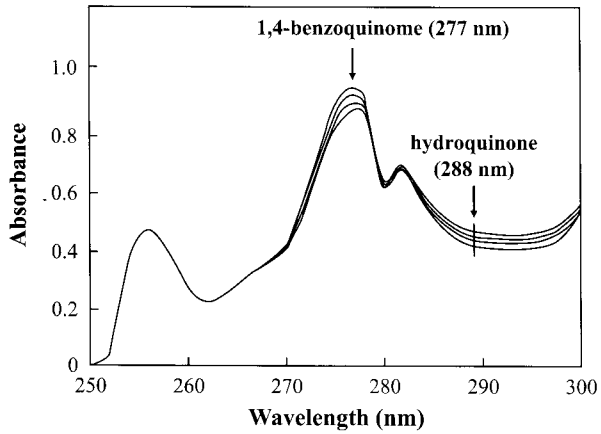

Fig. 5. Absorption spectra during reduction of 1,4-benzoquinone and formation of hydroquinone. Arrows indicate direction of change of absorption.

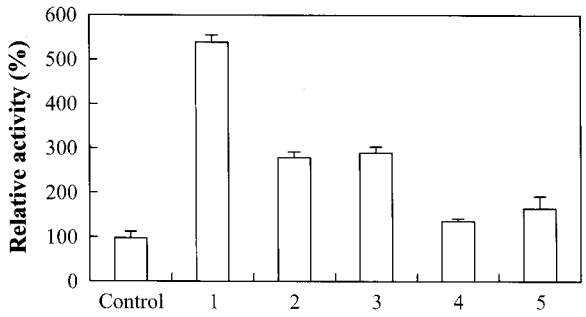

Fig. 6. Activity of intracellular quinone reductase from *T. versicolor* 951022 induced by different quinone compounds (100 μM). Lane 1, 1,4-benzoquinone; lane 2, menadione; lane 3, 2,6-dimethoxy-1,4-benzoquinone; lane 4, duroquinone; lane 5, 2-hydroxy-1,4-naphthoquinone.

reagent which involves in chemical oxidation of aromatic compounds called Fenton reaction (Jensen *et al.*, 2002). Therefore, quinone reductase is another important enzyme for degradation by wood rot fungi. Quinone compounds could also induce quinone reductase during fungal culture. All quinone compounds added into culture of *T. versicolor* could increase activity of quinone reductase (Fig. 6), and addition of 100 μM 1,4-benzoquinone showed the highest increase (550%) of enzyme activity.

Biochemical characteristics of quinone reductase of *T. versicolor* is quite different from that of white rot fungus *Phanerochaete chrysosporium* (Constam *et al.*, 1991; Stahl *et al.*, 1995), while it shares some properties with that of brown rot fungus *Gloeophyllum trabeum* (Jensen *et al.*, 2002; Qi and Jellison, 2004a, 2004b). The results obtained in this study show that quinone reductase of *T. versicolor* has some desirable characteristics as a catalytic agent for recalcitrant aromatic compounds. More studies on amino acid sequence, inducers and many other factors are necessary to assess its industrial applications.

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