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ORIGINAL ARTICLE

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NADPH-dependent ferrireductase produced by white-rot fungus *Phanerochaete sordida* YK-624

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Abstract An intracellular, soluble ferrireductase thought to be involved in the reduction of manganese dioxide by white-rot fungus *Phanerochaete sordida* YK-624 was purified for the first time. Two isoenzymes, NAD(P)H-dependent and NADPH-dependent, respectively, were detected by hydrophobic chromatography. The NADPH-dependent ferrireductase was purified to homogeneity by ammonium sulfate fractionation, hydrophobic interaction, gel permeation, and anion-exchange chromatography. The purified protein, which is monomeric, has a molecular mass of 35kDa (determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and pI 5.1 (determined by isoelectric focusing). The purified protein did not use cellobiose as an electron donor. The purified protein reduced Fe(III)–nitrilotriacetate complex, Mn(III)–malonate complex, methoxy-*p*-benzoquinone, and cytochrome c; veratraldehyde, 2-hydroxy-1,4-naphthoquinone, phenazine methosulfate, and plumbagin could not be reduced. Particularly, the protein showed the highest reducing rate for Fe(III)–organic acid complexes, such as Fe(III)–nitrilotriacetate, among these electron acceptors.

Key words NADPH-dependent ferrireductase · *Phanerochaete sordida* YK-624 · Manganese dioxide · Purification

Introduction

Several species of white-rot fungi have been studied intensively in recent years because of their ability to degrade and remove lignin from wood. In response to environmental concerns and increasingly stringent emissions standards, the pulp and paper industry is looking for ways to decrease the level of chlorinated lignin residues in its effluents through both production process changes and improved treatment technologies. The white-rot fungi *Phanerochaete chrysosporium* Burds.,[1,2] *Coriolus versicolor* (L. ex Fr.) Quel.,[3,4] *Phanerochaete sordida* Eriksson & Ryvarden YK-624,[5,6] IZU-154,[7,8] and unknown species SKB-1152[9] have the ability to bleach kraft pulp. Manganese peroxidase was noted to be a key enzyme in the biological bleaching of unbleached hardwood kraft pulp (UKP) with white-rot fungi, as the MnP activity detected in the culture linearly correlated with the brightness increase of the UKP treated with these fungi.[1,5,10] Moreover, Kondo et al. found that bleaching of UKP was successfully conducted with partly purified MnP secreted by *P. sordida* YK-624.[11]

The Mn(II) ion is necessary for production and function of MnP from *P. chrysosporium*[12,13] and *P. sordida* YK-624.[14,15] UKP contains the Mn element in a concentration of about 50mg/kg pulp,[10,14] and *P. sordida* YK-624 utilizes the Mn element during the biological bleaching of UKP because this fungus can produce MnP and brighten UKP in the culture containing only UKP and water.[5,6] An increase in the brightness of UKP during in vitro MnP treatment has not been observed without the addition of $MnSO_4$,[11] and the bleaching of UKP was successful with MnP without the addition of $MnSO_4$ by using oxalate as an effective Mn(III)-chelating and manganese dioxide-reductive agent.[16] These results suggest that the UKP dominantly contained Mn(IV), such as manganese dioxide, but has scarce Mn(II), and that *P. sordida* YK-624 reduces manganese dioxide present in UKP to Mn(II) during the biological bleaching of UKP.

Roy et al. reported that cellobiose:quinone oxidoreductase (CBQase) reduces manganese dioxide.[17] In our recent report, the reduction of manganese dioxide by *P. sordida* YK-624 was mediated by iron chelates, and the produced ferric chelates were reduced by NAD(P)H-dependent ferrireductase present in the fungus.[18] Some intracellular oxidoreductases such as 1,4-benzoquinone reductase,[19–21] aryl-alcohol dehydrogenase,[22] and 1,2,4-trihydroxybenzene 1,2-dioxygenase[23] can be isolated from *P. chrysosporium*.

