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Use of X-ray Absorption Spectroscopy and Esterification to Investigate Cr(III) and Ni(II) Ligands in Alfalfa Biomass

KIRK J. TIEMANN, JORGE L. GARDEA-TORRESDEY,* GERARDO GAMEZ, KENNETH DOKKEN, AND SALVADOR SIAS

Department of Chemistry, The University of Texas at El Paso, El Paso, Texas 79968

MARK W. RENNER AND LARS R. FURENLID

Department of Applied Science, National Synchrotron Light Source, Brookhaven National Laboratory, Upton, New York 11973-5000

Previously performed studies have shown that alfalfa shoot biomass can bind an appreciable amount of nickel(II) and chromium(III) ions from aqueous solution. Direct and indirect approaches were applied to study the possible mechanisms involved in metal binding by the alfalfa biomass. The direct approach involves investigations of the metal-bound alfalfa shoot biomass by X-ray absorption spectroscopic analysis (XANES and EXAFS). Results from these studies suggest that nickel(II) and chromium(III) binding mostly occurs through coordination with oxygen ligands. Indirect approaches consist of chemical modification of carboxylate groups that have been shown to play an important role in metal binding to the alfalfa biomass. An appreciable decrease in metal binding resulted after acidic methanol esterification of the biomass, indicating that carboxyl groups are entailed in the metal binding by the alfalfa biomass. In addition, base hydrolysis of the alfalfa biomass increased the binding of these metals, which further indicates that carboxyl groups play an important role in the binding of these metal ions from solution. Therefore, by combining two different techniques, our results indicate that carboxylate groups are the major ligands responsible for the binding of nickel(II) and chromium(III) by alfalfa biomass.

Introduction

Currently, a great deal of interest has been given to the use of biological systems for the removal of heavy metals from polluted sites (*1*−*6*). Several studies have shown that nonliving natural materials are effective for the removal of heavy metals from the environment (*7*−*10*). Gardea-Torresdey and co-workers demonstrated that carboxyl groups found on the cell walls of dead algal biomass are partially responsible for copper binding (*11*). This phenomenon has spurred interest in other natural materials that may contain similar functional groups such as higher plant cells. Some investigators have studied the binding of many metals to inactivated cells of *Datura innoxia*, and other researchers have shown that plant cells are capable of binding heavy metals (*10, 12*−*16*). Ke et al. have utilized spectroscopic techniques such as Eu^{3+} and UO_2^{2+} luminescence for determining the functional groups responsible for metal binding by *Datura innoxia* (*17*−*21*). In addition, chemical modification techniques have also been shown to be valuable for characterizing the metal binding functional groups (*11, 22, 23*). The mechanism of metal binding by plant biomass is still not entirely understood, and further research is necessary. Metal ion binding by plant biomass is believed to take place through chemical functional groups (such as carboxyl, amino, sulfhydryl, or hydroxyl groups among others) (*11*). By determining which functional groups are responsible for metal binding by plant cells, chemical modification of the biomass might increase these groups and allow for better recovery of toxic metal ions in a more efficient manner.

Medicago sativa (alfalfa) was identified as a potential biological material that can be used for metal adsorption since it has been found growing in fields irrigated with heavy-metal contaminated water and was reported to accumulate metal-concentrations above the tolerance levels for most plants (*24*−*27*). Previously performed studies have shown that the alfalfa shoot biomass can bind an appreciable amount of metal ions such as nickel(II) and chromium(III) from aqueous solution (*28*−*30*).

Although much information has been gained on the binding of metal ions to alfalfa biomass, no information is available in relation to the actual metal binding chemical groups. The objective of this study is to identify the possible mechanisms involved in metal binding by the alfalfa biomass. In this paper we discuss the characterization of Cr(III) and Ni(II) binding with alfalfa shoot biomass by X-ray absorption spectroscopic analysis (XANES and EXAFS), which was performed at Brookhaven National Laboratory. Also, results from the esterification of carboxyl groups and their effects on metal binding are reported. In addition, the effects of hydrolysis of alfalfa biomass on metal binding are reported. These studies are particularly important for determining the ligands that may be involved in the binding of metal ions to the alfalfa biomass, thus aiding in the development of a phytofiltration system to remove and recover metal ions from contaminated waters.

