APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1990, p. 3285–3291
0099-2240/90/113285-07$02.00/0

Vol. 56, No. 11

Isolation and Identification of the Coal-Solubilizing Agent Produced by *Trametes versicolor*

MARTIN S. COHEN,[1]* KATHLEEN A. FELDMAN,[1] CYNTHIA S. BROWN,[1] AND EDWARD T. GRAY, JR.[2]

Department of Biology[1] and Department of Chemistry,[2] University of Hartford, West Hartford, Connecticut 06117

Received 30 April 1990/Accepted 7 August 1990

Low-ranked coals were dissolved by using cell extracts derived from liquid cultures of *Trametes versicolor*. The coal-solubilizing agent (CSA) was separated from the broth components by a multistep isolation procedure including reverse-phase high-pressure liquid chromatography, size exclusion chromatography, ethanol fractionation, and recrystallization. Staircase voltammetry was used to show that two CSA moieties can coordinate to aqueous copper(II) ion. A molecular weight determination (using amperometry) gave an apparent molecular weight of $1.3_4 \times 10^2$ g/mol ± 8%. Nuclear magnetic resonance indicated that all protons on CSA are exchangeable in D_2O and that there is only one type of carbon in CSA. The infrared spectrum of recrystallized CSA is identical to that of ammonium oxalate, and X-ray studies confirmed the crystal structure and composition of CSA to be that of ammonium oxalate monohydrate. The equivalent weight of the coal in solution, when the coal was dissolved by ammonium oxalate, is 7,940 g of coal per mol of iron present in the coal.

Biological solubilization of low-rank coals has continued to be a subject of interest since 1982 when it was reported that lignite could be degraded to recoverable liquid products by the fungus *Polyporus versicolor* (*Trametes versicolor*) growing in solid agar cultures (4). Since that time, many authors have reported that these coals can be dissolved by additional microorganisms including *Paecilomyces* TLi (6), various species of *Penicillium* (10) and *Streptomyces* (8, 13, 14), *Phaerochaete chrysosporium* (12, 16), *Candida* sp. (11), and *Cunninghamella* sp. (11). Coal solubilization in cell-free liquid broths in which fungi have grown has also been reported (2, 3). However, there have been no reports of the direct isolation of a fungal or bacterial metabolite that is capable of coal solubilization. While several authors have reported that microbial coal solubilization may be nonenzymatic (13, 14) or may result from production of alkaline materials by the microorganisms (11), no single coal-solubilizing chemical product has been isolated or identified.

This paper describes the isolation and chemical identification of the first biologically produced compound from *T. versicolor* that dissolves low-rank coal and the first report of the chemical identification of a coal-solubilizing product produced by any microorganism.

MATERIALS AND METHODS

Culture conditions. *T. versicolor* ATTC 12679 was routinely maintained by growth on Sabouraud maltose agar (Difco Laboratories) plates at 30°C. Several agar blocks were aseptically cut out and added to 100-ml Fahraeus and Reinhammer medium (F&R) (5) in a 500-ml Erlenmeyer flask and incubated at 30°C unshaken until significant coal-solubilizing activity was detected, generally after about 30 to 40 days of growth. The broth was filtered through glass wool to remove hyphae. The filtered broth was used directly for subsequent processing or concentrated by rotoevaporation at 38°C. Distilled-deionized water was used in all culturing procedures and subsequent isolation and identification procedures of the coal-solubilizing agent (CSA).

Spectrophotometric coal solubilization assay. CSA activity was assayed by a modification of the method of Cohen et al. (2, 3). Appropriately diluted active broth (0.5 ml) and 0.5 ml of 0.1 M monosodium phosphate–0.1 M sodium acetate buffer at pH 5.4 (the standard buffer) were incubated with 10 mg (149 to 250 μm) of leonardite coal (American Colloid Co., Reeder, N.D.) at 30°C on a rotary shaker (175 rpm). A_{450} of each sample was determined at 24 h.

Chromatography. Concentrated (10×) F&R active filtrate (70 ml) was pretreated by mixing with approximately 70 g of C_{18} resin (552 to 105 μm; Waters Associates) in batch with stirring. The active filtrate was removed from the resin by filtering through a glass-fiber filter (Whatman GF/F), and the resin was washed with 500 ml of water. The filtrates were pooled and concentrated by rotoevaporation at 38°C to 20× the original concentration of whole filtrate. The pretreated concentrate (2 ml) was injected onto a preparative μBondapak C_{18} high-pressure liquid chromatography column (19 by 150 mm; Waters) and eluted with water at a flow rate of 6 ml/min. The fractions with highest CSA activity were pooled and concentrated by rotoevaporation at 40°C to 67× the concentration of the original broth. Two milliliters of this concentrate was then applied to a column (1.5 by 25 cm) packed with Sephadex G-15-120 resin. The column was eluted with water at a flow rate of 2 ml/min. The fractions were collected and assayed for coal-solubilizing activity. Active fractions were pooled and stored at −4°C. These fractions represented a 17× concentration of the original F&R broth.