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Extracellular cellobiose dehydrogenase[24–27] and intracellular 1,4-benzoquinone reductase[20,21] are recognized as ferrireductase in the white-rot fungus *P. chrysosporium*; and a transplasma membrane redox system of the fungus can reduce ferricyanide.[28] Cellobiose dehydrogenase was not detected in the culture of *P. sordida* YK-624.[18] Herein we report the purification of NADPH-dependent ferrireductase from *P. sordida* YK-624 to apparent homogeneity and determined the reduction of the ferric chelates lignin-related quinone and aldehyde by the ferrireductase.

Materials and methods

Fungus strain *P. sordida* YK-624 (ATCC 90872) was used in this study. The strain was maintained on potato dextrose agar (PDA; Difco Laboratories) slants at 4°C. PDA plates (diameter 9cm) inoculated with the strain were incubated for 3 days at 30°C. Three fungal disks punched from the growing edge of the wood-rotting fungus mycelium on a PDA plate were added to a petri dish (diameter 9cm) containing 15ml of a liquid medium. The liquid medium contained 1% glucose, 1.2mM ammonium tartrate, 20mM 2,2-dimethylsuccinate, 0.1% Tween 80, 14.7mM KH_2PO_4, 2.16mM nitrilotriacetate (NTA), 2.80mM $MgSO_4$, 1.72mM $MnSO_4$, 6.33mM NaCl, 0.13mM $FeSO_4$, 0.24mM $CoSO_4$, 1.14mM $CaCl_2$, 0.24mM $ZnSO_4$, 14.8μM $CuSO_4$, 14.3μM $AlK(SO_4)_2$, 60.7μM H_3BO_3, 17.8μM Na_2MoO_4, and 3μM thiamine-HCl, adjusted to pH 4.5. Five hundred plates were statically incubated at 30°C for 12 days.

Preparation of enzyme extract and purification of the ferrireductase

Mycelial mats were removed from the cultures and washed with ice-cold distilled water. All subsequent steps were carried out at 4°C. The mycelial mats were broken up with 700ml of an extraction buffer in a Waring blender 7010 (Waring Products Division, Dynamics Corporation of America). The blender was operated 15 times at 15200 rpm for 20s at 15-min intervals. The extraction buffer consisted of 20mM sodium phosphate (pH 7.0), 0.004% phenylmethylsulfonyl fluoride, and 0.05% Tween 80. The homogenate was centrifuged at 7000rpm for 30min, and the supernatant was filtered with 0.45μm pore size membrane filter (diameter 47mm; nitrocellulose; Toyo Roshi Kaisha, Japan) to produce cell-free extract.

The filtrate was fractionated by sequential additions of solid ammonium sulfate. Proteins that exhibited ferrireductase activity precipitated between 30% and 65% ammonium sulfate saturation and were redissolved in 20mM sodium phosphate (pH 7.0).

A column (1.6 × 10.0cm) of Phenyl Sepharose (Pharmacia Biotech, Sweden) was equilibrated with 20mM sodium phosphate (pH 7.0) containing 1M ammonium sulfate. The protein was applied in the equilibration buffer, and the column was washed with 100ml of the equilibration buffer; protein was then eluted with a linear gradient (total volume 120ml) of decreasing ammonium sulfate concentration from 1 to 0M. The fractions containing ferrireductase activity were pooled and concentrated by ultrafiltration (10kDa cutoff).

The active fraction from the Phenyl Sepharose column was applied to a column (2.0 × 50.0cm) of Superdex 75 (Pharmacia Biotech, Sweden), which was equilibrated in 20mM sodium phosphate (pH 7.0) containing 0.1M ammonium sulfate, with a flow rate of 0.5ml/min.

The active fractions from the Superdex 75 column were desalted by passage through a PD-10 (Pharmacia Biotech) column equilibrated with 10mM Tris-HCl buffer (pH 9.0) and concentrated by ultrafiltration (10kDa cutoff). The concentrated solution was loaded onto a Mono Q HR 5/5 (Pharmacia Biotech) column equilibrated with 10mM Tris-HCl buffer (pH 9.0). Unbound protein was washed off with 15ml of 10mM Tris-HCl buffer (pH 9.0), and the active fractions were eluted with a linear gradient of increasing ratio of 10mM Tris-HCl buffer (pH 7.0) at a flow rate of 0.3ml/min.

