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Biochemistry

Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase)

(detoxification/dipeptidyl transpeptidase/phytochelatin biosynthesis)

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ABSTRACT **An enzyme has been discovered and characterized from *Silene cucubalus* cell suspension cultures that catalyzes the transfer of the γ-glutamylcysteine dipeptide moiety of glutathione to an acceptor glutathione molecule or a growing chain of [Glu(-Cys)]$_n$-Gly oligomers, thus synthesizing phytochelatins, the metal-binding peptides of higher plants and select fungi. The enzyme was named γ-glutamylcysteine dipeptidyl transpeptidase and given the trivial name phytochelatin synthase. The primary reaction catalyzed is [Glu(-Cys)]-Gly + [Glu(-Cys)]$_n$-Gly → [Glu(-Cys)]$_{n+1}$-Gly + Gly. The enzyme is isoelectric near pH 4.8 and has temperature and pH optima at 35°C and 7.9, respectively. Phytochelatin synthase is constitutively present in cell cultures of various plant species and its formation is not noticeably induced by heavy metal ions in the growth medium. The enzyme (M_r 95,000) seems to be composed of four subunits, the dimer (M_r 50,000) being also catalytically active. Cd^{2+} is by far the best metal activator of the enzyme followed by Ag^+, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, and Au^+. The K_m for glutathione is 6.7 mM. The enzyme activity seems to be self-regulated in that the product of the reaction (the phytochelatins) chelates the enzyme-activating metal, thus terminating the enzyme reaction. The molar ratio of the γ-glutamylcysteine dipeptide in phytochelatin to Cd^{2+} in the newly formed complex was 2:1.**

Phytochelatins of the general formula [Glu(-Cys)]$_n$-Gly (n = 2 . . . 11) are the principal heavy-metal-detoxifying components in the plant kingdom (1–4). The phytochelatins can be viewed as linear polymers of the γ-glutamylcysteine (γ-Glu-Cys) portion of glutathione. Because of the repetitive γ-glutamic acid bonds, they cannot be regarded as primary gene products and must be formed by a ribosome-independent enzyme reaction. These peptides could be enzymatically produced by stepwise condensation of γ-Glu-Cys moieties to glutathione itself and the growing phytochelatin chain. The linkage of glutathione metabolism to phytochelatin synthesis has been shown clearly by kinetic experiments *in vivo* (4, 5) and elegantly by fungal mutants that lack both enzymes in glutathione biosynthesis (γ-Glu-Cys synthetase or glutathione synthetase) and that were, as a result, unable to produce both glutathione and [Glu(-Cys)]$_n$-Gly peptides and, therefore, became hypersensitive to Cd (6).

In this study we characterize the enzyme responsible for phytochelatin biosynthesis. Phytochelatins are synthesized by the action of a specific γ-Glu-Cys dipeptidyl transpeptidase (phytochelatin synthase). This enzyme was purified to apparent homogeneity from *Silene cucubalus* suspension culture (7) and is here characterized with respect to its physicochemical and polymerizing properties.

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MATERIAL AND METHODS

Plant Cell Cultures. Cell suspension cultures of *Silene cucubalus* were cultivated as described (4).

Substrates. [*glycine-2-*^{3}H]Glutathione was supplied by NEN and all other materials were of highest purity available from Sigma, Merck, and Boehringer Mannheim. *S*-Monobromo-bimaneglutathione was synthesized as described (8).

Enzyme Assay. The enzyme incubation mixture contained 200 mM Tris·HCl (pH 8.0), 10 mM 2-mercaptoethanol, 0.1 mM $Cd(NO_3)_2$, and 1 mM glutathione as well as various amounts of phytochelatin synthase in a total volume of 100 μl. Incubation was at 35°C for various periods of time. The reaction was stopped by removing 50 μl of the incubation mixture and adding to it 50 μl of water and 50 μl of NaOH (0.6 M containing 50 μg of $NaBH_4$). After 10 min the mixture was acidified with 20 μl of 3.6 M HCl and centrifuged for 5 min. The supernatant (100 μl) was separated by HPLC as described (4).

