Plant Physiol. (1995) 107: 515–521

Synthesis of Phytochelatins and Homo-Phytochelatins in *Pisum sativum* L.[1]

Sigrid Klapheck*, Sigrid Schlunz, and Ludwig Bergmann

Botanical Institute, University of Cologne, D-50923 Cologne, Germany

In the roots of pea plants (*Pisum sativum* L.) cultivated with 20 μM $CdCl_2$ for 3 d, synthesis of phytochelatins [PCs or $(\gamma EC)_nG$, where γEC is γglutamylcysteine and G is glycine] and homo-phytochelatins [h-PCs, $(\gamma EC)_n\beta$-alanine] is accompanied by a drastic decrease in glutathione (GSH) content, but an increase in homo-glutathione (h-GSH) content. In contrast, the in vitro activity of GSH synthetase increases 5-fold, whereas h-GSH synthetase activity increases regardless of Cd exposure. The constitutive enzyme PC synthase, which catalyzes the transfer of the γ-EC moiety of GSH to an acceptor GSH molecule thus producing $(\gamma EC)_2G$, is activated by heavy metals, with Cd and Cu being strong activators and Zn being a very poor activator. Using h-GSH or hm-GSH for substrate, the synthesis rate of $(\gamma EC)_2\beta$-alanine and $(\gamma EC)_2$-serine is only 2.4 and 0.3%, respectively, of the synthesis rate of $(\gamma EC)_2G$ with GSH as substrate. However, in the presence of a constant GSH level, increasing the concentration of h-GSH or hm-GSH results in increased synthesis of $(\gamma EC)_2\beta$-alanine or $(\gamma EC)_2$-serine, respectively; simultaneously, the synthesis of $(\gamma EC)_2G$ is inhibited. γEC is not a substrate of PC synthase. These results are best explained by assuming that PC synthase has a γEC donor binding site, which is very specific for GSH, and a γEC acceptor binding site, which is less specific and accepts several tripeptides, namely GSH, h-GSH, and hm-GSH.

In plants and certain fungi, heavy metals induce the synthesis of thiol-rich peptides of the general structure $(\gamma EC)_nG$ with n = 2 to 11, commonly known as PCs (Rauser, 1990; Steffens, 1990). Their function is assigned to metal detoxification and homeostasis of essential micronutrients (Kneer and Zenk, 1992). Among the common metals, Cd is the strongest inducer, whereas other metals such as Cu, Zn, Pb, and Ni are less effective and require higher external levels for induction (Grill et al., 1986b, 1987). PCs form complexes with the metals Cd and Cu, which ultimately are sequestered in the vacuole (Vögeli-Lange and Wagner, 1990; Ortiz et al., 1992). The complexes are variable in size (3–9 kD), consist of PCs of different length and also of other γEC peptides with related structure, have a Cd:Cys-SH ratio ranging from 1:2 to 1:3, and contain variable amounts of acid-labile sulfide (Rauser, 1990; Steffens, 1990).

Evidence from in vivo experiments supports the function of GSH as the precursor of PCs. The metal-induced synthesis of PCs is accompanied by a depletion of the GSH pool in roots (Rüegsegger et al., 1990; Tukendorf and Rauser, 1990; Rauser et al., 1991) and in cell cultures (Grill et al., 1987; Scheller et al., 1987; Delhaize et al., 1989; Schneider and Bergmann, 1995). GSH is synthesized by the action of γEC synthetase, which joins Glu with Cys, followed by GSH synthetase, which adds Gly to γEC. The activity of one or both of the enzymes increases due to exposure of the plants to Cd (Rüegsegger et al., 1990; Rüegsegger and Brunold, 1992; Chen and Goldsbrough, 1993; Schneider and Bergmann, 1994). Plant cells incubated with buthionine sulfoximine, a potent inhibitor of γEC synthetase, are unable to synthesize PCs and are hypersensitive toward Cd and Cu, whereas addition of GSH re-establishes PC synthesis (see refs. in Rauser, 1990; Rüegsegger et al., 1990).

An enzyme synthesizing PCs has been described for *Silene cucubalus* cell cultures (Grill et al., 1989; Loeffler et al., 1989) and for fission yeast (Yoshimura et al., 1990; Hayashi et al., 1991). This PC synthase catalyzes the transpeptidation of γEC moieties of GSH onto another GSH molecule or the growing PC peptide and is constitutively present in both organisms. PC synthase purified from *S. cucubalus* is strictly dependent on metal activation, with Cd being the best activator. The enzyme activity seems to be self-regulated in that newly synthesized PCs chelate the enzyme-activating metal at a ratio of Cd:PC-SH = 1:2, thus terminating the enzyme reaction. In addition to polymerization of GSH, the crude enzyme fraction from fission yeast also polymerizes $(\gamma EC)_n$ and GSH to form $(\gamma EC)_{n+1}$; addition of a Gly terminal residue catalyzed by GSH synthetase is suggested finally to produce PCs. Metal dependence of this PC synthase activity has not been shown.