Methodology

Esterification of Alfalfa Biomass. The plant tissues were collected from controlled agricultural fields at New Mexico State University near Las Cruces, NM, washed, and prepared as previously indicated (*28*−*30*). Esterification of the biomass was performed following similar methods as previously described (*11, 22*). In summary, the biomass (9.0 g) was suspended in an acidic methanol solution (633 mL of 99.9% methanol and 5.4 mL of concentrated HCl), which had a final acidic concentration of 0.1 M HCl. The solution was continuously stirred and heated to 60 °C for 48 h. In addition, controls were maintained from the original biomass; one control was reacted with 0.1 M HCl, another was reacted with DI (deionized) water, while a third was reacted with pure 99.9% methanol. The pelleted alfalfa biomass was then washed with DI water three times using centrifugation in order to quench the esterification reaction. The pelleted biomass was then lyophilized for further metal binding experiments.

Hydrolyzation of Alfalfa Biomass. Hydrolyzation of the biomass (9.0 g) was performed following similar methods as previously described (*11, 22*). The biomass was reacted with

* Corresponding author tel: (915)747-5359; fax: (915)747-5748; e-mail: jgardea@utep.edu.

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100 mL of 0.1 M sodium hydroxide (NaOH) for 1 h. The pelleted biomass was then washed with DI water three times and lyophilized for further metal binding experiments.

Metal Binding Experiment. For each of the hydrolyzed, esterified, and control samples described above, 10.0 mg was weighed and placed into clean test tubes. Separate metal solutions were made of 0.3 mM nickel [from $Ni(NO_3)_2 \cdot 6H_2O$] and 0.3 mM chromium [from $Cr(NO_3)_3 \cdot 9H_2O$] at pH 5.0. Two milliliters of 0.3 mM metal solution was added to each biomass-containing tube and equilibrated for 10 min by rocking (final biomass concentration was 5 mg/mL). The supernatants were kept for analysis, and the biomass was again reacted with fresh 0.3 mM metal solution. This process was continued for 10 times or until the biomass became saturated and was no longer able to bind more metal from the solution.

X-ray Absorption Spectroscopic Studies. The X-ray absorption spectra were measured at room temperature at beam lines X-18B and X-19A at the National Synchrotron Light Source. Data were collected with Si 111 (X-18B and X-19A) monochromator crystals with slits adjusted to give ~1−2 eV resolution. The $Ni(II)(NO_3)_2(H_2O)_6$ standard was measured as an aqueous solution in transmission mode using standard ion detectors. $K_2Cr(VI)_2O_7$ and $Cr(III)(NO_3)_3$ were measured as solids on tape in transmission mode. Alfalfa biomass immobilized in a silica support matrix as described by Gardea-Torresdey and co-workers was saturated with 1000 ppm [19 mM $Cr(III)(NO_3)_3 \cdot 9H_2O$ or 17 mM $Ni(II)(NO_3)_2(H_2O)_6$] of metal solution prior to the analysis (*28*−*30*). All of the metal biomass samples were then washed with DI water prior to use and run as solid powders in fluorescence mode using a Lytle ionization detector. The absolute energy positions were calibrated with Ni (8333 eV) and Cr (5989 eV) metal foils. The data were analyzed with the MacXAFS EXAFS analysis package using standard methods (*31*). The E_0 values were determined from the absorption edge step midpoint. A linear pre-edge background and a least-squares cubic spline EXAFS background were extrapolated to normalize the X-ray absorption near-edge structure (XANES) absorption intensities. Quantitative comparisons between unknown and standards were accomplished with nonlinear fits based on the general extended X-ray absorption fine structure (EXAFS) equation and verified with theoretical simulations carried out with FEFF 3.11, an ab initio curved-wave, single-scattering EXAFS simulation code (*32*). Analysis of the EXAFS data for standards were in good agreement with published X-ray crystallographic data.