Enzyme Isolation. Eight days after transfer into fresh medium, *Silene* suspension culture cells were harvested, deep frozen (liquid N_2), and stored at −20°C. One kilogram of cells was stirred in 600 ml of 30 mM Tris·HCl (pH 8.0) containing 10 mM 2-mercaptoethanol to give a homogeneous brei that was pressed through cheesecloth and centrifuged at 10,000 × g for 20 min to remove insoluble debris. The supernatant was adjusted to 15% (wt/vol) ammonium sulfate and centrifuged at 10,000 × g for 20 min. The supernatant was divided into two portions and loaded onto a column containing Phenyl Sepharose Cl-4B (3.0 × 15 cm; Pharmacia). The enzyme was eluted with 10 mM Tris·HCl (pH 8.0) containing 10 mM 2-mercaptoethanol and 10% (vol/vol) ethylene glycol. Both eluates were combined, 10% (vol/vol) glycerol was added to stabilize the enzyme, and the enzyme was adsorbed onto a hydroxylapatite column (2 × 12.5 cm; Bio-Rad) at a flow rate of 20 ml/hr. Elution was done with 100 mM potassium phosphate (pH 8.0) containing 10 mM 2-mercaptoethanol and 10% glycerol. Active fractions were chromatographed onto an AcA 34-Ultrogel column (2.5 × 109 cm; Serva) at a flow rate of 20 ml/hr in standard buffer (10 mM Tris·HCl, pH 8.0/10 mM 2-mercaptoethanol) supplemented with 10% glycerol.

The fractions containing enzyme activity were pooled, applied to a QAE Fast Flow column (1.0 × 10 cm; Pharmacia) and eluted with a 150–400 mM KCl gradient in standard buffer supplemented with 10% glycerol at a flow rate of 60 ml/hr. Active eluate to which ammonium sulfate had been added to 15% saturation was pumped onto a PhenylSuperose column (1.0 × 10 cm, Pharmacia). Material was eluted with an ammonium sulfate gradient (15–0%) in standard buffer. Enzyme-containing fractions were pooled and dialyzed against 25 mM potassium phosphate (pH 8.0) and subsequently applied onto a Zn-chelating Sepharose column (0.8 ×

10 cm; Pharmacia). Elution was done with 50 mM imidazole containing 25 mM potassium phosphate (pH 8.0).

Although a large loss in activity with little apparent gain in specific activity was observed up to the Zn-chelating Sepharose column, these steps were needed to remove several proteins that otherwise interfered with the quality of separation in the final step. Homogeneous enzyme was obtained after Sephadex G-25 gel filtration (PD-10 column; Pharmacia) equilibrated with standard buffer and chromatography on a Mono Q column (0.5 × 5 cm; Pharmacia). A 0.2–0.4 M KCl gradient in standard buffer was used to elute the phytochelatin synthase. The most active fractions were subjected to SDS/PAGE analysis. The enzyme isolation is summarized in ref. 7.

Molecular Weight Determination. The molecular weight of the protein was determined using a calibrated TSK G 3000 SW column (7.5 × 600 mm; Pharmacia-LKB). Samples (0.1 ml) were chromatographed in 25 mM potassium phosphate, pH 6.5/25 mM NaCl at a flow rate of 0.5 ml/min. The following standard proteins were used: catalase (M_r, 240,000); bovine serum albumin (M_r, 67,000); ovalbumin (M_r, 45,000); chymotrypsinogen A (M_r, 25,000); myoglobin (M_r, 17,800); cytochrome *c* (M_r, 12,300). These are proteins 1–6, respectively, in Fig. 1*A*.

Phytochelatin Isolation and Sequence Determination. Methods were used as described (2, 4).

