

Communication

Effects of Buthionine Sulfoximine on Cd-Binding Peptide Levels in Suspension-Cultured Tobacco Cells Treated with Cd, Zn, or Cu¹

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ABSTRACT

Inhibition of cell growth and accumulation of Cd-binding peptide were measured in cultured tobacco cells exposed to buthionine sulfoximine. This inhibitor of glutathione metabolism caused little or no reduction of growth (at 0.1 millimolar) in the absence of Cd, but growth was greatly reduced in cultures exposed to buthionine sulfoximine and ≥ 22 micromolar Cd. Decreased cell growth was directly correlated with decreased levels of Cd-binding peptide and increased levels of what is thought to be free Cd. Zinc inhibited growth of tobacco cells only at the highest levels examined (900–1800 micromolar Zn), but buthionine sulfoximine had no additional significant effect. Similar results were observed for Cu (45–90 micromolar). Results suggest that synthesis of plant Cd-peptide involves γ -glutamylcysteine synthetase or a related enzyme and that Zn accumulation in tobacco cells does not cause formation of significant Cd-peptide ligand.

It is generally recognized that vegetable foods account for $\geq 70\%$ of Cd intake in man and that smoking tobacco can substantially increase intake of this potentially toxic element (7, 19). Despite these facts, our understanding is incomplete regarding the mechanisms of acquisition and accumulation by plants of Cd and related metals. Experiments described here test the relationship between the formation of Cd-binding, sequestering peptides and glutathione metabolism, and compare effects of exposing plant cells to the pollutant-toxic metal Cd *versus* what are normally, at low concentration, nutrient metals, Zn and Cu.

Heavy metal binding proteins in plants have been studied intensively in recent years. In 1980, Bartolf *et al.* (1) showed that Cd induced a low molecular weight (10 kD) anionic Cd-binding protein in tomato. Similar proteins were subsequently found in tobacco (22), cabbage (22), *Agrostis* (13), and other plants (9, 23). Amino acid analyses (13, 21) showed that these proteins were composed of predominantly Glu, Cys, and Gly, and Wagner (21) demonstrated that these proteins, under denaturing conditions, were actually peptides <2 kD in size.

Earlier, Murasugi and co-workers (11, 12) described peptides

having small size and similar amino acid composition in fission yeast and showed that they contained 2 or 3 pairs of Glu-Cys groups attached to a single Gly residue. In addition, they demonstrated that the Gly-Cys pairs were not bound through standard peptide linkages, but were attached through the γ -carboxyl group of Glu residues, in a manner analogous to glutathione. Recent work by Grill *et al.* (6) using different methods for primary sequence determination showed that peptides (termed phytochelatins) formed by *Rauvolfia* cultured cells in the presence of Cd are structurally identical to those of fission yeast. Direct comparison of the properties of plant Cd-peptide and rat liver Cd, Zn-thionein show that these ligands differ greatly in composition, metal affinity, and metal selectivity (16).

The repeating γ -glutamylcysteine groups in Cd-binding peptides of higher plants and fission yeast suggest that their synthesis may involve enzymes of glutathione metabolism. The occurrence of this pathway has been documented in whole plants and cultured cells (see Rennenberg [17] for review) as have been numerous related pathways (10). BSO³ is a specific inhibitor of γ -glutamylcysteine synthetase (EC 6.3.2.2) in animal cells (3) and has been shown to inhibit glutathione synthesis in cultured tomato cells (2). Since this enzyme may be involved in the synthesis of plant Cd-binding peptide, we examined the effects of BSO on the synthesis of this peptide in suspension-cultured tobacco cells exposed to Cd. We also examined the effects of Zn and Cu on formation of ligand of Cd-binding peptide, as these metals have been reported to induce the ligand (albeit very low levels) in fission yeast (5) and *Rauvolfia* cultured cells (6).

MATERIALS AND METHODS

BSO, sucrose, vitamins, plant growth regulators, and inorganic salts were obtained from Sigma Chemical Co. Radionuclides were obtained from New England Nuclear (¹⁰⁹CdCl₂, 2.74 mCi/mg) and ICN (H₂³⁵SO₄, 43 Ci/mg). Ultrapure ZnSO₄ ($\leq 0.0001\%$ Cd) was purchased from Aldrich Chemical Co. All chemicals used in the culture medium were of the highest grade available.