Metal Analysis. Each metal studied was analyzed with a Perkin-Elmer model 3110 atomic absorption spectrometer with deuterium background subtraction. Analytical wavelengths used for the metals were 359.4 nm for chromium and 352.5 nm for nickel. Calibration of the instrument was performed within the range of analysis and a correlation coefficient for the calibration curve of 0.98 or greater was obtained.

Results and Discussion

Metal Binding Study with Esterified Biomass. Since carboxyl groups have been found to play a role in the binding of heavy metals, acidic methanol was used to chemically block the carboxyl groups by transforming them into methyl esters, which should be less capable to bind metal cations. A summary of the chemical esterification reaction is shown below (*11, 22*):

$$\text{R-COOH} + \text{CH}_3\text{OH} \overset{\text{H+}}{\rightleftharpoons} \text{R}-\text{COO-CH}_3 + \text{H}_2\text{O} \qquad (1)$$

To determine the extent to which the biomass had been esterified, it was necessary to have nonesterified controls for comparison. These controls consisted of washed biomass reacted with 0.1 M HCl, DI water, and pure 99.9% methanol, respectively. The methanol and acid controls indicated that the esterification of the biomass was neither due to the acid nor to the methanol alone but both combined (data not shown). Table 1 shows the milligrams of metal bound (at pH 5.0) by 1.0 g of alfalfa shoot biomass before and after the esterification reaction. It can be observed from this table that the addition of acidic methanol to the biomass caused a reduction of 100% of the binding of Cr(III) by the esterified biomass as compared to the nonesterified (DI) control. Since the acidic methanol-treated biomass did not bind any of the Cr(III) after esterification, carboxyl groups should be the main ligand involved in the binding of these metals. However, since Ni(II) binding by the modified biomass was reduced by 91% as compared to the nonesterified (DI) control, other groups may be involved in the binding of this metal. The reduction of the metal binding by the addition of acidic methanol suggests that the carboxyl groups were modified and therefore may play a significant role in the binding of Ni(II) and Cr(III) to the alfalfa biomass.

TABLE 1. Capacity for Metal Binding by Chemically Modified Alfalfa Shoots[a]

	mg of metal/g of alfalfa biomass		
metal	before modification	after esterification	after hydrolysis
nickel(II)	12.3 ± 1.6	0.2 ± 0.9	15.8 ± 1.8
chromium(III)	10.7 ± 1.6	0.0 ± 1.0	22.6 ± 3.4

[a] The biomass was reacted repeatedly until saturation was achieved with 0.3 mM solution of each metal at pH 5.0 for 10 min. All experiments were carried out in triplicate, and a 95% confidence interval was used to determine error.

Metal Binding Study with Hydrolyzed Biomass. The ligands present in the biomass that contains esters and is not active in the binding of metals may be hydrolyzed (or saponified) with 0.1 M NaOH, forming new carboxyl groups. A summary of the chemical hydrolyzation reaction is shown below (*11, 22*):

$$\text{R-COO-CH}_3 + \text{NaOH} \rightarrow \text{R-COO}^- + \text{CH}_3\text{OH} \qquad (2)$$

Therefore, by hydrolyzing these esters, we may form new binding sites that were previously unavailable to the metal ions. Table 1 shows the milligrams of metal bound by 1.0 g of alfalfa shoot biomass before and after the hydrolyzation reaction. It is observed in Table 1 that the metal binding was enhanced for the hydrolyzed biomass, 29% for Ni(II) and 111% for Cr(III) as compared to the non-hydrolyzed (DI) control. This indicates that the increased metal binding is due to the newly formed carboxylate groups. Thus, the inhibition of metal binding due to blockage of the carboxyl group by acidic methanol addition and enhancement of metal binding due to ester hydrolyzation implies that carboxyl groups are responsible for a great portion of the binding of Ni(II) and Cr(III) by the alfalfa biomass. These results are in agreement with metal binding studies performed with algal and *Datura innoxia* biomass (*11, 22*). Carboxyl groups were found to play an important role in metal binding by these biomasses.

