Phytochelatin Synthesis and Glutathione Levels in Response to Heavy Metals in Tomato Cells[1]

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ABSTRACT

Cell suspension cultures of tomato, Lycopersicon esculentum Mill. cv VFNT-Cherry, produce phytochelatins (poly[γ-glutamylcysteinyl]gly-cines) when exposed to cadmium. The synthesis of these peptides is accompanied by a decline in cellular levels of glutathione. Buthionine sulfoximine, an inhibitor of glutathione synthesis, inhibits the sustained production of phytochelatins. However, phytochelatin synthesis can occur in the presence of buthionine sulfoximine provided that sufficient gluta-thione is available. These results indicate that glutathione is a substrate for phytochelatin synthesis. The protein synthesis inhibitor cyclohexim-ide does not affect the initial production of phytochelatin.

Heavy metals are toxic to most organisms and a variety of mechanisms have evolved for coping with the toxic effects of these elements. In mammals and some fungi, proteins are synthesized that bind heavy metals within the cell. These metallothioneins are characterized by their low mol wt, induction by heavy metals, high cysteine content, and their ability to bind a number of heavy metals. There have been a number of reports in recent years that both differentiated plants and plant cells grown in culture produce heavy metal binding complexes when exposed to these metal ions (2, 4, 7, 13, 16, 20, 21). In general, these complexes have been poorly characterized and their structures unknown. However, it has been shown recently that the most abundant heavy metal binding complex in a number of higher plants and in Schizosaccharomyces pombe is comprised of a family of peptides that are structurally related to GSH[2] (10–12, 14, 15, 18). These peptides, termed PCs, have the structure (γ-Glu-Cys)n-Gly where $n = 2$ to 10; for GSH, $n = 1$. PCs are analogous to metallothioneins in that they are induced by, and bind, heavy metals. The mechanism of action of these two groups of compounds appears to be similar in their use of cysteine SH groups to bind heavy metals.

The structure of PCs indicates that these peptides are synthesized enzymically and are not primary translation products of mRNAs. The structural similarity between PCs and GSH suggests that GSH may be involved in the synthesis of these heavy metal binding peptides. We have examined cellular levels of GSH in tomato cell cultures during the induction of PC biosynthesis by cadmium. Inhibitors of protein synthesis and GSH

[2] Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; PC, phytochelatin; SH, sulfhydryl; BSO, buthionine sulfoximine.

production have been used to study the mechanism of PC synthesis.

MATERIALS AND METHODS

Cell Cultures. Cell suspension cultures of tomato, Lycopersicon esculentum Mill. cv VFNT-Cherry, were obtained from Dr. R. A. Bressan, Department of Horticulture, Purdue University, and maintained as described (3). Cultures were started with an inoculum of 20 mg cells (fresh weight) per ml of media and grown with shaking at 24 to 26°C. A typical growth curve is shown in Figure 1. The cells were subcultured weekly and all experiments were performed with cells 3 to 4 d after subculturing.

SH and GSH Assays. Cells to be assayed for SH and GSH were collected by filtration and frozen at −80°C. Extracts were prepared from 100 mg of cells by adding 0.3 ml of 6.67% (w/v) 5-sulfosalicylic acid and keeping the mixture on ice for 10 min, during which time the extracts were vortexed thrice. The lysate was centrifuged at 13,000g at 4°C for 4 min, and the acid-soluble supernatant was either assayed immediately for SH and GSH content or stored at −80°C for future analysis.

Nonprotein SH content was measured spectrophotometrically with Ellman's reagent (6). The acid-soluble supernatant was diluted 10 to 20 times with buffer (final concentration: 120 mM Na-phosphate [pH 7.5], 5 mM EDTA, 0.6 mM 5,5'-dithiobis[2-nitrobenzoic acid]) and absorbance measured at 412 nm. Total GSH equivalents (total GSH and GSSG measured as reduced GSH) were determined using the method of Anderson (1).

HPLC Assay for Phytochelatins. Cell extracts were prepared as described above and SH content determined with Ellman's reagent. The extracts were derivatized using p-chloromercuriben-zoate (8), added to give a final concentration of 3 times the SH concentration. Derivatization was carried out at room temperature for 10 min immediately before analysis. For each injection, an extract prepared from 20 mg of cells was used. The derivatives were separated on a Novapack RP 4 μm C-18 column using a gradient of 20 to 40% acetonitrile in 0.1% trifluoracetic acid over 20 min at 2 ml/min. The column eluant was monitored at 255 nm.