Ethanol fractionation. Absolute ethanol was added to pooled active fractions described above in a 3:1 (vol/vol) ratio. The mixture was centrifuged, and both the precipitate and supernatant were saved (75% ethanol cut). Additional absolute ethanol was added to give a final 6:1 (vol/vol) ratio of ethanol/active fraction. The mixture was centrifuged, and the precipitate was saved (86% ethanol cut). All of the

* Corresponding author.

precipitates and supernatants were dried and tested for CSA activity.

Recrystallization of the CSA fraction. The precipitate obtained from the 86% ethanol cut was dissolved in a minimal amount of water. The solution was allowed to evaporate slowly in a desiccator at room temperature until crystals formed. The crystals were removed by filtration through a 0.45-μm filter (Gelman Nylaflo) and washed with water and 95% ethanol. The filtrate was saved, additional active material from the 86% ethanol cut was added to the filtrate, and the process of recrystallization was repeated. Crystals of CSA obtained from this procedure were used throughout the chemical testing procedures.

Thin-layer electrophoresis. Thin-layer electrophoresis was performed on Whatman K-2 cellulose plates (5 by 20 cm) and Baker IB2 silica gel plates (20 by 20 cm) in a paper electrophoresis unit (Arthur H. Thomas Co.). Active F&R culture broth was concentrated 100× by rotoevaporation, and 5 μl was spotted onto cellulose or silica gel plates. The origin was located on a line bisecting the plate across the short axis. Additional plates were run concurrently without application of active broth. Electrophoresis was carried out for 45 min at a constant voltage of 300 V in freshly prepared 0.05 M sodium acetate buffer, pH 4.6. Following electrophoresis, half of the plates containing the active broth were stained with ninhydrin reagent. The reagent solution was sprayed uniformly onto the plates and allowed to dry. The plates were then heated at 110°C for 5 min (1).

The cellulose or silica gel coating was scraped off the anodic and cathodic half of each unstained plate (with and without the active fraction). Each cellulose or silica gel sample was placed in a separate beaker and eluted overnight with stirring at room temperature in 100 ml of water. The cellulose or silica gel was removed by filtration, and the filtrates were brought to dryness in a rotoevaporator, redissolved in 1 ml of 0.05 M monosodium phosphate–0.05 M sodium acetate buffer (pH 5.4), and tested for coal-solubilizing activity. Five microliters of the concentrated active broth was also tested for coal-solubilizing activity.

Aliquots (1 to 2 μl) of active fractions following high-pressure liquid and size exclusion chromatographies, ethanol precipitations, and recrystallization were also separated by thin-layer electrophoresis, using Whatman K2 cellulose thin-layer chromatography plates as described above.

Spectrophotometric and gravimetric coal-solubilizing assays of the recrystallized CSA. A sample of leonardite coal was brought to constant weight at 38°C and placed in a desiccator. Fifteen approximately equal (10 ± 0.1 mg) samples of the cooled leonardite were weighed rapidly in test tubes. To each coal sample, 0.5 ml of the standard phosphate-acetate buffer and 0.5 ml of CSA solution (recrystallized CSA dissolved in water) were added. The CSA solutions contained varying amounts of CSA ranging between 0 and 6.0 mg/ml. Each treatment was prepared in triplicate. The mixtures were shaken at 30°C for 24 h. The liquid fraction was removed from each test tube with a Pasteur pipette. The A_{450} of each sample was measured, and the liquid fraction was returned to the test tube. The contents of each test tube were filtered through nylon filters (Gelman Nylaflo, 0.45 μm). Each tube was washed with additional water and the washings were poured through the filters. The unsolubilized coal particles were dried on the filters at 38°C for 20 h, placed into a desiccator for 1 h to cool, and weighed.

Spectrophotometric titration of the CSA. Visible absorption data were obtained with a Cary 219 spectrophotometer whose cell compartment was thermostated at (25.0 ± 0.1)°C. For the titration, 5.00 ml of a solution at pH 11 which contained 1.698×10^{-3} M Cu^{2+} and 6.16×10^{-3} M glycylglycine was placed in a 2.000-cm quartz cell. Unconcentrated F&R active filtrate containing CSA was used as the titrant. Absorption measurements were made at 700 nm as 50.0-μl aliquots of CSA (pH 11) were added. Measurement of pH was accomplished with an Orion 701A meter, using a Beckman Futura glass electrode (0-14) and a calomel reference electrode which was bridged to the test solution through an $NaNO_3$-agar bridge. This same reference electrode was used in the voltammetry experiments.

Determination of the stoichiometry of the Cu-CSA complex. Staircase voltammograms of a 9.346×10^{-5} M solution of Cu^{2+} were obtained before the addition of CSA and after the addition of various amounts of CSA when the CSA was always in great excess. All voltammetry was performed on a computer-controlled unit built in-house (7). The DME-staircase voltammograms were obtained at (25.0 ± 0.1)°C with a 5,000-step ramp over the voltage range indicated for each experiment.

Amperometric titration of copper(II) ion with CSA. In the case of the amperometric titration, full voltammograms were obtained for each point from which the current at a specific voltage was determined. The potential at which the current was monitored was between the half-wave potential of the original copper solution and that of the 1:1 complex of copper and CSA.