In many plants of the family Fabaceae, structurally related h-PCs, $[(\gamma EC)_n\beta Ala]$ are found instead of or in addition to PCs (Grill et al., 1986a). Many species of the family Poaceae have γEC peptides of the structure $(\gamma EC)_nSer$, called hm-PCs (Klapheck et al., 1994). These peptides are assumed to be formed from h-GSH ($\gamma EC\beta Ala$) and hm-GSH ($\gamma ECSer$), respectively, but their mode of synthesis has not been elucidated.

In the present study we examined the pathway leading to PC and h-PC synthesis in Cd-exposed pea (*Pisum sativum* L.) roots. We compared the changes in thiol content

[1] This work was done with the support of the Deutsche Forschungsgemeinschaft.

* Corresponding author; fax 49–2214705181.

Abbreviations: desGly-PC, desGly-phytochelatin; γEC, γGlu-Cys; h-GSH, homo-glutathione; hm-GSH, hydroxymethyl-glutathione; hm-PC, hydroxymethyl-phytochelatin; h-PC, homo-phytochelatin; PC, phytochelatin (PC_n, where n is the number of γEC residues).

observed in vivo and the changes in GSH and h-GSH synthetase activity with the substrate specificity of PC synthase determined in vitro and analyzed the influence of metals on PC synthase activity.

MATERIALS AND METHODS

Plant Cultivation

Seeds of pea (*Pisum sativum* L. cv Kleine Rheinländerin; Schmitz and Laux, Hilden, Germany) were soaked for 1 d in aerated water at room temperature. After subsequent germination in damp vermiculite for 2 d, the seedlings were transferred to shallow plastic boxes with aerated nutrient solution (50 seedlings in 400 mL of Hoagland solution). Germination and growth were at a controlled temperature (21°C) with a 14-h light period at 120 μE m^{-2} s^{-1} and 85% RH.

The roots of 6-d-old plants were exposed to Cd by addition of 20 μM $CdCl_2$ to the nutrient solution. The roots of control and Cd-exposed plants were collected after 0, 1, 2, and 3 d, washed thoroughly, cut about 1 cm below the seed, and extracted at once.

Extraction of greater amounts of PC synthase was done with the roots of 7-d-old plants germinated for 3 d in vermiculite and grown for 3 d in nutrient solution. Metal activation was analyzed with control plants, and for analysis of substrate specificity plants were grown at 20 μM $CdCl_2$ for the last 2 d.

Determination of GSH, h-GSH, and γEC Peptide Content

One part of plant material was extracted with four parts (w/v) of 0.1 N HCl, using a mortar, pestle, and quartz sand. The homogenate was centrifuged at 40,000*g* for 10 min. The GSH and h-GSH contents were determined after reduction with DTE and derivatization with monobromobimane by HPLC separation on RP_{18} columns (Klapheck, 1988). Each extract was derivatized twice using different amounts of extract. γEC peptides were analyzed after reduction with DTE by HPLC using the 5,5′-dithiobis(2-nitrobenzoic acid) postcolumn reaction given by Klapheck et al. (1994). PC identification was based on the similarity in retention times of the detected peaks with those of γEC peptides characterized in *Oryza sativa* (Klapheck et al., 1994). h-PC identification was based on comparison with chromatograms from analysis of γEC peptides from *Glycine max*, which produces only h-PCs, and from enzymatic h-PC_2 synthesis. The γEC peptide concentrations are expressed as γEC units, based on peak areas of the GSH standard.

Determination of GSH and h-GSH Synthetase Activity

One part of plant material (root system of control or Cd-exposed plants) was ground on ice with 3 parts (w/v) of extraction buffer (50 mM Tris-HCl, 0.1 mM EDTA, 10% [v/v] glycerine, pH 8.0) and 0.1 part (w/w) of PVP, using a mortar, pestle, and quartz sand. The homogenate was centrifuged at 40,000*g* for 20 min. The supernatant was desalted using PD-10 columns (Pharmacia, Uppsala, Sweden) and 20 mM Tris-HCl, 0.1 mM EDTA, 10% (v/v) glycerine, pH 8.0, for elution buffer.

The activity of GSH and h-GSH synthetase was determined according to the method of Hell and Bergmann (1988). The assay system contained, in a final volume of 200 μL, 100 mM Tris-HCl (pH 9.0), 0.1 mM EDTA, 50 mM KCl, 20 mM $MgCl_2$, 4 mM ATP, 4 mM PEP, 10 units/mL pyruvate kinase, 2.5 mM DTE, 0.5 mM γEC, and 50 to 100 μL of enzyme solution. Reaction was started by addition of 5 mM Gly or β-Ala. After incubation for 0, 20, or 40 min at 30°C, the reaction was stopped by transferring an aliquot (20 μL) into derivatization solution containing 0.2 mL of 0.2 M Tris-HCl, pH 8.0, and 12 μL of 10 mM monobromobimane. After 15 min, 5% (v/v) acetic acid was added to a total volume of 1 mL. The reaction products were determined by HPLC analysis as given above.

