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Retention of Cadmium in Roots of Maize Seedlings[1]

Role of Complexation by Phytochelatins and Related Thiol Peptides

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Cd from roots of maize was partitioned in seedlings exposed to 3 μM $CdSO_4$ for 1 to 7 d. Most of the root Cd (92–94%) was buffer soluble and provided the classical metal-induced cysteine-rich, high-molecular-weight Cd-binding complex. This complex, however, bound only part of the Cd within the roots, from 19% after 1 d of exposure to 59% by d 7. Three families of peptides formed the Cd-binding complex: (γ-glutamic acid-cysteine)$_n$-glycine [(γ-Glu-Cys)$_n$-Gly], or phytochelatins, (γ-Glu-Cys)$_n$, and (γ-Glu-Cys)$_n$-Glu. The monothiols γ-Glu-Cys-Gly (glutathione), γ-Glu-Cys, and γ-Glu-Cys-Glu were absent from the complex. The n_2 oligomers of any peptide were the least concentrated, whereas the n_3 and n_4 oligomers increased in the complex with exposure to Cd. By d 7, 75% of (γ-Glu-Cys)$_4$-Gly, 80% of (γ-Glu-Cys)$_4$, and 73% of (γ-Glu-Cys)$_3$-Glu were complexed with Cd. The peptide thiol:Cd molar ratio for the complexes was 1.01 ± 0.07, as if the minimal amount of thiol was used to bind Cd. Acid-labile sulfide occurred in the complexes from d 1 onward at the low S^{2-}:Cd molar ratio of 0.18 ± 0.02.

Cd is a trace metal and potential toxin that is a concern when transferred from plant products to the human diet. At least 70% of the Cd intake by humans originates from plant foods (Wagner, 1993). Soils are the principal source of Cd for vascular plants, and roots are the major organ of entry. Roots of 23 species retained Cd at higher concentrations than did shoots and, in most cases, the total content of Cd in roots exceeded that in shoots (Jarvis et al., 1976). The ability of roots to retain Cd is accentuated in several inbreds of maize (Florijn and Van Beusichem, 1993) and some *Nicotiana* species but not in commercial *Nicotiana tabacum* (Wagner and Yeargan, 1986).

The basis for high retention of Cd by roots, largely desirable in the context of the food chain, is poorly understood. Up to 71% of the Cd in roots of maize seedlings occurred as an anionic Cd-binding complex of undetermined composition (Rauser, 1986). Prominent complexers of Cd are the Cd-inducible, Cys-rich peptides with the structure (γEC)$_n$G (Kondo et al., 1984; Grill et al., 1985). These peptides are the cadystins or phytochelatins (for reviews, see Rauser, 1990; Steffens, 1990). Examination of other species has revealed further related peptides. Certain legumes produce homo-phytochelatins, or (γ-Glu-Cys)$_n$-βAla (Grill et al., 1986), whereas some species of the family Poaceae (Gramineae) produce hydroxymethyl-phytochelatins or (γ-Glu-Cys)$_n$-Ser (Klaphecket al., 1994). Maize roots and shoots contain an abundance of (γEC)$_n$, as well as (γEC)$_n$E and (γEC)$_n$G (Meuwly et al., 1995). The variety of Cys-rich peptides is collectively designated γEC peptides.

To test the putative function of γEC peptides in Cd sequestration it is essential to study Cd-binding complexes. Gel filtration analyses of alkaline extracts from roots, cultured plant cells, and algae exposed to a variety of Cd concentrations for different times show that at least 70% and usually in excess of 90% of the buffer-soluble Cd occurs as Cd-binding complex (Jackson et al., 1984; Grill et al., 1985; Lue-Kim and Rauser, 1986; Steffens et al., 1986; Reese and Wagner, 1987; Verkleij et al., 1990; Gupta and Goldsbrough, 1991; Howe and Merchant, 1992; Kneer and Zenk, 1992). However, in most of these studies it is impossible to estimate how much cellular Cd occurs as Cd-binding complex, because the amount of Cd in the single initial extract analyzed is not related to the total Cd in the tissues or cells. Quantitating this form of Cd is fundamental to understanding the speciation of Cd within roots.

The characteristics and compositions of Cd-binding complexes vary considerably among organisms, concentrations of Cd used, exposure time, and nutrient medium. The Cd-binding complexes may be low and high molecular weight (Murasugi et al., 1983; Jackson et al., 1984; Kneer and Zenk, 1992). The high-molecular-weight complex is essential for sustained growth in high concentrations of Cd (Mutoh and Hayashi, 1988) and contains acid-labile sulfide (Murasugi et al., 1983; Speiser et al., 1992). Those complexes from the yeasts *Schizosaccharomyces pombe* and *Candida glabrata* with S^{2-}:Cd molar ratios exceeding 0.4 had CdS crystallites surrounded by γECG, (γEC)$_2$G, and (γEC)$_2$ (Dameron et al., 1989). The high sulfide fractions of tomato complex had characteristics of small CdS crystallites (Reese et al., 1992). The complexes from *S. pombe* and *C. glabrata*

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Abbreviations: (γEC)$_n$, (γ-Glu-Cys)$_n$; (γEC)$_n$G, (γ-Glu-Cys)$_n$-Gly; (γEC)$_n$E, (γ-Glu-Cys)$_n$-Glu; γEC peptide, γ-glutamylcysteinyl peptide; SH, thiol.