RESULTS

Characterization of Phytochelatin Synthase. Phytochelatin synthase is a metal-dependent transpeptidase and apparently a soluble enzyme. By means of hydrophobic-interaction, anion-exchange, gel-filtration, and metal-ion-interaction chromatographies, phytochelatin synthase was purified to apparent homogeneity—forming a single band in a silver-stained SDS/polyacrylamide gel. The purified enzyme showed a specific activity of 463 pkat/mg of protein (1 kat = the amount of enzyme that catalyzes a reaction rate of 1 mol of substrate per s) and was ≈160-fold enriched compared to the crude extract. This represents a minimal value. Under denaturing conditions, the enzyme had a molecular weight of 25,000. The molecular weight determination of the enzyme under nondenaturing conditions by gel-permeation HPLC yielded enzyme activity at M_r 95,000 and M_r 50,000 (Fig. 1*A*). However, enzyme preparations of an early purification step, the active fractions after Phenyl Sepharose chromatography, showed a single broad peak of activity at M_r 95,000. These observations suggest that the enzyme is an oligomeric protein

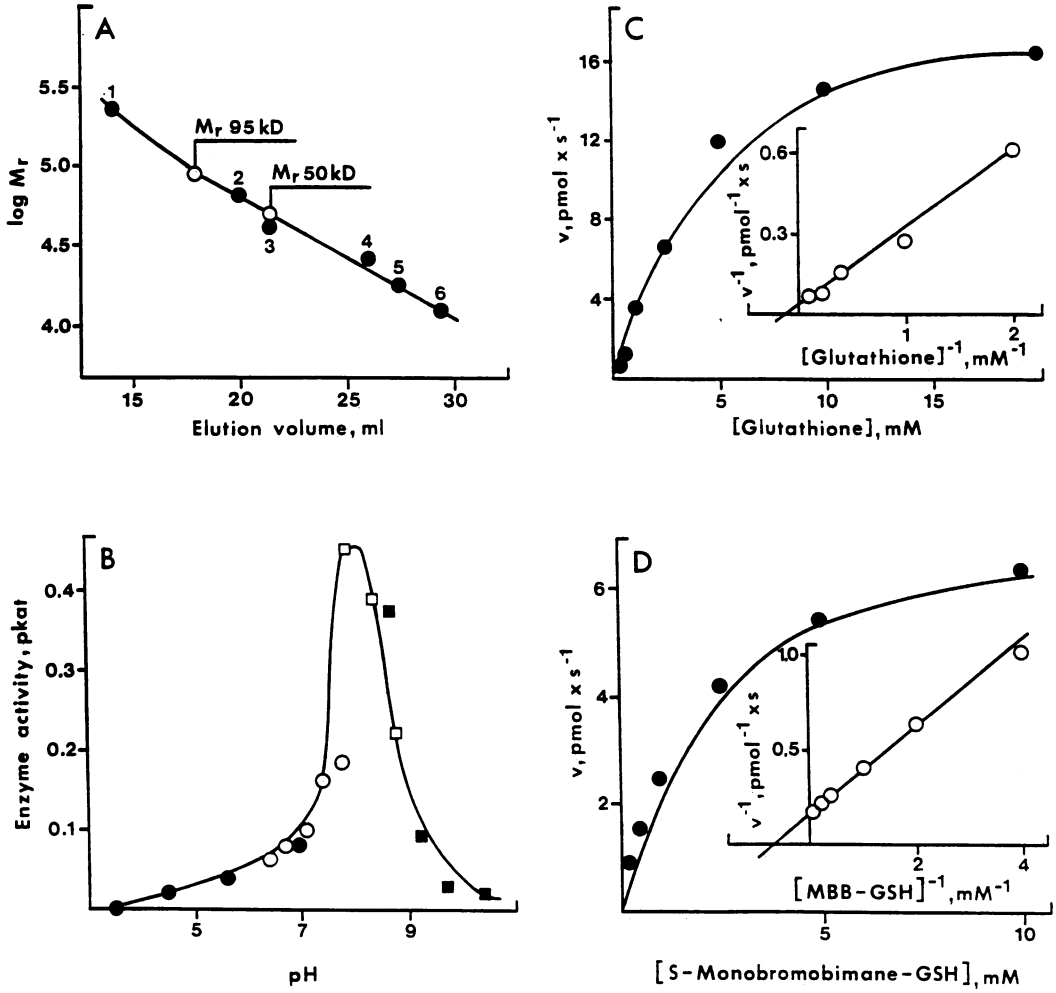

FIG. 1. Characterization of phytochelatin synthase from *Silene cucubalus*. (*A*) Determination of the molecular weight by gel filtration. Positions corresponding to M_r 50,000 and M_r 95,000 globular proteins are indicated by open circles; those of molecular weight standards are indicated by solid circles. The proteins used for calibration are listed in *Material and Methods*. (*B*) pH dependence of enzyme activity. Buffer solutions used were 0.2 M citrate·NaOH (●), potassium phosphate (○), and glycine·NaOH (■), in addition to the standard Tris·HCl buffer (□). (*C*) Determination of K_m values for glutathione. Cadmium nitrate concentration was maintained constant at 0.1 mM. (*D*) Determination of K_m values for *S*-monobromobimane-glutathione (MBB-GSH). Cadmium nitrate was maintained at 0.1 mM throughout the assays. Activity was monitored by HPLC (*A–C*) and by measuring the liberated labeled glycine (*D*).