Determination of PC Synthase Activity

PC synthase activity was analyzed using a modification of the methods of Grill et al. (1989) and Hayashi et al. (1991). One part of plant material (root system of at least 10 control or Cd-exposed plants) was ground on ice with 2 parts of buffer (30 mM Hepes-KOH, 10 mM DTE, pH 8.0) and 0.1 part of PVP, using a mortar, pestle, and quartz sand. The homogenate was centrifuged for 10 min at 40,000*g*. The supernatant was desalted using PD-10 columns and 30 mM Hepes-KOH, 1 mM DTE, pH 8.0, for elution.

For some experiments, the PC synthase was concentrated by adjusting the supernatant to 70% ammonium sulfate saturation at 0°C, followed by centrifugation at 40,000*g* for 10 min, resuspension of the pellet in elution buffer, centrifugation, and desalting of the supernatant as given above.

The standard incubation mixture contained 1 mM DTE, 0.18 M Hepes-KOH, 0.1 mM $Cd(NO_3)_2$, 5 mM GSH, and 300 μL of enzyme solution in a total volume of 600 μL. Incubation was at 25°C. The reaction was stopped by removing an aliquot of the incubation mixture (160 μL) and adding to it 40 μL of 20% (w/v) TCA. After incubation on ice for a few minutes, the mixture was centrifuged for 3 min and analyzed at once or stored at −20°C for several days before analysis (which was without influence on γEC peptide content). The supernatant (50 μL) was separated by HPLC as described above for γEC peptides, thus allowing quantification of synthesized γEC peptides (PC_2, h-PC_2, and hm-PC_2) as well as control of substrate and DTE concentration during the incubation period. The γEC peptide synthesis rate is expressed as transfer rate of γEC units, and HPLC quantification is based on peak areas of GSH standards. Each value of enzyme activity is based on determination of γEC peptide synthesis at three time points during an incubation period of 15 to 60 min.

Protein Determination

The protein content was measured according to Bradford (1976) with BSA as a standard.

Chemicals

PVP (insoluble polyvinylpolypyrrolidone) was from Sigma and γEC was from Nacalai Tesque, Inc. (Kyoto, Japan). h-GSH was isolated from seeds of *Phaseolus coccineus* according to Klapheck (1988), and hm-GSH was synthesized by Abimed (Langenfeld, Germany).

RESULTS

Addition of 20 μM Cd to the nutrient solution leads to a significant growth reduction of the roots of peas, which is more pronounced with respect to root length increase than to root fresh weight increase (35 and 13% inhibition, respectively, after 3 d of Cd exposure) (Fig. 1). In the roots of pea plants, both h-GSH and GSH are present. The content of both thiols changes during growth of the seedlings; starting with only GSH in the very young root, GSH and h-GSH reach equal concentrations after 12 d (nearly 100 μmol g^{-1} fresh weight) due to a steady increase in h-GSH content and a steady decrease in GSH content (Schlunz, 1991). Within 1 d after the addition of Cd to the growth solution of 6-d-old plants, the GSH content of pea roots

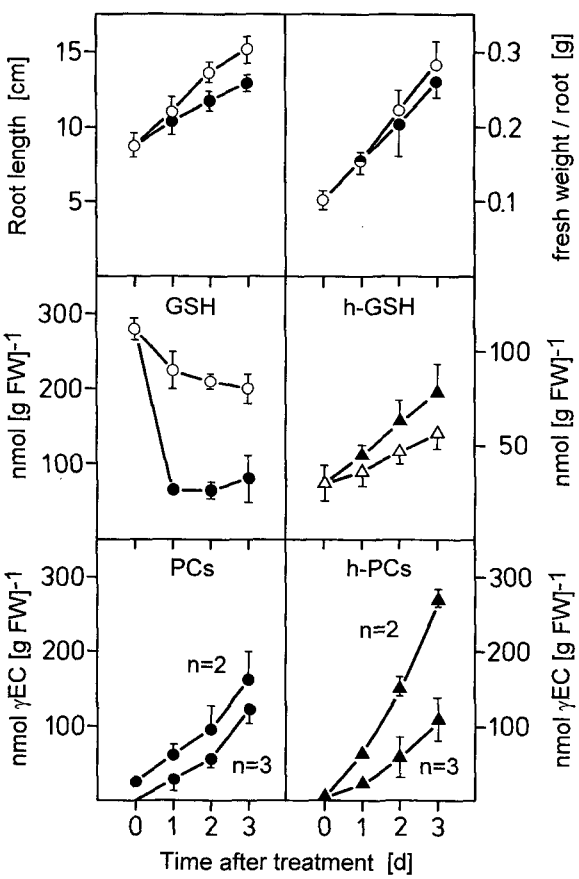

Figure 1. Influence of Cd on growth and thiol content of pea roots. Pea plants (6 d old) were grown hydroponically in a nutrient solution without (open symbols) or with (filled symbols) 20 μM $CdCl_2$ for 1 to 3 d. The roots of 10 plants each were harvested and analyzed for fresh weight and length increase and for thiol content. Values are means ± SE of four experiments.

