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Iron-binding compounds produced by white-rot fungus *Phanerochaete sordida* YK-624

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Abstract Iron-binding compounds were isolated from a culture of *Phanerochaete sordida* YK-624 and were found to bind to Fe(III) preferentially compared with Fe(II). Two iron-binding compounds were purified to near-homogeneity with gel permeation chromatography. Hydrolysis of the iron-binding compounds with 6N hydrochloric acid gave ninhydrin-negative products. The molecular weight of these compounds was 3–5kDa. These compounds may play an important role in the reduction of extracellular manganese dioxide to Mn(II) by intracellular ferrireductases for lignin degradation by manganese peroxidase.

Key words Iron-binding compound · *Phanerochaete sordida* YK-624 · Gel permeation chromatography · Ninhydrin-negative compound · White-rot fungi

Introduction

The accumulation of manganese dioxide [Mn(IV)] is sometimes observed in decayed wood.[1] This accumulation is probably triggered by the production and function of ligninolytic manganese peroxidase (MnP). Recently, we reported that white-rot fungus *Phanerochaete sordida* YK-624 reduced manganese dioxide to Mn(II), and that ferrous chelate is involved in the reduction.[2] Moreover, intracellular NADPH-dependent ferrireductase, reducing ferric chelate to ferrous chelate, was isolated from this fungus,[3] and various wood-rot fungi showed ferric chelate-reducing activity.[4] Thus, reduction of Fe(III) is an important reaction in wood-rotting fungi.

To utilize Fe(III) and other transition metals, many microorganisms have an efficient acquisition system. In the case of *P. sordida* YK-624, it is necessary that Fe(III), as the substrate for intracellular ferrireductase, passes through the cell membrane of the fungi. Components of a high-affinity iron system, probably the best understood of all metal acquisition systems, include extracellular low-molecular-weight chelators, termed siderophores, which bind to Fe(III).[5] Fekete et al. reported that all of the 10 wood-rot fungi were positive for chrome azurol-S, which can detect siderophores, in the solid and liquid cultivations, and that nine fungi produced fluorescent iron-binding compounds.[6] Jellison et al. isolated and partially purified the low-molecular-weight iron-binding compounds produced by the brown-rot fungus *Gloeophyllum trabeum*.[7] However, iron-binding compounds produced by white-rot fungi have not yet been clarified, although we purified ferrireductase from white-rot fungus *P. sordida* YK-624.[3] Herein, we primarily isolated, purified, and characterized iron-binding compounds produced by *P. sordida* YK-624.

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 (9cm diameter) were inoculated with the strain and incubated for 3 days at 30°C. Three fungal disks punched from the grown edge of the mycelium on a PDA plate were added to a petri dish (15cm diameter) containing 50ml of a liquid medium and statically incubated at 30°C for 12 days. The liquid medium contained 1% glucose, 1.2mM ammonium tartrate, 20mM 2,2-dimethylsuccinate, 1% Tween 80, 14.7mM KH_2PO_4, 2.16mM nitrilotriacetate (NTA), 2.80mM $MgSO_4$, 1.72mM $MnSO_4$, 6.33mM NaCl, 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

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Na_2MoO_4, and 3 μM thiamine HCl, adjusted to pH 4.5. Two hundred petri dishes were used.

Assay of iron-binding activity

Iron-binding activity of the compounds was confirmed by assay of Fe(II) with 1,10-phenanthroline (PHT). The reaction mixture (1 ml) consisted of 20 mM sodium phosphate buffer (pH 5.0), 0.1 mM $FeSO_4$, and sample solution (100 μl). The reaction was carried out at room temperature for 5 min, and 0.3 mM PHT was added to the mixture. After the reaction, the free Fe(II)–PHT complex was measured at 510 nm (12.11 $mM^{-1}cm^{-1}$).[8]