Nicotiana tabacum L. cv Wisconsin 38 suspension cultures were maintained on a 7 d cycle in a modified B5 medium (15). Experimental cultures were initiated on the first day of the culture period at a density of 200 μ l packed fresh cells/ml. CdSO₄, ZnSO₄, CuSO₄, BSO and, in some experiments, H₂³⁵SO₄ (20 μ Ci/25 ml culture) were added to the cultures at this time. All experiments were repeated at least 3 times. Cells were collected on d 5 by centrifugation (2000g for 7 min) and immediately frozen. Packed cell volume, dry weight, and statistical determi-

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³ Abbreviation: BSO, buthionine sulfoximine.

nations were as previously described (15).

Cadmium-binding peptide was prepared by grinding (Corning 12 ml glass tissue grinder) approximately 5 ml packed cells in an equal volume of N_2 purged 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4) which contained 10 mM 2-mercaptoethanol (KPO₄ buffer). Temperature was maintained at $<4^\circ C$ in an ice bath. The resulting brei was centrifuged at 100,000g for 1 h, 1 ml of the supernatant was removed and to it 0.05 μCi ^{109}Cd was added to effect ^{109}Cd exchange-binding (21). After 15 min at room temperature, labeled supernatant was fractionated on a 1.3×30 cm Sephadex G-15 column, equilibrated with half-strength KPO₄ buffer. Bound (void volume) and 'unbound' metals (the latter defined as that which elutes after glutathione standard, 300 D, and in the region where $^{109}CdCl_2$ eluted) were monitored by flame atomic absorption spectrophotometry and/or γ -scintillation spectrophotometry of ^{109}Cd . Unbound Cd, Zn, and Cu are assumed to occur as free metals.

Cd-binding peptide, which eluted in the void volume (bound metal) fraction was monitored as ^{109}Cd and quantitated by measuring *in vivo* associated ^{112}Cd . Control experiments showed that virtually all the ^{112}Cd in this fraction was recovered with peptide purified by gel filtration on Sephadex G-50 and subsequent electrophoresis (16, 21). Liquid scintillation measurements of ^{35}S -labeled ligand were also made in some experiments, using a quench corrected system. Results of these experiments confirmed the validity of using ^{109}Cd to monitor Cd-peptide levels (21). Estimates of Cd-peptide ligand formed in Zn treated cells were made from measurement of ^{109}Cd binding and ^{35}S incorporation.

RESULTS AND DISCUSSION

Effects of Cd, Zn, and Cu on cell growth in the presence and absence of BSO are shown in Figure 1. Dry weight values from replicate experiments were standardized using the mean dry weight of controls. BSO (0.1 mM) did not significantly inhibit cell growth in tobacco cell cultures. However, in combination with Cd, even at Cd levels which were not themselves inhibitory to growth ($\leq 11 \mu M$), a significant decrease in dry weight of cells was observed. With 45 and 90 μM Cd in the presence of BSO essentially no growth occurred in the cultures, which is shown by a comparison of the dry weight of cells after 5 d to that of the initial inoculum (see Fig. 1, 90 μM Cd). Significant inhibition of growth due to Zn was observed only at the highest levels used (900–1800 μM) and there was no additional decrease in growth due to the addition of 0.1 mM BSO with these or any of the Zn

treatments tested. Results similar to that found with Zn were observed for Cu at 45 and 90 μM . Results with 90, 180, and 450 μM Zn were similar to that of 45 μM (data not shown). The lack of significant Zn-BSO and Cu-BSO interaction is evident from the data in Figure 1. Differences in the Cd-, Zn-, and Cu-BSO interactions suggest that in this culture system the mechanisms involved in the control (sequestration) of high levels of these metals differ significantly.

Tobacco Cd-binding peptide has been characterized as to its mol wt, charge, and metal binding affinities and was shown to have an amino acid composition of (Glu-Cys)₄-Gly, per 1300 D peptide (16). Measurements of Cd-peptide formed in the presence of Cd or Zn are shown in Figure 2. As total Cd in cultures was increased in the absence of BSO, there was a corresponding increase in the amount of Cd-peptide formed. In contrast, addition of BSO (0.1 or 0.25 mM) caused a significant decrease in the level of peptide observed.

Small amounts of ligand of Cd-binding peptide were isolated from cultured cells exposed to 900 to 1800 μM Zn (Fig. 2, inset). The total peptide present, under these levels of Zn, corresponds roughly to that found in tobacco cells exposed to more than 250-fold less Cd. These results are comparable to those of Grill *et al.* (5) who showed that, in fission yeast, 2000 μM Zn induced $<2\%$ of the total ligand formed in the presence of 200 μM Cd. On the basis of available data, one can question whether the level of ligand formation observed with Zn would be significant in plants growing in environments polluted or not polluted with Zn.