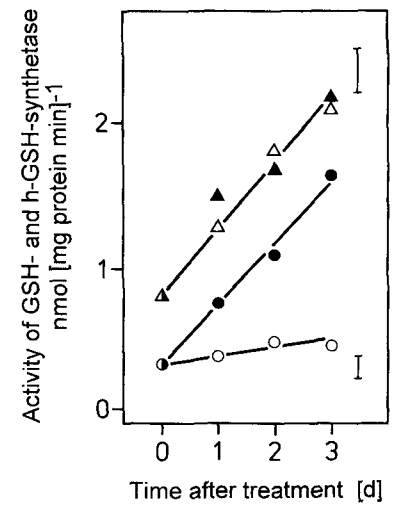

Figure 2. Influence of Cd on extractable GSH and h-GSH synthetase activity. Pea plants (6 d old) were grown hydroponically in a nutrient solution without (open symbols) or with (filled symbols) 20 μM $CdCl_2$ for 1 to 3 d. The roots of the plants were extracted and the GSH synthetase activity (circles) or h-GSH synthetase activity (triangles) were determined using Gly or β-Ala for substrate. Values are the means of three experiments. The bars denote the mean SE of enzyme activity, in the upper corner for h-GSH synthetase, in the lower corner for GSH synthetase.

decreases to one-fourth of that of the control and is not restored significantly during the next 2 d. The h-GSH content, however, increases constantly and at a higher rate during the Cd incubation time compared to the control plants (Fig. 1). Incubation with Cd leads to synthesis of two isoforms of γEC peptides, PCs and h-PCs, with two or three γEC units. PC_2 is already present before Cd addition and increases constantly, but its content is soon exceeded by the h-PC_2 content, which, after 3 d, is the most prominent γEC peptide (Fig. 1). PC_3 and h-PC_3 are found in similar amounts.

The extractable activity of h-GSH synthetase, which is 2.5 times higher than the GSH synthetase activity in the roots of 6-d-old plants, increases nearly 3-fold during the next 3 d. Cd exposure of the plants has no influence on this increase. In contrast, GSH synthetase activity remains low in control plants but rises severalfold due to the Cd incubation (Fig. 2).

Cell-free and desalted extracts of pea roots are able to synthesize PC_2 peptides. Using standard assay [0.1 mM $Cd(NO_3)_2$ and 5 mM GSH], PC synthase activity is 1.26 nmol (mg protein $min)^{-1}$ in 6-d-old control plants and remains constant during the next 3 d. After Cd exposure of the plants the activity increases to 2.14 nmol (mg protein $min)^{-1}$ (mean value of the 3-d period) (Fig. 3). Omission of Cd from the assay has only a very minor influence on the activity of the enzyme from Cd-exposed plants, but in extracts prepared from control plants PC synthase activity is hardly detectable without Cd (Fig. 3). Similar results are found after ammonium sulfate precipitation (70% saturation) of the enzyme. Although the recovery of precipitated PC synthase activity from Cd-exposed plants is low (37 ±

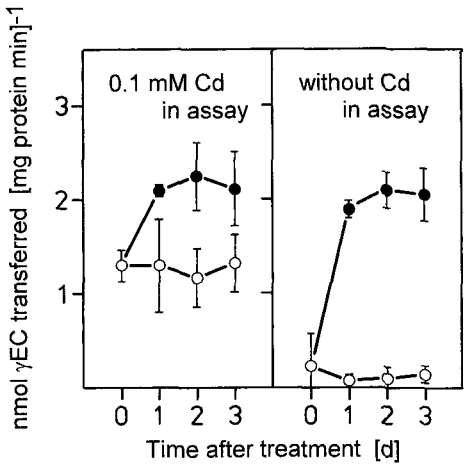

Figure 3. Activity of PC synthase in pea roots. Pea plants (6 d old), grown hydroponically, were exposed to 20 μM $CdCl_2$ for 1 to 3 d. The PC synthase was extracted from the roots of control (open symbols) and Cd-exposed (filled symbols) plants. The activity was determined at 5 mM GSH substrate concentration, with or without addition of 0.1 mM $Cd(NO_3)_2$ to the assay. Values are the means ± SE of three independent experiments.