consisting of four M_r 25,000 subunits. During purification the tetrameric form of the enzyme seems to dissociate into an active dimeric form.

Properties of the Enzyme. The purified enzyme exhibited increased stability. It has a half-life at 35°C of 6 hr, at 22°C of 34 hr, at 4°C of 140 hr, and at −20°C only a small decrease of activity was observed after 500 hr in 30% glycerol. The enzyme was stored in 10 mM Tris·HCl, pH 8.0/10 mM 2-mercaptoethanol supplemented with 0.01% NaN_3 to prevent microbial growth. Under standard incubation conditions [11 pkat of enzyme/1 mM glutathione/0.1 mM $Cd(NO_3)_2$], phytochelatin formation was linear for 120 min. The enzyme was functional over a relatively narrow pH range [maximal activity at pH 7.9 (Fig. 1*B*)] and a broad temperature range (maximal rate of conversion at 35°C). Half-maximal activity was observed at 20°C and 47°C. The isoelectric point was ≈pH 4.8 with minor activities comprising <20% at pH 4.1 and pH 6.5, possibly due to multiple forms of the enzyme.

The dependence of the rate of phytochelatin formation on glutathione yielded a K_m value of 6.7 mM in the presence of 0.1 mM Cd^{2+} (Fig. 1*C*). Due to the presence of some catalytically inactive enzyme in the highly purified enzyme preparations a minimal $k_{cat} = 0.2\ s^{-1}$ was determined. *S*-Monobromobimane-glutathione, harboring a sulfhydryl protective group, yielded a K_m value of 1.5 mM (Fig. 1*D*). The fact that S-substituted glutathione is a substrate (with an even lower K_m value than the natural substrate glutathione) suggests that a glutathione-*S*–metal complex is not necessary for peptide cleavage.

Substrate Specificity. An initial characterization of the substrate specificity of phytochelatin synthase was performed by incubating the purified enzyme with $[Glu(-Cys)]_n$-Gly oligomers (where n was 2 to 4 or 5) in the presence of glutathione. In each case the formation of the next higher homologue, $n + 1$, was observed upon analysis by HPLC with sulfhydryl-specific detection. This result implies that phytochelatin synthase acts on phytochelatins of various chain lengths by adding one γ-Glu-Cys unit and concomitantly liberating one glycine residue.