Reduction of Cd-peptide levels and cell growth in the presence of BSO and Cd (Figs. 1 and 2) was coincident with an increase in unbound Cd (Fig. 3). These results are consistent with data that show BSO did not reduce total Cd uptake into the cells (not shown), but only reduced the levels of Cd-peptide available to bind cytosolic Cd. Levels of unbound Zn were not significantly affected by BSO at any level of Zn examined (Fig. 3), again suggesting that ligand of Cd-peptide is not involved in Zn sequestration. The low levels of ligand formed by tobacco cells in response to high levels of Zn and the lack of a significant BSO-Zn interaction found here are consistent with our findings that ligand of Cd-binding peptides formed in response to Cd, does not bind Zn *in vitro* (16). Regarding Cu, a peptide having size and amino acid composition similar but not identical to Cd-peptide was observed in cultures when 45 and 90 μM $CuSO_4$ was substituted for $CdSO_4$. However, the amount of Cu recovered with this component was only 20% the level of Cd recovered with Cd-peptide in cultures containing 45 and 90 μM Cd. While

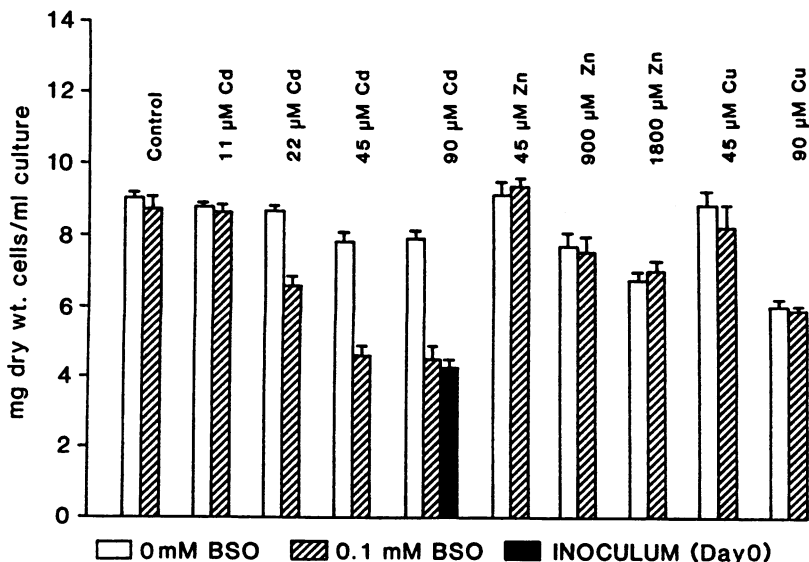


FIG. 1. Effects of Cd, Zn, and Cu on growth of suspension-cultured tobacco cells in the presence or absence of 0.1 mM BSO ($\bar{X} \pm SE$, $n=3$, $LSD_{P=0.05}=0.981$).

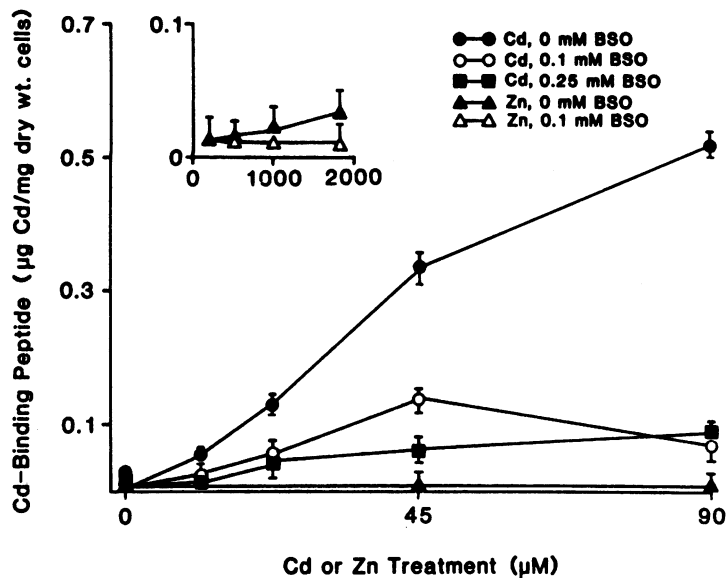


Fig. 2. Effects of Cd and Zn on levels of Cd-binding peptide isolated from cultured tobacco cells grown in the presence or absence of BSO ($\bar{X} \pm SE$, $n=3$).