Enzyme assay

Ferrireductase activities were determined by the formation of Fe(II)–1,10-phenanthroline (PHT) complex at 510nm (an extinction coefficient of $12.11\,cm^{-1}\,mM^{-1}$).[29] These assays were carried out at 30°C with a Beckman DU 640 spectrophotometer unless otherwise indicated. Standard reaction mixtures in 1ml consisted of 20mM sodium phosphate (pH 7.0), 100μM Fe(III)–NTA complex, 1.5mM PHT, 100μM NADPH, and enzyme. Reactions were initiated by the addition of NADPH.

Oxidation of various electron donors by the purified protein

To determine the oxidation of NADH, NADPH, cellobiose, and succinate (electron donors) by the purified protein, the formation of Fe(II)–PHT complex at 510nm with various electron donors was observed. The reaction mixtures in 1ml consisted of 100μM Fe(III)–NTA complex, 1.5mM PHT, the purified protein (127ng), and 50μM electron donors.

Reduction of various electron acceptors by the purified protein

Oxidation of NADPH at 340nm by the purified protein with various electron acceptors was carried out to determine the reduction of various electron acceptors by the purified protein. The reaction mixtures (20mM sodium phosphate buffer, pH 7.0) contained 100μM electron acceptors, the purified protein (127ng), and 50μM NADPH. For reduction of the Fe(III)–NTA complex, 1.5mM PHT was added. To determine the reduction of cytochrome c, the reaction mixture contained 100μM cytochrome c, the

purified protein (2.6 μg), and 50 μM NADPH; the reduction was measured by following the increase in absorbance at 550 nm (ferrocytochrome c; an extinction coefficient of $21.1\,cm^{-1}mM^{-1}$).[21]

Steady-state kinetics

To determine steady-state kinetic parameters for ferric chelates, ferrireductase activities were determined by the formation of the Fe(II)–PHT complex at 510 nm in 20 mM sodium phosphate buffer (pH 7.0). The parameter for 1,4-benzoquinone was determined by the oxidation of NADPH at 340 nm.

Analytical methods

Protein concentration was measured by the method of Bradford,[30] with bovine serum albumin as a standard. The native molecular weight was determined by gel filtration on the Superdex 75 column and the molecular weight of the denatured form by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) performed in 11% polyacrylamide gels. Isoelectric focusing (IEF) was performed using an Ampholine PAGplate (Pharmacia Biotech).

Chemicals

Cytochrome c (from horse heart), PHT, 2-hydroxy-1,4-naphthoquinone, phenazine methosulfate, plumbagin, dicumarol, and veratraldehyde were obtained from Wako Pure Chemical Industries. Cibacron blue F3G-A was purchased from Fluka Biochemika. Ferric chelates were formed by the addition of ferric chloride and 5.0 equivalent of chelators such as NTA in distilled water.

Results

Purification of NADPH-dependent ferrireductase

In our previous report,[18] the cell-free extract from the white-rot fungus *P. sordida* YK-624 contained ferrireductase activity. The protein precipitated at 30% to 65% ammonium sulfate saturation was prepared from the cell-free extract and applied to a Phenyl Sepharose column (Fig. 1). Two types of ferrireductase (I and II) were detected by chromatography. At 135 ml of elution volume, ferrireductase I, which reduced the Fe(III)–NTA complex with NADH and NADPH was observed; and ferrireductase II, which oxidized NADPH more than NADH, was revealed near 180 ml of elution volume. Because ferrireductase I was almost completely inactivated within 24 h after the chromatography, we tried to purify ferrireductase II.

Table 1 summarizes the purification procedure from 260 g (wet weight) of mycelial mats grown in the liquid medium. After Mono Q chromatography, SDS–PAGE and IEF–PAGE analysis of the active fraction revealed that the protein consisted of a single band (Fig. 2). The overall enzyme yield was 4.8%, with a concomitant 347-fold purification.

