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Short Communication

Biosolubilization of low-rank coal by a *Trametes versicolor* siderophore-like product and other complexing agents

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SUMMARY

A heat stable, low molecular weight (< 1000) extracellular product in *Trametes versicolor* (= *Coriolus versicolor* = *Polyporous versicolor*) cultures was demonstrated to be a principal factor in the solubilization of leonardite and other low-rank coals. The solubilization of leonardite by *T. versicolor* cell-free cultures and active fractions was inhibited by Fe^{3+} and was mimicked by the siderophore desferal mesylate and the iron chelating agents EDTA and 8-hydroxyquinoline. Leonardite solubilization by these later compounds was also inhibited by Fe^{3+}. The ferrated and unferrated form of the partially purified active component from *T. versicolor* cultures demonstrated absorption spectra that were similar to the ferrated and unferrated form of desferal mesylate.

INTRODUCTION

The microbial solubilization of coal, first reported by Cohen and Gabriele [3], is a phenomenon that can be mediated by a number of microorganisms [11,12,14]. Despite the numerous reports of microbial solubilization of coal, there has not been agreement on a general mechanism of biosolubilization. Strandberg and Lewis [12] and Gupta et al. [4] demonstrated that coal solubilization by *Streptomyces* sp. was nonenzymatic and suggested that solubilization was mediated by microbial alkaline products, a hypothesis further supported by Quigley et al. [10].

Since Cohen and Gabriele [3] first reported the solubilization of lignite by *Polyporous versicolor* and *Poria monticola,* it has been hypothesized, because of similarities in structure, that the metabolic capacity of these organisms for degrading wood was also responsible for coal solubilization. Sub-

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sequent work by Pyne et al. [9] and Cohen et al. [2] demonstrated that extracellular products of *T. versicolor* were responsible for the biosolubilization of leonardite and presented data suggesting that these components were enyzmes.

We describe in this report evidence that solubilization of low-rank coals by *T. versicolor* is mediated by a low molecular weight extracellular component that has siderophore-like properties.

MATERIALS AND METHODS

Media, culturing, and chemicals

T. versicolor was routinely cultured in Sabourauds maltose broth (Difco, Detroit, MI) and maintained on agar slants of the same medium. Broth cultures were incubated at 27°C under static conditions. To obtain quantities of culture required for purification of extracellular product, *T. versicolor* was also cultured in a 20-l fermentor (Virtis, model 43-100). Static and fermentor cultures were harvested after 21–28 days following appearance of leonardite solubilizing activity.

The siderophore desferal mesylate was a gift from CIBA-GEIGY Corp. (Summit, NJ), EDTA was purchased from Sigma Chemical Co. (St. Louis, MO), and 8-hydroxyquinoline was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Quantification of coal solubilization

Coal was crushed and seived to obtain a uniform size fraction between 150–295 μm and dried at 50°C for 24 h in a vacuum oven. Ten mg of the dried coal were weighed into 16 × 100-mm glass test tubes, which were stopped, autoclaved, and allowed to dry at room temperature. Into duplicate test tubes, 0.5 ml of 0.1 M sodium phosphate-sodium acetate buffer (pH 5.4–5.5) and either 0.5 ml of culture filtrate (0.2 μm) or sterile Sabourauds maltose broth (blank) were added. Test tubes were incubated at 27°C for 24 h in a shaking incubator. After 24 h, the assay mixture was pipetted into a microfuge tube and centrifuged for 1 min to pellet the coal particles. The absorbance of the supernatant or dilution thereof at 450 nm was measured spectrophotometrically (Beckman model DU-8, Beckman Instruments, Inc., Fullerton, CA). A standard curve, prepared using acid-precipitated biosolubilized coal, was used to calculate the amount of solubilized coal. Analytical standards were prepared from leonardite solubilized by *C. versicolor* Sabouraud maltose agar plate cultures. Solubilized coal was removed by pipette directly from agar plates, filtered through a 0.22 μm membrane filter, freeze-dried, and placed in a drying pistol over P_2O_5, using toluene as the refluxing agent. After drying, coal standards were carefully weighed, dissolved in water, and their absorbance at 450 nm determined. All values are reported as leonardite solubilizing units (LSU), which are defined as the weight in mg of leonardite converted to water soluble product in 24 h at 27°C.