NMR spectra of the CSA. Nuclear magnetic resonance (NMR) spectra were obtained on a GE 200-MHz spectrometer at 25.0°C. A single solution of CSA was prepared by dissolving 100 mg of the purified material in D_2O, which is prepared with 0.1% (wt/wt) DSS as standard (MSD Canada Limited).

Crystallographic molecular structure determination of the CSA. A series of X-ray crystallographic studies were performed at room temperature and −110°C on single crystals of CSA which had been isolated as described above. The X-ray crystallographic work was performed through the Electric Power Research Institute (EPRI) in the laboratory of D. van der Helm at the University of Oklahoma.

IR spectra of isolated CSA and commercial ammonium oxalate. Infrared (IR) spectra were obtained with a Perkin-Elmer model 1310 infrared spectrometer. The CSA samples and ammonium oxalate monohydrate reagent (Mallinckrodt) were prepared as KBr (spectroscopic grade [Buck Scientific]) pellets.

Reagent preparation. Ninhydrin (4.0 g) was dissolved in 100 ml of 95% ethanol. Copper nitrate was prepared by the quantitative dissolution of the metal (electrolytic foil; J. T. Baker Chemical Co.) in nitric acid (Fisher Scientific), followed by the evaporation of any excess nitric acid and dilution of the resultant crystals of copper nitrate. Glycylglycine (Sigma Chemical Co.) was used without further purification.

RESULTS

Multistep procedure for CSA isolation. The protocol of passing the concentrated, pretreated F&R broth through the preparative μBondapak C_{18} column followed by a small-volume column filled with a low-molecular-weight cutoff resin resulted in concentrating the coal-solubilizing activity into one 4-ml fraction. Thin-layer electrophoresis followed by staining with ninhydrin showed that the active fraction still contained at least asparagine (one component of the F&R medium), aspartic acid, and one unidentified spot as

TABLE 1. Coal-solubilizing activity of filtrates of F&R media in which *T. versicolor* had grown and of fractions of the media after several steps in the purification protocol for CSA

Treatment	Dry wt (g)	% Dry wt recovered	Wt of CSA (g)[a]	% CSA recovered	Wt % CSA
F&R filtrate	2.86	100	0.74	100	25
Filtrate pretreatment	2.28	80	0.60	81	26
After C_{18} chromatography	1.05	37	0.28	38	27
After Sephadex chromatography	0.54	19	0.20	27	37
CSA crystals	0.09	3	0.09	12	100

[a] These values are based on the fact that 1.2 mg of CSA produces an A_{450} of 10 and the assumption that all of the coal-solubilizing activity is caused by CSA.

contaminants. The aspartic acid was produced from asparagine when the medium was sterilized in the autoclave. The presence of asparagine and aspartic acid was verified by comparison against standard samples.

Ethanol precipitation was used to separate CSA selectively from the contaminants. Most (69%) of the coal-solubilizing activity was associated with the precipitate from the 86% ethanol cut. The thin-layer electrophoretic chromatograms of this precipitate showed no spots after staining with ninhydrin. When a very large sample (10 to 20 μl) was applied to the thin-layer plate and the procedure was repeated, one very faint spot appeared after staining with ninhydrin.

When a small amount of solid from the ethanol precipitation was dissolved in water and the water was allowed to evaporate slowly, small, clear, colorless, needlelike crystals formed. One of these crystals was used in the coal-solubilizing assay described above. The weight of the crystal was <100 μg. The resulting assay gave an absorbance of 0.4 AU over the background. Analysis of the crystals by thin-layer electrophoresis showed no ninhydrin-active species. The results of the various separation procedures are summarized in Table 1.

It is noteworthy that when thin-layer electrophoresis was performed on cellulose plates, CSA activity migrated toward the anode and was recovered. However, no CSA activity was recovered from silica gel plates after electrophoresis. One difference between the cellulose plates and the silica gel plates is that the silica gel plates contain calcium sulfate as a binder. It is likely that the CSA, which is negatively charged under these conditions, is coulombically attracted and/or coordinated to the calcium ion in the binder. These interactions could inhibit the elution of CSA from the plate. The CSA was not localized to a ninhydrin-positive spot.

Spectrophotometric and gravimetric assays of the recrystallized CSA. Spectrophotometric assay of the recrystallized CSA showed a progressive linear absorbance with added amounts of CSA (Fig. 1). Results of gravimetric analysis of the recrystallized CSA are shown in Fig. 9. Loss in coal weight is linear up to about 1.5 mg of CSA added, with each unit of CSA dissolving twice its weight in coal. The amount of coal dissolved per unit of CSA added decreases with higher amounts of CSA, since the CSA can dissolve approximately one half of the coal.

