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Reduction of ferric chelate caused by various wood-rot fungi

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Abstract The reduction of ferric chelate caused by various wood-rot fungi was analyzed. Ferric chelate reductive activity was detected in cell-free extracts of seven wood-rot fungi: *Phanerochaete chrysosporium*, *P. sordida* YK-624, *Ganoderma* sp. YK-505, *Coriolus versicolor*, *Bjerkandera adusta*, *Tyromyces palustris*, and *Gloeophyllum trabeum*. These fungi produced NADPH- or NADH-dependent ferric chelate reductive enzymes (or both) of different molecular weight. In the liquid culture of *P. sordida* YK-624 and *C. versicolor*, a positive correlation was observed between extracellular MnP activity and intracellular NADPH-dependent ferric chelate reductive activity.

Key words Wood-rot fungus · Ferric chelate reduction · Manganese peroxidase · Gel permeation chromatography

Introduction

Iron is an essential element for life in all living organisms. The importance of iron in biochemical processes is due in part to its ability to exist in two redox states, ferric and ferrous. Iron found in iron-sulfur proteins or heme proteins has been reported to have oxidation reduction potentials between −500 and +300 mV.[1] This redox potential difference allows iron to function in a wide variety of electron transfer reactions. Particularly, the hydroxyl radical, produced by the reduction of hydrogen peroxide with Fe(II), is the strongest oxidizing agent in aqueous systems.[2] The hydroxyl radicals which brown-rot fungi produce cause selective cleavage of non-crystalline regions of cellulose and increase the contents of carbonyl and carboxyl groups.[3,4] In the white-rot fungus *Phanerochaete sordida* YK-624, ferrous chelates are involved in the reduction of manganese dioxide.[5] The iron in nature is in the oxidized [Fe(III)] form, which may form insoluble polymers of hydroxides, carbonates, and silicates under aerobic conditions around neutral pH.[6]

The first step in the uptake of iron by *Saccharomyces cerevisiae* involves the reduction of ferric iron at the cell surface via a transplasma membrane electron transfer system, and ferrireductase activity is increased when the cells are grown under iron/copper-deficient conditions.[7] The two proteins encoded by the *FRE1* and *FRE2* genes contribute to the cell ferrireductase activity.[8] Extracellular cellobiose dehydrogenase[9–12] and intracellular 1,4-benzoquinone reductase[13,14] in wood-rot fungi show ferric chelate (including cytochrome c) reductive activities; and a transplasma membrane redox system of white-rot fungus *Phanerochaete chrysosporium* can reduce ferricyanide.[15] Recently, we detected intracellular ferrireductases from the white-rot fungus *P. sordida* YK-624.[16] The reduction of Fe(III) is thought to play an important role in the degradation of wood components by wood-rot fungi. In this study, ferric chelate reductive activities of various wood-rot fungi were determined, and the characteristics of the ferric chelate reductive activity were analyzed.

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Materials and methods

Preparation of cell-free extracts

Five white-rot and two brown-rot fungi were used. *Phanerochaete sordida* YK-624 (ATCC 90872)[16] and *Ganoderma* sp. YK-505[17] were isolated from decayed woods. *Coriolus versicolor* (IFO 6482), *Bjerkandera adusta* (IFO 5307), *Tyromyces palustris* (IFO 30339), and *Gloeophyllum trabeum* (IFO 6268) were purchased from

the Institute for Fermentation, Osaka. *Phanerochaete chrysosporium* (ME446) was obtained from the American Type Culture Collection.

Three disks punched from the growing edge of the wood-rot fungal mycelium on a PDA plate were inoculated into a petri dish (9cm diameter) containing 15ml of a liquid medium.[16] Twenty petri dishes were statically incubated at 30°C for 9 days. 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 homogenized with an extraction buffer in a Waring blender 7010 (Waring Products Division, Dynamics Corporation of America). Homogenization at 15200rpm for 20s was repeated 15 times at 15-min intervals. The buffer solution 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 a 0.45-μm membrane filter (47mm diameter; nitrocellulose; Toyo Roshi Kaisha, Japan) to prepare the cell-free extract.