Metal Binding Studies by X-ray Absorption Spectroscopy. X-ray absorption spectroscopy (XAS) was also used to investigate the metal ion binding mechanism to the alfalfa biomass. XAS is based on the absorption of high-energy monochromatic X-rays by an element in the region of a characteristic absorption edge (*33*). X-ray absorption near-edge structure (XANES) contains chemical and structural information about the metal's oxidation state, coordination

number, and geometry. Extended X-ray absorption fine structure (EXAFS) is produced when photoelectrons leaving the absorbing atom(s) are backscattered by the surrounding nearest neighbor atoms. EXAFS data contain information about the absorbing atom's environment, e.g., coordination number, nearest neighbor, distances, and, in ideal cases, bond angles. Analysis of EXAFS oscillations typically provide accuracies for the coordination number of ±1 and distances of ±0.02 Å. XAS experiments were performed for both nickel(II)- and chromium(III)-exposed alfalfa biomass.

The edge position for the Ni biomass and Ni(II)$(H_2O)_6(NO_3)_2$ are similar, within 0.5 eV, thus indicating that the nickel binds to the biomass as Ni(II), see Figure 1A. The absence of a 1s−4pz peak and a weak 1s−3d peak in both XANES spectra is characteristic of a six coordinate octahedral environment for the nickel (*33*−*35*). The isolated EXAFS and the Fourier transform (FT)-EXAFS data are shown in Figure 1, panels B and C, respectively. As observed in Figure 1, panels B and C, the Ni biomass with the Ni(II) standard containing oxygen ligands appears qualitatively similar. Good fits were obtained using six nitrogens at 2.08 Å or five oxygens at 2.05 Å, see Table 2. In addition, Figure 2 shows the isolated first-shell EXAFS oscillations and nonlinear least-squares fits for $Ni(NO_3)_2$ and Ni biomass with six oxygens at 2.05 Å and six nitrogens at 2.08 Å, respectively. In both cases, good fits were obtained. Fits using only sulfur or a mixture of sulfur and oxygen or nitrogen all gave unreasonable results; data not shown. From the XAS studies, nickel is six-coordinate with oxygen or nitrogen ligands at ∼ 2.05 Å. The lack of multiple scattering enhanced peaks at 2.9 and 4.2 Å, which are normally observed for metal−imidazole complexes, suggests that histidine is not involved in the nickel binding (*36*).