RESULTS

Cell suspension cultures of tomato rapidly synthesize GSH within the first 2 d after subculturing at an initial density of 20 mg/ml (Fig. 1). Thereafter, cellular concentrations of GSH decline over the course of the 7 d culture period. During this time the cultures grow to a final density of 135 to 160 mg/ml. In order to standardize the experimental conditions, most induction experiments were performed 4 d after subculturing when the cell density was approximately 50 mg fresh weight per ml. However, in experiments where a higher cellular concentration of GSH was desirable, cells were used 3 d after subculturing.

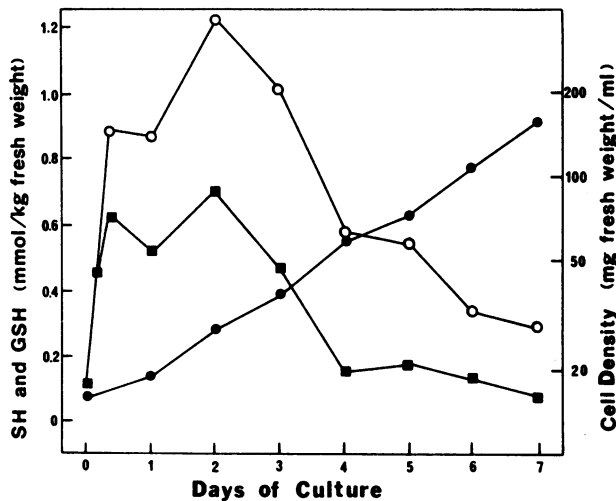In Figures 1, 2, and 3, GSH refers to total GSH and GSSG. It

FIG. 1. Changes in cell density (fresh weight) (●), GSH content (○), and nonprotein SH content (■), during the cell culture cycle.

Table I. *Recovery of GSH by Extraction with 5-Suflosalicylic Acid*

Aliquots of tomato cells (100 mg), 4 d after subculturing, were extracted as described with the addition of various amounts of Cd^{2+} or GSH to the extraction buffer. GSH levels in these extracts were measured as described. The results are the means of three replicates (±SE)

Extraction Buffer	GSH content	Recovery
	mmol/kg fresh wt	*%*
6.67% 5-Sulfosalicylic acid	0.672 ± 0.055	
+0.5 mM CdCl₂	0.702 ± 0.061	
+0.4 mM GSH/kg cells	1.023 ± 0.052	87.7
+1.2 mM GSH/kg cells	1.753 ± 0.248	90.1

is evident that not all of the GSH is measured as SH. This is presumably because at least some of the GSH is present in the oxidized form GSSG. In order to check the efficiency of the procedure for extracting GSH from cells, known amounts of GSH were added to the buffer before extracting cells and the proportion of this additional GSH that was recovered was determined (Table I). Approximately 90% of the added GSH was measured using this procedure. The addition of 0.5 mM CdCl₂ to the extraction buffer had no effect on the measured GSH content, indicating that Cd^{2+} does not interfere with the extraction or assay procedures.

Induction of SH Accumulation with Different Heavy Metals. It has been demonstrated that cells of a number of plant species, including tomato (18), respond to cadmium by synthesizing PCs. These peptides are rich in cysteine and give a positive reaction with Ellman's reagent (10). We have used Ellman's reagent to assay nonprotein (*i.e.* acid soluble) extracts of tomato cells for SH groups, as a measure of PC production. Grill *et al.* (10) have estimated that, in cells of *Rauvolfia serpentina*, at least 90% of the nonprotein SH groups accumulated in response to Cd^{2+} are accounted for by PCs. Apart from Cd^{2+}, a number of other heavy metal ions are known to induce the synthesis of PC in *R. serpentina* (12). When tomato cells are grown in the presence of various heavy metal ions for 24 h, increased concentrations of nonprotein SH groups are detected (Table II). Buthionine sulfoximine has been shown to inhibit PC production in plant cells (12, 18). In the presence of this inhibitor the accumulation of nonprotein SH groups induced by these heavy metal ions is decreased. Although the measurement of SH content is not specific for PC, the effect of BSO on the response to heavy metals indicates that the increase in SH concentration is probably the

Table II. *Accumulation of Nonprotein SH Groups in Response to Various Metals*

Tomato cells were grown in the presence of various heavy metals for 24 h, either with or without 200 μM BSO. Cells were assayed for their nonprotein SH content. The results are the means of three replicates (± SE).

