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Partial Characterization of a Cadmium-binding Protein from the Roots of Cadmium-treated Tomato[1]

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ABSTRACT

A Cd-binding protein has been isolated from the roots of Cd-treated tomato plants cv. Rutgers. Almost all the Cd from a high-speed supernatant fraction was recovered in a 10,000-dalton fraction from a gel filtration column coincident with 250-nanometer absorbing material. DEAE-cellulose chromatography of this 10,000-dalton material yielded one major component, which eluted at 0.34 molar NaCl, had an absorption spectrum characteristic of metallothionein, and showed absorption changes upon acidification typical of metallothionein. Although the Cd-binding protein did not behave like metallothioneins from animal sources during gel electrophoresis at pH 8.9, a single band containing Cd and staining with Coomassie brilliant blue could be detected following electrophoresis at pH 6.9. Synthesis of the Cd-binding protein appeared to be "induced" by treatment of the plants with Cd^{2+}.

Recent concern over Cd as an environmental contaminant (23) has stimulated research on the response of plants to Cd (7, 15, 16). Although dose-response data have been obtained for many plant species, little is known concerning the biochemical mechanisms of Cd toxicity or tolerance (19, 22, 23). Because metallothionein is of great importance in animals as a Cd scavenger (3, 10), several groups have considered the possibility that it might play a similar role in plants.

Premakumar *et al.* (17) found a Cu-containing protein of low mol wt in a mixture of leaves and shoots from Cu^{2+}-treated *Phaseolus aureus*. Curvetto and Rauser (5) found two low mol wt Cu-binding proteins in the roots of *Agrostis gigantea* exposed to Cu^{2+}. One of the proteins had a cysteine content of 20.8% and was designated a Cu-thionein. Brenmer and Young (1) isolated Cu-thioneins containing approximately 30% cysteine from the livers of Cu^{2+}-injected rats and suggested that the low-cysteine "Cu-chelatins" of Premakumar *et al.* (17) were artifacts produced during isolation. Although it is apparent that plants produce low mol wt Cu-binding proteins in response to Cu^{2+} exposure, it is not clear that these proteins are metallothioneins.

In the only report of a Cd-thionein in higher plants, Casterline and Barnett (2) found 13,000-dalton Cd-containing material in the high-speed supernatants from leaf, stem, and root homogenates from soybean plants exposed to Cd^{2+}. These molecules, although not characterized further, appeared similar to metallothionein in having a high A at 254 nm and a low A at 280 nm.

We have found a Cd-binding protein in the roots of tomato plants treated with Cd^{2+}.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill.) cv. Rutgers were grown in washed quartz sand in the greenhouse with a modified Hoagland solution. After the plants were approximately 6 weeks old, they received a daily application of $CdCl_2$ at 2 μg Cd^{2+}/ml. The plants were harvested after at least 2 weeks of Cd^{2+} treatment. Control plants received no Cd.

Tissue Fractionation. The roots were harvested, rinsed with deionized H_2O, and chilled. Approximately 15 g roots were cut into small pieces with scissors and ground in a chilled mortar containing 10 ml 50 mM Tris (pH 7.8)-5 mM Na ascorbate and sand. The brei was squeezed through two layers of Miracloth (Chicopee Mills) and centrifuged at 10,000g for 5 min, and the supernatant was centrifuged again at 150,000g for 45 min. The final supernatant was collected.

Preparation of Cd-Thionein. The high-speed supernatant was fractionated via gel filtration using Sephadex G-50 fine (Pharmacia Fine Chemicals). The column was equilibrated and the sample eluted with 50 mM Tris (pH 7.8). The appropriate fractions were combined and then subjected to DEAE-cellulose chromatography (Whatman DE52). The DEAE-cellulose column was equilibrated with 50 mM Tris (pH 7.8) and, after sample application, washed with the same buffer. The sample was eluted with 200 ml of a linear gradient of 0.20 to 0.40 M NaCl in 50 mM Tris (pH 7.8). The appropriate fractions were combined and then desalted with coarse Sephadex G-25 using deionized H_2O as eluant. The sample then was concentrated by ultrafiltration using an Amicon UM-2 filter. Column dimensions are indicated in the figure legends.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was via tube gels, 10 cm long and 0.5 cm in diameter. The running gel was 12% acrylamide (0.4% bisacrylamide) and contained 0.03% (v/v) TEMED[5], 0.0175% (w/v) ammonium persulfate, and 0.38 M bis-Tris adjusted to pH 6.9 with HCl. The stacking gel was 2.5% acrylamide (0.625% bisacrylamide) and contained 23% (w/v) sucrose, 0.0625% (v/v) TEMED, 0.07% (w/v) ammonium persulfate, and 62.5 mM bis-Tris adjusted to pH 5.9 with HCl. The reservoir buffer was 20 mM bis-Tris adjusted to pH 6.5 with glutamic acid. Electrophoresis was at room temperature with DNP-aspartate as "tracking dye." Prior to electrophoresis, samples were mixed with an equal volume of sample buffer consisting of 32% (w/v) sucrose and 100 mM bis-Tris adjusted to pH 5.9 with HCl. Duplicate gels were run. One was stained at

