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Comparison of coal solubilization by bacteria and fungi

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Abstract

Coal-solubilizing agents produced by *Trametes versicolor, Phanerochaete chrysosporium, Aspergillus* sp., a bacterial consortium, and a bacterial isolate, *Arthrobacter* sp., from that consortium were compared in terms of pH dependence, thermostability, molecular mass, mechanism of action, and product diversity. The thermostability and low molecular weights exhibited by the coal-solubilizing agents indicated a non-enzymatic mechanism of action. Competition studies using cupric copper indicated that coal solubilization by these agents involved metal chelation. Results demonstrated that oxalate could account for some but not all of the coal solubilization observed for *T.versicolor* and *P.chrysosporium.* The very low levels of oxalate detected in *Aspergillus* sp. and the bacterial cultures indicated that oxalate is not an important factor in coal solubilization by these microbes. When subjected to gel permeation chromatography, the soluble coal products generated by each microbial coal-solubilizing agent yielded unique molecular mass profiles suggesting substantial product diversity. Such diversity increases the possibility of identifying potentially valuable compounds and extending the commercial utilization of coal.

Abbreviations: A_{450}, A_{260} – absorbances respectively at 450 nm and 260 nm, CSA – coal-solubilizing agent, CSU – coal-solubilizing unit, GPC – gel permeation chromatography, MEA – malt extract agar, PDA – potato dextrose agar, SDA – Sabouraud dextrose agar, SDB – Sabouraud dextrose broth, SEM – standard error of the mean, Tris – tris(hydroxymethyl)aminomethane, TSA – trypticase soy agar, TSB – trypticase soy broth

Introduction

Coal is the most abundant fossil energy resource in the United States. In light of growing economic, environmental, and energy import concerns, there has been increased interest in alternative coal-processing methods that would extend the utilization of coal as a fuel and as a source of valuable industrial chemicals. Although direct thermochemical liquefaction can convert coal into a cleaner liquid fuel, it is an energy intensive process requiring high temperatures and pressures, and, therefore, not cost effective (Lumpkin 1988). The microbial production of water soluble products from low-rank coals under ambient temperatures and pressures (conditions representing potential savings in processing costs) may be a commercially viable alternative.

A number of mechanisms for coal biosolubilization have been suggested, including enzymatic modification (Cohen et al. 1987; Pyne et al. 1987), alkaline solubilization (Quigley et al. 1989a; Strandberg & Lewis 1987), and metal ion chelation (Fredrickson et al. 1990; Quigley et al. 1989b). These studies have not been definitive and in some cases are contradictory. Only in the case of the white rot fungus *Trametes versicolor* has a coal-solubilizing agent (CSA) been chemically identified (Cohen et al. 1990). Initially, the coal-solubilizing ability of this fungus was attributed to enzymatic activity by Cohen et al. (1987) and Pyne et al. (1987). Later, Cohen et al. (1990) identified the CSA from *T. versicolor* as ammonium oxalate monohydrate. At the same time, Fredrickson et al. (1990) provided evidence indicating that the CSA produced by *T. versicolor* was not ammonium oxalate but a siderophore-like compound. This compound was not, however, fully characterized or identified. Consequently, some confusion exists in the literature regarding the nature of the CSA(s) produced by *T. versicolor*, with even less definitive information available for other fungal or bacterial CSAs.

Products of coal biosolubilization have potential as fuels, as substrates for microbial conversions to methane and alcohols, and as feedstocks for the synthesis of commercially valuable chemicals. Since different microorganisms may produce different CSAs, the potential exists for generating a wide range of soluble products, thereby increasing the possibilities for the successful application of biosolubilization technology in the fuel and chemical industries.

In the experiments reported here, we investigated the mechanisms by which different microbial systems solubilize coal in order to determine the nature of the compounds serving as CSAs for these microbes. In addition, a preliminary assessment was made of the soluble coal product diversity generated by the different CSAs.