were composed of $(\gamma EC)_{2-3}G$ and lesser quantities of $(\gamma EC)_2$ (Hayashi et al., 1988; Mehra and Winge, 1988; Dameron et al., 1989; Barbas et al., 1992); in plant Cd-binding complexes the n_3, n_4, and sometimes the n_5 oligomers of $(\gamma EC)_nG$ predominated (Gupta and Goldsbrough, 1991; Strasdeit et al., 1991; Reese et al., 1992). In the complex from *Rauvolfia serpentina*, Cd was coordinated through the SH of four Cys's (Strasdeit et al., 1991). A Cd-hypersensitive mutant of *S. pombe* lacking the high-molecular-weight sulfide-rich complex regained Cd resistance with enhanced expression of an ATP-binding cassette-type transporter protein located in the vacuolar membrane (Ortiz et al., 1992). In vitro, this protein transports the sulfide-depleted but not the sulfide-rich complex (Ortiz et al., 1995). It is thus inferred that the high-molecular-weight sulfide-rich complex is made in the vacuole. A similar subcellular compartmentation occurs in plants. Virtually all of the Cd and γEC peptides in protoplasts from tobacco leaf cells occurred within the vacuoles (Vögeli-Lange and Wagner, 1990). Tonoplast vesicles from oat roots transport $(\gamma EC)_2G$, $(\gamma EC)_3G$, and Cd-$(\gamma EC)_3G$ by a mechanism typical of ATP-binding cassette-type transporters (Salt and Rauser, 1995). Direct measurements of Cd-binding complex in purified vacuoles are lacking.

A previous study of the accumulation of $(\gamma EC)_nG$, $(\gamma EC)_n$, and $(\gamma EC)_nE$ in roots of maize exposed to Cd (Meuwly et al., 1995) did not establish the role of the peptides in Cd sequestration. The goals of this study were (a) to establish, through time of exposure, the proportion of Cd in maize roots that occurred as Cd-binding complexes and (b) to characterize the nature of these complexes in terms of the γEC peptides and acid-labile sulfide.

MATERIALS AND METHODS

Plant Material

Seedlings of maize (*Zea mays* L., Cargill hybrid 37701) were grown hydroponically as described previously (Meuwly and Rauser, 1992). Caryopses were germinated for 3 d, and 50 seedlings were transplanted into plastic vessels with 4 L of aerated one-half-strength Hoagland solution No. 2 and kept at 22 to 23°C with a 16-h/d light period beginning at 7 AM. Roots were exposed to 3 μM $CdSO_4$ in nutrient solution, which was changed daily, from 5 to 12 d after planting.

Seedlings were collected after 1, 2, 4, 5, and 7 d at 9 AM, and the roots were washed for 10 min in ice-cold 5 mM $CaCl_2$ solution to displace extracellular Cd (Rauser, 1987). After blotting the primary root systems were excised, the fresh weights determined, and the tissues frozen in liquid N_2 for storage at −70°C.

Extraction Procedures

Frozen roots were pulverized in a mortar and pestle with liquid N_2 and homogenized under a stream of N_2 in equal parts (w/v) of ice-cold N_2-purged 100 mM Tris-HCl (pH 8.6), 1 mM PMSF, and 1% (v/v) Tween 20. The homogenate was centrifuged at 4°C and 48,000*g* for 6 min. The volume of supernatant, designated extract 1, was measured, a subsample was withdrawn for Cd analysis, and the remainder was retained for anion-exchange chromatography. The mortar was rinsed with a volume of N_2-purged wash buffer (10 mM Tris-HCl [pH 8.6] and 1% [v/v] Tween 20) 1.5 times the fresh weight and added to the pellet for resuspension while on ice. The suspension was centrifuged as before, and the supernatant (extract 2) was retained. Resuspension and centrifugation were repeated another four times. After extract 6 was decanted, the pellet was suspended in ice-cold 100 mM HCl using a volume two-thirds that of the root fresh weight. The suspension was centrifuged as before, and the supernatant (extract 7) was retained. The pellet was suspended two more times in 100 mM HCl, using a volume 1.5 times the fresh weight, to give extracts 8 and 9. The pellet was transferred to a test tube and ashed at 500°C for approximately 20 h. The ash was suspended in 3.0 mL of 0.4 M HCl, filtered, and used for Cd analysis. The fresh weight of roots used varied from 13.6 to 17.6 g for 75 seedlings treated with Cd for 1 d to 12.3 to 16.1 g for 50 seedlings treated for 2 d, 8.4 g for 20 seedlings treated for 4 d, 8.1 to 8.5 g for 20 seedlings treated for 5 d, and 9.6 to 9.9 g for 20 seedlings exposed for 7 d. With the exception of the 4-d exposure, all extractions were replicated three times from seedlings grown on different occasions.

Total SHs in roots were extracted with 100 mM HCl as described previously (Meuwly and Rauser, 1992). Extracts were divided into 500- to 800-μL portions, frozen in liquid N_2, and stored at −70°C no longer than 4 weeks before analysis by HPLC. For quantitation, the contribution of tissue fluids was corrected afterward by adding 0.92 times the fresh weight to the extractant volume.