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[5] Abbreviations: bis-Tris: bis(2-Hydroxyethyl)imino-tris(Hydroxymethyl) methane; DNP-aspartate: 2,4-dinitrophenyl aspartate; TEMED: *N,N,N',N'*-tetramethylethylenediamine.

least 24 h with Coomassie brilliant blue (21) and then scanned at 560 nm in a Gilford model 2520 gel scanner. The other was frozen in dry ice, sliced into 2-mm sections, digested with 30% H_2O_2, and analyzed for Cd.

Other Techniques. Cd was determined on a Perkin-Elmer atomic absorption spectrophotometer model 303. Absorption spectra were obtained from a Beckman model UV 5270 spectrophotometer.

RESULTS AND DISCUSSION

A distinctive indication of the presence of metallothionein in tissue extracts is the presence of a zone of UV-absorbing material corresponding to about 10,000 daltons in a gel filtration profile (4, 18). When we subjected a high-speed supernatant fraction of the roots of tomato plants that had been treated with 10 μg $CdCl_2$/ml to preparative gel filtration, we observed such a prominent band of absorption at 250 nm (Fig. 1). This zone also contained almost all of the Cd of the extract applied to the gel column. Analytical gel filtration of the fractions within this zone yielded a mol wt of approximately 10,000 (Fig. 2). Inasmuch as no such zone of UV-absorbing or Cd-containing material was detected in control tomato roots, synthesis of this 10,000-dalton material appears to be "induced" by the Cd^{2+} treatment.

Metallothioneins may be further characterized by anion exchange chromatography; DEAE-cellulose chromatography yields either one (13, 14, 20) or two (9, 25) Cd-containing species. When the 10,000-dalton material purified via gel filtration from Cd^{2+}-treated tomato roots was chromatographed on DEAE-cellulose, we observed a single major zone of Cd-containing material (Fig. 3). This material required 0.34 M NaCl for its elution, indicative of a highly anionic molecule. There was also a shoulder of Cd-containing material eluting at a somewhat lower ionic strength. Shallower gradients of NaCl resolved the shoulder somewhat from the major zone of Cd-containing material but did not reveal any additional zones of Cd-containing material.

Another feature of metallothionein is its characteristic low absorption at 280 nm, due to a lack of aromatic amino acids (10), and its relatively high absorption at 250 nm, attributed to metal-mercaptide chromophores (18, 25). When the material eluting at high ionic strength from the DEAE-cellulose column was desalted, concentrated, and brought to 50 mM Tris (pH 7.8), it yielded a UV-absorption spectrum characteristic of metallothionein (Fig. 4,

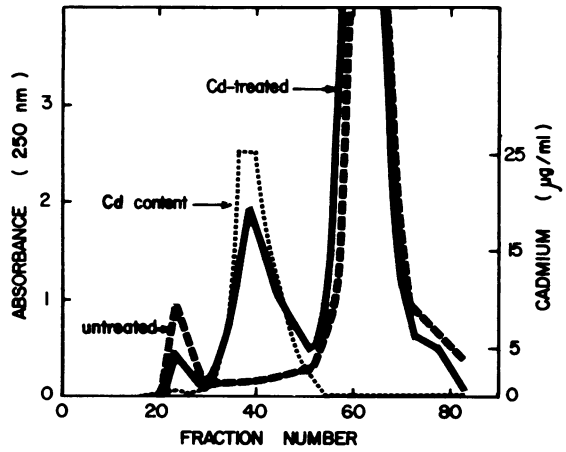

FIG. 1. Preparative gel filtration of a high-speed supernatant from Cd^{2+}-treated tomato roots. The high-speed supernatant was fractionated via Sephadex G-50 fine in a column 14 × 4.2 cm in diameter at 151 ml/h with 50 mM Tris (pH 7.8) as elution buffer. The fractionation was at room temperature. Each fraction was approximately 5 ml. ——, A at 250 nm for Cd^{2+}-treated tomato roots; - - -, A at 250 nm for untreated roots; • • •, Cd in μg/ml for Cd^{2+}-treated tomato roots.

