PERGAMON

Phytochemistry 62 (2003) 423–431

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

# Phytochelatin synthase catalyzes key step in turnover of glutathione conjugates

Andreas Beck[a], Klaus Lendzian[a], Matjaz Oven[b,1], Alexander Christmann[a], Erwin Grill[a,*]

[a]*Lehrstuhl für Botanik, Technische Universität München, Am Hochanger 4, D-85350 Freising, Germany*
[b]*Leibniz-Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle, Germany*

Received 9 September 2002; received in revised form 11 October 2002

This contribution is dedicated to Professor Meinhart H. Zenk on the occasion of his 70th birthday

**Abstract**

Conjugation of xenobiotic compounds and endogenous metabolites to glutathione is an ubiquitous process in eukaryotes. In animals, the first and rate-limiting step of glutathione-*S*-conjugate metabolism is characterized by the removal of the aminoterminal glutamic acid residue of glutathione. In plants, however, glutathione-*S*-conjugates are generally metabolized by removal of the carboxylterminal glycine residue of the tripeptide glutathione to give rise to the *S*-glutamylcysteinyl-derivative. Purification of the glutathione-conjugate catabolizing activity from cell suspension cultures of the plant *Silene cucubalus* indicated that phytochelatin synthase catalyzes the first step of the pathway. Heterologously expressed phytochelatin synthase from *Arabidopsis* efficiently converted *S*-bima ne-glutathione to *S*-bimane-glutamylcysteine, the formation of which was unequivocally identified by mass spectrometry. No further products, such as *S*-derivatives of phytochelatins, were observed. Several different glutathione-*S*-conjugates served as substrates for the enzyme and were processed to the corresponding glutamylcysteinyl-adducts. Affinity-purified phytochelatin synthase preparations required divalent heavy metal ions such as $Cd^{2+}$, $Zn^{2+}$ or $Cu^{2+}$ for detectable turnover of glutathione-*S*-conjugates. Characterization of the enzymatic properties of phytochelatin synthase argues for both cellular functions of the γ-glutamylcysteinyl-dipeptidyltransferase: (1) formation of heavy-metal binding peptides and (2) degradation of glutathione-*S*-conjugates. Mechanistically, the former role is the result of γ-glutamylcysteinyl transpeptidation onto glutathione or derivatives thereof, while the catabolic function reflects transpeptidation of *S*-glutamylcysteinyl-adducts onto the acceptor molecule water. Thus, phytochelatin synthase seems to fulfil a second crucial role in glutathione metabolism.


*Keywords:* Xenobiotic; Detoxification; Heavy metal; Transpeptidation; GSH; Herbicide

## 1. Introduction

Glutathione (GSH) is ubiquitous in eukaryotes and the tripeptide serves a plethora of physiological functions including redox regulation, conjugation of metabolites, and detoxification of xenobiotics (Meister, 1995). In addition, GSH is converted into heavy metal-binding peptides by the action of phytochelatin synthase (PCS) that has been characterized as a specific γ-glutamyl-cysteinyl (EC) dipeptidyltransferase (Grill et al., 1989). The products of the enzymic reaction, the phytochelatins (PCs), have the general structure $(\gamma\text{-EC})_n$-glycine ($n = 2$–11) (Zenk, 1996). Recent cloning of PCS from plants, selected fungi, and invertebrates revealed a unique enzyme structure rich in cysteine residues in its carboxylterminal domain (Clemens, 2001; Cobbett and Goldsbrough, 2002).

Plants synthesizing GSH isoforms (γ-EC)-Xaa, where Xaa signifies a β-alanine, serine, or glutamic acid residue, generate respective PC-isoforms, (γ-EC)n-Xaa during heavy metal exposure (Zenk, 1996). In soybean plants, the PCS is able to use homoglutathione as an acceptor molecule for the generation of the β-alanine containing PC-isoform (Oven et al., 2002). The dithiolic to polythiolic PC peptides have a higher affinity to heavy-metal ions such as $Zn^{2+}$, $Cd^{2+}$ or $As^{3+}$ than the monothiol GSH (Zenk, 1996; Schmöger et al., 2000).

* Corresponding author. Tel.: +49-8161-715433; fax: +49-8161-715471.
*E-mail address:* grill@botanik.biologie.tu-muenchen.de (E. Grill).
[1] Present address: School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK.