Chromatographic separations and ultrafiltration

Gel permeation chromatography was used to define the molecular weight range of the extracellular coal-biosolubilizing *T. versicolor* product. Approximately 35 ml of culture, which had been concentrated 20-fold by freeze-drying and filtered through a 0.45 μm membrane filter (Acrodiscs, low protein binding, Gelman Sciences, Inc., Ann Arbor, MI) was applied to a Sephadex G-50 (fine) 2.5 × 26-cm column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The column was eluted with 0.05 M 2,2-dimethylsuccinic acid at pH 5.5, and 2 ml fractions were collected. The absorbance at 280 nm and leonardite solubilizing activity of each fraction were determined. Molecular weight standards were also eluted on the Sephadex G-50 column to obtain an estimate of the molecular weight of the extracellular product. These standards included ovalbumin (molecular weight = 43 000), chymotrypsinogen (molecular weight = 25 000) and ribonuclease A (molecular weight = 13 700).

Ultrafiltration was also used to define the molecular weight of the coal biosolubilizing extracellular product. Approximately 100 ml of active *T. versicolor* culture filtrate was sequentially ultrafiltered (Amicon) through membranes with molecular weight cutoffs of 10 000, 5 000, 1000, and 500. Retentates and filtrates were adjusted to the original

culture volume prior to assaying for coal biosolubilization.

The procedure used for isolation of the component(s) responsible for coal biosolubilization activity was similar to that described by Teintze et al. [13]. Approximately 1 g of ferric nitrate was added to 1 l of *T. versicolor* culture filtrate and the suspension saturated with ammonium sulfate. The resulting slurry was extracted with benzyl alcohol using a procedure described by Neilands [8]. The red-brown aqueous extracts were concentrated to dryness in a vacuum. The residue was dissolved in a mixture of pyridine and acetic acid (1:5, v/v), and the pH of the resulting solution adjusted to 5.5. The solution was then chromatographed on a column containing CM-Sephadex C-25. Two distinct bands were separated, and the predominant red-brown band was concentrated to dryness. The residue was dissolved in 10 ml of pyridine and 30 ml of water, and then chromatographed at 4°C on a Bio-Gel P-2 (Bio-Rad Laboratories) column equilibrated in 0.2 M pyridine–acetic acid, pH 7.4. The red-brown band was concentrated to dryness and the residue stored at 4°C in the dark. Approximately 0.1 g of the residue was dissolved in 3 ml of water, and the UV absorption spectrum was determined with a Varian DMA 200 UV-visible spectrophotometer.

Approximately 0.5 of the red-brown residue was deferrated with 8-hydroxyquinoline as described by Meyer and Abdallah [6]. The resulting yellow-green extract was concentrated to dryness, the residue dissolved in 5 ml of 0.2 M pyridine–acetic acid (pH 5.5), and the resulting solution chromatographed on a Bio-Gel P-2 column equilibrated with the same buffer. The resulting yellow-green band was concentrated to dryness. Approximately 0.1 g of this residue was dissolved in 3 ml of distilled water and the UV absorption spectrum determined.

RESULTS AND DISCUSSION

To determine the approximate molecular weight of the *T. versicolor* extracellular component responsible for leonardite solubilization, concentrated (20-fold) culture filtrate was chromatographed on a Sephadex G-50 (fine) column. The peak leonardite solubilizing activity eluted in the same fractions as the peak 280 nm absorbance (Fig. 1). Further characterization of these fractions by thin layer chromatography revealed up to six ninhydrin-positive bands (data not shown), most of which were also present in the Sabouraud maltose broth medium control. This suggests that the Ab_{280} may have been due, in part, to amino acids and peptides in the medium.