Metal Specificity. There is an absolute requirement of heavy metal ions for the enzyme activity. Since there is evidence that, in addition to the main enzyme activity at pI 4.8, there may be two minor forms of the enzyme (pI 4.1 and pI 6.5) present in crude homogenates, the metal activation of the dipeptidyl transpeptidase was studied using a protein fraction resulting from PhenylSepharose chromatography that should still contain all the enzyme species. The activation of the enzyme by metal ions is shown in Table 1. The strongest activation was observed with Cd^{2+} as compared to 0.5 mM Bi^{3+}, 0.01 mM Cu^{2+}, and other heavy metal ions at 0.1 mM. This finding is consistent with our experience (1–4) and that of others (9) for the *in vivo* systems thus far studied.

Table 1. Activation of γ-Glu-Cys dipeptidyl transpeptidase by metal ions under standard assay conditions using the HPLC assay

Metal ion compound	Concentration, mM	Relative activity
$Cd(NO_3)_2$	0.1	100
$AgNO_3$	0.1	58
$Bi(NO_3)_3$	0.5	56
$Pb(NO_3)_2$	0.1	43
$ZnCl_2$	0.1	33
$CuCl_2$	0.01	27
$HgCl_2$	0.1	26
$NaAuCl_4$	0.1	12
None	—	0

The crude extract was concentrated ≈15-fold by PhenylSepharose chromatography prior to the assays. The 100% activity value is 4.1 nmol of phytochelatin formed in 70 min.

Besides Cd^{2+}, Ag^+, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, and Au^+ revealed (in this order) a decreasing activation of phytochelatin synthase. These are exactly the ions known to induce phytochelatin synthesis in plant cells (1–4). No enzyme activation was detected with Al^{3+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^+, and K^+.

Kinetic Analysis of the Transpeptidase Reaction. Insight into the mode of phytochelatin biosynthesis was originally provided by kinetic analysis of the *in vivo* formation of the metal-binding peptides (4). *In vitro* experiments were performed with a 15-fold purified enzyme and substantiated these results. As outlined in Fig. 2, phytochelatin synthesis was observed immediately after administration of 0.1 mM Cd^{2+} ions. $[Glu(-Cys)]_2$-Gly appeared without a noticeable lag period. Fifteen minutes after the administration of Cd^{2+}, synthesis of the heptapeptide was detected, and, after a further 20 min, the nonapeptide was also detected. Finally, after 100 min the reaction came to a halt. The administered Cd^{2+} was completely complexed by the synthesized peptides and, thus, not available to the enzyme. A second addition of Cd^{2+} resulted in additional phytochelatin synthesis at the same level (data not shown). Computation of these results (Fig. 2) yielded 23.7 nmol of γ-Glu-Cys units in phytochelatin, the formation of which was initiated by 10 nmol of Cd^{2+}. In the second experiment, this value was 19.8 nmol of dipeptide in phytochelatin. This means, if all the Cd^{2+} were chelated by the peptides, the molar ratio of Cd to cysteine in the phytochelatin complex is 1:2, exactly the number determined in Cd–phytochelatin isolated from exposed plants (1). In fact, chelation of Cd^{2+} by EDTA as well as metal-free phytochelatin instantaneously inhibited phytochelatin formation (data not shown), supporting the hypothesis that phytochelatin synthesis proceeds until the metal ions are complexed and are no longer accessible to the synthase. The mechanism of phytochelatin biosynthesis was further elucidated by incubation of $[Glu(-Cys)]_2$-Gly with purified enzyme and glutathione. Upon addition of Cd^{2+}, formation of the heptapeptide occurred immediately and efficiently (Fig. 3*A*) and subsequently, synthesis of the nonapeptide was observed.