Isolation and purification of iron-binding compounds

Mycelial mats were separated from the cultures, and the culture fluid was filtered with a 0.45 μm pore size membrane filter (47 mm diameter) (nitrocellulose; Toyo Roshi, Japan). The filtrate was lyophilized and dissolved in 100 ml distilled water. The precipitate was removed by filtration with filter paper. Acetone was added to the solution to 70% (v/v), and after centrifugation (5000 *g*, 20 min) the precipitate was dissolved in 10 ml distilled water. A 2-ml aliquot of the solution was loaded onto a Superdex 75 (Pharmacia Biotech, Sweden) column (2.0 × 50 cm) equilibrated with distilled water. The column was eluted with distilled water at a flow rate of 0.5 ml/min. Fractions having iron-binding activity were collected and lyophilized; they were then dissolved in 5 ml distilled water. The solution (2 ml) was loaded onto a Sephadex G-25 (fine; Pharmacia Biotech) column (2.6 × 40 cm) eluted with distilled water at a flow rate of 1 ml/min. The active fractions were lyophilized and then dissolved in 2 ml distilled water. The solution (0.2 ml) was applied to a Sephadex G-25 (superfine; Pharmacia Biotech) column (1.0 × 200 cm) eluted with distilled water at a flow rate of 0.3 ml/min. The purification with Sephadex G-25 (superfine) was repeated twice.

Metal-binding specificity

To clarify iron [Fe(II) or Fe(III)]-binding specificity, 0.1 mM $FeSO_4$ or 0.1 mM $FeCl_3$ was mixed with 70% acetone-insoluble fraction prepared from the culture filtrate containing 20 mM sodium phosphate buffer (pH 5.0). The reaction mixture was applied to the column (2.6 × 40 cm) of Sephadex G-25 (fine) equilibrated with distilled water, then eluted with distilled water at a flow rate of 1 ml/min. The amount of iron in each fraction was measured by atomic absorption analysis.

To determine metal-binding specificity, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 mM $MnSO_4$, 0.1 mM $CoSO_4$, or 0.1 mM $ZnSO_4$ was added to the reaction mixture containing 20 mM sodium phosphate buffer (pH 5.0), 0.1 mM $FeSO_4$, and a purified iron-binding compounds solution. The reaction was carried out at room temperature for 5 min, and the free Fe(II)–PHT complex was measured at 510 nm.

Results and discussion

Detection of iron-binding compounds

Phanerochaete sordida YK-624 was incubated in the synthetic liquid medium without Fe(II) for 12 days at 30°C; then 70% acetone-soluble and 70% acetone-insoluble fractions were prepared from the culture filtrate. Iron-binding activity was detected in the 70% acetone-insoluble fraction. The iron-binding activity was greatly decreased by the addition of Fe(II) (2 mM) to the medium. These results were coincident with the result that the production of siderophores by fungi is suppressed with iron-sufficient medium (>1 mM Fe ion).[5]

To clarify iron-binding specificity to Fe(II) or Fe(III), the same concentration of Fe(II) or Fe(III) was added to the solution of the 70% acetone-insoluble fraction dissolved in distilled water, and the mixtures were applied to the Sephadex G-25 (fine) column. Figure 1 shows the concentration of iron bound to the compound eluted from the column. The iron concentration measured by atomic absorption spectrophotometry was higher in the case of the Fe(III) addition. This suggests that iron-binding compounds from *P. sordida* YK-624 bind to Fe(III) preferentially compared with Fe(II). However, we assayed iron-binding activity with Fe(II) and PHT in the further experiments because Fe(II) bound to the compounds can be easily detected with PHT at 510 nm.

Purification of iron-binding compounds

Iron-binding compounds were isolated from 800 ml of culture fluid [without Fe(II)] inoculated with *P. sordida*