Anion-Exchange and Gel Filtration Chromatography

Premeasured quantities of extracts 1 through 6 were added quantitatively to a 0.5-mL bed of Q Sepharose Fast Flow equilibrated with 10 mM Tris-HCl (pH 8.6). The exchanger was washed with 24 mL of 10 mM Tris buffer. The fluid passing through the exchanger was collected for Cd analysis. Anionic material was eluted with 4.0 mL of 10 mM Hepes (pH 8.0) and 1 M KCl. A second elution with 5.0 mL of Hepes-KCl buffer yielded only a small amount of Cd. The recovery of Cd from anion-exchange chromatography was 90 to 96%.

Preparation grade Superose 12 was used for gel filtration chromatography of anionic material. The column (1.6 × 50.5 cm) was equilibrated with N_2-purged 10 mM Hepes (pH 8.0) and 300 mM KCl. The sample (2.8–2.9 mL) was applied and the column developed in equilibration buffer at a flow rate of 0.8 mL/min at 4°C. The A_{254} was recorded, and fractions (2.74–2.79 mL) were collected every 3.5 min in an N_2 atmosphere. Gel filtrations had Cd recoveries of 92 to 104%.

After Cd was measured, the fluid remaining in the fractions of interest was combined and diluted 3-fold with water and passed over a 0.25-mL bed of Q Sepharose Fast Flow equilibrated with 10 mM Tris-HCl (pH 8.6). After the exchanger was washed with 8 mL of 10 mM Tris buffer, anionic material was eluted with 1.5 mL of 10 mM Hepes

(pH 8.0) and 1 M KCl. Such concentrates were subsampled for Cd and acid-labile sulfide and analyzed for SH by HPLC.

Analytical Procedures

SHs in 500- to 800-μL samples of HCl extracts of roots were separated by HPLC in reverse phase on a C_{18} column and quantified by postcolumn derivatization with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) according to an adaptation (Rauser, 1991) of the original method (Grill et al., 1985). For Superose 12 fractions concentrated by Q Sepharose chromatography, ice-cold portions (500–1000 μL) of eluate were titrated to pH 2 with 6 M HCl, held on ice for 15 min, centrifuged for 10 min at 4°C in a microcentrifuge, and applied immediately to the C_{18} column.

Acid-labile sulfide in Superose 12 fractions concentrated by Q Sepharose chromatography was determined according to the method of King and Morris (1967). The amount of Cd present in the subsamples did not interfere with the assay. The assays were calibrated with fresh approximately 0.1 mM Na_2S solution, where the precise sulfide concentration was determined by isocratic HPLC in aqueous 0.1% (v/v) TFA and detection with Ellman's reagent.

Concentrations of Cd in extracts and column effluents were measured by atomic absorption spectroscopy (model AA-475; Varian, Inc., Mississauga, Ontario, Canada).

RESULTS

Partitioning of Cd in Roots of Maize

Buffer extract 1 yielded 63 to 74% of the Cd present in roots. The five additional extractions yielded diminishing amounts of Cd. The six consecutive buffer extractions together consistently yielded 92 to 94% of the total Cd in roots. These high yields were irrespective of the times of exposure to Cd. The Cd remaining in the root pellet (<8% of the root Cd) was largely extracted with ice-cold 100 mM HCl (extracts 7–9); little Cd occurred in the ash. Detergent was included in the extraction and wash buffers to maximize the extraction of buffer-soluble Cd. Homogenizing roots in buffer without detergent yielded 48% of the root Cd in extract 1 versus 63% with detergent for 2-d samples or 51% versus 65% for 5-d samples.

Passage of extracts 1 to 6 through an anion-exchange column concentrated the anionic forms of buffer-soluble Cd for gel filtration. The proportion of root Cd not bound to the anion exchanger decreased from 61% after 1 d to 28% by 7 d. The proportion of root Cd in anionic form increased sharply early during exposure to Cd (33 and 44% on d 1 and 2, respectively) and more slowly later (57–66% on d 4–7, respectively).

Two examples of gel filtration profiles of anionic buffer-soluble Cd are shown in Figure 1. The largest proportion of Cd in each separation appeared in region II (59% on d 1, 75% on d 2 [Fig. 1A], to 90% by d 7 [Fig. 1B]) and corresponded to the classical metal-induced Cd-binding complex (see below). Declining proportions occurred in region I, from 38% in d-1 samples to 23% by 2 d (Fig. 1A) and 7% by 7 d (Fig. 1B). However, in terms of concentration in the root, the Cd in region I increased 5-fold from 0.58 ± 0.02 on d 1 to 2.87 ± 0.12 μg Cd g^{-1} fresh weight by d 7. The Cd in region I peaked at fraction 21 on d 1 and 2 (Fig. 1A), well after the column void volume and yet before Cyt *c* (M_r 12,400) at fraction 28. A small amount of Cd in each chromatogram (2–3%) eluted late in region III, which encompassed the total volume and represented 0.9 to 1.1% of the Cd in the roots.