0031-9422/03/$ - see front matter 
PII: S0031-9422(02)00565-4

In plants, GS-conjugates comprise adducts of secondary metabolites such as auxin, anthocyanins and sterols as well as a diverse range of xenobiotics including the herbicides atrazin and chlorinated compounds (Lamoureux and Rusness, 1986, 1993; Marrs, 1996). The wide spectrum of conjugated compounds is generated by GSH transferases, which are encoded by a small gene family. GS-conjugates frequently accumulate in the vacuole via the action of specific ABC-type transporters in the tonoplast (Martinoia et al., 2000).

The first step in the plant's biotransformation of GS-xenobiotic conjugates involves removal of the glycine residue, thus yielding the corresponding γ-EC-adducts (Lamoureux and Rusness, 1986, 1993; Lamoureux et al., 1991), while in animal systems the turnover of GS-conjugates starts with the removal of the γ-glutamyl residue and leads to the generation of the corresponding cysteinyl-glycine-conjugate (Meister, 1995). After an additional cleavage reaction the xenobiotic is frequently excreted as the *N*-acetylated cysteinyl adduct, the so-called mercapturic acid derivative. The critical control point of the pathway is the initial truncation of the GS-conjugate by γ-glutamyl-transpeptidase.

In plants, the cleavage of the first amino acid residue from the GS-xenobiotic conjugate is also considered the key step of the catabolic pathway (Lamoureux and Rusness, 1986, 1993). A carboxypeptidase has been assigned to be responsible for the reaction (Steinkamp and Rennenberg, 1985; Wolf et al., 1996).

In this contribution we demonstrate that purification of the GS-conjugate specific carboxypeptidase activity yielded fractions enriched in PCS. Characterization of heterologously expressed PCS from *Arabidopsis thaliana* (L.) Heynh. confirms the efficient conversion of GS-conjugates into the corresponding EC-product. Thus, PCS appears to catalyze a key step in the turnover of glutathione-conjugates in plants.

## 2. Results

### 2.1. Identification of GS-conjugate catabolism in vitro

In order to screen for GS-conjugate catabolizing enzymes, the well-established analytical system for bimane-derivatised GSH (bimane-GS) and metabolites was employed (Newton and Fahey, 1995). Incubation of cell-free extracts from various plant cell suspensions in the presence of bimane-GS and subsequent HPLC analyses resulted in the detection of bimane-EC generation by extracts from *Silene cucubalus* Wib. (not shown, comparable to Fig. 1). In addition, minor levels of bimane-cysteine were observed. The assignment of bimane-EC and cysteinyl-bimane formation was preliminarily based on co-elution of corresponding reference samples after HPL chromatography. Interestingly, the addition of $Cd^{2+}$ ions in the range from 3 to 100 μM to the enzymatic fraction stimulated the removal of glycine residues from the GS-conjugate in the range of 2- to 3-fold (data not shown).

The conversion of GS-conjugate into bimane-EC was employed as an assay for enrichment of the catalytic activity. The optimized isolation procedure including hydrophobic interaction chromatography, ion exchange chromatography as well as size exclusion chromatography (Table 1) yielded a 50-fold enzyme enrichment