Materials and methods

Culture methods

The fungi *Trametes versicolor* (ATCC 12679), *Phanerochaete chrysosporium* (ATCC 24725), and *Aspergillus* sp. (strain CP4, isolated from a coal compost) were maintained on PDA (ATCC medium # 337, pH = 5.6), 2% MEA (pH = 5.5) and SDA (pH = 5.6), respectively. For the production of CSAs, all fungi were cultured at pH 5.5 in the same defined liquid medium (Fahraeus & Reinhammer 1967). Iron was omitted from this medium in the case of *Aspergillus* cultures since this was found to promote CSA production by this fungus. For *T. versicolor*, the inoculum was prepared by aseptically homogenizing SDB cultures that had been incubated at 25° C without agitation for 10 days. Inocula were added to 500 ml Erlenmeyer flasks containing 250 ml of defined medium at a concentration equal to 40 mg (dry weight)/l of medium. These cultures were incubated at 25° C with agitation (150 rpm). Inocula for *P. chrysosporium* and *Aspergillus* sp. were prepared by harvesting spores in sterile, distilled water from 3 to 4 week-old MEA and SDA cultures, respectively. Spore concentrations were adjusted to give 2.5×10^8 spores/l of defined medium. For *P. chrysosporium*, inocula were added to 500 ml Erlenmeyer flasks containing 150 ml of defined medium and incubated at 37° C, with agitation (200 rpm). For *Aspergillus* sp., inocula were added to 2800 ml Fernbach flasks containing 300 ml defined medium and incubated at 25° C with no agitation. All CSAs were harvested from 3 week-old cultures by vacuum filtration through glass fiber filters (Whatman, 934-AH).

A bacterial consortium (CP1 + 2) exhibiting coal solubilizing activity was isolated from a coal compost and consisted of *Pseudomonas, Bacillus,* and *Arthrobacter* species. Both the consortium and a pure culture of *Arthrobacter* sp. (strain CP3) isolated from the consortium were studied. Bacterial CSA was produced by inoculation of TSB with cells from a stock TSA slant followed by incubation at 25° C with agitation (120 rpm). Active, cell-free preparations were obtained by centrifugation after 6 days growth.

Standard spectrophotometric assay for coal solubilization

For the fungi, one ml of CSA buffered with 0.1 M imidazole, pH 8 was added to 10 mg of distilled water-washed Leonardite, a highly oxidized North Dakota lignite coal (150 μ particle size; American Colloid Co., Reeder, N.D.), in 13 × 100 mm screw-capped glass test tubes and mixed on a Glas-Col (Terre Haute, IN) laboratory rotator at 25° C for 20 h. Reaction mixtures then were centrifuged for 4 min in a Beckman Microfuge E and the absorbances of resulting supernatants measured on a Gilford Response Spectrophotometer at 450 nm, after appropriate dilutions were prepared. Control reference blanks were supernatants of reaction mixtures prepared with 10 mg Leonardite and 1 ml of 0.1 M imidazole buffer adjusted to pH 8. The pH 8 imidazole buffer solubilizes relatively small amounts of Leonardite compared with other buffers, such as phosphate or Tris, tested at pH 8. All buffers used in this study contained 0.002% chlorhexidine to inhibit bacterial contamination. The bacterial assay procedure was the same except that 50 mg of Leonardite was used when particularly high levels of solubilizing activity rendered 10 mg rate-limiting. A minimum of two replicates were run for each assay. Coal-solubilizing activity was expressed as the A_{450} of reaction mixture supernatants or as CSUs. A CSU was defined as the amount of CSA producing 100 μg of soluble product in 20 h under the standard assay conditions. Soluble product was acid-precipitated, dried, aliquots weighed, and redissolved in order to prepare a standard curve for converting A_{450} to μg product.