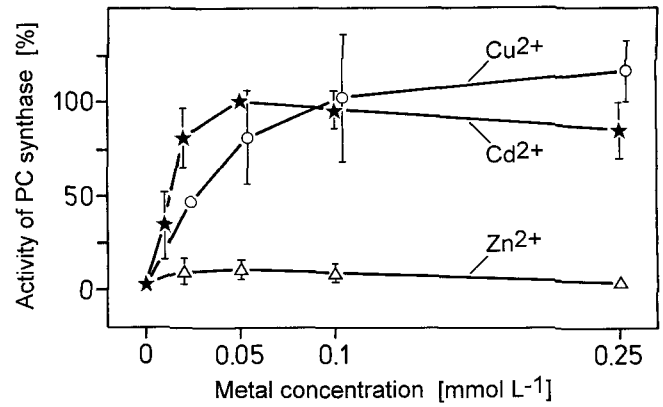

Figure 4. Metal activation of PC synthase. Enzyme solution [70% $(NH_4)_2SO_4$ cut from an extract of control pea roots] was incubated in an enzyme assay with 5 mM GSH. Concentrations of $Cd(NO_3)_2$, $CuSO_4$, or $ZnSO_4$ were as indicated. All activities are related to the activity with 0.05 mM $Cd(NO_3)_2$ in the assay. Values are means ± SE of three (Cu) or four (Cd, Zn) experiments with three different batches of enzyme.

17% recovery, $n = 3$), the enzyme activity is still independent of Cd addition to the assay. In the control plants, the precipitation leads to a severe loss of activity (9 ± 4% recovery, $n = 3$) and activity is completely absent without Cd in the assay.

Maximal activation of PC synthase, using the enzyme extracted and precipitated from control plants, is reached at 0.05 mM Cd in the assay (Fig. 4). Higher concentrations are inhibitory. The ratio of Cd added to the enzyme assay and PC_2 produced reaches values of Cd:PC-SH = 1:8 after 1 h of incubation at Cd concentrations of 10 and 25 μM without noticeable loss of the time linearity of the enzyme reaction. Zn is only a poor activator of the PC synthase reaction. The addition of Cu increases enzyme activity to levels slightly higher than those found at the optimal Cd concentration. This metal, however, leads to severe oxidation, indicated by the complete loss of DTE during the incubation time of 1 h, a loss of time linearity of PC_2 synthesis, and a decline in GSH content in the assay after longer incubation times at concentrations of 0.1 and 0.25 mM Cu. Therefore, activation by Cu may be even greater than shown in Figure 4.

Substrate specificity of PC synthase was analyzed using ammonium sulfate precipitates of Cd-exposed plants. With GSH for substrate, the enzyme exhibits saturation kinetics, and an apparent K_m value of 51 ± 30 mM ($n = 5$) was found. With h-GSH for substrate instead of GSH, there was a very low synthesis rate of h-PC_2, which reached 2.4 ± 0.2% ($n = 3$) of the PC_2 synthesis rate with GSH at 5 mM substrate concentrations in the assays. Addition of h-GSH to enzyme assays containing 2.5 mM GSH increases the h-PC_2 synthesis rate exponentially with increasing h-GSH concentrations (Fig. 5). There is a corresponding decrease in the rate of synthesis of PC_2. If GSH and h-GSH are both added at 2.5 mM, the h-PC_2 synthesis rate is 81% of the PC_2 synthesis rate. The PC synthase from pea roots also accepts hm-GSH (γECSer) for substrate. hm-GSH is found in several plants of the family Poaceae (Klapheck et al., 1992). The synthesis rate of hm-PC_2 also increases exponentially with increasing hm-GSH concentrations at a constant concentration of 2.5 mM GSH in the assay, and the PC_2 synthesis rate is reduced correspondingly (Fig. 5). If both tripeptides are added at 2.5 mM, the hm-PC_2 synthesis rate is twice as high as the PC_2 synthesis rate. With 5 mM hm-GSH alone for substrate, hm-PC_2 synthesis is 0.3 ± 0.3% ($n = 3$) of the PC_2 synthesis rate at 5 mM GSH alone.

The dipeptide γEC seems not to be a substrate of PC synthase. PCs without terminal Gly's (desGly-PCs) are not found, either with any combination of GSH and γEC or with γEC alone for substrate. Addition of the dipeptide,

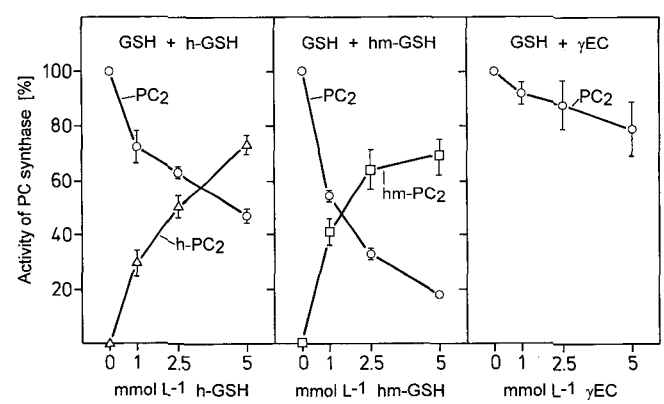

Figure 5. Synthesis of different γEC peptides by PC synthase. Enzyme solution [70% $(NH_4)_2SO_4$ cut from an extract of Cd-exposed pea roots] was incubated in an enzyme assay with 0.1 mM $Cd(NO_3)_2$. GSH concentration was 2.5 mM for all assays. Addition of h-GSH, hm-GSH, or γEC was as indicated. Synthesis of PCs, h-PCs, or hm-PCs was analyzed by HPLC. All activities are related to the activity with 2.5 mM GSH only, which was 0.58 ± 0.24 nmol (mg protein min)$^{-1}$ (mean ± SE, $n = 3$).

however, leads to an increasing inhibition of PC_2 synthesis with increasing γEC concentrations (Fig. 5).