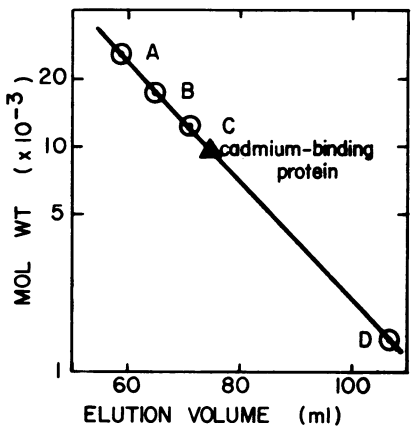

FIG. 2. Mol wt estimation of Cd-binding protein via gel filtration. Analytical gel filtration on Sephadex G-50 fine (97 × 1.2 cm diameter) at 17 ml/h with 50 mM Tris, 50 mM NaCl, pH 7.8. Standards (mol wt): A, chymotrypsinogen (25,700); B, myoglobin (17,200); C, Cyt c (12,300); D, bacitracin (1,400).

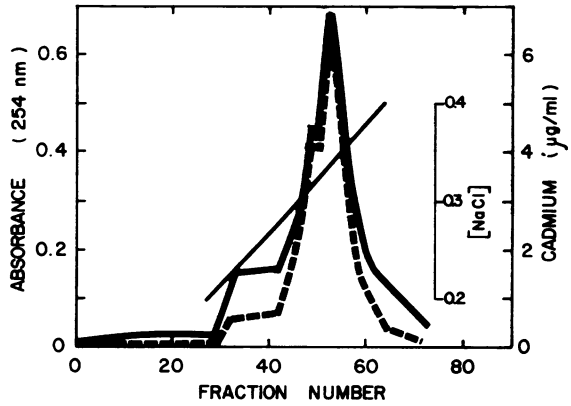

FIG. 3. DEAE-cellulose chromatography of the 10,000-dalton fraction from a Sephadex G-50 column. The column was equilibrated with 50 mM Tris (pH 7.8); after sample application the column was washed with 50 mM Tris (pH 7.8), and the sample was eluted with a linear gradient of 0.2 to 0.4 M NaCl in 50 mM Tris (pH 7.8). The fractions were approximately 5 ml. The column was 20 cm in length and 1.5 cm in diameter with a flow rate of approximately 55 ml/h. ——, A at 254 nm; - - -, Cd (μg/ml).

solid line). In addition, the characteristic absorption in the 250-nm region was significantly reduced upon titration to pH 2 (Fig. 4, broken line). In the case of metallothionein the decrease in absorption upon acidification is known to be due to the breaking of the Cd-mercaptide bonds (18). The absorption at 250 nm returned upon titration to pH 7.8. The absorption in the 280-nm region of the spectrum is higher than in authentic metallothionein and may be due either to contaminants in the preparation or to the reaction of phenolic oxidation products with the protein during tissue grinding (11).

When the material eluting at high ionic strength from the DEAE-cellulose column was subjected to electrophoresis at pH 8.9 in a standard Davis gel (6), the Cd-binding protein moved with the bromophenol blue tracking dye. When the sample was run without tracking dye, it moved as a sharp brown band the same distance as the tracking dye of a control gel. A similar observation has been made with Cu-chelatin from mung bean (17), although the band of protein was reported to be broader than the one we observed. Varying the acrylamide concentration from 8 to 18% did not resolve the Cd-binding protein from the electrophoretic front. The Cd-binding protein from tomato roots thus differs from that in animals, where electrophoresis in alkaline gels