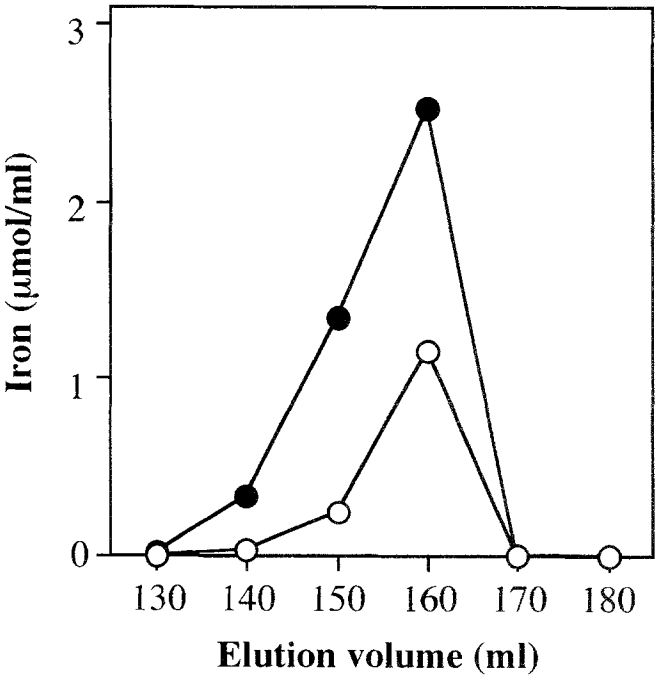

Fig. 1. Iron-binding specificity of each fraction in a Sephadex G-25 (fine) column. *Open circles*, Fe(II) added; *filled circles*, Fe(III) added

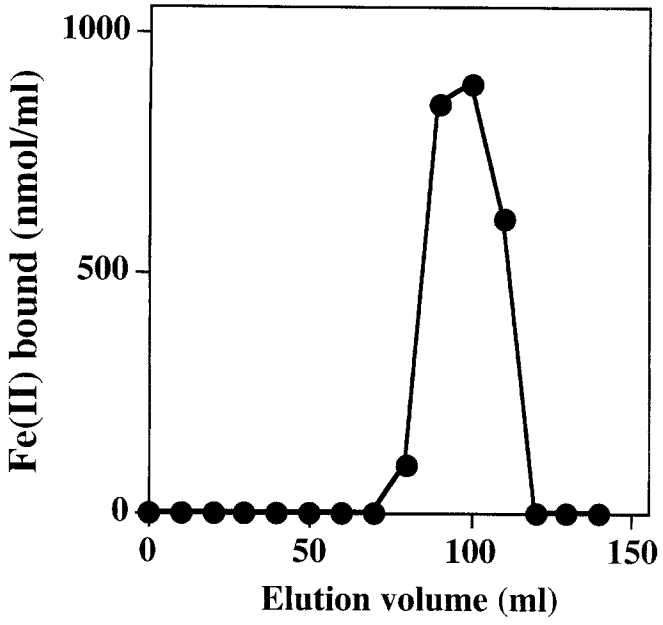

Fig. 2. Superdex 75 chromatography of the 70% acetone-insoluble fraction prepared from the culture of *P. sordida* YK-624

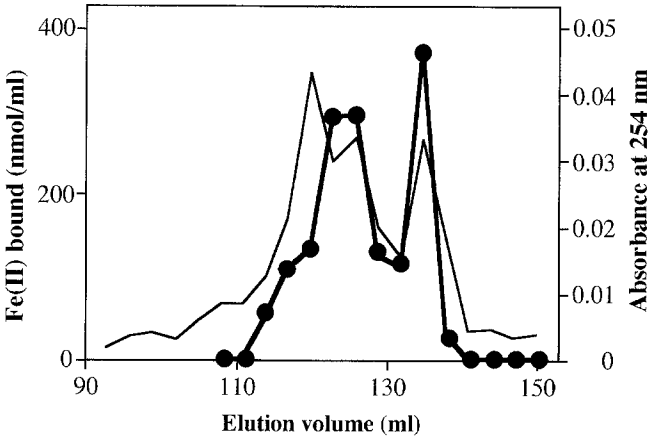

Fig. 3. Sephadex G-25 (superfine) chromatography of the crude iron-binding compounds eluted from a Sephadex G-25 (fine) column. *Circles*, iron-binding activity; *fine line*, absorbance at 254 nm