XANES spectra for Cr biomass, $Cr(III)(NO_3)_3{\cdot}6H_2O$, $Cr(III)_2S_3$, and $K_2Cr(VI)_2O_7$ are shown in Figure 3A. From the edge position and the absence of a strong pre-edge peak, associated with tetrahedral Cr(VI), it can be concluded that the Cr biomass is Cr(III) in an octahedral environment (*37*). The maximum peak height for $Cr(III)_2S_3$ is smaller than $Cr(III)(NO_3)_3{\cdot}6H_2O$, and the position is shifted to lower energy for $Cr(III)_2S_3$. Similar results were noted for octahedral Ni(II) compounds with sulfur versus nitrogen or oxygen ligands (*38*). The edge shift is thought to arise from the covalent nature of the Ni−S compound as compared to the more ionic Ni−O/N species. These features correlated with the number of Ni−S bonds (*38*). The isolated EXAFS and FT-EXAFS chromium data are shown in Figure 3, panels B and C, respectively. In addition, Figure 4 shows the isolated first-shell EXAFS oscillations and nonlinear least-squares fits for $Cr(NO_3)_3$ and Cr biomass with six oxygens at 1.97 Å and five oxygens at 2.00 Å, respectively. The similarity between the EXAFS oscillations and transforms for the Cr biomass and the $Cr(III)(NO_3)_3{\cdot}6H_2O$ standard suggests that Cr binds to the biomass by oxygen ligand(s). The best single-shell fits for the Cr biomass was five oxygens at 2.00 Å, see Table 2. Two-shell fits using four oxygens at 2.01 Å and one nitrogen at 1.93 Å afford better fits (data not shown). The short bond does not seem rational for a Cr−N bond, but there are examples in the literature of Cr−O bonds at ∼1.93 Å (*39, 40*). The XANES spectra clearly indicates that chromium binds as Cr(III) and six coordinate octahedral. The fitting results afford Cr−O distances of 2.00 Å for the Cr biomass. The small Debye−Waller factors determined for the Ni and Cr biomass indicate that both metals bind in a homogeneous manner. Most likely only 2−3 biomass functional groups are involved in metal binding with the rest of the metal's ligation sites occupied by water. This would also explain the similarity between the hexa-aquo metal standards and the metal biomass materials.

The combination of chemical modification and XAS experiments provides clues to the mechanisms involved in metal binding to alfalfa biomass. Chemical modification of the alfalfa biomass by esterification of available carboxyl ligands with acidic methanol has shown a dramatic reduction in the biomass metal interaction for Ni(II) and Cr(III). In addition, hydrolysis of the alfalfa biomass can appreciably increase the binding of these metals, which further indicates that carboxyl groups play an important role in the binding of Ni(II) and Cr(III) from solution.