Spectrophotometric titration of the CSA. Figure 2 is a spectrophotometric titration of Cu^{2+} in the presence of excess glycylglycine, using CSA as the titrant. The intersection of the two linear regions of the titration curve gives an empirical concentration, subject to the determination of the

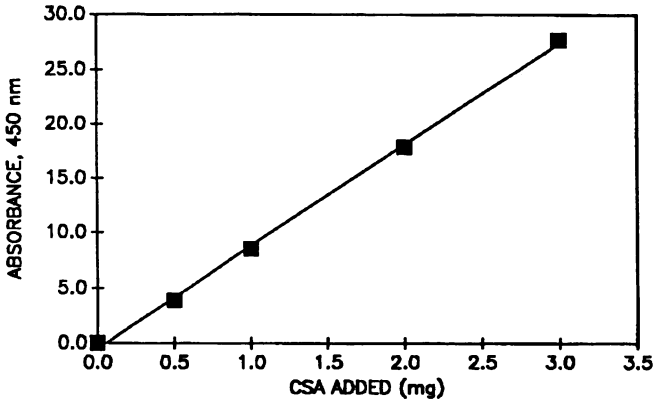

FIG. 1. Spectrophotometric assay of coal solubilization by recrystallized CSA.

stoichiometry of the Cu-CSA complex. In this case, assuming CSA is the only coordinating agent in significant concentration, the concentration of CSA is 0.027 M in the case of a 1:1 complex and 0.054 M in the case of a 1:2 complex of copper-CSA.

Determination of the stoichiometry of the Cu-CSA complex. The determination of the stoichiometry of the Cu-CSA complex, in consort with the titration presented above, permits the calculation of the amount of CSA present in any solution. Values of $E_{1/2}$ were calculated from plots of $E_{applied}$ versus $\log[i/(i_d - i)]$. A plot of $(E_{1/2})_c$ versus $\log(g_{CSA})$ from equation 2 for various amounts of added CSA is shown in Fig. 3.

Amperometric titration of copper(II) ion with CSA. It is possible to observe the addition of the first CSA molecule to copper through an amperometric titration by judicious choice of the potential at which the current is to be monitored. In this case, that potential was between the half-wave potential of the original copper solution and that of the 1:1 complex of copper and CSA. Figure 4 is the plot of current at −0.08 V versus added (purified) CSA.

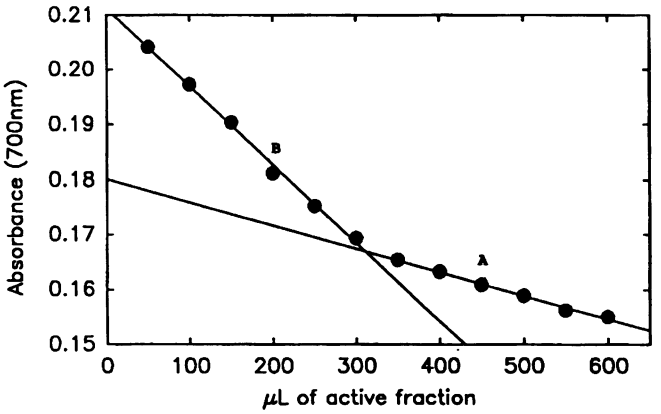

FIG. 2. Spectrophotometric titration of Cu^{2+} with CSA. ($[Cu^{2+}] = 1.698 \times 10^{-3}$ M, and [glycylglycine] = 6.16×10^{-3} M, pH 11; the CSA titrant brought to pH 11; pathlength, 2.000 cm.) The solid lines are the results of linear regression of the data points through which the lines pass (the point at 300 μl is not used). The intersection occurs at 314 μl.

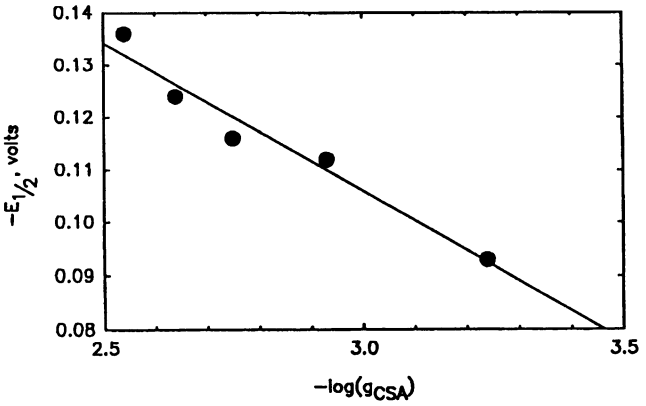

FIG. 3. A plot of the half-wave potential of solutions of the Cu-CSA complex versus $-\log(g_{CSA})$. ($[Cu^{2+}] = 9.346 \times 10^{-5}$ M; $[NaNO_3] = 0.10$ M; 0.002% Triton X-100 and 1.025×10^{-3} M acetate buffer, pH 5.50.) The $E_{1/2}$ of each voltammogram (a scan of +0.2 to −0.8 V) was determined from a linear regression of $E_{applied} = E_{1/2} - 0.0292\log[i/(i_d - i)]$. The slope of the regression line in the plot is −0.056.