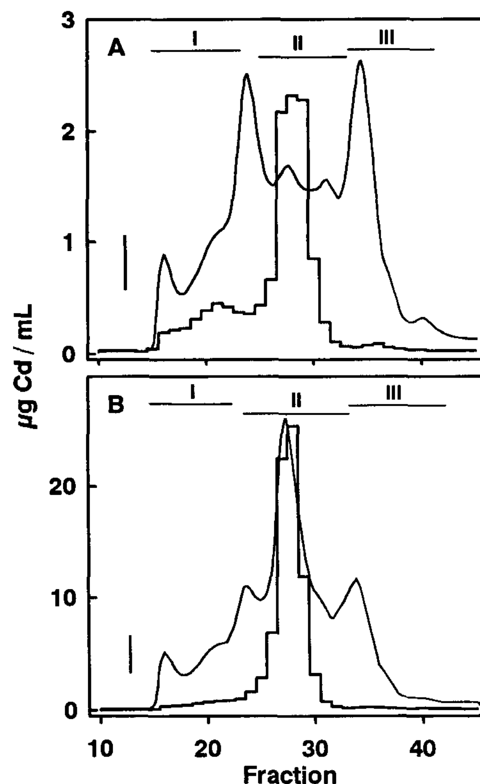

Figure 1. Gel filtration chromatography of extracts from maize roots exposed to 3 μM $CdSO_4$. The anionic fraction from buffer extracts 1 to 6 was chromatographed on Superose 12 in 10 mM Hepes (pH 8) and 300 mM KCl. A, Material equivalent to 9.36 g fresh weight of roots exposed for 2 d; B, material equivalent 6.45 g fresh weight of roots exposed for 7 d. The histograms show the concentration of Cd in the 2.7- to 2.8-mL fractions; the continuous trace indicates the A_{254}, with the vertical calibration bar over fraction 13 representing 0.01. Regions I, II, and III indicate the fractions that were pooled for peptide and sulfide analyses. Column characteristics were: void volume, 42 mL at fraction 16; total volume using acetone, 98 mL at fraction 36; and elution volume of $CdSO_4$, 103 mL at fractions 37 to 38.

The partitioning of root Cd in exposures up to 7 d is summarized in Figure 2. The total concentration of Cd in roots increased linearly at 9.54 μg g^{-1} d^{-1} (r^2 = 0.9873). The amount of root Cd found as Cd-binding complex of region II increased from 19% after 1 d of exposure to 59% by 7 d (Fig. 2). The best fitting linear regression of Cd as Cd-binding complex versus time was for 2 to 7 d (6.39 μg Cd $g^{-1}d^{-1}$, r^2 = 0.9776, *x* intercept 1.5 d). The lag period in linear response was indicative of the increasing rate of Cd retention early after transfer to 3 μM $CdSO_4$ and of biosyn-

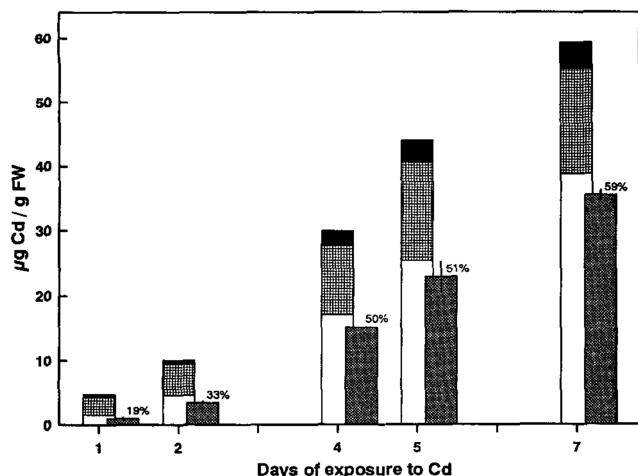

Figure 2. Partitioning of Cd in maize roots exposed to 3 μM $CdSO_4$ for 7 d. Root extracts 1 to 6 constituted the buffer-soluble Cd; the Cd remaining in the pellet (■) was acid soluble (extracts 7–9) and in the ash. The buffer-soluble Cd was divided by anion-exchange chromatography on Q Sepharose into the nonbound (▦) and bound Cd (□). The Cd-binding complex (▩) was that part of the bound Cd found in region II of Superose 12 gel filtrations. The percentages are the amounts of root Cd found as Cd-binding complex. Means ± SE of three experiments, except for d 4, are indicated.

thesis and assembly of constituents into the major Cd-binding complex.

SH Peptides Complexing Cd

Materials from regions I and III of gel filtrations yielded few or no SH peaks. The Cd-binding complexes from region II yielded a variety of SHs. Two representative HPLC separations are shown in Figure 3, A and B, with the total SHs of the roots shown in Figure 3, C and D. The latter two separations are similar to those in prior studies in which the SH structures were determined (Meuwly et al., 1993, 1995). The compounds in peaks a, b, c, and d were the monothiols Cys, γECG (GSH), γEC, and γECE, respectively. The numbered peaks made up three families of polythiols: (a) $(EC)_nG$: peak 1 = n_2, 5 = n_3, 8 = n_4; (b) $(\gamma EC)_n$: 2 = n_2, 6 = n_3, 9 = n_4; and (c) $(\gamma EC)_nE$: 3 = n_2, 7 = n_3. SH peak 4 is perhaps a variant of $(\gamma EC)_4$. Peaks 10 and 11 were tentatively identified as $(\gamma EC)_5G$ and $(\gamma EC)_5$, respectively, based on the linear relationship between the log of the number n of γEC units and the retention time of the peptides in each family. The sulfide in Figure 3, A and B, was the remnant not volatilized.