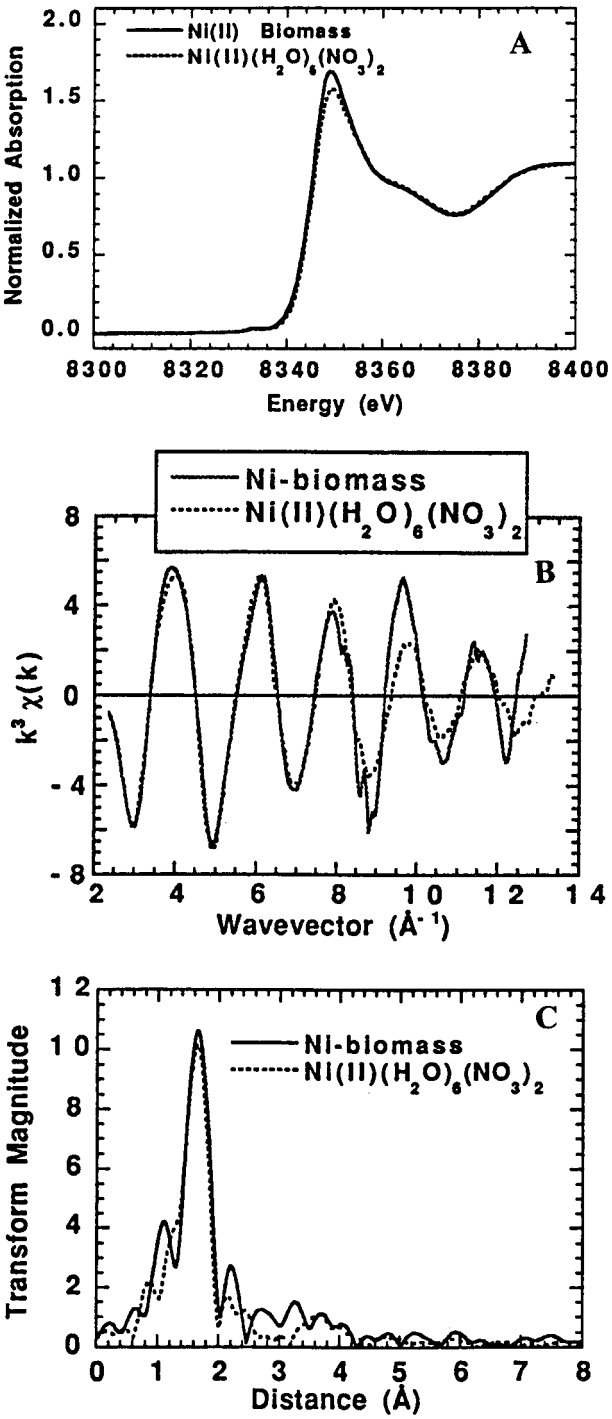

FIGURE 1. (A) X-ray absorption near-edge spectra for nickel-bound biomass (solid) and $Ni(NO_3)_2{\cdot}6H_2O$ (solution) at room temperature. (B) Isolated k^3-weighted EXAFS oscillation for nickel-bound biomass (solid) and $Ni(NO_3)_2{\cdot}6H_2O$ (solution) at room temperature. (C) Non-phase-shift-corrected Fourier transform magnitudes of the k^3-weighted EXAFS oscillation for nickel-bound biomass (solid) and $Ni(NO_3)_2{\cdot}6H_2O$ (solution) at room temperature.

TABLE 2. Nickel and Chromium Biomass EXAFS Results (Using FEFF 3.11 Generated Standards)[a–e]

compound	N (± 1)	R, Å (± 0.02)	$\Delta\sigma^2$ (Å^2) (± 0.001)	χ^2_{gof}
ni biomass	6 (N)	2.08	0.0026	0.035
	5 (O)	2.05	0.0030	0.006
$Ni(NO_3)_2(H_2O)_6$	6 (O)	2.05	0.0057	0.008
Cr biomass	5 (O)	2.00	0.0033	0.025
	5 (N)	2.00	0.0039	0.042
$Cr(NO_3)_3(H_2O)_6$	6 (O)	1.97	0.0028	0.100

[a] N is the coordination number per metal. [b] The metal-coordinating atom distance (R) is determined from fits of the first-shell EXAFS data. [c] $\Delta\sigma^2$ is a relative mean square deviation in r (the square of the Debye–Waller factor), $\Delta\sigma^2 = \sigma^2_{unknown} - \sigma^2$ std. [d] χ^2_{gof} is a relative goodness-of-fit statistic, defined as $\sum(\text{exp-fit})^2/\sum \text{exp}^2$. [e] Experimental details: k range ~2.8–13.4, Hanning window = 0.5 Å^{-1}; weighted = k^3, R window ~1.0–2.2 Å, Hanning window ~1.0 Å.