Partial purification of CSAs

Coal solubilizing agents were subjected to GPC on Sephadex G10 (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) columns (2.5 cm × 34 cm) prepared according to the manufacturer's instructions in 0.03 M imidazole, pH 7.0. For each chromatographic run, approximately 10 ml of culture filtrate (concentrated 10 × by lyophilization for the fungi) was applied to the drained bed of the G10 column. Reagent grade ammonium oxalate monohydrate (1 ml at 45 mg/ml) also was subjected to chromatography. Fractions were eluted in imidazole buffer at a rate of 1.6 ml min^{-1} and assayed for Leonardite solubilization using the standard spectrophotometric assay. Solubilizing activity recovered from G10 chromatography of the different CSAs ranged from 76% to 89% of the total activity applied.

Gravimetric determination of coal solubilization

Duplicate reaction mixtures (155 mg of Leonardite plus 7 ml of CSA in 0.1 M imidazole buffer, pH 8) were prepared in preweighed 50 ml capped plastic centrifuge tubes. The activities of partially purified (Sephadex G10 GPC) CSAs were adjusted to that of the least active CSA in order to give approximately equal solubilizing activities with Leonardite. Controls consisted of 155 mg Leonardite and 7 ml of buffer without CSAs. Reaction mixtures and controls were agitated in the centrifuge tubes at approximately a 45 ° angle at 200 rpm on a rotary culture shaker for 42 hours. After incubation, tubes were centrifuged at 1085 × g in a Sorval RC5B centrifuge and the supernatants (soluble products) transferred to separate tubes from which aliquots were removed for determining A_{450}. Pellets (residual Leonardite) were subjected to one 5 ml wash with distilled water before drying at 60° C and weighing. The percent of Leonardite solubilized was determined both from the weight of residual Leonardite and from spectrophotometric measurements of reaction mixture supernatants.

Dialysis of CSAs

All CSAs were subjected to exhaustive dialysis against distilled water at 4° C using dialysis tubing with a molecular weight cut-off of 6000–8000 Da (Spectrum Medical Industries, Inc., Los Angeles, CA). The solutions from inside and outside the tubing then were lyophilized, resuspended in the original volume of imidazole buffer and assayed for coal solubilization using the standard spectrophotometric method.

Determination of pH optima for CSAs

For each fungus, standard assay reaction mixtures were prepared with 2X concentrated culture filtrates buffered with 0.05 M CH_3COOH and 0.05 M imidazole and adjusted to the appropriate pH with HCl or KOH. Filtrates were concentrated by lyophilization. Duplicate reaction mixtures were adjusted to specific hydrogen ion concentrations over the pH range of 5 to 8. Duplicate bacterial reaction mixtures containing partially purified CSAs were prepared in 0.1 M imidazole and were adjusted to specific hydrogen ion concentrations ranging from pH 6.5 to pH 8. Control reaction mixtures consisted of Leonardite with buffered, uninoculated, 2X concentrated culture medium (for fungal CSAs) or 0.1 M imidazole buffer (for bacterial CSAs), adjusted to the appropriate hydrogen ion concentration. The A_{450} of resulting supernatants were determined using the supernatants from the control reaction mixtures as reference blanks.

Oxalate and $NH_4{}^+$ determinations

Oxalate and ammonium ion concentrations were measured using the Sigma Diagnostics test kits specific for these compounds (Sigma Chemical Co., St. Louis, MO).

Air sparging for NH_4^+ removal

Partially purified CSAs from the bacteria and the fungi were assayed for Leonardite-solubilizing activity at pH 8 and for ammonium ion concentration. The CSAs then were adjusted to a pH of approximately 11 with KOH and sparged for 18 h with compressed air provided through plastic tubing fitted with Pasteur pipettes in order to drive off ammonia. After sparging, the CSA solutions were returned to the solubilization assay pH of 8 with HCl, adjusted to their original volume with distilled water, and assayed for Leonardite-solubilizing activity and ammonia ion concentration. Oxalate concentrations for *T. versicolor* and *P. chrysosporium* were also determined before and after sparging.