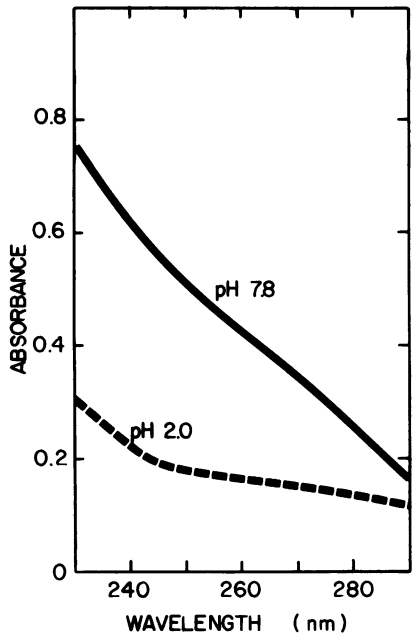

FIG. 4. Absorption spectrum of Cd-binding protein. The Cd-containing material under the major peak from the DEAE-cellulose fractionation was desalted via Sephadex G-25 course, concentrated via ultrafiltration, and brought to 50 mM Tris (pH 7.8). ——, absorption spectrum at pH 7.8; - - -, absorption spectrum after adjusting pH to 2.0 with HCl.

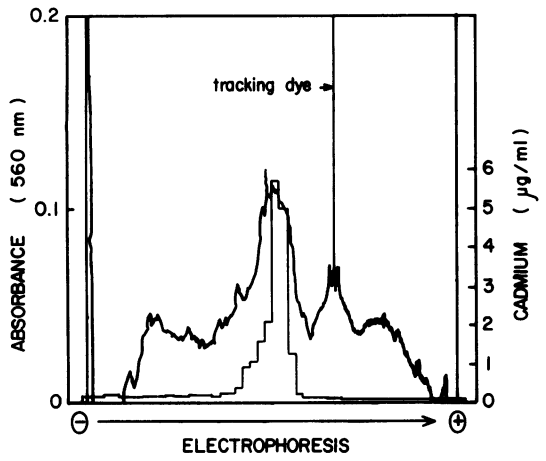

FIG. 5. Polyacrylamide gel electrophoresis of tomato root Cd-binding protein at pH 6.9. The material eluting at high ionic strength from the DEAE-cellulose column was desalted, concentrated, mixed with sample buffer, and run on duplicate gels (12% acrylamide at 0.5 mamp/gel for 1 h and 1.5 mamp/gel for 4.5 h. One gel was stained for 48 h, destained, and scanned at 560 nm (——). The other gel was analyzed for Cd (bar graph). The DNP-aspartate tracking dye was marked with a copper wire.

yielded either one (20, 24) or two species (12, 25) behind the front. Inasmuch as it moved with the electrophoretic front at pH 8.9 and required high ionic strength for elution from DEAE-cellulose, tomato root Cd-binding protein must be a very anionic molecule. We decided to alter the electrophoretic conditions in order to reduce the negative charge on the Cd-binding protein and perhaps cause it to migrate slower than the electrophoretic front. When the Cd-binding protein was subjected to polyacrylamide gel electrophoresis at pH 6.9, we observed one major Coomassie-blue staining zone in the gels (Fig. 5, solid line). The protein had a low affinity for the dye and, to reduce background staining, the gels had to be thoroughly destained prior to scanning at 560 nm. The DNP-aspartate "tracking dye" was marked by a Cu wire which was responsible for the "line" in the gel scan (Fig. 5). When the dye front was not so marked, it was not detectable above the background absorption in the gel scan. When an identical gel was analyzed for Cd, the metal was found in only one narrow region of the gel. This Cd band was contained within the protein band but did not encompass it (Fig. 5). This indicates that there were probably contaminating proteins in our preparation.

In the pH 6.9 gels, brown material in the sample stacked into a tight band and then became diffuse during migration in the electric field, remaining in the top half of the gel. It was distinct from the Cd-binding protein and was probably the result of the polymerization of phenolic compounds during root extraction (11). We worked with roots rather than stems or leaves because we observed less "browning" with root preparations. We had also added Na ascorbate to our grinding buffer because it reduced the extent of "browning."

It is possible that our Cd-binding protein was modified during tissue grinding by reaction with phenolic oxidation products. Jones and Lyttleton (8) reported that ribulose bis-P carboxylase prepared from red clover in the absence of a polyphenol oxidase inhibitor had a higher electrophoretic mobility than when it was prepared in the presence of an inhibitor. They suggested that the oxidized phenolics reacted with amine and sulfhydryl groups on the protein molecule, resulting in a more negatively charged species at elevated pH. This may be the reason our Cd-binding protein had a higher electrophoretic mobility than animal metallothionein.

Although we have provided strong evidence that our Cd-binding protein is a metallothionein, conclusive evidence requires that the protein be purified to homogeneity so that its amino acid composition can be compared to that of the proteins from animal sources.