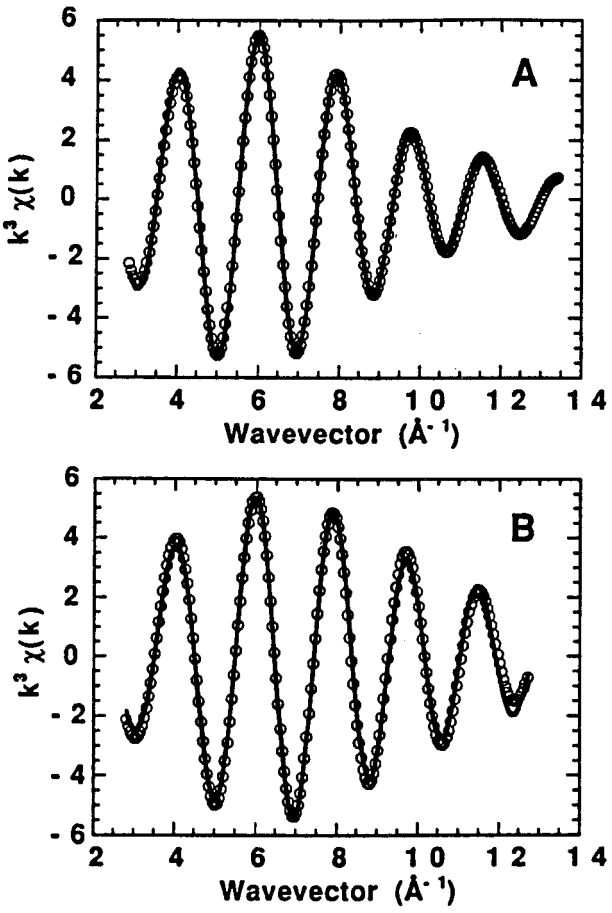

FIGURE 2. Isolated first-shell EXAFS oscillations (○) and nonlinear least-squares fits (–) for (A) $Ni(NO_3)_2(H_2O)_6$, 6 O at 2.05 Å, and (B) Ni biomass, 6 N at 2.08 Å.

The XAS experiments afford information about the metals coordination number, geometry, and, more importantly, the oxidation state of the metal. From the XANES spectra, we have determined that both nickel and chromium are in octahedral environments and that the oxidation states of the metals are Ni(II) and Cr(III). EXAFS data analysis provides information about the coordinating atoms, and in some cases the functional groups can be determined. From the EXAFS fitting results, we determined that the metals are bound via oxygen atoms at 2.05 Å for Ni biomass and 2.00 Å for Cr biomass. Similarity between the metal hexa-aquo and metal biomass XANES and EXAFS data as well as the small Debye–Waller factors indicates homogeneous metal biomass binding via ~2–3 functional groups, and the rest of the metal coordination sites are occupied by water molecules. Since carboxyl