Physical properties

Ferrireductase II exhibited a pI value of 5.1, determined by isoelectric focusing, as shown in Fig. 2. The enzyme exhibited a native molecular mass of approximately 35 kDa as determined by gel filtration on a Superdex 75 column

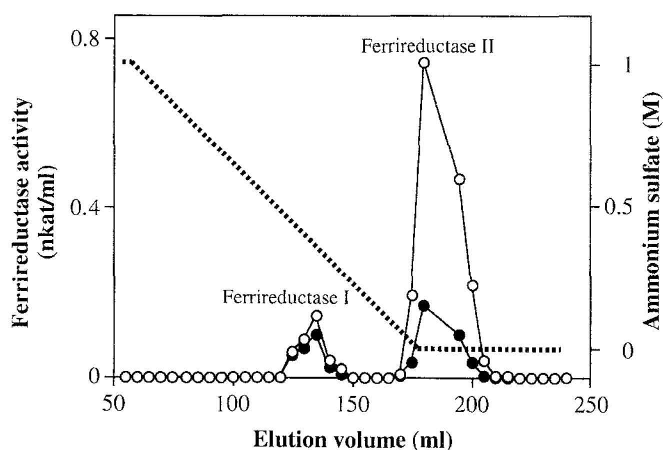

Fig. 1. Phenyl Sepharose chromatography of the cell-free extract from *P. sordida* YK-624. *Open circles*, NADPH-dependent activity; *close circles*, NADH-dependent activity; *dotted line*, ammonium sulfate

Table 1. Purification of ferrireductase II from *P. sordida* YK-624

Step	Volume (ml)	Protein (mg)	Activity Total (nkat)	Activity Specific (nkat/mg)	Yield (%)	Purification (fold)
Cell-free extract	830	703	2130	3	100	1.0
$(NH_4)_2SO_4$ precipitate	250	293	1290	4	60	1.5
Phenyl Toyopearl	50	22	360	16	17	5.5
Superdex 75	20	3.7	220	60	10	19.8
Mono Q	7.5	9.8×10^{-2}	102	1040	4.8	347

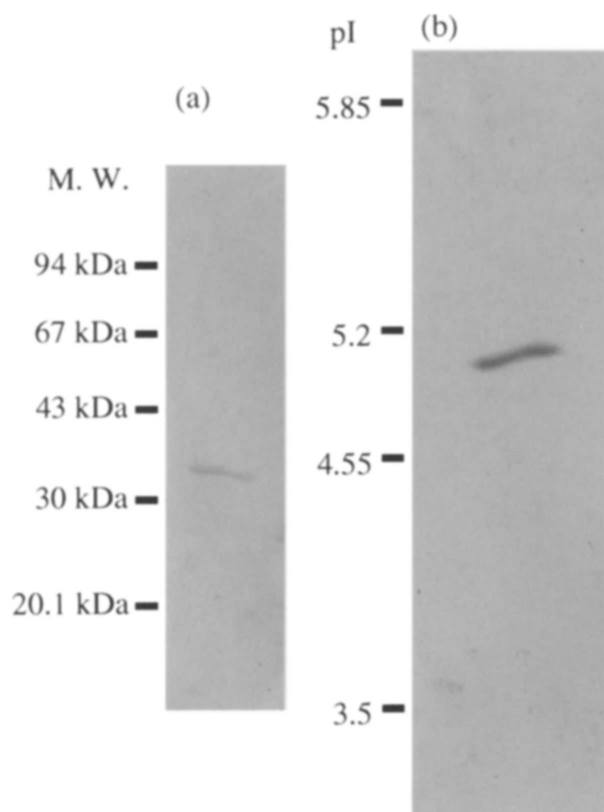

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (**a**) and isoelectric focusing (IEF) – PAGE (**b**) of ferrireductase II from *P. sordida* YK-624. Molecular mass markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa). IEF markers were bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.2), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.5). These proteins were visualized in gels by Coomassie brilliant blue staining