Gel permeation chromatography

The cell-free extracts were applied to a Superdex 75 column (2 × 50cm; Pharmacia Biotech, Sweden) equilibrated in 20mM sodium phosphate (pH 7.0) containing 0.1 M ammonium sulfate. The flow rate was 0.5ml/min. The molecular weight of ferric chelate reductive enzymes from various wood-rot fungi were estimated by the use of markers: bovine serum albumin (67kDa), ovalbumin (43kDa), chymotrypsinogen A (25kDa), and ribonuclease A (13.7kDa).

Enzyme assays

Ferric chelate reductive activities were determined by the increase in absorbance at 510nm using Fe(II)-1,10-phenanthroline (PHT) complex (extinction coefficient 12.11 $cm^{-1}mM^{-1}$).[18] The standard reaction mixture (1ml) consisted of 20mM sodium phosphate pH 7.0, 100μM Fe(III)–NTA complex, 1.5mM PHT, 100μM NADPH or NADH, and the cell-free extract. Reactions were initiated by the addition of NADPH or NADH. Extracellular manganese peroxidase (MnP) activities were determined by a modification of the method of Wariishi et al.[19] The reaction mixture (1ml) contained culture liquid, 1.0mM $MnSO_4$, 1.0mM 2,6-dimethoxyphenol, and 0.2mM hydrogen peroxide. These reactants were measured at 30°C with a Beckman DU640 spectrophotometer.

Results

Ferric chelate reductive activities of various wood-rot fungi

Figure 1 shows ferric chelate reductive activities in cell-free extracts from various wood-rot fungi grown for 9 days. All white-rot fungi exhibited higher NADPH-dependent ferric chelate reductive activity than NADH-dependent ferric chelate reductive activity. Higher ferric chelate reductive activities were detected in the cell-free extracts from *B. adusta* and *P. sordida* YK-624 than in those from other white-rot fungi. On the other hand, *G. trabeum* produced much higher NADH-dependent ferric chelate reductive enzyme than NADPH-dependent ferric chelate reductive enzyme.

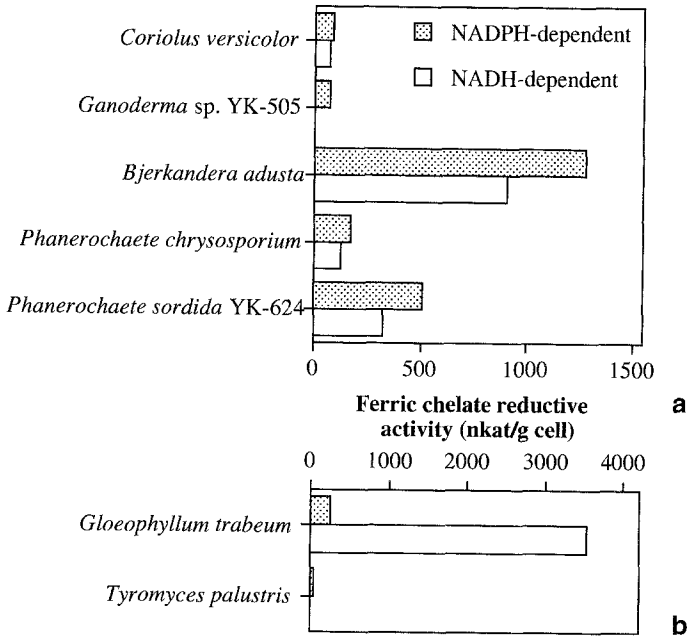

Fig. 1. Ferric chelate reductive activities in cell-free extracts from white-rot fungi (**a**) and brown-rot fungi (**b**)

Gel permeation chromatography of cell-free extracts from various wood-rot fungi

The cell-free extracts from *P. sordida* YK-624, *B. adusta*, and *G. trabeum*, which contained high ferric chelate reductive activity, were analyzed with gel permeation chromatography (Fig. 2). Elution volumes of ferric chelate reductive enzymes produced by the three fungi were different from each other, and molecular weights of NADPH-dependent ferrireductase produced by *P. sordida* YK-624, *B. adusta*, and *G. trabeum* were estimated to be 35, 42, and 34kDa, respectively. The molecular weights of NADH-dependent ferric chelate reductive enzyme from *G. trabeum*, *P. sordida*, and *B. adusta* were estimated to be 51, 35, and 42kDa, respectively.