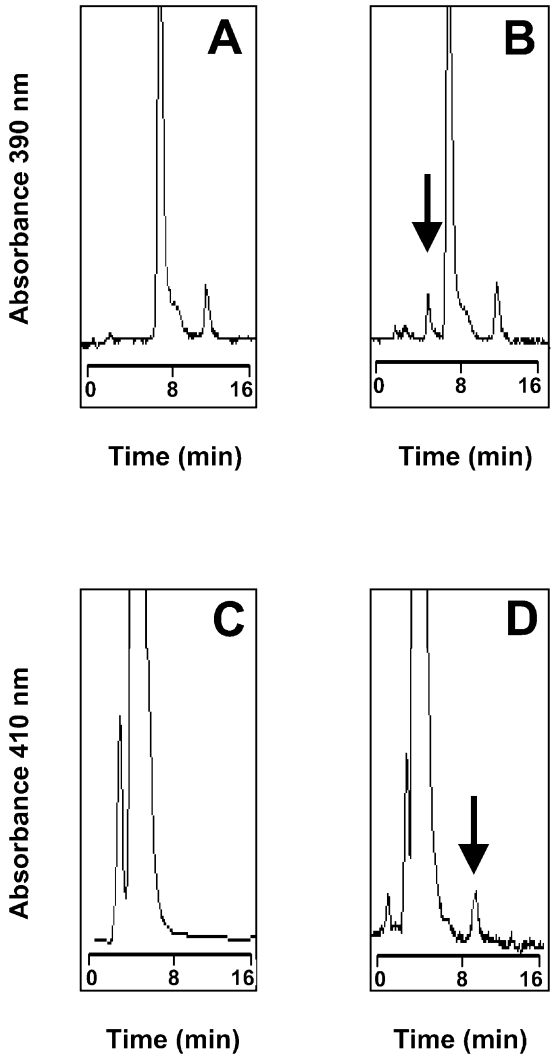


Fig. 1. Turnover of bimane-*S*-glutathione and glutathione by *Silene cucubalus* enzyme fraction. 50-fold enriched GS-conjugate catabolizing activity (2.2 pkat) was immediately stopped by addition of ethanol (A) or incubated in the presence of 0.1 mM $Cd^{2+}$ and 1 mM bimane-*S*-conjugate for 1 h (B). The same enzyme fraction synthesized $PC_2$, $(EC)_2$glycine, in the presence of GSH (10 mM) instead of the GS-conjugate (C, D). No PC was detected prior incubation (C) while PC formation occurred with an activity of 2.6 pkat (D). The detection of biman-EC (B) and $PC_2$ (D) is indicated by an arrow. The main peak fraction represents unmetabolized substrate. The additional peak fractions in (A, B) and in (C, D) represent an internal standard (bimane-mercaptoethanol) and cysteine, respectively.

Table 1
Enrichment of glutathione-*S*-conjugate metabolizing enzyme activity from *Silene cucubalus* (0.3 kg fresh wt.)

| Fractionation | Protein (mg) | Volume (ml) | Activity (nkat) | Yield (% activity) | Specific activity (pkat/mg) | Enrichment factor |
|---|---|---|---|---|---|---|
| Extract concentrate | 356 | 44.5 | 5.1 | 100 | 14.4 | 1 |
| Phenylsepharose | 156 | 55 | 4.8 | 94 | 31.1 | 2.2 |
| Gelfiltration | 36.4 | 27.5 | 3.1 | 61 | 83.9 | 5.8 |
| Hydroxyapatite | 9.7 | 59 | 1.1 | 22 | 111.7 | 7.8 |
| QAE-chromatography | 1.1 | 6 | 0.8 | 16 | 730.6 | 51 |

with a specific activity of 0.7 nkat/mg protein. The procedure was similar to a previous protocol established for partial purification of PCS from *S. cucubalus* (Grill et al., 1989). The enriched enzyme fraction was able to catalyze bimane-EC formation (Figs. 1A and B) as well as to synthesize PCs in the presence of GSH and 0.1 mM $Cd^{2+}$ (Figs. 1C and D). In fact, the specific activity of PCS in that fraction was 45-fold enriched compared to the crude extract. Thus, either the bimane-EC generating enzyme has been co-purified with PCS or PCS itself is responsible for the conversion of the GS-conjugate as indicated previously (Grill et al., 1989). To further follow up the idea of a dual enzymatic function of PCS the competition of bimane-GS by GSH for a common binding site was examined. Indeed, the bimane-GS conversion was efficiently reduced by increasing GSH concentrations (Fig. 2). The $IC_{50}$ value of GSH was approximately 1.5 mM corresponding to a 15-fold molar excess of GSH over bimane-GS. Hence, GSH interferes with the turnover of the *S*-alkylated substrate indicating either allosteric inhibition or competition for substrate binding of the catabolizing enzyme.