DISCUSSION

Several different isoforms of γEC peptides are formed in different plants after exposure to Cd or other heavy metals. In legumes, h-PCs are present either alone or in addition to PCs (Grill et al., 1986a), and in grasses hm-PCs, PCs, and desGly-PCs are found (Klapheck et al., 1994). The results presented here show that the mechanism by which h-PC_2 and hm-PC_2 are formed is the same as for PC_2 synthesis: the γEC moiety of a donor tripeptide is transferred to an acceptor tripeptide. For PC_2 synthesis, both donor and acceptor tripeptide are GSH. However, synthesis of h-PC_2 and hm-PC_2 using the pea root enzyme seems to depend on the presence of GSH as well as h-GSH or hm-GSH. With either h-GSH or hm-GSH alone the γEC transfer rate is only 2.4 and 0.3%, respectively, of that found with GSH alone. When either h-GSH or hm-GSH is added together with GSH, high activities of h-PC_2 and hm-PC_2 synthesis are found. The results presented in Figure 5 are best explained assuming that PCs, h-PCs, and hm-PCs are synthesized by one enzyme, which has a γEC donor and a γEC acceptor binding site with differing substrate specificities: the γEC donor binding site has a high specificity for GSH, whereas the γEC acceptor binding site has a low specificity for γEC tripeptides and will accept GSH, h-GSH, and hm-GSH. Inhibition of GSH-dependent PC_2 synthesis due to the addition of h-GSH or hm-GSH and simultaneous synthesis of h-PC_2 and hm-PC_2 reveals that both tripeptides compete with GSH at the acceptor binding site. Using 2.5 mM substrate concentrations of each of GSH and h-GSH or GSH and hm-GSH, more hm-PC_2 is formed compared to PC_2, but less h-PC_2, which implies that the γEC acceptor binding site has a higher affinity for hm-GSH and a lower affinity for h-GSH than for GSH at this concentration. It is possible that both GSH homologs also exert a competitive inhibition on GSH binding at the donor binding site, but they are only very poor γEC donors. The larger C-terminal amino acid (β-Ala or Ser instead of Gly) may prevent binding of the tripeptides due to steric hindrance, or the ability to split off the C-terminal amino acid may be restricted to Gly for other reasons. Grill et al. (1989) have shown that the PC synthase from *S. cucubalus* also accepts PC_2 and PC_3 as the γEC acceptor as well as the γEC donor. It seems reasonable to assume that the PC synthase from pea is also a dipeptidyl transpeptidase that uses longer-chain-length γEC peptides with C-terminal Gly's as γEC donors but uses all isoforms of γEC peptides as γEC acceptors.

The dipeptide γEC is not a suitable substrate of the PC synthase of pea roots. At the concentrations we have used no desGly-PCs are formed, but PC_2 synthesis is inhibited. γEC polymerization by dipeptidyl transfer from GSH to $(\gamma EC)_n$ has been shown by Hayashi et al. (1991) at concentrations of 12 μM GSH and 1000 μM $(\gamma EC)_n$ (n = 1–3) with a crude enzyme fraction from *Schizosaccharomyces pombe*. It seems possible that this high concentration of γEC in the assay enables the dipeptide to replace GSH at the acceptor binding site and thus facilitate $(\gamma EC)_2$ synthesis. In vivo, however, the GSH concentration is always higher than the γEC concentration, even when the GSH content has declined and the γEC content has increased due to Cd exposure (Rauser et al., 1991; Meuwly and Rauser, 1992; Rüegsegger and Brunold, 1992; Schneider and Bergmann, 1995). Therefore, it is unlikely that the desGly-PCs found in many organisms after Cd exposure (see refs. in Klapheck et al., 1994) are synthesized by γEC transfer from GSH to γEC. The occurrence of desGly-PCs may be explained by the cleavage of the C-terminal amino acids from PCs, h-PCs, or hm-PCs by a peptidase. By analogy to the reactions catalyzed by carboxypeptidase C, a proteolytic enzyme that also acts as dipeptidyl transpeptidase (Würz et al., 1962), desGly-PCs may also arise from a hydrolytic activity of PC synthase, i.e. a cleavage of the Gly after binding of a PC molecule at the donor binding site and transfer to water instead of to a γEC acceptor.