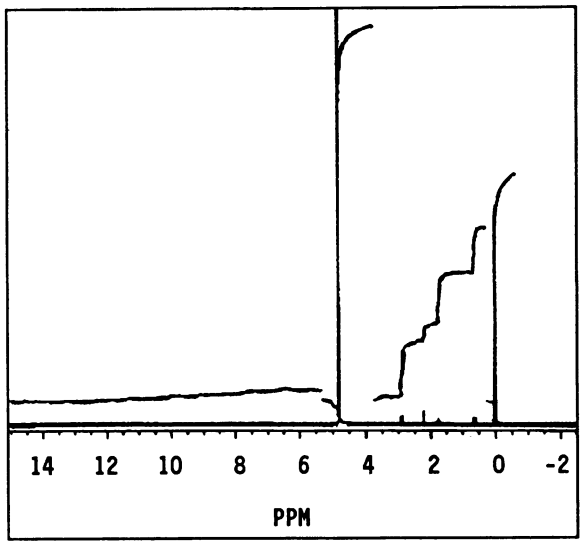

FIG. 5. ^{1}H-NMR of purified CSA dissolved in D_2O.

NMR spectra of CSA. The proton- and ^{13}C-NMR spectra of purified CSA are presented in Fig. 5 and 6, respectively.

Crystallographic molecular structure determination of CSA. Figure 7 shows the hydrogen bonding in the unit cell of the single crystals of the isolated CSA. The complex network is formed from five independent hydrogen bonds. The ammonium ion and water molecule are shown in their tetrahedral environments, and the two carboxylic oxygen atoms are in trigonal arrangements.

IR spectra of isolated CSA and commercial ammonium oxalate. The mid-IR spectrum of CSA and the mid-IR spectrum of ammonium oxalate monohydrate are presented in Fig. 8, in which the solid line is the spectrum of CSA and the dotted line is the spectrum of ammonium oxalate.

DISCUSSION

Analysis of the isolation protocol. The filtrate pretreatment removes most of the yellow color from the medium with little loss in CSA activity (Table 1). Reversed-phase C_{18} chromatography separates the hydrophilic CSA from the more nonpolar components of the broth. Size exclusion chromatography (Sephadex G-15-120 resin) separates the CSA from higher-molecular-weight species. The ethanol precipitation results in the production of CSA crystals with a large increase in CSA activity per milligram of dry weight. The results of the analyses by thin-layer electrophoresis indicate that the isolation protocol results in a highly purified CSA sample.

The spectrophotometric assay indicates that the original filtered F&R broth contains 25% CSA by weight. However, only 12% of this activity was recovered by this isolation scheme. The low percent recovery of original CSA activity resulted primarily from loss of CSA during C_{18} chromatography. Initially, it was decided to sacrifice percent recovery for CSA purity to allow chemical identification of the CSA. Subsequently, the procedure was modified with increased recovery (up to 26%). This was accomplished by pooling the fractions from the tailing CSA peak in the C_{18} chromato-

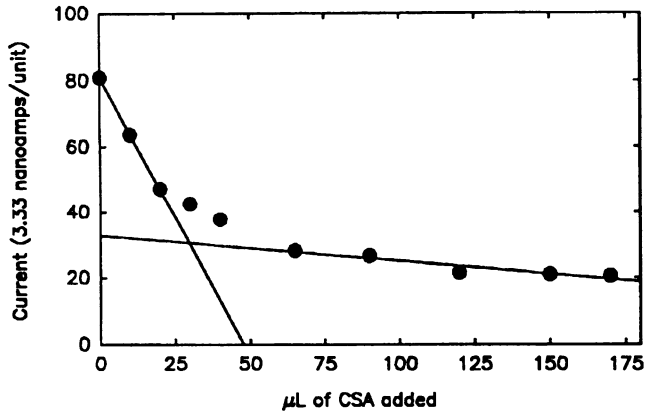

FIG. 4. An amperometric titration of Cu^{2+} with CSA. ($[Cu^{2+}] = 3.738 \times 10^{-4}$ M; $[NaNO_3] = 0.10$ M; 0.002% Triton X-100; 1.025×10^{-3} M acetate buffer, pH 5.50; the current was measured at an $E_{applied}$ of −0.08 V.) The two linear regression lines intersect at 29.8 μl of a CSA solution whose concentration is 1.5 mg/100 μl. This indicates a molecular weight of $1.3_4 \times 10^2$ g/mol ± 8% for CSA.

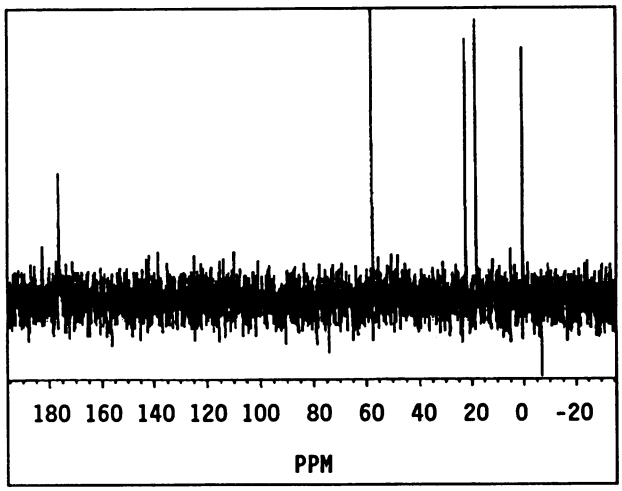

FIG. 6. ^{13}C-NMR of purified CSA dissolved in D_2O.