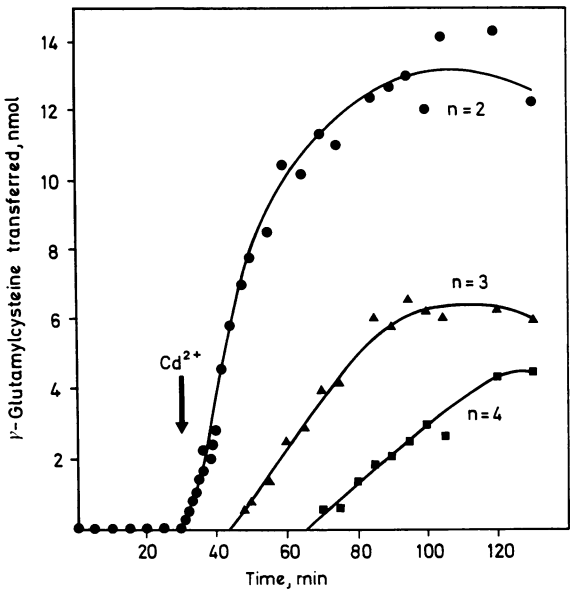

FIG. 2. Time course of phytochelatin synthesis from glutathione before and after administration of 10 nmol of $Cd(NO_3)_2$ (see arrow) to the incubation mixture containing 2.9 pkat of 15-fold purified enzyme and 1 mM glutathione. Quantities of individual phytochelatins with n (no. of γ-Glu-Cys units per molecule) = 2 (●), 3 (▲), or 4 (■) are expressed as nmol of γ-Glu-Cys per 0.1 ml, as quantitated by HPLC.

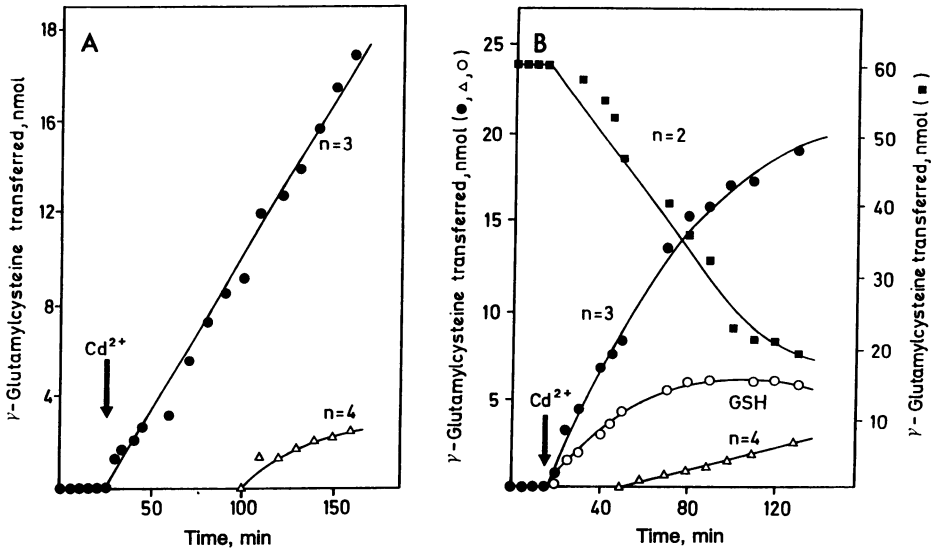

FIG. 3. Time course of phytochelatin synthesis from $[Glu(-Cys)]_2$-Gly to $[Glu(-Cys)]_3$-Gly and $[Glu(-Cys)]_4$-Gly in the presence (*A*) and absence (*B*) of glutathione. The standard incubation mixture contained in *A* was 1.5 pkat of 15-fold purified enzyme, 0.5 mM $[Glu(-Cys)]_2$-Gly, and 1 mM glutathione and in *B* 1.9 pkat of 12-fold purified enzyme, 0.6 mM $[Glu(-Cys)]_2$-Gly. Exposure to 35 nmol of $Cd(NO_3)_2$ was performed after 20 min (*A*) and to 40 nmol after 15 min (*B*). Glutathione (GSH, ○) and individual phytochelatins with *n* (no. of γ-Glu-Cys units per molecule) = 2 (■), 3 (●), or 4 (△) are expressed as nmol per 0.1 ml, as quantitated by HPLC.