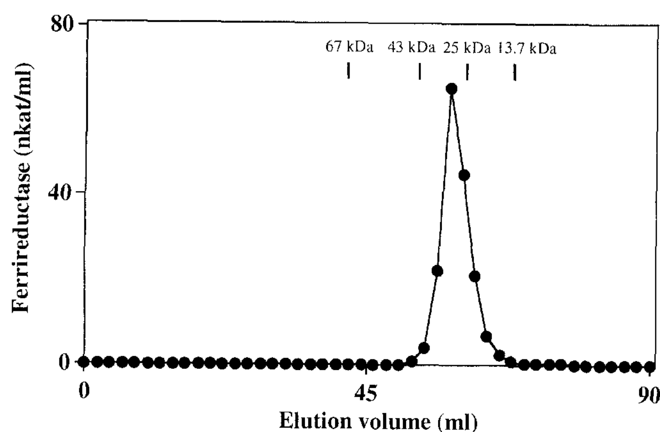

Fig. 3. Gel permeation chromatography of ferrireductase II from *P. sordida* YK-624. Molecular mass markers were bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). *Solid circles*, ferrireductase activity

(Fig. 3). With SDS–PAGE (Fig. 2), a single band that corresponded to a molecular mass of 35 kDa was observed.

Substrate specificity

Several electron donors were tested as substrates for ferrireductase II (Table 2) in 20 mM sodium phosphate buffer (pH 7.0). When NADPH was used as electron donor, Fe(III)–NTA was significantly reduced by ferrireductase II, but the reduction hardly occurred with NADH as electron donor. In 20 mM sodium phosphate buffer (pH 5, 6, and 8), NADPH-dependent reduction of Fe(III)–NTA was also observed. No reduction of Fe(III)–NTA by ferrireductase II was observed when cellobiose and succinate were used as electron donors.

Oxidation of NADPH was observed when Fe(III)–NTA, Mn(III)–malonate, and methoxy-*p*-benzoquinone were used as electron acceptors (Table 3). Particularly, ferrireductase II showed a greater rate of NADPH oxidation for Fe(III)–NTA than for the other electron acceptors. Ferrireductase II could reduce ferricytochrome c without a specific ferrous chelator, such as PHT. No oxidation of NADPH was observed when 2-hydroxy-1,4-naphthoquinone, phenazine methosulfate, plumbagin, and veratraldehyde were used (Table 3).

Table 2. Oxidation of electron donors by ferrireductase II from *P. sordida* YK-624

Substrate	Relative activity (%)
NADPH	100.0
NADH	1.4
Cellobiose	0
Succinate	0

Table 3. Reduction of electron acceptors by ferrireductase II from *P. sordida* YK-624

Electron acceptor	Relative activity (%)
Fe(III)-NTA	100.0
Mn(III)-malonate	46.1
Methoxy-*p*-benzoquinone	35.8
Cytochrome c	1.5
Veratraldehyde	0
2-Hydroxy-1,4-naphthoquinone	0
Phenazine methosulfate	0
Plumbagin	0

Steady-state kinetic parameters for ferric chelates and 1,4-benzoquinone, which were reduced by ferrireductase II, were determined (Table 4). Three ferric chelates showed much higher substrate specificity for ferrireductase II than methoxy-*p*-benzoquinone. We could not determine the K_m and k_{cat} values of Mn(III)–malonate for ferrireductase II because the Mn(III)–malonate complex was unstable under aerobic conditions.[17]

Table 4. Steady-state kinetic parameters for ferrireductase II[a]

Compound	Apparent K_m (μM)	Apparent k_{cat} (s^{-1})	k_{cat}/K_m
Fe(III)–PHT	4.2	1.3×10^4	3.1×10^6
Fe(III)–citrate	7.3	9.2×10^3	1.3×10^6
Fe(III)–NTA	9.7	7.6×10^3	7.8×10^5
Methoxy-*p*-benzoquinone	36.8	1.0×10^4	2.7×10^5

[a] Reaction mixtures: 30 μM PHT, 50 μM NADPH, and 127 ng ferrireductase II

Table 5. Inhibition of ferrireductase II by various compounds

Compound	Concentration	Inhibition rate (%)
Cu(II)–NTA	100 μM	4.5
Sodium azide	1 mM	7.7
EDTA	1 mM	66.2
Dicumarol	10 μM	100
Cibacron blue	10 μM	100

Inhibitors

Table 5 shows the inhibition of ferrireductase activity by various compounds. Sodium azide (1 mM) and Cu(II)–NTA (100 μM) inhibited the enzyme less than 10%, whereas the enzyme activity was lowered 34% in the presence of 1 mM EDTA. The enzyme activity was not detected in the presence of 10 μM dicumarol and 10 μM cibacron blue, which were inhibitors of NAD(P)H:(quinone acceptor) oxidoreductase (DT diaphorase).[31]