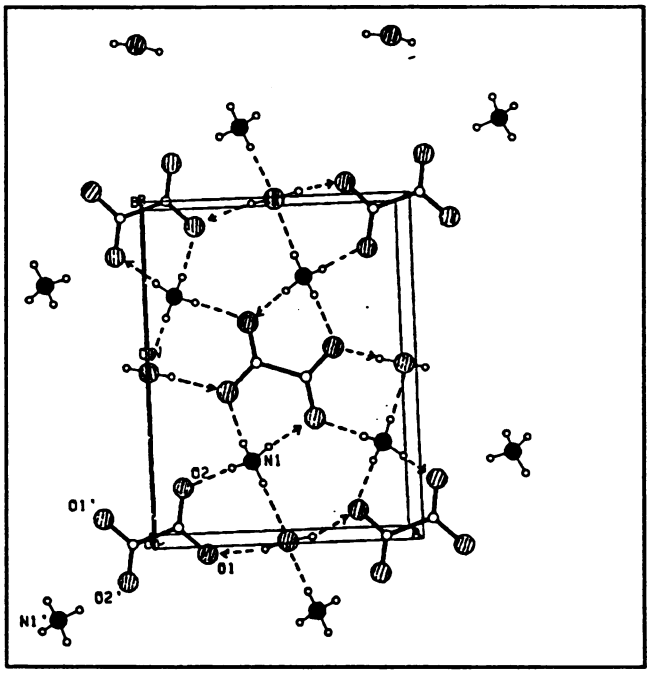

FIG. 7. Hydrogen bond network in the crystal of CSA (ammonium oxalate). Arrows indicate bond to a translationally related molecule.

gram, concentrating these fractions by rotoevaporation, and subsequently separating the CSA by a second pass on the C_{18} column. The Sephadex fractionation step was also eliminated because the ethanol precipitation and recrystallization procedures effectively removed any high-molecular-weight material along with other contaminants.

Spectrophotometric titration of the CSA. The first step in the isolation and identification of any chemical species from a mixture is the determination of the amount of that species present at any stage in the separation. Ideally, the method used should be rapid and require minimal manipulation. In this case, the apparently large stability constant of CSA with metal ions has been exploited to analyze even the filtered, but unprocessed, medium in which *T. versicolor* was grown. Furthermore, this method is the first method to be presented which does not require coal, thus eliminating the uncertainties endemic in an inhomogeneous reagent.

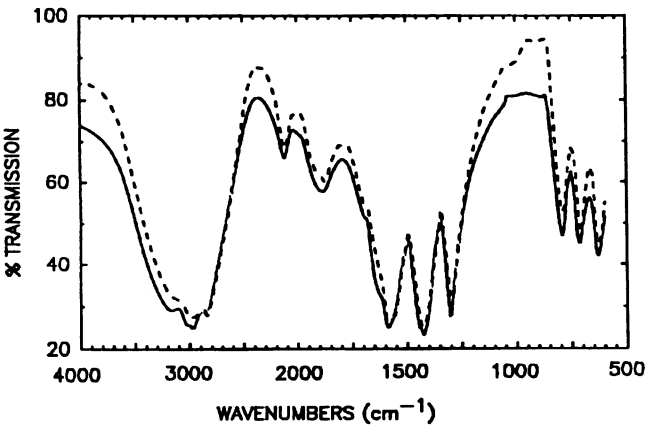

FIG. 8. Mid-IR spectrum of CSA (solid line) and mid-IR spectrum of ammonium oxalate monohydrate (dotted line).

Determination of stoichiometry of the Cu-CSA complex. Laitinen et al. (9) initially presented a rearrangement of the steady-state application of the Nernst equation for the determination of the number of identical ligands coordinated to a metal ion:

$$(E_{1/2})_c - (E_{1/2})_s = -0.0296\log K - p(0.0296)\log C \quad (1)$$

where $E_{1/2}$ refers to the half-wave potential of the polarogram, the subscripts c and s refer to complex and simple ions (or, as in this study, the solutions with and without CSA, respectively), K is the conditional stability constant, p is the number of ligands per metal ion, and C is the concentration of the complexing agent (which must be in strong excess) in units of molarity. This equation can be manipulated to the form

$$(E_{1/2})_c = \text{constant} - p(0.0296)\log(g_{CSA}) \quad (2)$$

where $(E_{1/2})_c$ is the half-wave potential of the copper-CSA complex. Thus, the data plotted in Fig. 3 follow a linear relationship. A linear regression of the data gives a slope of -0.056. Thus, p, the coordination number, is $1.9 \approx 2$. Since two CSA molecules are apparently coordinating to the copper ion under these conditions, CSA is likely acting as a bidentate or tridentate ligand.

Amperometric titration of copper(II) ion with CSA. The two linear regions of the titration in Fig. 4 can be subjected to a linear regression, and the number of microliters of titrant added at the intersection can be determined from these two linear equations. The intersection at 29.8 μl in this case gives an apparent molecular weight of CSA of $1.3_4 \times 10^2$ g/mol, with an uncertainty of 8%.