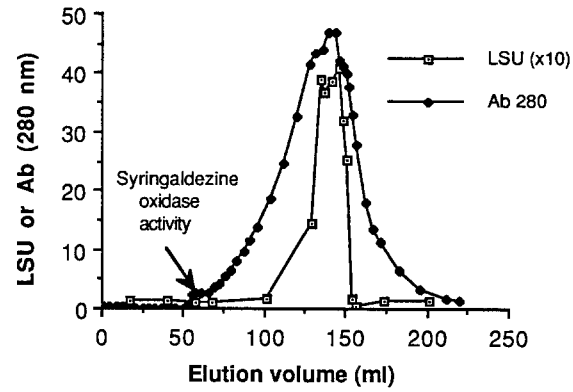

Fig. 1. Gel permeation chromatography of *T. versicolor* concentrated (20 times) filtrate on Sephadex G-50 (fine). Leonardite biosolubilization is expressed in leonardite solubilizing units (LSU), which are defined as the weight in mg of leonardite converted to water soluble product in 24 h at 27°C.

A molecular weight standard curve was constructed using the elution volumes of the protein molecular weight standards. Calculation of the elution parameter K_{av} for the *T. versicolor* active fraction indicated that the molecular weight of this product was less than 1000. This value was extrapolated as the active component eluted at a volume less than the lowest molecular weight standard, ribonuclease A (molecular weight = 13 700). The syringaldezine oxidase activity of the individual fractions was determined qualitatively and was found to elute in a fraction that contained little leonardite solubilizing activity (Fig. 1). Syringaldezine oxidase from non-induced cultures of *T. versicolor* is estimated to have a molecular weight of approximately 70 000 [1]. Heating the active fractions to 100°C for 40 min had no effects on the coal biosolubilizing activity, indicating that the extracellular product

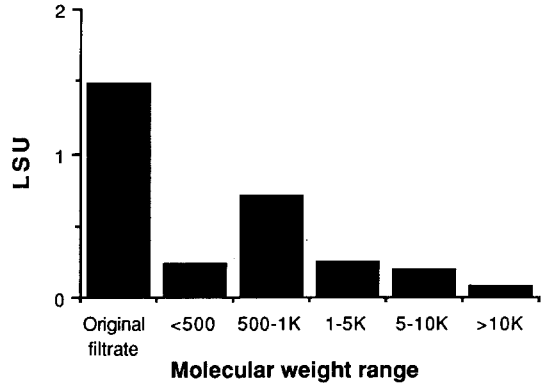

Fig. 2. Leonardite biosolubilized by *T. versicolor* culture ultrafiltration fractions.

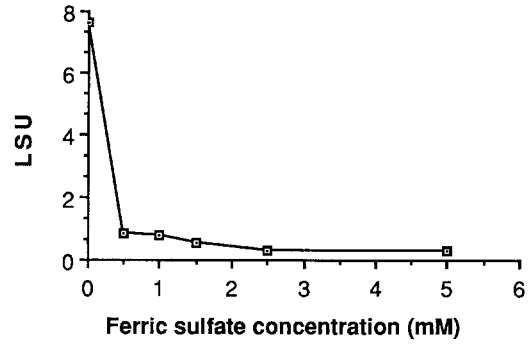

Fig. 3. Effect of ferric sulfate on leonardite solubilization by concentrated (4 times) *T. versicolor* culture filtrate.

was not an enzyme, in contrast to earlier suggestions that coal biosolubilization by *T. versicolor* was enzymatic [2,9].

The active *T. versicolor* extracellular coal-biosolubilizing product with a molecular weight less than 1000 is supported by culture filtrate ultrafiltration experiments. Fig. 2 shows the relative coal solubilizing activity of the various ultrafiltered fractions. Almost no activity is present in either the $>10\,000$ or the 5 000–10 000 fractions. There is some activity in the 1000–5 000 fraction, the greatest activity in the 500–1000 fraction, and little again in the <500 fraction.