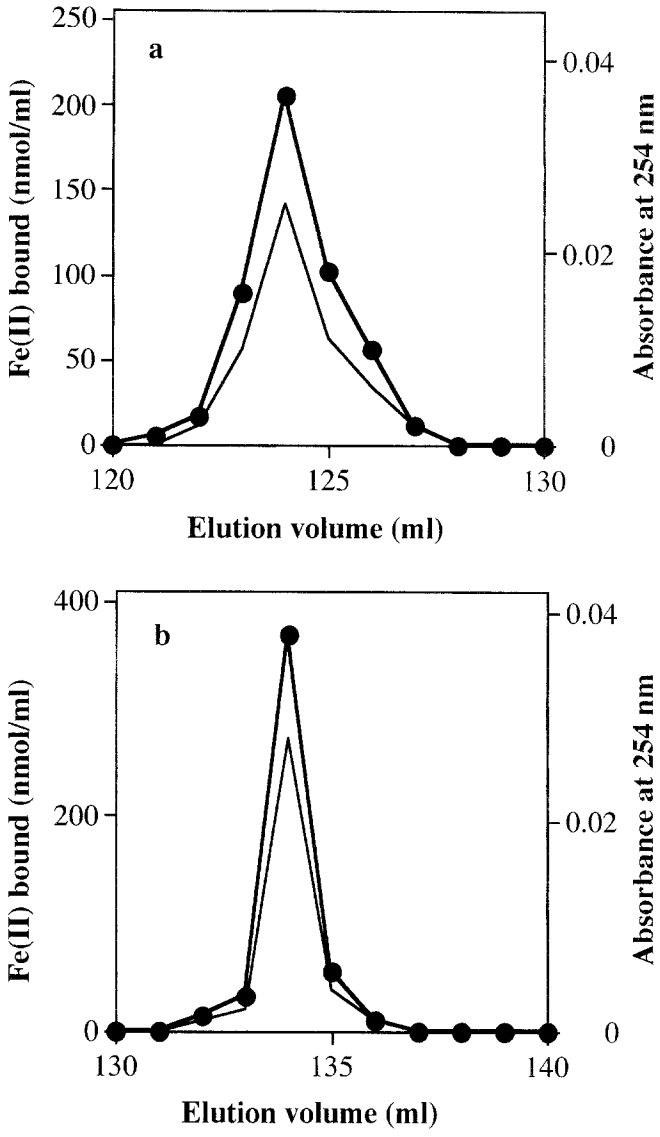

Fig. 4. Sephadex G-25 (superfine) chromatography of iron-binding compounds I (**a**) and II (**b**). *Circles*, iron-binding activity; *line*, absorbance at 254 nm

YK-624. Figure 2 shows a Superdex 75 chromatogram of the 70% acetone-insoluble fraction. Fractions (80–110 ml of elution volume) containing iron-binding activity were lyophilized and then applied to a Sephadex G-25 (fine) column. The active fractions eluted from the column were lyophilized and then applied to a Sephadex G-25 (superfine) column (Fig. 3). The fraction containing iron-binding compounds was separated into two fractions. Each active fraction was purified again with the same column. Finally, two iron-binding compounds, namely compounds I (2.0 mg) and II (1.5 mg), were isolated. The elution profile of iron-binding activity was coincident with that of absorbance at 254 nm, suggesting that compounds I and II were purified to near-homogeneity (Fig. 4).

Chemical and physical properties

Hydrolysis of iron-binding compounds (I and II) with 6N hydrochloric acid gave ninhydrin-negative products. These results indicate that iron-binding compounds from *P. sordida* YK-624 were not polypeptides. Moreover, iron-binding compounds from *P. sordida* YK-624 were not dissolved in any organic solvents, although siderophores from *Fusarium roseum* were dissolved in methanol.[9] These results suggest that iron-binding compounds from *P. sordida* YK-624 differed from extracellular glycopeptide from wood-rot fungi[10,11] and siderophore from yeast.[5]

The iron-binding compounds (I and II) had a molecular weight of approximately 3–5 kDa as determined by gel permeation chromatography and ultrafiltration. The molecular weights of iron-binding compounds isolated from *G. trabeum* and siderophores from microorganisms are less