NMR spectra of CSA. The HDO peak at 4.7 ppm is the only form of hydrogen from CSA which is observed in the spectrum. The resonances between 0 and 3 ppm are from the DSS internal standard. Thus, all protons on CSA are rapidly exchangeable in the D_2O solvent. The ^{13}C spectrum again shows the DSS resonances between 0 and 60 ppm, plus one resonance at 175.8 ppm. The latter resonance implies that a single form of carbon is present in CSA and that this is likely a carbonyl or organic acid functionality.

Crystallographic molecular structure determination of CSA. All of the crystallographic evidence indicates that the orthorhombic, $P2_12_12$, crystal system of CSA is that of ammonium oxalate monohydrate. The oxalate group is nonplanar and twisted around the central C—C bond, with a dihedral angle of 27° between the two COO^- planes. The length of the C—C single bond was determined to be 1.562 Å at −110°C and 1.564 Å at room temperature, consistent with the slightly elongated single bond of oxalate.

IR spectra of isolated CSA and commercial ammonium oxalate. The IR spectra of the isolated CSA and ammonium oxalate monohydrate are clearly identical.

The active agent in the dissolution of low-rank coals by the action of *T. versicolor* (CSA) is the oxalate anion. This has been confirmed by the chemistry exhibited by CSA, its NMR and IR spectra, and its crystal structure, as the monohydrated ammonium salt.

Coal solubilization using purified CSA. Absorbance in the spectrophotometric CSA coal-solubilizing assay remains linear (Fig. 1), while loss of coal weight in the gravimetric CSA coal-solubilizing assay deviates from linearity over the same range of coal added (Fig. 9). This implies that, during the time of the assay, molecules too light to measure but

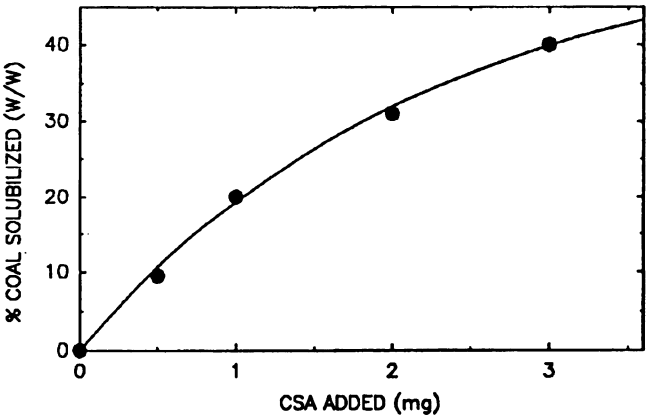

FIG. 9. Gravimetric assay of coal solubilization by recrystallized CSA. The solid line is a calculated fit to equation 6.

containing chromophores which absorb at 450 nm are released from the coal at a constant rate.

As described above, there is a limit to the percentage of the coal which can be dissolved by the action of the CSA obtained from *T. versicolor*. Considering the shape of the curve (Fig. 9), if CSA were able to bind transition metals very strongly under the conditions used here, the percentage of the coal solubilized would increase linearly with the addition of CSA and then turn over sharply to a horizontal line indicative of the independence of the percent solubilization to the amount of added CSA. The gradual increase of the percentage of coal solubilized with added CSA seen in Fig. 9 is typical of a system in which the conditional equilibrium constant is not large enough to make the reaction essentially quantitative under all levels of added CSA.

The fit shown in Fig. 9 is obtained if one assumes that oxalate is acting as a bidentate ligand (L) and the general form of the solubilization reaction is:

$$\text{coal}_{\text{with iron}} + 2L \rightleftarrows \text{Fe}L_2 + \text{coal}_{\text{aqueous}} \qquad K_{\text{CSA}} \quad (3)$$

where L is the oxalate ion. The hydroxide ions present on the iron-ligand complex have been left out for the sake of simplicity, but would have to be taken into account if this analysis were to be used for the determination of solubility at some other pH. It follows that

$$K_{\text{CSA}} = \frac{[\text{Fe}L_2][\text{coal}_{\text{aqueous}}]}{[\text{coal}_{\text{with iron}}][L]^2} \quad (4)$$

Since the inherent (if small) solubility of the coal without CSA can be expressed:

$$\text{coal}_{\text{solid}} \rightleftarrows \text{coal}_{\text{with iron}} \qquad K_{\text{sp}} \quad (5)$$