Multivalent metal ions are known to be important components in the structure of low-rank coals and are hypothesized to link organic moieties by salt bridging [5]. Therefore, we hypothesized that metal binding by an extracellular component of *T. versicolor* may be involved in the solubilization of leonardite. To test this hypothesis, various concentrations of Fe^{3+}, as ferric sulfate, were added to the biosolubilization assay mixture. Even at the lowest concentration, 0.5 mM, ferric sulfate inhibited coal solubilization by almost tenfold (Fig. 3).

Since metal ions can bind to protein and other organic compounds that are not produced specifically to complex metals, the ability of known Fe chelating agents to solubilize leonardite was also examined. Desferal, a hydroxamate-type siderophore with an Fe^{3+} formation constant (log K_f) of 30.6 produced by an *Actinomyces* [7], was a potent agent for solubilizing leonardite (Fig. 4) as was the synthetic chelator EDTA. The leonardite biosolubilizing values obtained with these Fe binding agents are in the range of those obtained with *T. versicolor* culture filtrates or active gel permeation chromatography fractions. Both desferal and EDTA solubilized significant quantities of leonardite, even at the lowest concentration tested (5 mM). The synthetic metal binding agent 8-hydroxyquinoline also solubilized leonardite but to a much lesser degree than either desferal or EDTA. Desferal (50 mM) also solubilized weathered Alabama and Mississippi lignites, but to a lesser degree than leonardite (data not shown). These results support earlier observa-

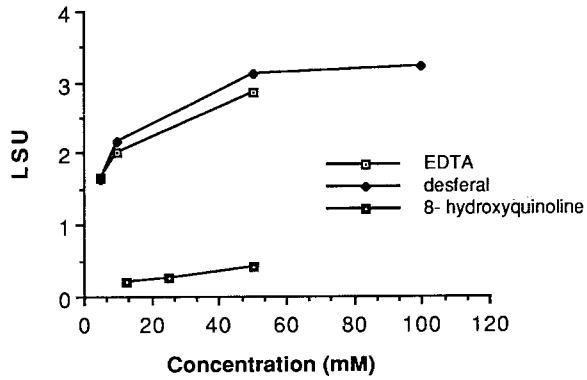

Fig. 4. Solubilization of leonardite by the hydroxamate-type siderophore desferal and the synthetic chelators EDTA and 8-hydroxyquinoline.

tions that synthetic chelating agents solubilized leonardite [9].

The addition of 1 mM ferric sulfate to leonardite solubilization assay mixtures with 5 mM desferal reduced solubilization by 37%, similar to the Fe^{3+} inhibition of leonardite solubilization by *T. versicolor* culture filtrate. The pH of the reaction mixture, buffered with 0.1 M sodium phosphate-sodium acetate at pH 5.5, was measured at the beginning, during and at the end of the 24-h assay period and was found not to fluctuate more than 0.1 unit from the buffer pH, thus ruling out base solubilization as a mechanism for leonardite biosolubilization by *T. versicolor* extracellular products.

The purification protocol used for separation of the extracellular leonardite-biosolubilizing product was that employed by Teintze et al. [13] for the purification of the bacterial siderophore pseudobactin. The use of this purification scheme with *T. versicolor* cultures filtrate resulted in a red-brown band similar in description to that of ferric pseudobactin. When 0.5 ml (380 mg in 2 ml of distilled water and adjusted to pH 5.5) of this material was assayed for leonardite biosolubilization, the fraction was found to have an activity of 1.8 leonardite solubilizing units. Although this was the ferrated form, the addition of 10 mM ferric sulfate to the reaction mixture reversed the coal solubilizing activity of this material. It may have been that the other ninhydrin-positive compounds in this fraction (i.e. amino acids, peptides) were binding some of the Fe^{3+} so that not all of the *T. versicolor* siderophore was complexed. This material, after deferration with 8-hydroxyquinoline [6], no longer solubilized leonardite. However, when desferal was deferrated using this same procedure, it also no longer solubilized leonardite, indicating that the coal solubilizing activity is destroyed during the process of deferration with 8-hydroxyquinoline.