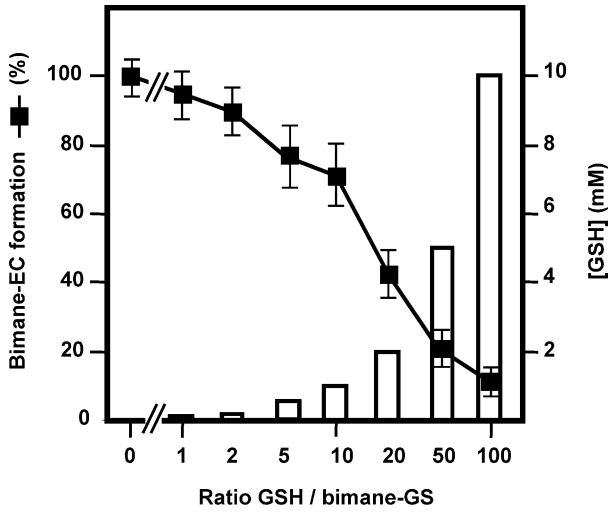


Fig. 2. Dependence of bimane-*S*-glutathione turnover on the presence of glutathione. The partially purified *Silene cucubalus* enzyme fraction (2.1 pkat) was incubated in the presence of 0.1 mM bimane-GS and increasing concentrations of GSH in the absence of $Cd^{2+}$. Subsequently, the formation of the bimane- EC was quantified. The assay was carried out in duplicate (±SE).

### 2.2. Phytochelatin synthase efficiently generates bimane-*S*-glutamyl-cysteine

We used recombinant PCS of *Arabidopsis* (AtPCS1) that had been heterologously expressed in *E. coli*, and subsequently affinity-purified by virtue of a carboxyl-terminal histidine tag, in order to investigate whether PCS catalyzes bimane-GS catabolism. Incubation of the purified PCS in the presence of the substrate bimane-GS yielded bimane-EC (Fig. 3). Surprisingly, the enzymatic reaction was strongly dependent on the $Cd^{2+}$ concentration. In contrast to the partially purified enzyme from *S. cucubalus*, there was no detectable AtPCS1 activity in the absence of $Cd^{2+}$ (detection limit below 0.2% of maximal rate), while optimal conversion of the GS-conjugate by AtPCS1 was observed at 30 μM $Cd^{2+}$. The combined recovery rate for bimane-GS and bimane-EC was 100% (±3%) of initial substrate values (Fig. 3) indicating that no or only very low levels of other products were formed. In particular, we tested whether bimane-derivatives of PCs were generated as a side product. No PC-derivatives were found. In contrast, monobromobimane-derivatized PC peptides chromatographed as positive controls were detected with high sensitivity. The unequivocal identification of bimane-EC as the product generated by PCS was carried out by mass spectrometry. The bimane-EC and the bimane-GS fraction were collected after HPLC separation and subjected to ESI-MS analysis. The isolated bimane-GS and bimane-EC yielded a single prominent signal at 498 ($m+H^+$) and 441 ($m+H^+$), respectively (Fig. 3, insets). Thus, the dipeptidyl-aminotransferase catalyzes both reactions, PC formation and GS-conjugate hydrolysis. Turnover of bimane-GS by AtPCS1 occurred 2.2 times more efficient than the transpeptidation of GSH to PCs (3.9±0.4 pkat versus 1.8±0.2 pkat) under comparable conditions, that is peptidic substrates were provided at 1mM concentration in the presence of 30 μM $Cd^{2+}$. Taken together, the results demonstrate an efficient catabolism of the GS-conjugate to the corresponding EC-derivative by the action of AtPCS1.