Treatment	SH content	
	−BSO	+BSO
	mmol/kg fresh wt	
Control	0.708 ± 0.056	0.305 ± 0.062
107 μM Cd²⁺	3.926 ± 0.627	1.206 ± 0.159
90 μM Cu²⁺	1.777 ± 0.036	0.222 ± 0.007
4.2 mM Zn²⁺	6.555 ± 0.105	0.715 ± 0.064
2 μM Hg²⁺	1.084 ± 0.187	0.312 ± 0.029

result of PC synthesis.

Induction of SH Accumulation with Different Concentrations of Cd^{2+}. The proposed function of PCs is that they chelate heavy metals, thereby ameliorating the toxic effects of these elements within the cell. It might be predicted, therefore, that there would be a positive correlation between the concentration of heavy metal ions in the culture medium and the accumulation of PCs. This was tested by exposing cells to different concentrations of CdCl₂ and monitoring the rate of increase in SH groups. The results show that, at all concentrations tested, there is an increase in SH levels over the first 4 h (Fig. 2A). At Cd^{2+} concentrations between 50 and 200 μM the SH accumulation continues for at least 48 h, and there is a correlation between the concentration of Cd^{2+} in the medium and the cellular levels of SH groups. In five experiments, the SH content of cells exposed to 200 μM Cd^{2+} for 48 h was 3.424 mmol/kg fresh weight (SE ± 0.299). However, at very high concentrations (400 and 800 μM Cd^{2+}), the rate of SH accumulation soon levels off, presumably because the cells are dying. At 800 and 400 μM Cd^{2+}, the majority of the cells are lysed after 24 and 48 h, respectively. At lower concentrations, the cells survive and continue to grow (data not shown).

Under all the induction conditions examined, the increase in SH content is accompanied by a rapid decline in cellular GSH levels (Fig. 2B). The extent of GSH depletion is dependent on the concentration of Cd^{2+} to which the cells are exposed. Higher Cd^{2+} concentrations produce a more rapid and greater decline in cellular GSH. In cells exposed to nonlethal concentrations of Cd^{2+} (below 400 μM), GSH remains at a reduced level between 2 and 6 h after the addition of Cd^{2+} but does recover somewhat after 6 h. Similar results of SH accumulation and GSH depletion after exposure to Cd^{2+} have been observed in at least six experiments.

Inhibition of SH Accumulation with BSO. The results presented above suggest that GSH is involved in the synthesis of PC. To further investigate this, the effects of BSO on the accumulation of SH groups in response to Cd^{2+} were examined. BSO inhibits GSH synthesis in animal cells and specifically affects γ-Glu-Cys synthetase, the first enzyme in the GSH biosynthetic pathway (9). In plant cells, BSO has been shown to reduce GSH levels (5), although the precise mode of action of this inhibitor in plants has not been determined. When BSO is added to tomato cell cultures at 200 μM, cellular GSH declines to less than 10% of normal levels in 12 h (Fig. 3B), showing that BSO does inhibit GSH synthesis in tomato cells.