To enable precise differentiation of the γEC peptides in the Cd-binding complex from the total in the roots, the respective concentrations are shown in Figure 4 for 1- to 7-d exposures. The concentrations of total γEC peptides (Figs. 3D and 4, E and F) are similar to prior results (Meuwly et al., 1995). For peaks 4, 10, and 11, for which the molecular structure has not been defined, the concentrations are given in Table I based on the SH measured. At each harvest the peptides forming the complex were a subset of the total γEC peptides present in the roots (Fig. 4, A, B, and C, versus D, E, and F). The monothiols γECG, γEC, and γECE were not detected in Cd-binding complexes, and the n_2 oligomers of all γEC peptides were least concentrated, ranging from 0.003 to 1.00 nmol peptide g^{-1} fresh weight. The complexes contained nearly equimolar concentrations of the n_3 and n_4 oligomers of $(\gamma EC)_nG$, the phytochelatins. However, at each harvest the concentra-

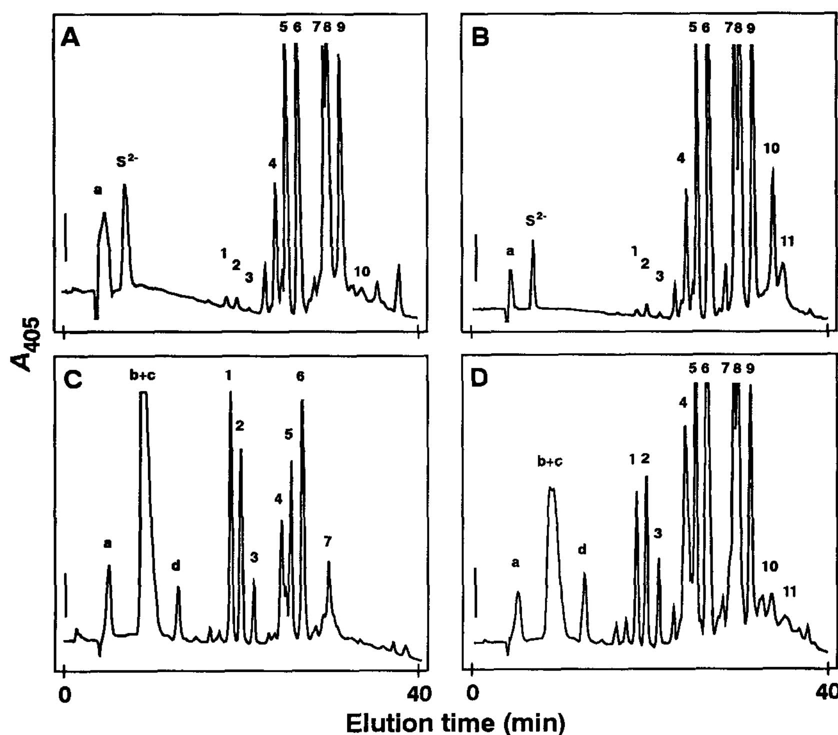

Figure 3. HPLC chromatograms of SHs from roots of maize exposed to 3 μM $CdSO_4$ for 2 d (A and C) and 7 d (B and D). A and B, SHs occurring in Cd-binding complex (region II of Superose 12 gel filtrations) equivalent to 4.20 and 1.61 g fresh weight of roots, respectively. C and D, Total SHs occurring in the roots equivalent to 0.42 and 0.51 g fresh weight, respectively. The vertical calibration bar at elution time 0 represents an A_{405} of 0.02 in A and 0.04 in the B to D. Peak a contains reactive material at the column breakthrough and Cys; the other peaks are identified in the text.

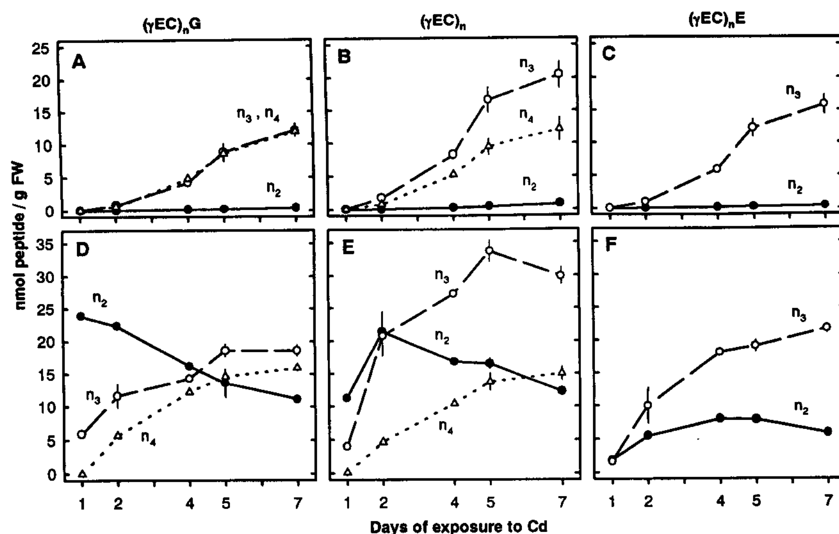

Figure 4. Concentrations of SH peptides in roots of maize exposed to 3 μM $CdSO_4$ for 7 d. A, B, and C, Peptides $(\gamma EC)_nG$, $(\gamma EC)_n$, and $(\gamma EC)_nE$, respectively, occurring in the high-molecular-weight Cd-binding complex (region II of Superose 12 gel filtrations). D, E, and F, Total concentrations of the respective peptides in acid extracts of the roots. The size of the oligomers is identified by n_2, n_3, and n_4, the number of γEC repeats in the molecules. Means ± SE of three experiments, except for d 1 and 4, are indicated.