Cu^{+2} inhibition studies

Partially purified CSAs from *T. versicolor* and *P. chrysosporium* were diluted to give a final oxalate concentration of 5 mM. The partially purified *Aspergillus* sp. and bacterial CSAs (< 0.07 mM oxalate) were used without dilution. Cupric sulfate was added to CSAs to yield reaction mixtures containing $CuSO_4$ concentrations ranging from 0 to 10 mM. One ml aliquots of the CSA/copper mixtures were assayed in duplicate using the standard spectrophotometric assay for coal solubilization. Controls consisted of 5 mM reagent grade ammonium oxalate monohydrate in 0.1 M imidazole, pH 8.

Molecular mass profiles of soluble products

Soluble products generated from Leonardite were chromatographed on Sephadex G25, G50 and G200 prepared in distilled water using K9/15 columns. Flow rates were maintained at 2 ml min^{-1} for G25 and G50 colums and at 0.15–0.18 ml min^{-1} for G200 columns. Fractions were eluted with 0.02 M NaCl, pH 8 and monitored spectrophotometrically at 260 nm.

Results and discussion

Effect of pH on CSA-mediated coal solubilization

All CSAs exhibited increasing levels of solubilization as the pH was raised to 8, with the fungal CSAs as a group causing more solubilization at neutrality and in the acidic range than the bacterial CSA (Fig. 1). The effect of pH on CSA solubilizing capacity was ambiguous above pH 8 due to high levels of solubilization attributable to increasing alkalinity which causes solubilization independent of CSA concentration.

CSA thermostability and molecular mass

All CSAs were stable to 15 min of autoclaving at 121°C and were able to pass through dialysis tubing with a molecular mass cut-off of 6000–8000 Da. Furthermore, when subjected to Sephadex G10 GPC column chromatography (fractionation range of up to 700 Da), the CSAs eluted close to ammonium oxalate (MW = 142.11). Since this was at the low (non-linear) end of the fractionation range for Sephadex G10, accurate molecular mass determinations were not possible. Nevertheless, both dialysis and Sephadex G10 GPC clearly demonstrated low molecular masses for all CSAs. This, together with the thermostability results, precluded an enzymatic mechanism of action for these CSAs (including the CSA from *T. versicolor*) in contrast to the report by Pyne et al. (1987) that the coal-solubilizing activity from *Coriolus versicolor* (*T. versicolor*) was due to a laccase-like oxidase.

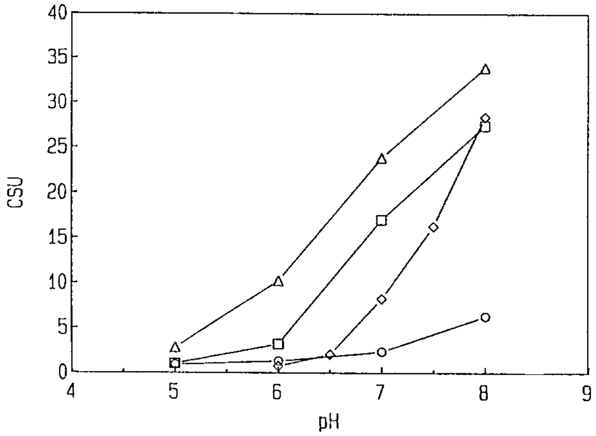

Fig. 1. Effect of pH on CSA solubilizing activity. Symbols: △, *T. versicolor*; □, *P. chrysosporium*; ○, *Aspergillus* sp.; ◇, CP1 + 2.