When BSO and Cd^{2+} are added simultaneously to tomato cells, there is an initial increase in SH content (Fig. 3A). However, this increase is not as great as observed in cells treated with Cd^{2+} alone, and is sustained for only 2 to 4 h as opposed to at least 12 h in the absence of BSO. Cellular GSH levels decline rapidly in response to Cd^{2+}, both in the presence and absence of BSO (Fig.
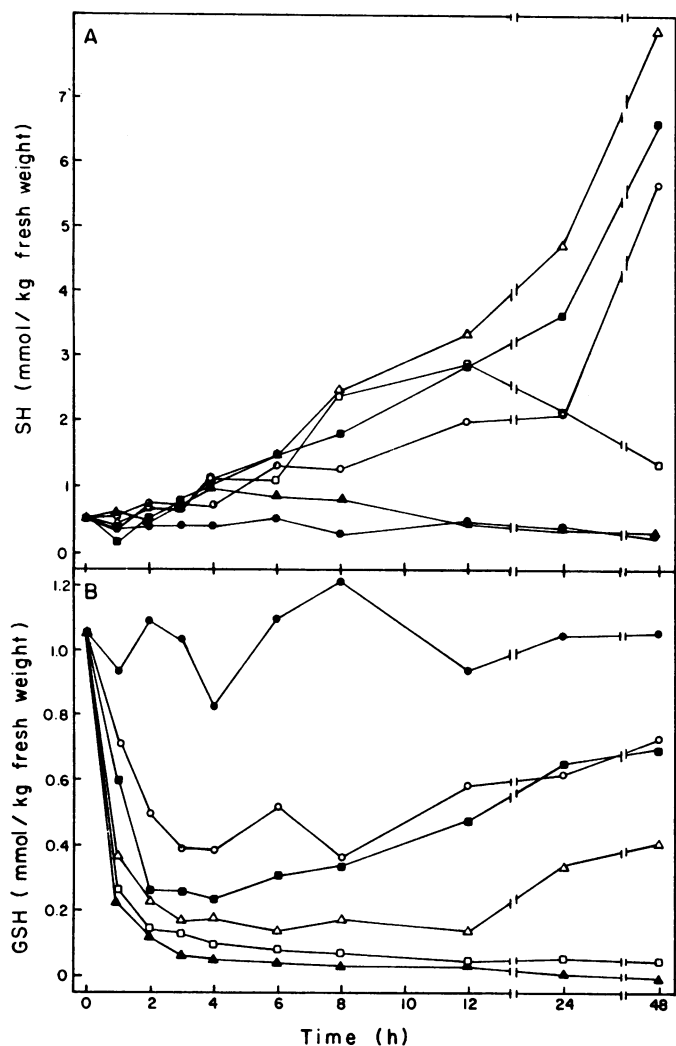
FIG. 2. Nonprotein SH accumulation (A) and GSH depletion (B) in tomato cells in response to different concentrations of $CdCl_2$. Control (●); 50 μM (○); 100 μM (■); 200 μM (△); 400 μM (□); 800 μM (▲).

FIG. 3. Effect of buthionine sulfoximine on cadmium induced SH accumulation (A) and GSH depletion (B). Control (■); 100 μM $CdCl_2$ (●); 100 μM $CdCl_2$ + 200 μM BSO (○); 200 μM BSO (□).

3B). After 3 h exposure to Cd^{2+} in the absence of BSO, GSH declines to approximately 40% of the initial GSH concentration and shows some recovery after 12 h. In the presence of BSO, GSH declines more rapidly after the addition of Cd^{2+} and does not recover during the course of the experiment. BSO inhibition of SH accumulation in response to Cd^{2+} has been observed in four separate experiments.

BSO has the same effect on GSH levels in both plants and animal cells. As synthesis of GSH follows the same pathway in both kingdoms (22), it is probable that BSO inhibits the activity of γ-Glu-Cys synthetase in plants, as it does in animals. If this is the case, these results show that γ-Glu-Cys synthetase is normally required for the continued production of PCs.