In the absence of glutathione, surprisingly, the formation of first the hepta- and later the nonapeptide also occurred (Fig. 3*B*). Simultaneously and correspondingly, the pentapeptide substrate decreased in the reaction mixture with the concomitant release of glutathione. Twenty minutes after addition of Cd^{2+} to the reaction mixture, 8 nmol of Glu-Cys units of $[Glu(-Cys)]_2$-Gly had disappeared and 6.2 nmol were incorporated in $[Glu(-Cys)]_3$-Gly. In addition, 2.7 nmol of glutathione were formed representing values that are within experimental error. After prolonged exposure unspecific oxidation of compounds bearing -SH groups occurred, upsetting the equilibrium. The higher phytochelatin members were synthesized at the expense of the pentapeptide substrate. This is an indication for the occurrence of an "autotranspeptidase" reaction in which the pentapeptide molecule can serve as a donor as well as an acceptor for γ-Glu-Cys units.

These data provide evidence that phytochelatin molecules can serve as acceptors for additional Glu-Cys units primarily supplied by glutathione and that phytochelatin synthase is a Glu-Cys dipeptidyl transpeptidase.

In plant cells, this reaction is probably capable of transferring up to 10 dipeptidyl units onto glutathione, generating the longest phytochelatin peptide identified thus far (3).

Occurrence of Phytochelatin Synthase in Plants. The ubiquity of the phytochelatin system in plants suggests that phytochelatin synthase occurs generally in plants. A simple preliminary examination of this assumption was hampered by the glutathione and phytochelatin degrading activities present in most plant extracts tested. Partial purification of the enzyme with every species seems necessary to solve this problem. Nonetheless, in 5 extracts of 21 tested from various cell suspension cultures, little or no degradation was detected. In all 5 tissue homogenates, phytochelatin synthase activity could be identified (Table 2). In control incubation mixtures containing extracts without glutathione or Cd^{2+}, no phytochelatin formation was observed. In addition to *Silene cucubalus*, plants from four families of higher plants as well as the phylogenetically more primitive pteridophyte *Equisetum* were capable of synthesizing the heavy-metal-binding peptides. In the *Podophyllum* extract, peptides containing two to five γ-Glu-Cys units were formed. In all the other cell cultures as well as in all the differentiated plants tested, the glutathione substrate was rapidly degraded so that no transpeptidase activity could be assayed with certainty, in spite of the fact that these plants or cell cultures were capable of forming phytochelatins when challenged with heavy metals (3).

DISCUSSION

At least three possible mechanisms can be visualized by which phytochelatins, the heavy-metal-binding peptides of plants, are formed: (*i*) γ-Glu-Cys units, synthesized by action of γ-Glu-Cys synthetase [EC 6.3.2.2], are transferred by an energy requiring process to glutathione; (*ii*) γ-Glu-Cys units are polymerized to $[Glu(-Cys)]_n$ oligomeric molecules (10, 11) that are subsequently transferred in a similar fashion to glycine as is known from the glutathione synthase [EC 6.3.2.3] reaction; or (*iii*) the peptides arise by transfer of the γ-Glu-Cys moiety of glutathione to an acceptor glutathione molecule or an oligomeric phytochelatin peptide. The characterization of the phytochelatin synthesizing enzyme reported here is only compatible with the third model. The biosynthesis of phytochelatin requires only enzyme, glutathione, and heavy metal ions and proceeds by stepwise addition of dipeptidyl units. After each dipeptidyl transfer, the product is released from the enzyme with exclusive formation of the pentapeptide from glutathione. The heptapeptide is only synthesized after the pentapeptide has

Table 2. Occurrence of γ-Glu-Cys dipeptidyl transpeptidase in plant cell cultures free of a glutathione degradation activity