Cd and possibly other metals have influence on the PC synthase activity of pea roots in several ways. First, they are necessary to activate the enzyme, which is present in control plants in an inactive state. In this respect, Cd is the best activator at low concentrations, whereas Cu is better at higher concentrations and Zn is a weak activator in general. These results are in general agreement with those found by Grill et al. (1989), who observed 100 and 33% activity at 0.1 mM Cd and Zn, respectively, and 27% activity at 0.01 mM Cu for the PC synthase of *S. cucubalus*. They also are consistent with the experience from in vivo systems: Cd and Cu are effective inducers of the γEC peptide synthesis and are constituents of the peptide complexes (Rauser, 1990; Steffens, 1990); the amounts of PCs found in plants after exposure to Zn are low, which has caused several investigators to question whether PCs play a role in Zn tolerance at all (Reese and Wagner, 1987; Krotz et al., 1989).

In addition to activation, Cd seems to increase the amount of PC synthase, which is indicated by the fact that Cd-exposed plants have 1.7-fold higher extractable activity than control plants (Fig. 3). This increase, however, could be due to higher recovery of enzyme activity during extraction, because the Cd-activated enzyme proved to be more stable during ammonium sulfate precipitation than the enzyme of control plants. An increase in the PC synthase activity due to Cd exposure was not observed for enzyme fractions of *S. cucubalus* (Loeffler et al., 1989) or *S. pombe* (Hayashi et al., 1991).

The activated state of the enzyme extracted from Cd-exposed pea plants is not abolished by ammonium sulfate precipitation and desalting, as has been shown for the crude enzyme fraction of *S. pombe* (Hayashi et al., 1991). This observation may be explained by insufficient removal of metals during the procedure or even metal enrichment due to the simultaneous precipitation of PC complexes. According to Grünhage et al. (1985), Cd-binding proteins of pea are precipitated by ammonium sulfate without loss of their Cd, whereas Gekeler et al. (1988) use this step to remove other proteins from the soluble phytochelatin complexes in the supernatant. However, there remains the

possibility of a very tight binding of Cd to the PC synthase. This is supported by the fact that enzyme activity is not reduced after synthesis of PC_2 in 8-fold excess of SH groups to the Cd initially added to the enzyme assay. This result differs from the observation of Grill et al. (1989) that PC synthase stops as soon as a molar ratio of Cd:PC-SH of 1:2 has been reached. However, it is in accord with reports that the Cd:SH ratio for PCs in maize root extracts varied from 1:10 to about 1:130, indicating that PCs are produced in excess of the amount required to chelate Cd (Tukendorf and Rauser, 1990; Meuwly and Rauser, 1992). Purification and further characterization of the PC synthase of pea roots are needed to elucidate how PC synthesis is regulated by metals.

The influence of Cd exposure on the GSH and h-GSH content and the GSH and h-GSH synthetase activities of pea roots, shown in Figures 1 and 2, is an additional indication for the substrate specificities of PC synthase as discussed above. If GSH is the only γEC donor, then three molecules of GSH are required for every one molecule of h-GSH to produce one molecule each of PC_2 and h-PC_2. Replenishment of the GSH pool should be more urgent than replenishment the h-GSH pool. Indeed, the GSH content drops drastically and the GSH synthetase activity increases 5-fold in the pea roots after Cd exposure, whereas the h-GSH synthetase activity is not influenced and the h-GSH content increases. A Cd-induced increase of GSH synthetase activity has been found for pea (Rüegsegger et al., 1990). Probably it is accompanied by a rise in the γEC synthesis rate, because an increase in the γEC content and/or the γEC synthetase activity has been observed for many plants (Meuwly and Rauser, 1992; Rüegsegger and Brunold, 1992; Chen and Goldsbrough, 1993; Schneider and Bergmann, 1995). Therefore, the Cd-induced increase of the h-GSH content found in pea roots may reflect a higher h-GSH synthesis rate due to higher substrate concentration, combined with only small h-GSH removal for γEC peptide synthesis and a high and Cd-independent activity of h-GSH synthetase. On the other hand, the high consumption of GSH for γEC peptide synthesis causes a reduced pool of GSH in the Cd-exposed plants, which does not recover to the control level despite the 5-fold increase of GSH synthetase activity. Exposure of rice plants to Cd has a similar effect on the thiol content of the roots and shoots, namely a drastic drop in the GSH content but an increase in the hm-GSH content (Klapheck et al., 1994). This effect may be caused by a similar substrate specificity of the PC synthase of rice plants, resulting in different patterns of consumption of GSH and hm-GSH for γEC peptide synthesis.

ACKNOWLEDGMENTS

The authors are grateful to Ms. I. Zimmer for her excellent technical assistance and to Dr. R. Hell for his critical comments on the manuscript.

Received August 15, 1994; accepted October 26, 1994.