Restoration of BSO-Inhibited PC Accumulation with Exogenous GSH. The effect of BSO on SH accumulation in response to Cd^{2+} and other heavy metals provides further evidence that GSH is required for the expression of this stress response. BSO clearly inhibits GSH synthesis and this alone might prevent PC production. However, γ-Glu-Cys synthetase might also be required for another part of PC synthesis. To investigate this, we added GSH to cells that had been pretreated with BSO and challenged them with Cd^{2+}. The assay of SH content is not specific for PC and so we developed an HPLC method to study PC accumulation. PCs were purified from cadmium tolerant
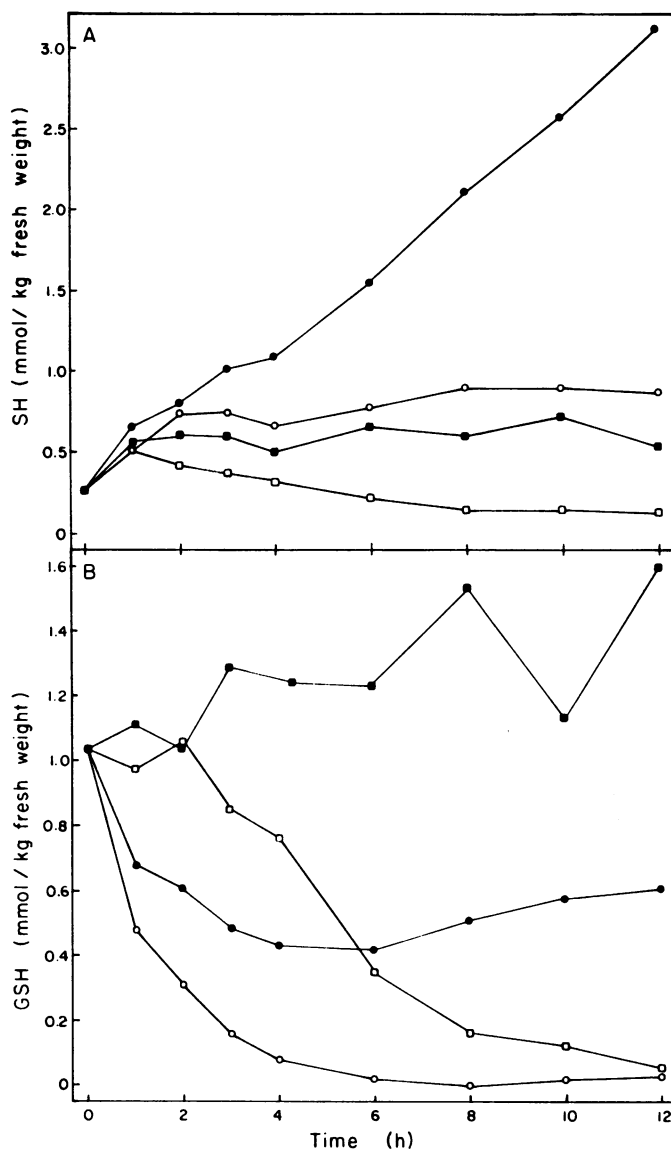
tomato cells by HPLC and their amino acid compositions were determined. The inset to Figure 4A shows the separation of PC 3-5, cysteine, and GSH by reverse phase HPLC after derivatization with p-chloromercuribenzoate (8).

Tomato cells that are treated with BSO for 16 h contain little GSH (Fig. 4A). When Cd^{2+} is added to these cells, no PCs are detected (Fig. 4B). Exogenous GSH is taken up by the cells under these conditions, as previously described for tobacco (17). The availability of GSH restores the ability of cells treated with BSO to synthesize PCs in response to Cd^{2+} (Fig. 4D) and this has been demonstrated in two other experiments. This experiment further demonstrates that GSH is essential for PC synthesis. In addition, the activity that is inhibited by BSO (presumably γ-Glu-Cys synthetase) is not required for the production of PC provided that sufficient GSH is available.