tions of $(\gamma EC)_3G$ or $(\gamma EC)_4G$ were exceeded by $(\gamma EC)_3$ (by 1.7- to 2.1-fold) and $(\gamma EC)_3E$ (by 1.2- to 1.4-fold). Of the total SHs available, the largest contributors to complexing Cd were the $(\gamma EC)_n$ peptides (61% on d 1 to 42% by d 7, peptide 11 included) with contributions from phytochelatins increasing with time (24% on d 1 to 36% by d 7, peptide 10 included). After d 2, $(\gamma EC)_nE$ provided 14 to 17% of the SHs.

Sulfide in Cd-Binding Complexes

The Cd-binding complexes formed through γEC peptides in region II (Fig. 1) contained increasing concentrations of acid-labile sulfide from d 1 onward (Table I). Molar ratios of acid-labile sulfide, peptide SH, and their sum on Cd in complexes were obtained by regression analyses during the 1 to 7 d of exposures. All regressions had high r^2 values and projected through the origin. The molar ratios (mean ± SE of 11 observations) were S^{2-}:Cd, 0.18 ± 0.03; peptide SH:Cd, 1.01 ± 0.07; and peptide SH plus S^{2-}:Cd, 1.19 ± 0.08. The material in region I of gel filtrations (Fig. 1) contained acid-labile sulfide. However, the concentrations were much lower than those associated with the Cd-binding complex of region II (Table I). The concentrations in region I were 0.69 nmol S^{2-} g^{-1} fresh weight on d 1 and 1.43 ± 0.17, 3.3, 2.23 ± 0.41, and 2.88 ± 0.35 on d 2, 4, 5, and 7, respectively. No consistent relationship was evident between acid-labile sulfide and Cd or the occasional SH peptides present in region I. Acid-labile sulfide was not detected in the material from region III of gel filtrations.

DISCUSSION

The focus of this study was to evaluate the role of γEC peptides in sequestering Cd in roots of maize. The identity and kinetics of appearance of three families of γEC peptides were reported for maize seedlings exposed to 3 μM Cd (Meuwly and Rauser, 1992; Meuwly et al., 1993, 1995). The choice of 3 μM Cd is at the high end of concentrations mimicking polluted soils (Wagner, 1993) yet much lower than the 100 to 6000 μM concentrations used for studies with cultured plant cells and yeasts. Since responses can vary with organism, Cd concentration, exposure time, and nutrient medium, our data from a single concentration of Cd serve as signposts for effects in other situations.

The roots used to quantitate the classical Cd-binding complex were desorbed to remove Cd from the surface film and to exchange the Cd in cell walls (Rauser, 1987). We assumed that the Cd remaining in the roots was intracellular Cd. The fact that 92 to 94% of this root Cd was extracted with buffer containing detergent regardless of the time of exposure to Cd (Fig. 2) attested to the efficiency of the protocol used. The positive influence of detergent during extraction was seen as simply increased extractability through solubilization of membranes. Since these experiments were completed, 0.2% Tween 20 has been found to be as effective as the 1% used here. The high percentages of root Cd in extract 1 (61–69%) and continued solubilization through five successive reextractions differ markedly from a similar extraction of maize roots (Florijn et al., 1993). The nature of the root-Cd interaction that yielded the Cd extracted with 100 mM HCl (fractions 7–9, Fig. 2) is unknown. Part of it is buffer-soluble Cd from extract 6 occluded in the pellet; the rest is perhaps from unbroken cells and entities that bind Cd very tightly.

The classical Cd-binding complex was quantitated by concentrating the anionic material from buffer extracts 1 to 6 and separating it by gel filtration. The separations on Superose 12 (Fig. 1) were similar to those from previous reports with other gels (Jackson et al., 1984; Grill et al., 1985; Lue-Kim and Rauser, 1986; Reese and Wagner, 1987; Verkleij et al., 1990; Gupta and Goldsbrough, 1991; Howe and Merchant, 1992; Kneer and Zenk, 1992). The identity of the agent(s) binding Cd in region I (Fig. 1A) is unclear. SH peptides were scarce, even though acid-labile sulfide was present, but at lesser concentrations than in region II. A similar situation was found in roots of *Silene vulgaris* (Verkleij et al., 1990). Region II (Fig. 1) comprised the classical

Table I. *Concentrations of other SHs, Cd, and sulfide in Cd-binding complexes and acid extracts of maize roots*

For peptides b+c and d, the nmol SH/g fresh weight equals the nmol peptide/g fresh weight. For peptides 4, 10, and 11, the conversion to nmol peptide/g fresh weight is not warranted because of the uncertain number of SHs/mol. Means ± SE of three replicates where available. n.d., Not detected.