Relation between extracellular MnP activity and intracellular NADPH-dependent ferric chelate reductive activity in white-rot fungi

Extracellular MnP activity and intracellular NADPH-dependent ferric chelate reductive activity by *P. sordida* YK-624 and *C. versicolor* in the liquid culture were analyzed

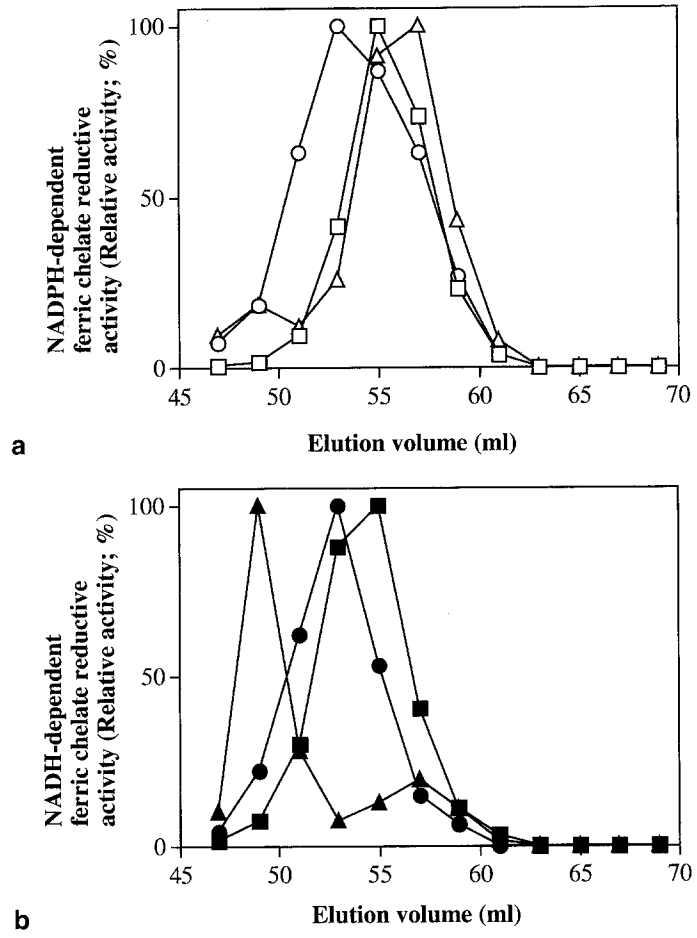

Fig. 2. Gel permeation chromatograms of the cell-free extracts from three wood-rotting fungi. (**a**) NADPH-dependent ferric chelate reductive activity. (**b**) NADH-dependent ferric chelate reductive activity. *Squares*, *Phanerochaete sordida* YK-624; *circles*, *Bjerkandera adusta*; *triangles*, *Gloeophyllum trabeum*

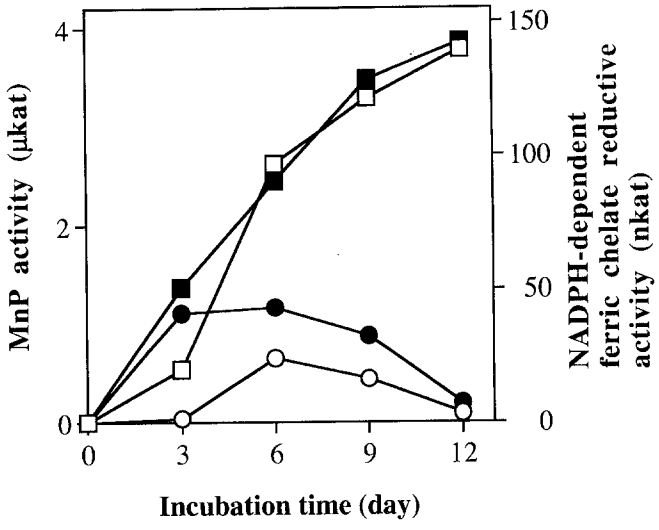

Fig. 3. Extracellular MnP and intracellular NADPH-dependent ferric chelate reductive activities of two white-rot fungi. Extracellular fluids and cell-free extracts were prepared from 15 plates inoculated with each fungus. *Open squares*, MnP of *P. sordida* YK-624; *open circles*, MnP of *Coriolus versicolor*; *filled squares*, NADPH-dependent ferric chelate reductive activity of *P. sordida* YK-624; *filled circles*, NADPH-dependent ferric chelate reductive activity of *C. versicolor*