The value $[\text{coal}_{\text{with iron}}]$ can be defined in terms of the total available mass of coal (C_{so}). If the total mass of coal available for solubilization is given as C_{wi}, and a value Q is defined as $(C_{\text{wi}})/(C_{\text{so}})$, then the effective initial concentration of coal without the iron removed is C_{wi}/MW/1.0 ml, which is equal to QC_{so}/MW/1.0 ml, where MW is the mass of dissolved coal associated with 1 mol of iron and 1.0 ml is the volume of solution. Similarly, if the mass of $\text{coal}_{\text{aqueous}}$ is given as C_a, $[\text{coal}_{\text{aqueous}}]$ is equal to C_a/MW/1.0 ml and is also equal to $[\text{Fe}L_2]$, since the initial concentration of both products is zero. Thus, if the total concentration (molarity) of oxalate is given as $[L_t]$ (which is mass [milligrams] of ammonium oxalate monohydrate divided by its molecular weight and the 1.0-ml volume), the equilibrium expression for any added mass of oxalate is:

$$K = Q \cdot K_{\text{CSA}} = \frac{[x]^2}{\{(C_{\text{so}}/\text{MW}/1.0\text{ ml}) - [x]\}([L_t] - 2[x])^2} \quad (6)$$

where $[x]$ is the concentration of either product. From the elemental analysis of the coal, an effective molecular weight (equivalent weight) of the coal would be 14,000 g of coal per mol of iron, if all the coal were dissolved in this process (15). Thus, the two variables needed to fit the equation to the data are the K and the fraction of the coal which actually dissolves. The minimization of the residuals using equation 6 (Σ[|experimental mass − calculated mass|]) gives a K of 2.9 and a fraction of coal dissolved of 0.57 (and thus an equivalent weight of 7,940 g of dissolved coal per mol of iron).

ACKNOWLEDGMENTS

This research was supported by contract RP8003-6 from the EPRI.

We acknowledge the assistance of L. Crane of Trinity College, Hartford, Conn., where the NMR experiments were performed. We also acknowledge the contributions made by D. van der Helm. The X-ray crystallographic experiments were sponsored by the EPRI and performed by D. van der Helm at the University of Oklahoma.

LITERATURE CITED

1. **Alexander, R. R., J. M. Griffiths, and M. L. Wilkinson.** 1985. Basic biochemical methods, p. 192. John Wiley & Sons, Inc., New York.
2. **Cohen, M. S., H. Aronson, K. A. Feldman, and E. T. Gray, Jr.** 1989. Recent progress in cell-free solubilization of coal, p. 9-1 to 9-13. *In* H. E. Lebowitz (ed.), Proceedings: Thirteenth Annual EPRI Conference on Fuel Science and Conversion. Electric Power Research Institute, Palo Alto, Calif.
3. **Cohen, M. S., W. C. Bowers, H. Aronson, and E. T. Gray, Jr.** 1987. Cell-free solubilization of coal by *Polyporus versicolor*. Appl. Environ. Microbiol. **53**:2840–2843.
4. **Cohen, M. S., and P. D. Gabriele.** 1982. Degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola*. Appl. Environ. Microbiol. **44**:23–27.
5. **Fahraeus, G., and B. Reinhammer.** 1967. Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chem. Scand. **21**:2367–2378.
6. **Faison, B. D., and S. N. Lewis.** 1989. Production of coal-solubilizing activity by *Paecilomyces* Sp. during submerged growth in defined liquid media. Appl. Biochem. Biotechnol. **20/21**:743–752.
7. **Gray, E. T., Jr., and H. J. Workman.** 1981. Fundamentals of a Z-80-based electrochemical system, p. 48–57. *In* P. Lykos (ed.), Personal computers in chemistry. Wiley-Interscience, New York.
8. **Gupta, R. K., J. K. Spiker, and D. L. Crawford.** 1987. Biotransformation of coal by ligninolytic *Streptomyces*. Can. J. Microbiol. **34**:667–674.
9. **Laitinen, H., E. Onstott, J. Bailar, and S. Swann.** 1949. Polarography of copper complexes. I. Ethylenediamine, propylenediamine, diethylenetriamine, and glycine complexes. J. Am. Chem. Soc. **71**:1550–1552.
10. **Moolick, R. T., J. C. Linden, and M. Nazmul Karim.** 1989. Biosolubilization of lignite. Appl. Biochem. Biotechnol. **20/21**: 731–742.
11. **Quigley, D. R., B. Ward, D. L. Crawford, H. J. Hatcher, and P. R. Dugan.** 1989. Evidence that microbially produced alkaline materials are involved in coal solubilization. Appl. Biochem. Biotechnol. **20/21**:753–763.
12. **Scott, C. D., and S. N. Lewis.** 1988. Biological solubilization of coal using both in vivo and in vitro processes. Appl. Biochem.

Biotechnol. **18**:403–412.

13. **Strandberg, G. W., and S. N. Lewis.** 1987. Solubilization of coal by an extracellular product from *Streptomyces setonii* 75Vi2. J. Ind. Microbiol. **1**:371–375.
14. **Strandberg, G. W., and S. N. Lewis.** 1988. Factors affecting coal solubilization by the bacterium *Streptomyces setonii* 75Vi2 and by alkaline buffers. Appl. Biochem. Biotechnol. **18**:355–361.
15. **Wilson, B. W., R. M. Bean, J. A. Franz, B. Thomas, M. S. Cohen, H. Aronson, and E. T. Gray, Jr.** 1987. Microbial conversion of low-rank coal: characterization of biodegraded product. Energy Fuels **1**:80–84.
16. **Wondrak, L., M. Szanto, and W. A. Wood.** 1989. Depolymerization of water soluble coal polymer from subbituminous coal and lignite by lignin peroxidase. Appl. Biochem. Biotechnol. **20/21**:765–780.