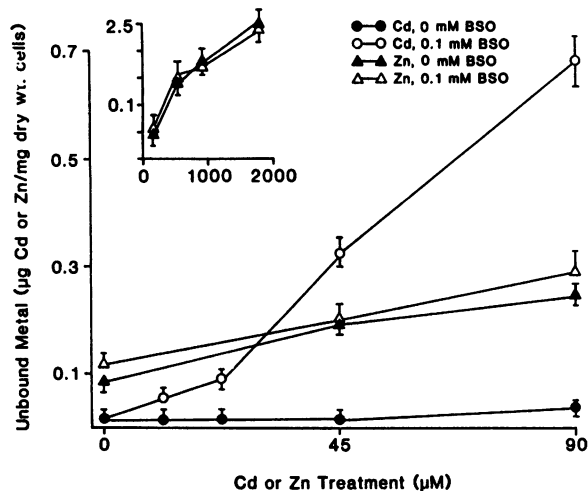


FIG. 3. Effects of BSO on unbound Cd and Zn (metals not associated with Cd-binding peptide) in extracts of suspension cultured tobacco cells ($\bar{X} \pm SE$, $n=3$).

a BSO effect on formation of Cu component was suggested, it was not clearly established (data not shown). Origin, composition and metal binding properties of the Cu component are not yet clear.

Through analogy with animal metallothionein, several functions have been proposed for plant Cd-binding peptide, including heavy metal sequestration and Zn/Cu homeostasis. At present, there are no unequivocal data which support these suggestions. There are no data which support a role for this peptide in Zn or Cu homeostasis. However, Jackson *et al.* (8) have correlative data linking the formation of Cd-binding peptide to Cd sequestration and detoxification. Their data show a direct correlation between the production of Cd-binding peptide and Cd-tolerance in cultured *Datura* cells. The data presented here suggest that Cd-toxicity results in part from an increase in unbound Cd ($R^2=0.75$, $P < 0.001$), which is inversely correlated with the amount of Cd-binding peptide present and therefore support the notion that Cd-peptide—formed after exposure to high levels of Cd—sequesters free Cd to lower its toxicity. The relationship of Zn to Cd-peptide is not like that of Zn to Cd, Zn metallothionein (16).

It is well documented that metal tolerance in plants can be highly metal specific (Woolhouse [24] for review). Therefore, a

lack of a significant formation of Cd-binding peptide ligand by Zn (or Cu) is not surprising. In addition, our data on the lack of *in vitro* Zn binding to ligand of Cd-binding peptide indicate that the peptide does not bind Zn at physiological pH. Data concerning Zn binding (or that of other metals) to fission yeast Cd-binding peptide is unavailable as the HPLC purification method utilized by Grill *et al.* (5) to detect ligand involved use of acidic conditions which result in loss of endogenously bound metal.

As in tomato (2), BSO inhibits glutathione metabolism in cultured tobacco cells (data not shown). Detailed studies of effects of BSO on glutathione metabolism are underway as are experiments to substantiate our belief that unbound Cd and Zn are as free metals (Fig. 3).

In summary, our results show that there is a correlation between BSO inhibition of Cd-binding peptide levels and cell growth in tobacco cells exposed to Cd. Given the presence of γ -glutamylcysteine pairs in plant Cd-binding peptide (6) and the specificity of BSO for γ -glutamylcysteine synthetase in animals (3), our data support the conclusion that this enzyme, or a closely related one, is involved in the synthesis of plant Cd-binding peptide and that this peptide may play a role in Cd-sequestration and detoxification. Also, the very low levels of ligand of Cd-binding peptide formed in the presence of 900 and 1800 μM Zn, the lack of BSO inhibition of cell growth in the presence of low or high Zn, and the lack of Zn binding to Cd-peptide ligand *in vitro* suggest that Zn-binding *in vivo* and amelioration of Zn toxicity do not involve ligand of the peptide formed in response to Cd. While Cu is known to cause the formation of Cu-binding peptides/proteins in plants (14, 18), their composition is not yet adequately defined. Results shown here do not support the conclusion that amelioration of Cu toxicity in tobacco cultured cells is associated with glutathione metabolism. Since the preparation of this manuscript, Steffens *et al.* (20) and Grill *et al.* (4) reported that BSO inhibits Cd-peptide formation in tomato and *Rauvolfia* suspension cells, respectively. In the latter paper, it is claimed that Cd-binding peptide, termed phytochelatin, is functionally analogous to metallothionein.

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