LITERATURE CITED

1. BREMNER I, BS YOUNG 1976 Isolation of (copper, zinc)-thioneins from the livers of copper-injected rats. Biochem J 157: 517–520
2. CASTERLINE JL, NM BARNETT 1977 Isolation and characterization of cadmium-binding components in soybean plants. Plant Physiol 59: S-124
3. COUSINS RJ 1979 Metallothionein synthesis and degradation: relationship to cadmium metabolism. Environ Health Persp 28: 131–136
4. COUSINS RJ, AD BARBER, JR TROUT 1973 Cadmium toxicity in growing swine. J Nut 103: 964–972
5. CURVETTO NR, WE RAUSER 1979 Isolation and characterization of copper-binding proteins from roots of *Agrostis gigantea* tolerant to excess copper. Plant Physiol 63: S-59
6. DAVIS BJ 1974 Disc electrophoresis. II. Method and application to human serum proteins. Ann N Y Acad Sci 121: 404–427
7. JARVIS SC, LHP JOSER, MD HOPPER 1976 Cd uptake from solution by plants and its transport from roots to shoots. Plant Soil 44: 179–191
8. JONES WT, JW LYTTLETON 1972 The importance of inhibiting polyphenol oxidase in the extraction of fraction 1 leaf protein. Phytochemistry 11: 1595–1596
9. KOJIMA Y, C BERGER, BL VALLEE, JHR KÄGI 1976 Amino-acid sequence of equine renal metallothionein-1B. Proc Natl Acad Sci USA 73: 3413–3417
10. KOJIMA Y, HRK KÄGI 1978 Metallothionein. Trends Biochem Sci 3: 90–93
11. LAM TH, M SHAW 1970 Removal of phenolics from plant extracts by grinding with anion exchange resin. Biochem Biophys Res Commun 39: 965–968
12. NÖEL-LAMBOT F, C GERDAY, A DISTECHE 1978 Distribution of Cd, Zn, and Cu in liver and gills of the eel *Anguilla anguilla* with special reference to metallothioneins. Comp Biochem Physiol 61C: 177–187
13. OLAFSON RW, K ABEL, RG SIM 1979 Prokaryotic metallothionein: preliminary characterization of a blue-green alga heavy metal-binding protein. Biochem Biophys Res Commun 89: 36–43
14. OVERNELL J, IA DAVIDSON, TL COOMBS 1977 A cadmium-binding glycoprotein from the liver of the plaice (*Pleuronectes platessa*). Biochem Soc Trans 5: 267–269
15. PAGE AL, FT BINGHAM, C NELSON 1972 Cadmium absorption and growth of various plant species as influenced by solution cadmium concentration. J Environ Qual 1:288–291
16. PETIT CM, SC VANDEGEIJN 1978 *In Vivo* measurement of cadmium (^{115m}Cd) transport and accumulation in stems of intact tomato plants (*Lycopersicum esculentum*, Mill). I. Long distance transport and local accumulation. Planta 138: 137–143
17. PREMAKUMAR R, DR WINGE, RD WILEY, KV RAJAGOPALAN 1975 Copper-

chelatin: isolation from various eucaryotic sources. Arch Biochem Biophys 170: 278–288
18. PULIDO P, JHR KÄGI, BL VALLEE 1966 Isolation and some properties of human metallothionein. Biochemistry 5: 1768–1777
19. RUSSO F, E BRENNAN 1979 Phytoxicity and distribution of cadmium in pin oak seedlings determined by mode of entry. For Sci 25: 322–333
20. SOKOLOWSKI G, U WESER 1975 Formation, circular dichroism and x-ray photoelectron spectroscopy of hepatic Zn-thionein. Z Physiol Chem 356: 1715–1726
21. SWANK RT, KD MUNKRES 1971 Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal Biochem 39: 462–477
22. TURNER MA 1973 Effect of cadmium treatment on Cd and Zn uptake by selected vegetable species. J Environ Qual 2: 118–119
23. VARMA MM, HM KATZ 1978 Environmental impact of cadmium. J Environ Health 40: 308–314
24. WESER U, H RUPP, F DONAY, F LINNEMANN, W VOELTER, W VOETSCH, G JUNG 1973 Characterization of Cd, Zn-thionein (metallothionein) isolated from rat and chicken liver. Eur J Biochem 39: 127–140
25. WINGE DR, R PREMAKUMAR, KV RAJAGOPALAN 1975 Metal-induced formation of metallothionein in rat liver. Arch Biochem Biophys 170: 242–252