Species (family)	Age, days	pkat per liter of medium
Silene cucubalus (Caryophyllaceae)	7	637
Podophyllum peltatum (Berberidaceae)	4	365
Eschscholtzia californica (Papaveraceae)	6	145
Beta vulgaris (Chenopodiaceae)	5	100
Equisetum giganteum (Equisetaceae)	10	34

accumulated and can compete both with glutathione or the previously formed pentapeptide as a Glu-Cys acceptor. Formation of the nonapeptide depends on accumulation of levels of the heptapeptide that can then in turn compete with the pentamer as the acceptor molecule, etc. The observed transformation of the pentapeptide into the heptapeptide in the absence of glutathione (Fig. 3*B*) implies that the enzyme is also able to transfer the dipeptidyl residue of γ-Glu-Cys peptides to thus form longer chain length phytochelatins. This enzyme responsible for the formation of phytochelatins is clearly a γ-Glu-Cys dipeptidyl transpeptidase. The reaction sequence catalyzed by the phytochelatin synthase follows the equation: $[Glu(-Cys)]_n$-Gly + $[Glu(-Cys)]_n$-Gly → $[Glu(-Cys)]_{n+1}$-Gly + $[Glu(-Cys)]_{n-1}$-Gly, where $n = 1, 2, 3, \ldots$.

Two transpeptidases studied in detail are γ-glutamyl transpeptidase [EC 2.3.2.2] and cathepsin C [EC 3.4.14.1], a dipeptidyl transpeptidase (12). Cathepsin C resembles phytochelatin synthase in catalyzing the polymerization of specific dipeptidylamides into the corresponding hexa- and octaamides. Cathepsin C, however, did not polymerize glutathione (unpublished result), which is not surprising since transpeptidases are generally substrate specific. The decisive factor in phytochelatin biosynthesis is, most likely, the heavy metal ions that act (*i*) by activating the enzyme and (*ii*) possibly by protecting the synthesized phytochelatin peptides from becoming a dipeptidyl donor by complex formation. It is conceivable that the reaction equilibrium of such a mechanism favors polymerization over hydrolysis in the presence of heavy metal ions.

Phytochelatin peptides, heavy metal ions, and phytochelatin synthase form a self-regulating loop. The enzyme is activated and forms phytochelatins in the presence of "free" heavy metal ions that are subsequently complexed by the peptides and are no longer able to provoke phytochelatin synthesis. This conclusion is based on the *in vitro* studies of phytochelatin formation (Fig. 2), supported by the fact that phytochelatin synthesis was completely and instantaneously prevented after addition of metal-free phytochelatin, an ideal metal-ion complexor. Thus, phytochelatin peptides appear to be the most efficient Cd^{2+} chelators in plants, which supports the proposed and observed detoxification function of these molecules for heavy metal ions (1, 4, 5, 13).

In addition to detoxification, phytochelatins probably fulfill a role in metal ion homeostasis since plant cells synthesize the peptides after transfer into fresh culture medium (4, 14). The level of phytochelatin formation was primarily dependent on the Zn^{2+} concentration. In culture medium free of essential heavy metal ions, no phytochelatin induction was observed.

It should be noted that several authors have claimed the lack of formation of phytochelatins in the presence of Zn^{2+} ions either in plants (15) or in fungi (16), which is in sharp contrast to our own observations (1–4, 14, 17). The data given in Table 1, however, demonstrate unequivocally that Zn^{2+} is a good activator of the dipeptidyl transpeptidase and, therefore, able to initiate phytochelatin formation.

Thus, phytochelatin and phytochelatin synthase appear to have an essential role in the regulation of the cellular balance of "free" and complexed heavy metal ions by a simple and efficient mechanism. In animals, the excess of heavy metal ions is counteracted by the induction of the metallothionein gene, whereas in plants, heavy metals mediate conversion of glutathione into phytochelatin catalyzed by a constitutive enzyme.

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