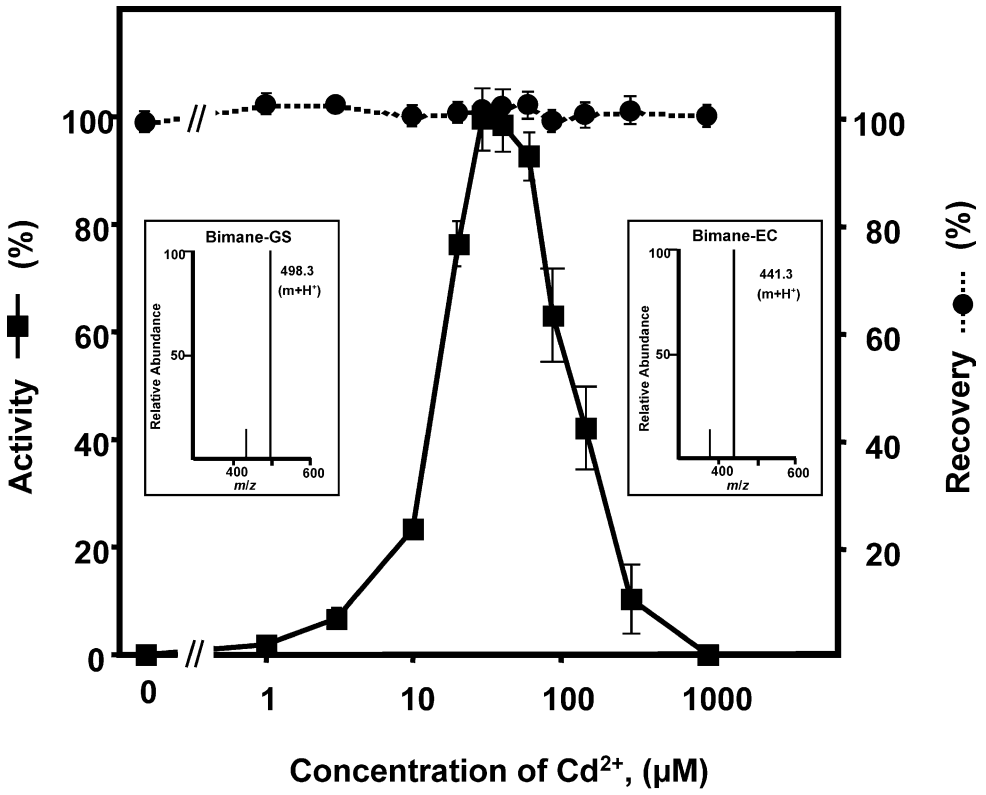


Fig. 3. The phytochelatin synthase from *Arabidopsis* catalyzes the breakdown of bimane-*S*-glutathione. Catabolism of bimane-GS by heterologously expressed and purified AtPCS1 is dependent on the $Cd^{2+}$ concentration. The conversion of the GS-derivative into bimane-EC was confirmed by electrospray ionization mass spectrometry (ESI-MS) which yielded the major signal ($m+H^+$) at 498 and 441, respectively (insets). Without $Cd^{2+}$ no enzyme activity was detected. Mean values (±SE) are given. The combined recovery rate of unmetabolized substrate and bimane-EC product was 100%±3. No formation of PC-derivatives was observed. Maximal activity was 4.4±0.3 pkat and was set to 100%.

The pH-dependence of the PCS-catalyzed reaction was optimal between pH 7.9 and 8.5 (Fig. 4). Little or no catalysis occurred below pH 6 or above pH 9. The $Cd^{2+}$ required for full activation of PCS could be replaced by the heavy metal ions $Cu^{2+}$ and $Zn^{2+}$ (Fig. 5), while the divalent metal ions $Mg^{2+}$ and $Ca^{2+}$ failed to activate the enzyme (not shown). Half-maximal activation of PCS was observed at 3 µM and 50 µM $Cu^{2+}$ and $Zn^{2+}$ concentrations, respectively. Administration of $Cu^{2+}$ to a final concentration of 40 µM yielded PCS activity that was 3-fold higher than at optimal $Zn^{2+}$ concentrations (100 µM) and which even surpassed maximal $Cd^{2+}$-mediated PCS stimulation by approximately 50%.

### 2.3. Phytochelatin synthase catabolizes various GS-conjugates

Xenobiotic compounds such as the herbicides atrazin, propachlor, and fluorodifen are detoxified as GS-conjugates (Lamoureux and Rusness, 1993). In order to test whether GS-adducts serve as general substrates for PCS we synthesized a number of diverse GS-conjugates. The sulfhydryl group of GSH was covalently attached to structural different moieties such as uracil, acetamidofluorescein, nitrobenzyl, benzyl, and phenylbenzyl. Incubation of affinity-purified PCS in the presence of these xenobiotic substrates resulted in the generation of the corresponding EC derivatives (Table 2). In several cases the conversion was less efficient than that of bimane-GS, which was catabolized to 98% under the conditions employed (set to 100%). Removal of the glycine residue from other GS conjugates ranged from 28% for uracil-GS to approximately 100% for benzyl- and nitrobenzyl-GS under these conditions, indicating a broad substrate specificity of PCS.