Effect of Cycloheximide on Phytochelatin Synthesis. The increase in nonprotein SH groups in response to Cd^{2+} can be detected within 15 min of adding the metal ion. The rapid nature of this response suggests that the enzymes required for PC synthesis are constitutively present. To determine if *de novo*
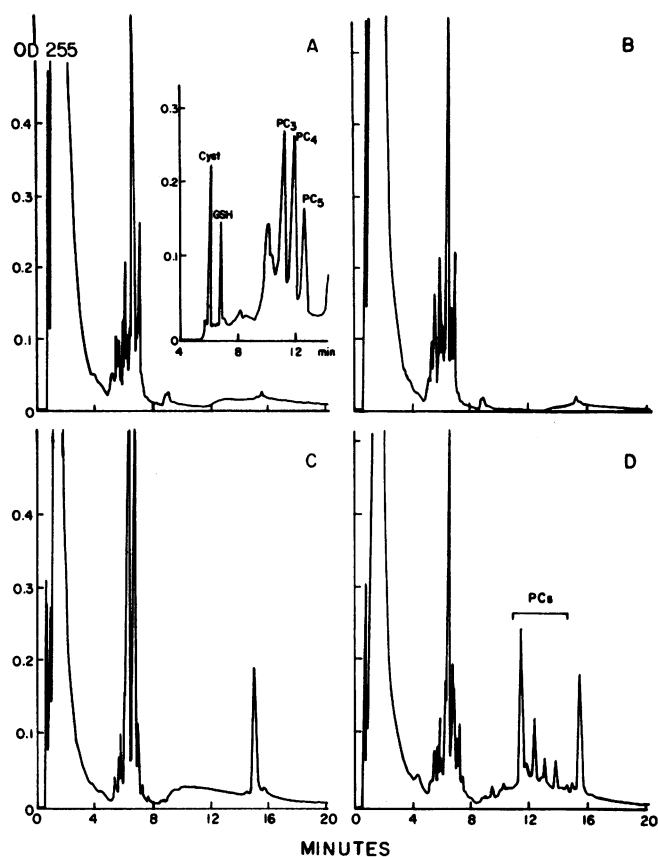
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FIG. 4. Restoration of PC synthesis by GSH in tomato cells treated with BSO. Cells were pretreated with 200 μM BSO for 16 h and then given no further treatment (A), 100 μM CdCl$_2$ (B), 1 mM GSH (C), or 100 μM CdCl$_2$ and 1 mM GSH (D) for 2 h. Extracts were prepared from these cells, derivatized and analyzed by HPLC as described. The inset to Figure 4A shows the separation of cysteine, GSH, and PC$_{3-5}$: 10 nmol of SH of each compound was injected.

protein synthesis is required for this response, the effects of cycloheximide on PC synthesis were examined. Incubation of tomato cells with 1 μg/ml cycloheximide reduces the incorporation of ^{35}S-methionine into TCA-precipitable material to less than 10% of that observed in untreated cells (data not shown). Under normal growth conditions and after exposure to cycloheximide for 2 h, no PC can be detected using this HPLC assay (Fig. 5, A and C). After growth in the presence of 100 μM Cd^{2+} for 2 h cellular GSH has declined and PCs are detected (Fig. 5D), as expected from previous experiments. Cells that are pretreated with cycloheximide for 1 h before the addition of Cd^{2+} are still able to produce PCs (Fig. 5B). Under these conditions, however, the level of GSH has declined more than observed in cells treated with Cd^{2+} alone. This observation is in agreement with other results that we have obtained, showing that cycloheximide inhibits GSH synthesis (data not shown). The results presented in Figure 5 are representative of those obtained in three experiments. PC accumulation, however, ceases after the available GSH is depleted. As with cells treated with BSO, PC synthesis can be maintained in the presence of cycloheximide by the addition of GSH to the medium (data not shown). The results of these experiments show that the activity required to produce PCs in tomato cells is not dependent on *de novo* cytoplasmic protein synthesis.

DISCUSSION

Tomato cells that are exposed to cadmium synthesize large amounts of phytochelatins (18). Similar responses have been
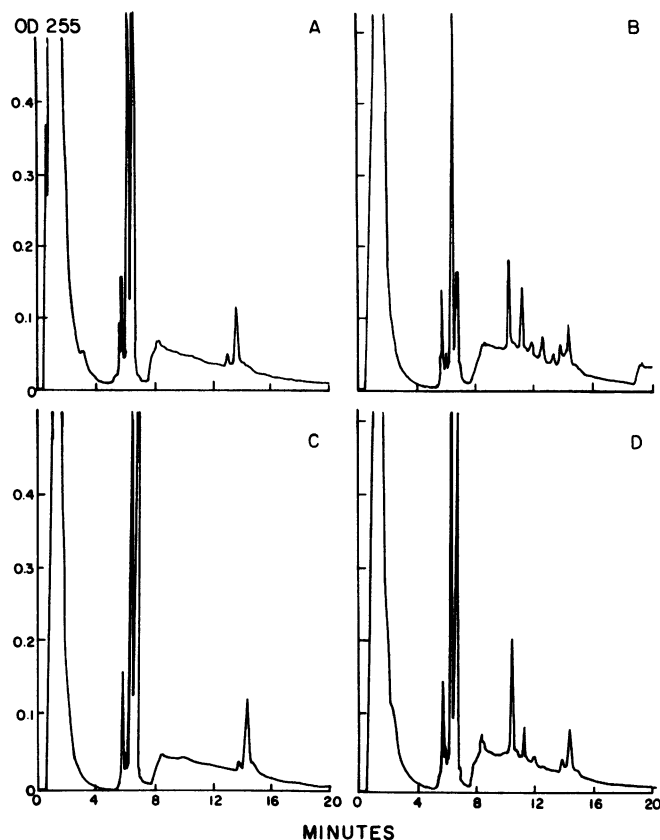
FIG. 5. Effect of cycloheximide on PC synthesis. Tomato cells, 3 d after subculturing, were given no treatment (A), 1 μg/ml cyclohexamide for 3h (C), 1 μg/ml cycloheximide for 1 h followed by 100 μM CdCl$_2$ for 2 h (B), and 100 μM CdCl$_2$ for 2 h (D). Cell extracts were analyzed by HPLC as described.