Day	Amount in Cd-Binding Complex					Amount of Peptide in Acid Extract				
	Peptide			Cd	S^{2-}	b+c	d	4	10	11
	4	10	11							
	nmol/g fresh wt									
1	0.033	0.025	0.012	7.30	3.63	205.33	5.71	13.37	n.d.	n.d.
2	0.81 ± 0.15	0.30	n.d.	29.60 ± 1.65	10.23 ± 0.62	191.46 ± 8.39	12.53 ± 0.30	32.84 ± 4.49	2.04 ± 0.50	n.d.
4	5.45	12.00	12.08	133.44	31.08	114.06	18.39	51.94	10.54	8.06
5	10.21 ± 1.15	19.69 ± 4.85	17.11 ± 4.70	201.46 ± 20.40	48.03 ± 5.99	111.12 ± 10.15	17.06 ± 1.56	58.71 ± 1.58	15.09 ± 2.32	6.87 ± 1.92
7	15.83 ± 1.20	21.65 ± 2.99	14.69 ± 4.66	312.84 ± 618	59.23 ± 4.25	86.36 ± 1.62	14.32 ± 0.29	59.00 ± 1.93	19.47 ± 1.13	17.76 ± 2.62

Cd-binding complex reported upon extensively (Rauser, 1990; Steffens, 1990). This material contained γEC peptides (Figs. 3, A and B, and 4, A–C), acid-labile sulfide (Table I), and a peptide SH:Cd molar ratio near 1 and eluted a little later than Cyt *c*. These features permit designation of this complex as the high-molecular-weight complex found in yeasts and other plants (Murasugi et al., 1983; Jackson et al., 1984; Mutoh and Hayashi, 1988; Reese et al., 1992; Speiser et al., 1992). Nothing in regions II and III indicated the presence of the less abundant sulfide-free, low-molecular-weight Cd-binding complex (Murasugi et al., 1983; Jackson et al., 1984; Kneer and Zenk, 1992). Perhaps a matrix other than Superose 12 would increase resolution in the low-molecular-weight range or the putative low-molecular-weight Cd-binding complex was not retained during anion-exchange chromatography and was absent for fractionation on Superose 12.

The prevailing view that most Cd is sequestered by γEC peptides in the Cd-binding complex originates from the distribution of buffer-soluble Cd within gel filtration profiles. About 90% of the Cd is found as Cd-binding complex (Jackson et al., 1984; Grill et al., 1985; Lue-Kim and Rauser, 1986; Steffens et al., 1986; Reese and Wagner, 1987; Verkleij et al., 1990; Gupta and Goldsbrough, 1991; Howe and Merchant, 1992; Kneer and Zenk, 1992). In the same manner, the high-molecular-weight complex from maize roots sequestered 59% of the Cd in the gel filtration profile after 1 d and increased to 88 to 92% by d 4 to 7. However, when the sequestered Cd is expressed as a proportion of the total Cd in the roots, the high-molecular-weight complex accounted for 19% of the root Cd on d 1 to a maximum of 59% by d 7 (Fig. 2). These estimates are fundamental to modeling Cd speciation within roots and suggest that additional mechanisms retain Cd in this tissue.

Our evidence tempers the absolute role of γEC peptides in complexing cellular Cd without discounting the Cd status in the whole maize root. Data in two reports can be recalculated for comparison to maize. In *S. vulgaris* 90% of the Cd in the root extract occurred as Cd-binding complex during gel filtration; yet when based on the total tissue Cd the complex bound 52% of the Cd present in water-rinsed roots exposed to 40 μM $CdSO_4$ for 21 d (Verkleij et al., 1990). The extract of cultured cells of *R. serpentina* had 97% of its ^{109}Cd as Cd-binding complex (high- and low-molecular-weight forms combined); however, the proportion decreased to 52% when based on total ^{109}Cd in the cells exposed to 100 μM $CdCl_2$ for 72 h and washed to remove ^{109}Cd from cell walls (Kneer and Zenk, 1992). Since both groups only used the equivalent of extract 1 for gel filtration, it is possible that the value of 52% is an underestimate. The proportions of root Cd as a high-molecular-weight Cd-binding complex based on γEC peptides (Fig. 2) agree well with the uncharacterized "Cd-binding protein" obtained for small root samples of maize (Rauser, 1986). This verifies the usefulness of that simple technique as a potential tool in screening programs for maize.

The high-molecular-weight Cd-binding complexes isolated during the 1 to 7 d of exposure (Fig. 2) were composed of peptides from three families: $(\gamma EC)_nG$, or phyto-

chelatins, $(\gamma EC)_n$, and $(\gamma EC)_nE$ (Fig. 4; Table I). The $(\gamma EC)_n$ peptides, particularly the n_3 oligomer, were in highest concentrations, followed by the $(\gamma EC)_nG$ and $(\gamma EC)_nE$ peptides. The preponderance of n_3 and n_4 oligomers in Cd-binding complexes from maize corroborates the increasing affinity of Cd for longer $(\gamma EC)_nG$ peptides (Hayashi et al., 1988). The complexes from maize differed from those of other sources through the preponderance of $(\gamma EC)_n$ peptides and the presence of $(\gamma EC)_nE$. The n_3 and n_4 oligomers of phytochelatins are most abundant in other plant complexes, and additional unidentified SHs may be present (Gupta and Goldsbrough, 1991; Strasdeit et al., 1991; Reese et al., 1992). The $(\gamma EC)_{2,3}$ peptides also occur in Cd-binding complexes of the yeasts *S. pombe* and *C. glabrata;* however, the $(\gamma EC)_{2-4}G$ peptides are usually more concentrated (Mehra and Winge, 1988; Barbas et al., 1992).