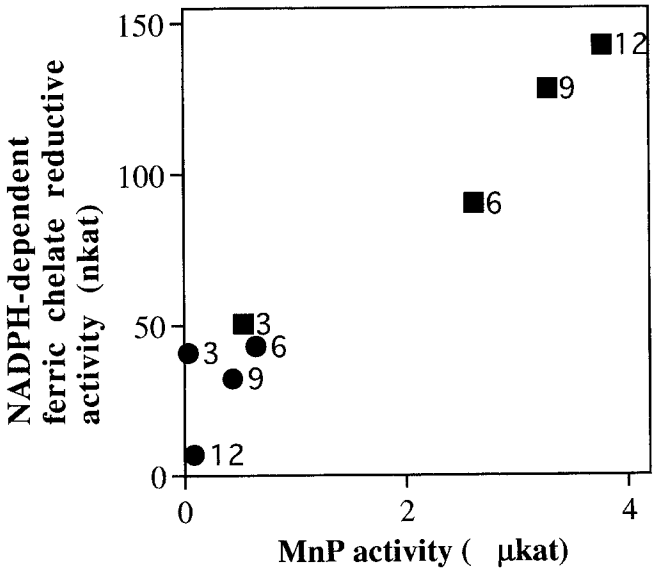

Fig. 4. Relation between extracellular MnP activity and intracellular NADPH-dependent ferric chelate reductive activity of two white-rot fungi grown in liquid culture. The numbers indicate the incubation day. *squares*, *P. sordida* YK-624; *circles*, *C. versicolor*

(Fig. 3). The enzyme activities of *P. sordida* YK-624 increased with increasing incubation time, whereas the maximum activity of *C. versicolor* was detected on day 6. Figure 4 shows the relation between extracellular MnP activity and intracellular NADPH-dependent ferric chelate reductive activity by the two white-rot fungi in the liquid culture. The higher the MnP activity that was detected, the higher was the NADPH-dependent ferric chelate reductive activity.

Discussion

Extracellular cellobiose dehydrogenase[9–12] and intracellular 1,4-benzoquinone reductase[13,14] in wood-rot fungi and intracellular ferrireductase isolated from *P. sordida* YK-624[15] are able to reduce ferric iron. Ferric chelate reductive activities were detected in cell-free extracts from almost all the wood-rot fungi tested. Three wood-rot fungi – *P. sordida* YK-624, *B. adusta*, *G. trabeum* – produced both NADPH-dependent and NADH-dependent ferric chelate reductive enzymes with different molecular weights.

Hyde and Wood reported that the critical Fe(II)/hydrogen peroxide combination (Fenton's reaction) is generated at a distance from the hyphae of brown-rot fungi and that hydroxyl radicals are formed without damage to the fungi.[20] The Fe(II)–hydrogen peroxide combination is involved in the depolymerization of cellulose by brown-rot fungi. With white-rot fungi manganese dioxide is accumulated in the decayed wood,[21] and the phenomenon is probably triggered

by the production of MnP. We previously reported that the white-rot fungus *P. sordida* YK-624 reduced manganese dioxide, and that the ferrous chelate was involved in the reduction.[5] In the present experiment, the production of extracellular MnP was linearly correlated with the production of intracellular NADPH-dependent ferric chelate reductive enzyme. This result suggests that the intracellular ferric chelate reductive enzyme has an important role in the production of MnP, because the rates of production of MnP and the oxidation by MnP are decreased by the accumulation of manganese dioxide [the decrease in the concentration of Mn(II)].

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