Contribution of ammonium ion

Several studies have suggested that microbial solubilization of coal results from the production of basic metabolites such as ammonia, polyamines, or basic peptides (Quigley et al. 1988; Strandberg & Lewis 1987, 1988). It has been presumed that these organic bases neutralize carboxylic acid functional groups in coal thereby rendering it soluble in water (Quigley et al. 1989a). This would explain why low rank, relatively oxidized coals (containing greater numbers of carboxylic acid groups) are more susceptible than higher rank coals to alkaline solubilization. Although cultures of the fungi and bacteria exhibited increases in pH into the alkaline range during growth, the CSAs partially purified from these cultures showed solubilizing activity at pH 7 and below (Fig. 1) indicating a mechanism other than alkaline solubilization. Furthermore, the solubilization values determined using the standard spectrophotometric assay at a pH of 8 were corrected for the effect of pH by using control reaction mixtures containing only pH 8 buffer as spectrophotometric references. Therefore, while the reduction in the hydrogen ion concentration that occurs in cultures during growth presumably contributes to the solubilization observed for the crude filtrates, particularly those from the bacteria, the fungi and bacteria are nonetheless producing

Table 1. Effect of air-sparging on CSA ammonium ion concentration and coal-solubilizing activity.

	NH_4^+ (mM)		Solubilization (A_{450})	
	Sparging		Sparging	
CSA Source	Before	After	Before	After
T. versicolor	63.6 ± 2.8[a]	14.7 ± 0.1	19.5 ± 0.2	27.1 ± 1.4
P. chrysosporium	32.8 ± 0.0	15.2 ± 0.2	13.7 ± 1.8	23.1 ± 0.6
Aspergillus sp.	14.5 ± 0.1	3.5 ± 0.0	9.7 ± 0.6	14.3 ± 1.0
CP1 + 2	33.8 ± 1.9	1.4 ± 0.0	6.4 ± 0.9	11.4 ± 2.2
Arthrobacter sp.	30.3 ± 3.2	2.0 ± 0.0	6.7 ± 0.8	11.5 ± 2.3
5 mM $(NH_4)_2C_2O_4$	9.9 ± 0.1	1.6 ± 0.0	4.2 ± 0.2	6.4 ± 0.2

[a] Mean ± SEM.

CSAs that solubilize coal by a mechanism distinct from alkaline solubilization.

All of the CSA preparations tested positively for ammonium ion. In order to determine if ammonium ion was involved in coal solubilization in a way other than a possible pH effect via NH_4OH, CSAs were sparged with air to strip ammonium ion from solution and then tested for coal solubilization at pH 8. Sparging greatly decreased the ammonium ion concentration for all microbial CSAs and reagent grade ammonium oxalate without decreasing solubilizing activity (Table 1). In fact, solubilization was somewhat greater following the sparging treatment suggesting that ammonium ion is inhibitory. As a control for any losses in CSAs during the experimental manipulations, the oxalate concentrations of ammonium oxalate and the CSAs from *T. versicolor* and *P. chrysosporium* were measured before and after sparging and found to be unchanged.

Solutions of 10 mM ammonium chloride and 5 mM ammonium sulfate (equimolar in ammonium ion) and 10 mM potassium chloride at pH 8 showed no solubilizing activity against coal whereas 5 mM ammonium oxalate and 5 mM potassium oxalate did (A_{450} = 3.9±0.1 and 5.2±0.3, respectively). These results indicate that the solubilizing activity of ammonium oxalate resides solely with the oxalate moiety of the molecule. Note that the potassium oxalate showed higher levels of solubilization than did the ammonium oxalate, again suggesting an inhibitory effect for ammonium ion.