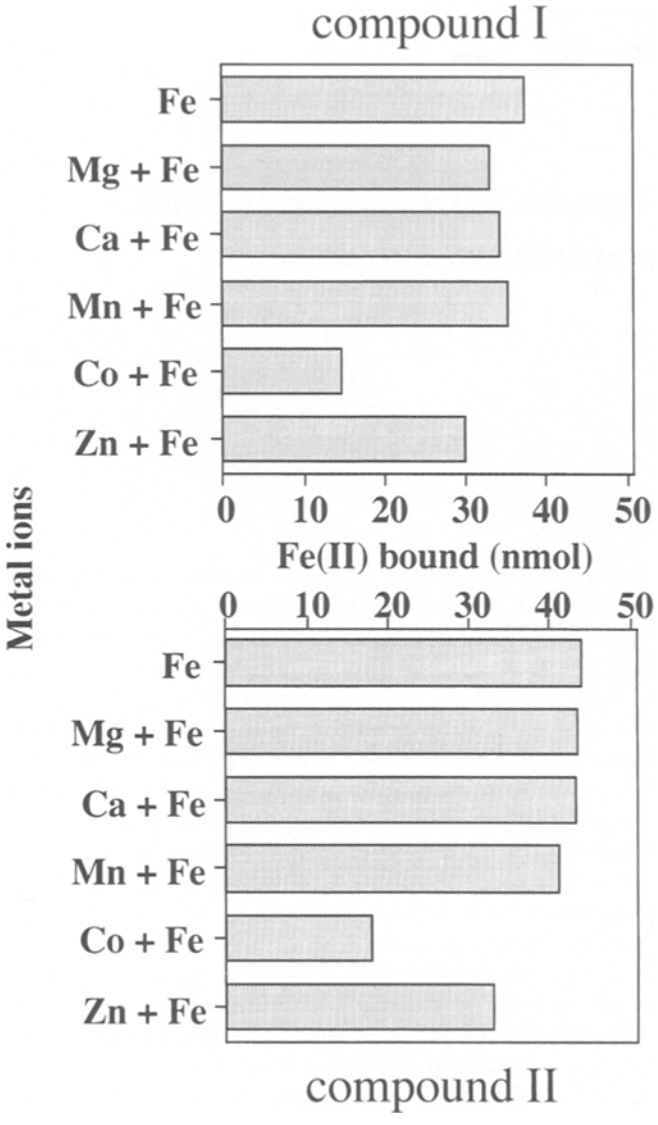

Fig. 5. Effect of metal ions on the Fe(II)-binding activities of compounds I and II

than 1 kDa.[5,6] Thus, the molecular weights of the iron-binding compounds from *P. sordida* YK-624 were higher than that from other microorganisms.

Metal-binding specificity

The metal-binding specificities of compounds I and II in the presence of Fe(II) were determined. As shown in Fig. 5, Fe(II)-binding activity was decreased by the addition of Co(II), although the addition of other metal ions did not affect the activity extensively. This suggested that iron-binding compounds could also bind to iron or cobalt.

Conclusions

We primarily isolated, purified, and characterized iron-binding compounds from white-rot fungus *P. sordida* YK-624, which are necessary for incorporating iron in the cell and reducing incorporated Fe(III) by intracellular NAD(P)H-dependent ferrireductases.[3] Two iron-binding compounds were purified by gel permeation chromatography. The iron-binding compounds were not polypeptides, as hydrolysis of iron-binding compounds with 6N hydrochloric acid gave ninhydrin-negative products. The iron-binding compounds exhibited molecular weights of approximately 3–5 kDa. Iron-binding compounds could bind Fe(III) preferentially, compared with Fe(II), and also bound to cobalt. It is possible that iron-binding compounds are involved in the incorporation of Fe(III) in the cell. Probably the Fe(III)–compound complex is directly reduced by intracellular NAD(P)H-dependent ferrireductases.[3] The structure of the iron-binding compounds is being examined in detail and will be reported on a forthcoming paper.

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