The ferrated and deferrated complexes from *T. versicolor* gave absorbance maxima at 430 and 360 nm, respectively (Fig. 5a). These absorption maxima are comparable to those of other siderophores. The ferrated and deferrated forms of desferal have absorption maxima at 424 and 370 nm, respectively (Fig. 5b). Also, the Fe complex of pyoverdine and

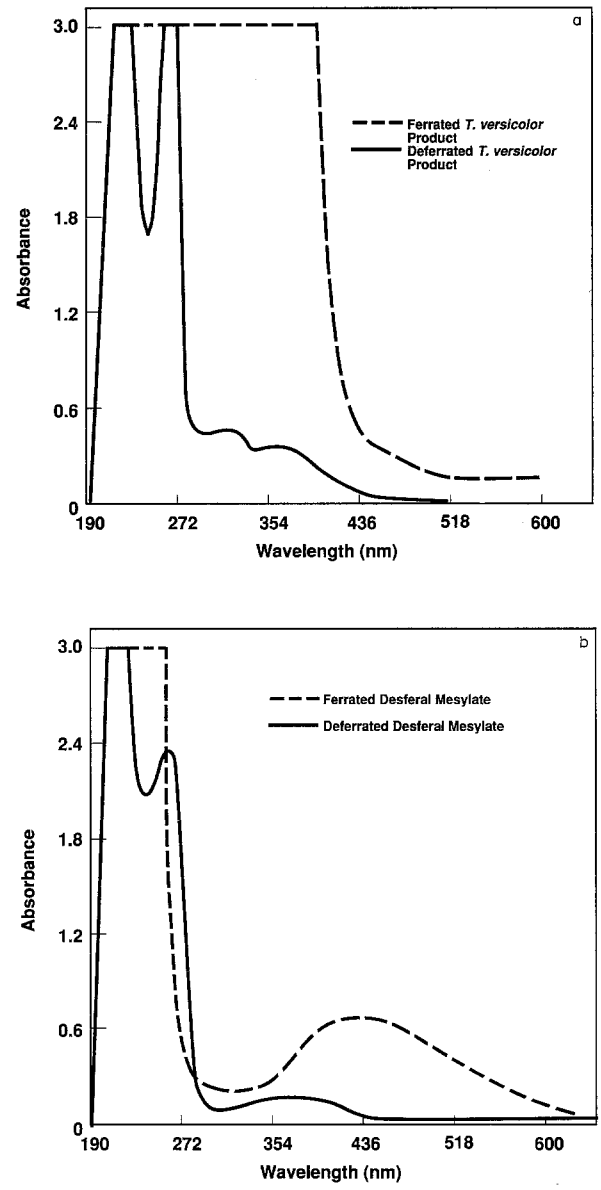

Fig. 5. Absorption spectra of (a) ferrated and deferrated *T. versicolor* coal biosolubilizing product and (b) ferrated and deferrated desferal.

pyoverdine have absorbance maxima at approximately 420 nm and 380 nm, respectively [6]. From these preliminary results it appears that the extracellular product produced in cultures of *T. versicolor* responsible for leonardite biosolubilization is chemically similar to siderophores produced by other microorganisms.

The Fe^{3+}-mediated inhibition of leonardite solubilization by cell-free fractions from *T. versicolor* and other Fe chelating agents, both microbially produced and synthetic, suggests that the major product contributing to biosolubilization of low-rank coals by *T. versicolor* is a siderophore. In addition, it appears that the mechanism of coal biosolubilization by this organism is, at least in part, by complexation of Fe or other metal cations that may act as bridges between the organic moieties. Further studies are needed to fully characterize the *T. versicolor* siderophore-like product and its mechanism of action in biosolubilizing coal.

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