In our HPLC analyses of SHs in complexes and in acid extracts, high sample loadings were used where no individual peptide exceeded the linear response in the postcolumn reaction (Rauser, 1991). This procedure reduced the bias against detection of those peptides present in low amounts. The origin of the $(\gamma EC)_n$ and $(\gamma EC)_nE$ peptides remains unclear. The concentrations of individual oligomers at different times (Fig. 4) cannot indicate whether they originate via anabolism from γEC and γECE or from catabolism of $(\gamma EC)_nG$ prior to or after formation of the complex. Specific studies of the biological origin are required. Artifactual formation of $(\gamma EC)_n$ and $(\gamma EC)_nE$ through acid hydrolysis of $(\gamma EC)_nG$ is deemed unlikely (Meuwly et al., 1993, 1995).

The γEC peptides forming Cd-binding complexes were at all times a subset of the total γEC peptides in the roots of maize (Fig. 4; Table I). Starting with the n_2 peptides, increasing proportions of the larger oligomers in the root participated in forming Cd-binding complex. The highest for individual peptides was by d 7 when 75% of the total $(\gamma EC)_4G$, 80% of the total $(\gamma EC)_4$, and 73% of the total $(\gamma EC)_3E$ formed Cd-binding complex. The monothiols γECG, γEC, and γECE were not detected in the Cd-binding complexes from maize. Under these circumstances it is clear that quantitation of total γEC peptides in acid extracts, even after subtracting GSH and other monothiols, is an unreliable measure of their participation in binding Cd. Assessment of this function is best achieved by quantitative isolation of the complex(es) and determination of the constituent peptides. Some of the γEC peptides (n_2 and greater) not involved in forming Cd-binding complex yet measurable in total acid extracts (Fig. 4; Table I) may be peptides in the process of becoming larger oligomers; others may be in the putative low-molecular-weight Cd-binding complex not observed here.

Acid-labile sulfide occurred at all times in the high-molecular-weight Cd-binding complex from roots exposed to 3 μM Cd, and the concentration increased from d 1 to 7 (Table I). The maize complexes had an S^{2-}:Cd molar ratio of 0.18 ± 0.02. This ratio resembled that in roots of *S. vulgaris* (0.20–0.21), irrespective of whether Cd-sensitive plants were exposed to 10 and 30 μM Cd or Cd-tolerant plants were exposed to 30 and 180 μM Cd for 3 and 7 d (de Knecht et al., 1994). *Brassica juncea* grown in synthetic medium with 100 μM Cd for 7 d produced a high-molecular-weight complex with an S^{2-}:Cd molar ratio of 1.0 and a small complex with a ratio of 0.42 (Speiser et al., 1992). Incompletely resolved complexes from roots of tomato exposed to 100 μM Cd for 4 weeks had a continuum of S^{2-}:Cd molar ratios ranging from 0.15 to 0.41 for the higher- and 0.04 to 0.13 for the lower-molecular-weight forms (Reese et al., 1992). The yeasts *S. pombe* and *C. glabrata* grown in different media for 16 to 48 h and exposed to 500 or 1000 μM Cd had complexes with S^{2-}:Cd molar ratios of 0.11 to 0.55 (Reese et al., 1988; Dameron et al., 1989). How sulfide, Cd, and peptides interact within the complex is unclear for the cases in which the ratio is low. Only at S^{2-}:Cd ratios greater than 0.40 are CdS crystallites surrounded by γEC peptides (Dameron et al., 1989; Reese et al., 1992).

The molar ratios of peptide SH:Cd and peptide SH plus S^{2-}:Cd were near unity for the Cd-binding complexes from maize and yet lower than those of the complexes from roots of *S. vulgaris* (1.81–1.95, de Knecht et al., 1994). The soluble low-sulfide complex from *R. serpentina* (peptide SH:Cd ratio 3.78) coordinated Cd atoms through four sulfurs from phytochelatins (Strasdeit et al., 1991). When the same complex was intentionally saturated with $CdSO_4$ the material precipitated and had a peptide SH:Cd ratio of 1.01. In this form a Cd atom was coordinated by one sulfur and three to four O,N atoms (Strasdeit et al., 1991). If the same situation were to hold for the maize complexes (peptide SH:Cd ratio 1.01 ± 0.07), it would mean that at all sampling times the roots produced a complex binding the maximal amount of Cd with the minimal amount of SH while remaining soluble at pH 8.6. It is unclear whether sulfide or the particular complement of peptides affords solubility to the putatively highly Cd-charged complexes from maize roots. Studies of the molecular interactions among Cd, sulfide, and γEC peptides are required for the high-molecular-weight complex from maize.

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