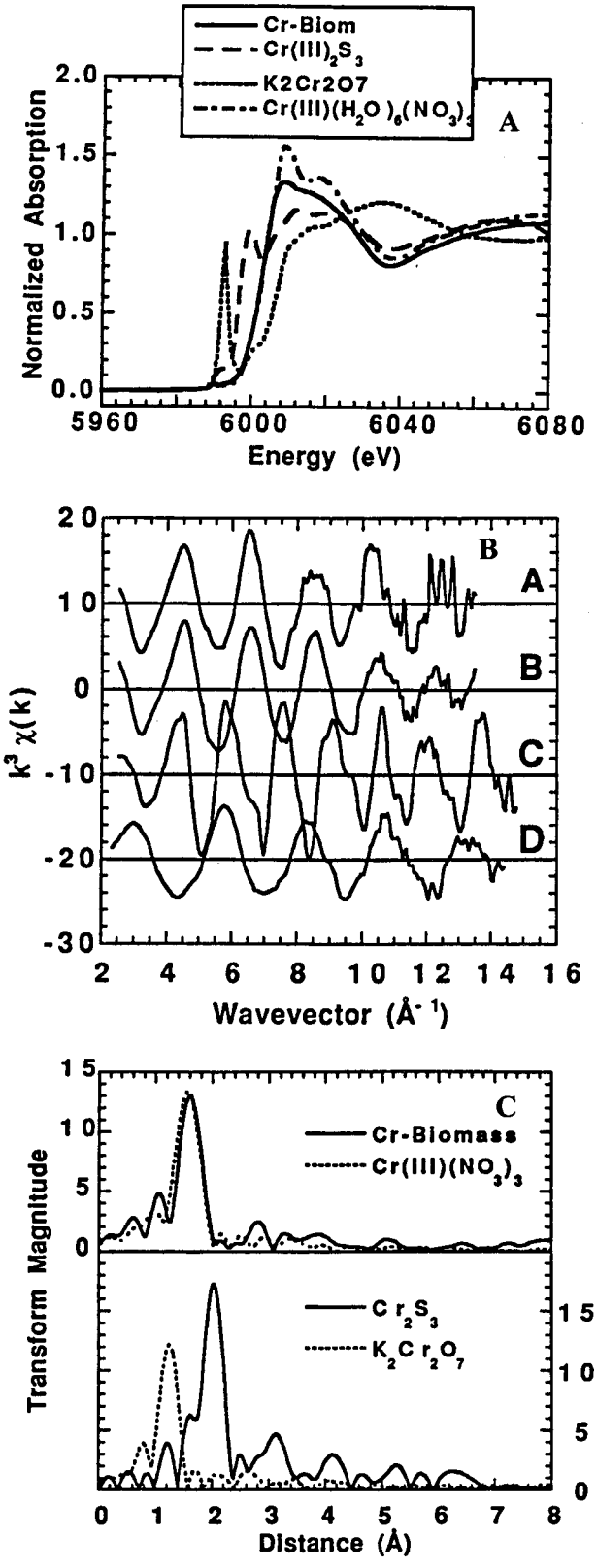

FIGURE 3. (A) X-ray absorption near-edge spectra for chromium-bound biomass (solid), $K_2Cr_2O_7$, $Cr(III)_2S_3$, and $Cr(III)(NO_3)_3$ (solids) at room temperature. (B) Isolated k^3-weighted EXAFS oscillation for chromium-bound biomass (solid), spectral line A, $K_2Cr_2O_7$, spectral line D, $Cr(III)_2S_3$, spectral line C, and $Cr(III)(NO_3)_3$ (solids), spectral line B, at room temperature. (C) Non-phase-shift-corrected isolated k^3-weighted EXAFS oscillations for Cr-bound biomass (solid), $K_2Cr(VI)_2O_7$, $Cr(III)_2S_3$, and $Cr(III)(NO_3)_3$ (solids) at room temperature.

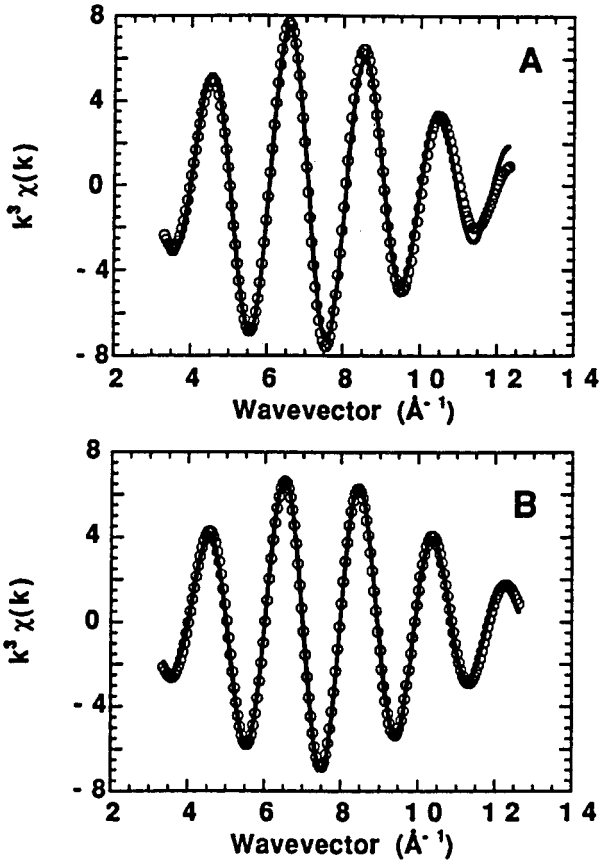

FIGURE 4. Isolated first-shell EXAFS oscillations (○) and nonlinear least-squares fits (−) for (A) $Cr(NO_3)_3$, 6 O at 1.97 Å, and (B) Cr biomass, 5 O at 2.00 Å.

groups have shown to play a major role in the binding of nickel(II) and chromium(III), modification of the biomass through hydrolyzation of possible methyl esters, thus producing more carboxyl groups, will result in more efficient recovery of toxic metals from contaminated waters.

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