Contribution of chelators

Our CSA preparations from both *T. versicolor* and *P. chrysosporium* contained significant levels of oxalate which can function as a Lewis base or chelator of cations. Removal of multivalent cations from coal has been shown to increase coal solubilities in alkaline solutions and in microbial culture media, probably by disrupting cation-carboxylic acid cross linkages that form large insoluble complexes from smaller, soluble coal moieties (Quigley et al. 1989b). Cohen's group (1990) identified the *T. versicolor* CSA as ammonium oxalate monohydrate, whereas Fredrickson et al. (1990) suggested instead that it was an iron-binding siderophore. Relevant to this, the levels of solubilization observed for our CSA preparation from *T. versicolor* were significantly greater than those observed for equivalent concentrations of reagent grade ammonium oxalate. This was revealed in an experiment comparing the oxalate concentrations of partially purified CSAs with their coal-solubilizing capacities. Duplicate reaction mixtures for each CSA were incubated and the percent solubilization was determined both spectrophotometrically and gravimetrically. As seen in Table 2, both techniques were in close agreement, therefore validating the use of the more convenient spectrophotometric assay. The CSA preparation from *T. versicolor* with an oxalate concentration of 5.2 mM solubilized approximately 12% of the Leonardite. However in an assay not shown here, a 5.2 mM concentration of reagent grade ammonium oxalate monohydrate, in the same buffer with the same concentration of Leonardite, was able to solubilize only 5.2% of the Leonardite (supernatant A_{450} = 6.8±0.4 SEM). A similar observation was made for the CSA from *P. chrysosporium* but to even a greater extent as it solubilized approximately 16% of the Leonardite while having an oxalate concentration of only 2.5 mM. These data suggest that something

Table 2. CSA solubilization of Leonardite.

Source	Supernatant A_{450}	Oxalate (mM)	% Solubilized	
			Spectrophotometric	Gravimetric
T. versicolor	16.5 ± 0.4[a]	5.2 ± 0.2[a]	12.8	10.8
P. chrysosporium	21.5 ± 2.3	2.5 ± 0.0	16.7	15.2
Aspergillus sp.	28.8 ± 0.2	0.2 ± 0.0	21.8	18.0
CP1 + 2	25.5 ± 0.4	0.4 ± 0.0	19.7	17.6

[a] Mean ± SEM.

Table 3. Effect of Cu^{+2} on solubilization of Leonardite by CSAs.

	Solubilization (A_{450})					
mM Cu^{+2}	*T. versicolor*[a]	*P. chrysosporium*[a]	*Aspergillus sp.*[b]	CP1 +2[b]	*Arthrobacter sp.*[b]	oxalate[a]
0	9.0 ± 0.2[c]	20.3 ± 0.3	22.7 ± 0.3	14.2 ± 0.0	14.0 ± 0.1	5.3 ± 0.4
0.02	–[d]	–	21.6 ± 0.2	15.0 ± 0.5	14.7 ± 0.1	–
0.05	–	–	21.3 ± 0.9	13.4 ± 0.4	13.2 ± 0.4	–
0.5	–	–	3.9 ± 0.1	1.8 ± 0.4	0	–
1.0	1.8 ± 0.05	10.0 ± 0.1	0	0.1 ± 0.0	0	2.1 ± 0.1
2.0	0.7 ± 0.1	2.5 ± 0.1	–	–	–	0.7 ± 0.1
2.5	0.4 ± 0.1	1.8 ± 0.3	–	–	–	0
3.0	0	0.9 ± 0.1	–	–	–	0
5.0	–	0.2 ± 0.0	–	–	–	–
10.0	–	0	–	–	–	–

[a] oxalate concentration adjusted to 5 mM.

[b] oxalate concentration <0.07 mM.

[c] mean ± SEM.

[d] not assayed.

in addition to ammonium oxalate is contributing to the solubilization of Leonardite by these fungi. As for *Aspergillus* sp. and CP1 + 2, it appears that their ability to solubilize Leonardite depends very little, if at all, on oxalate since the concentrations of oxalate observed in these CSA preparations were relatively low (Table 2).