observed in a number of other plant species (10, 12, 14). The structural similarities between PC and GSH suggest that the synthesis of these compounds may be related. Our results indicate that GSH is a substrate for PC synthesis. When PCs are synthesized in response to the addition of Cd^{2+} to the medium, the cellular concentration of GSH declines. The degree of GSH depletion is dependent on the concentration of Cd^{2+}. Further evidence to support the involvement of GSH in PC production is demonstrated by the effect of BSO, an inhibitor of GSH synthesis, which prevents PC accumulation. This inhibition by BSO can be overcome by supplying GSH in the medium, thus restoring PC synthesis.

The pathway of PC biosynthesis has not been determined. Tomato cells that are treated with BSO and GSH synthesize PC in response to Cd^{2+}; under these conditions, however, ^{35}S-cysteine is not incorporated into PC (B Huang, PB Goldsbrough, unpublished data). This observation indicates that the sequential addition of cysteine and glutamate to GSH (or preformed PC) is not the method of synthesis of PC. An alternative mechanism would involve the addition of γ-Glu-Cys to GSH. Subsequent additions of γ-Glu-Cys moieties would result in the family of peptides that have been described. Such a mechanism is in agreement with the kinetics of synthesis of larger PCs in *S. pombe* (11) and *R. serpentina* (12). There are at least two mechanisms for producing γ-Glu-Cys in plant cells: either by γ-Glu-Cys synthetase or by the activity of a carboxypeptidase on GSH (19). Transfer of γ-glutamyl residues from GSH to free cysteine by γ-glutamyl transpeptidase is another mechanism, but may be unlikely in plants (19) and would not be in agreement with the data obtained on labeling of PC with ^{35}S-cysteine.

It has not been demonstrated that BSO inhibits the activity of plant γ-Glu-Cys synthetase. However, the fact that the pathway of GSH synthesis is the same in plants and animals, and that BSO does inhibit GSH production in plants (5), suggests that BSO specifically inhibits γ-Glu-Cys synthetase in plants. If this is indeed the case, our results indicate that this activity is not required for PC synthesis provided there is sufficient GSH. Our experiments have not examined the importance of a carboxypeptidase activity, nor have they eliminated the possibility that γ-Glu-Cys synthetase is utilized in the absence of BSO. It is also possible that PC can be assembled from 2 molecules of GSH alone (or 1 molecule each of GSH and PC) without the participation of free γ-Glu-Cys.

PC synthesis is induced very rapidly by cadmium. This initial response is not inhibited by cycloheximide, indicating that the enzymes required for this process are constitutively present. However, PCs are present only at very low concentrations under normal growth conditions (18). This raises the possibility that Cd^{2+} may directly induce the synthesis of PCs. The binding of Cd^{2+} to an enzyme might alter its activity thereby inducing PC synthesis. Alternatively, a substrate for PC synthesis may be modified, perhaps by binding Cd^{2+}, before it can be utilized for PC production.

Experiments are in progress to further examine the pathway of PC synthesis and its regulation by heavy metals. In addition, we are studying the production of PCs in cell lines that have been selected for resistance to increased concentrations of Cd^{2+}. These studies will be relevant to determining the mechanisms of resistance to cadmium in cell culture.

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