LITERATURE CITED

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Chen J, Goldsbrough PB (1993) Characterization of a cadmium tolerant tomato cell line (abstract No. 933). Plant Physiol **102:** S-162

Delhaize E, Jackson PJ, Lujan LD, Robinson NJ (1989) Poly(γ-glutamylcysteinyl)glycine synthesis in *Datura innoxia* and binding with cadmium. Plant Physiol **89:** 700–706

Gekeler W, Grill E, Winnacker E-L, Zenk MH (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch Microbiol **150:** 197–202

Grill E, Gekeler W, Winnacker E-L, Zenk MH (1986a) Homophytochelatins are heavy metal-binding peptides of homoglutathione containing Fabales. FEBS Lett **205:** 47–50

Grill E, Löffler S, Winnacker E-L, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamyldipeptidyl transpeptidase (phytochelatin synthase). Proc Natl Acad Sci USA **86:** 6838–6842

Grill E, Winnacker E-L, Zenk MH (1986b) Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells. FEBS Lett **197:** 115–120

Grill E, Winnacker E-L, Zenk MH (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci USA **84:** 439–443

Grünhage L, Weigel H-J, Ilge D, Jäger H-J (1985) Isolation and partial characterization of a cadmium-binding protein from *Pisum sativum*. J Plant Physiol **119:** 327–334

Hayashi Y, Nakagawa CW, Mutoh N, Isobe M, Goto T (1991) Two pathways in the biosynthesis of cadystins $(\gamma EC)_nG$ in the cell free system of the fission yeast. Biochem Cell Biol **69:** 115–121

Hell R, Bergmann L (1988) Glutathione synthetase in tobacco suspension cultures: catalytic properties and localization. Physiol Plant **72:** 70–76

Klapheck S (1988) Homoglutathione: isolation, quantification and occurrence in legumes. Physiol Plant **74:** 727–732

Klapheck S, Chrost B, Starke J, Zimmermann H (1992) γ-Glutamylcysteinylserine: a new homologue of glutathione in plants of the family Poaceae. Bot Acta **105:** 174–179

Klapheck S, Fliegner W, Zimmer I (1994) Hydroxymethyl-phytochelatins [$(\gamma$-Glu-Cys$)_n$-Ser] are metal-induced peptides of the Poaceae. Plant Physiol **104:** 1325–1332

Kneer R, Zenk MH (1992) Phytochelatins protect plant enzymes from heavy metal poisoning. Phytochemistry **31:** 2663–2667

Krotz RM, Evangelou BP, Wagner GJ (1989) Relationship between cadmium, zinc, Cd-peptide, and organic acid in tobacco suspension cells. Plant Physiol **91:** 780–787

Loeffler S, Hochberger A, Grill E, Winnacker E-L, Zenk MH (1989) Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. FEBS Lett **258:** 42–46

Meuwly P, Rauser WE (1992) Alteration of thiol pools in roots and shoots of maize seedlings exposed to cadmium. Plant Physiol **99:** 8–15

Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J **11:** 3491–3499

Rauser WE (1990) Phytochelatins. Annu Rev Biochem **59:** 61–86

Rauser WE, Schupp R, Rennenberg H (1991) Cysteine, γ-glutamylcysteine, and glutathione levels in maize seedlings. Plant Physiol **97:** 128–138

Reese RN, Wagner GJ (1987) Effects of buthionine sulfoximine on Cd-binding peptide levels in suspension-cultured tobacco cells treated with Cd, Zn, or Cu. Plant Physiol **84:** 574–577

Rüegsegger A, Brunold C (1992) Effect of cadmium on γ-glutamylcysteine synthesis in maize seedlings. Plant Physiol **99:** 428–433

Rüegsegger A, Schmutz D, Brunold C (1990) Regulation of glutathione synthesis by cadmium in *Pisum sativum* L. Plant Physiol **93:** 1579–1584

Scheller HV, Huang B, Hatch E, Goldsbrough (1987) Phytochelatin synthesis and glutathione levels in response to heavy metals in tomato cells. Plant Physiol **85:** 1031–1035

Schlunz S (1991) Glutathion- und Homoglutathion-Synthese in *Pisum sativum* L. PhD thesis. University of Cologne, Germany

Schneider S, Bergmann L (1995) Regulation of glutathione synthesis in suspension cultures of parsley and tobacco. Bot Acta (in press)

Steffens JC (1990) The heavy metal-binding peptides of plants. Annu Rev Plant Physiol Plant Mol Biol **41:** 553–575

Tukendorf A, Rauser WE (1990) Changes in glutathione and phytochelatins in roots of maize seedlings exposed to cadmium. Plant Sci **70:** 155–166

Vögeli-Lange R, Wagner GJ (1990) Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Plant Physiol **92:** 1086–1093

Würz H, Tanaka A, Fruton JS (1962) Polymerization of dipeptide amides by cathepsin C. Biochemistry **1:** 19–29

Yoshimura E, Kabuyama Y, Yamazaki S, Toda S (1990) Activity of poly(γ-glutamylcysteinyl)glycine synthesis in crude extract of fission yeast, *Schizosaccharomyces pombe*. Agric Biol Chem **54:** 3025–3026