To investigate this further, we tested the effect of Cu^{+2}, a Lewis acid competitor of chelation, on Leonardite solubilization by the different microbial CSAs and reagent grade ammonium oxalate. As expected, based on the theoretical complexing of one mole of copper with two moles of oxalate, the solubilizing activity of 5 mM ammonium oxalate was completely inhibited in the presence of 2.5 mM cupric sulfate (Table 3). In contrast, 2.5 mM cupric sulfate did not completely inhibit the solubilizing activity of CSAs from *T. versicolor* or *P. chrysosporium* when the CSAs were adjusted to an oxalate concentration of 5 mM. Oxalate concentrations for the other microbial CSAs were negligible (less than 0.07 mM) and not expected to make a significant contribution to coal solubilizia-tion. Thus, a 0.05 mM cupric sulfate solution, more than enough to completely eliminate any solubilization due to oxalate in these CSAs, caused only a slight diminution in solubilization. These results corroborate the earlier conclusion that ammonium oxalate concentrations cannot account for all the coal solubilization generated by the CSAs. The fact that all the remaining solubilization was eliminated at higher cupric sulfate concentrations indicates that solubilization by these CSAs can be fully explained by a chelation/Lewis base mechanism and that in the case of at least two of the

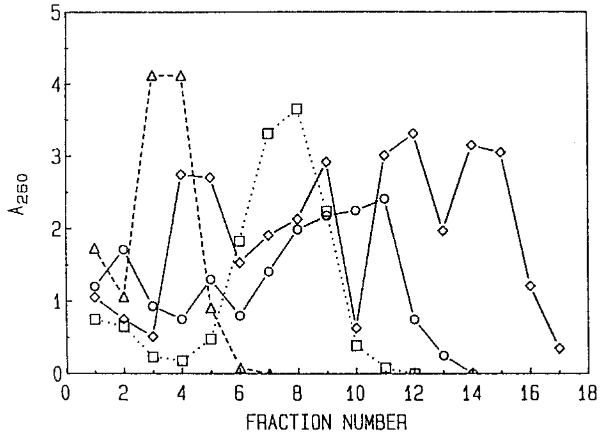

Fig. 2. Sephadex G200 gel permeation chromatography of soluble Leonardite products generated by fungal and bacterial CSAs. Symbols: △, *T. versicolor*; □, *P. chrysosporium*; ○, *Aspergillus* sp.; ◇, CP1 + 2.

fungi more than one chelator is involved. Given this, the inhibitory effect of ammonium ion noted earlier can be attributed to its action as a competitive Lewis acid. The presence of more than one chelator would explain why the descriptions of the *Trametes* CSA by Cohen et al. (1990) and Fredrickson et al. (1990) differ. It also raises the possibility for synergistic or competitive interactions among the different chelators during the coal solubilization process. We currently are developing methods for the purification of these multiple chelators to permit identification and subsequent study of these chelators relative to their individual vs. combined coal solubilizing activities.

The results reported here indicate that both the fungi and bacteria can produce CSAs that function by a chelation/Lewis base mechanism and that in some cases multiple chelators are involved. Although a chelation mechanism has been reported for the fungus *T. versicolor*, it has not been demonstrated in other fungi nor has it been reported in any bacterial system. Regarding the bacteria, standard coal solubilization assays of parallel cultures have shown that virtually all of the coal solubilizing activity observed for the consortium ($A_{450} = 113 \pm 3$) can be accounted for by *Arthrobacter* sp. ($A_{450} = 108 \pm 4$). The similarity in data for the consortium and *Arthrobacter* sp. in experiments reported here (Tables 1 and 3) is consistent with this observation.

Product molecular mass distribution

In order to make a preliminary assessment of the heterogeneity of products generated by the different CSAs, soluble products were subjected to Sephadex GPC. The most complete fractionation was achieved with Sephadex G200, most of the higher molecular mass products being excluded from G25 and G50 columns. Distinct, reproducible differences in the product profiles from the different microbial CSAs were observed (Fig. 2). Molecular masses for the various peaks were not determined because of possible interactions between the Leonardite products and the Sephadex gel (Polman & Quigley 1991). Although product-column interactions may have contributed to the observed profiles, such interactions also may reflect product differences generated by the different CSA treatments. The product diversity suggested here warrants further chemical analysis to evaluate the potential application of CSAs for the production of alternative fuels and/or commercially important chemicals. The development of analysis procedures for the separation and identification of soluble coal products is currently underway.

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