Discussion

In the present study, ferrireductase II, an NADPH-dependent enzyme, was purified for the first time from an intracellular component of the white-rot fungus *P. sordida* YK-624. Several oxidoreductases were purified from *P. chrysoporium*,[20–23] and extracellular cellobiose dehydrogenase[24–27] and intracellular 1,4-benzoquinone reductase[20] were recognized as ferrireductase. A transplasma membrane redox system of the fungus also reduces ferricyanide.[28] Cellobiose-dependent oxidoreductase activity was not detected in our previous study,[18] and the addition of NAD(P)H stimulated the manganese dioxide-reducing activity of the washed mycelia, although the reduction of quinones by a transplasma membrane redox system of *P. chrysosporium* was not affected by the addition of NAD(P)H and cellobiose.[28] These results suggested that cellobiose dehydrogenase and a transplasma membrane redox system are not involved in the reduction of manganese dioxide by *P. sordida* YK-624.

Ferrireductase II showed high activity on the Fe(III) complex, although the Mn(III) complex and methoxy-*p*-benzoquinone were also reduced to a lesser extent. This enzyme was not able to reduce veratraldehyde, 2-hydroxy-1,4-naphthoquinone, phenazine methosulfate, or plumbagin, as shown in Table 3. Brock et al. reported that steady-state rate constants, k_{cat}/K_m, of 1,4-benzoquinone reductase were 1.8×10^8 for methoxy-*p*-benzoquinone and 1.6×10^4 for ferricyanide.[20] In our present study, steady-state rate constants of ferrireductase II were 7.8×10^5 for Fe(III)–NTA and 2.7×10^5 for methoxy-*p*-benzoquinone. NAD(P)H:quinone acceptor oxidoreductase (DT diaphorase) is able to reduce 2-hydroxy-1,4-naphthoquinone, phenazine methosulfate, and plumbagin.[32–34] It is concluded that ferrireductase II purified from *P. sordida* YK-624 differs from alcohol dehydrogenase,[22] 1,4-benzoquinone reductase, and DT diaphorase.

Addition of 1 mM EDTA inhibited reduction of the Fe(III)–NTA complex by ferrireductase II, probably owing to the formation of the Fe(III)–EDTA complex, which is hardly reduced by ferrireductase II (data not shown). As is true for DT diaphorase of animal tissues,[31] the reduction of Fe(III) complex by ferrireductase II was inhibited by the addition of 10 μM dicumarol and cibacron blue. On the other hand, sodium azide, which inhibited peroxidases containing heme, hardly inhibited the ferrireductase activity. It is therefore expected that the active site of the ferrireductase is similar to that of DT diaphorase, being flavoprotein. To confirm this point, we tried to isolate a large quantity of the ferrireductase for analyzing the UV-VIS spectrum of its concentrated solution. However, the concentration of the protein was too low to confirm the active site. Further studies are planned to elucidate the active site of the ferrireductase.

In the present study, ferrireductase II could not reduce Fe(III)–NTA using NADPH without the Fe(II)-specific chelator PHT (data not shown). It suggests that certain Fe(II)-specific chelators involved in the release of Fe(II) from the active site of ferrireductases are produced by *P. sordida* YK-624, and that the Fe(II)-specific chelators are high-molecular-weight compounds such as siderophores,[35] as ferrireductase II reduced ferricytochrome c (Table 3).

Regarding the transport of iron in certain bacteria and yeasts, siderophores have an important role.[35] Siderophores are biosynthesized by the organisms under negative iron control and are released to the environment where the ferrisiderophore complexes are formed.[35] The complexes are subsequently taken up by the microorganisms[35] and are directly reduced by intracellular ferrireductase. Probably the compounds involved in the uptake of Fe(III) and the release of Fe(II) from ferrireductases are produced by white-rot fungi, although siderophores produced by certain bacteria and yeasts exhibit high specificity for Fe(III). The extracellular low-molecular-weight polypeptide from *Tyromyces palustris*[36] may be this kind of compound.

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