## 3. Discussion

GS-conjugates of endogenous compounds are involved in metabolism, transport and storage in plants (Marrs, 1996). In addition, conjugation of xenobiotics to GSH initiates a detoxification pathway that generally leads to the secretion or compartmentation of the biotransformed compound. Metabolism of GS-xenobiotics such as atrazin and other halogenated herbicides is typically initiated by removal of the carboxylterminal glycine residue of the GSH tripeptide to give rise to the *S*-EC-derivative (Lamoureux and Rusness, 1993). The

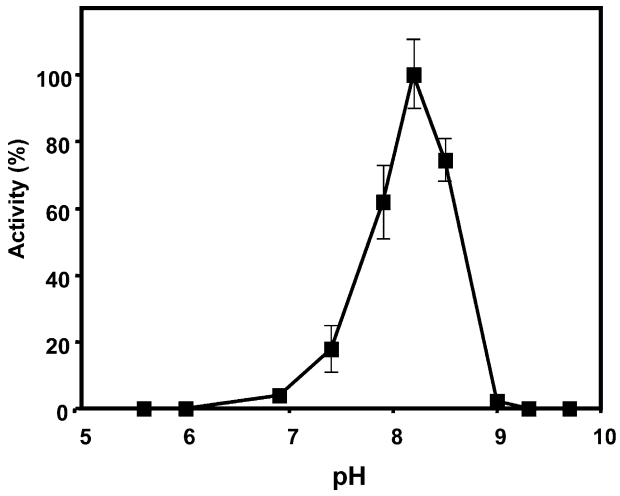


Fig. 4. pH-dependence of bimane-*S*-glutamylcysteine formation. AtPCS1 activity was determined at the indicated pH values. Mean values (±SE) are given. Maximal activity was 4.3±0.4 pkat at pH 8.2 and was set to 100%.

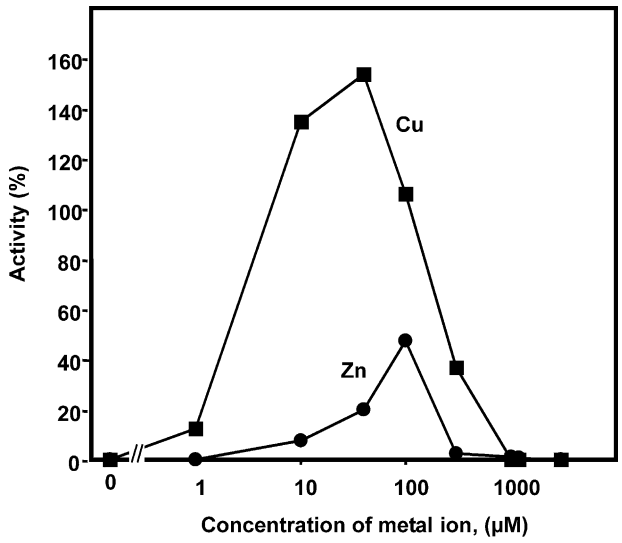


Fig. 5. Copper and zink ions are capable of activating AtPCS1. AtPCS1 was activated by administration of increasing levels of $Zn^{2+}$ and $Cu^{2+}$. Activity is expressed relative to maximal activation by $Cd^{2+}$ at 40 μM concentration (corresponding to 4.4 pkat; set to 100%). Mean values of duplicates are given (SE:±8%).

pathway is different from *S*-conjugate metabolism of animals and man where the first and rate-limiting step is the removal of the aminoterminal glutamic acid residue (Meister, 1995).

Purification of the GS-conjugate metabolizing activity from cell suspension cultures of *Silene cucubalus* indicated that phytochelatin synthase catalyzes the generation of *S*-EC-adducts. In line with this observation, heterologously expressed and purified AtPCS1 from *Arabidopsis* efficiently converted bimane-GS to bimane-EC, the product being unequivocally identified by mass spectrometry. No further products such as *S*-derivatives of phytochelatins were observed. Several different GS-*S*-conjugates served as substrates and were processed by PCS to the corresponding EC-adduct (in the case of phenyl benzyl-GS, respective PC-derivatives might have been formed as a minor product as judged by HPLC analysis). Interestingly, preliminary studies of a carboxypeptidase from vacuoles of barley support an EC-conjugate generation by this enzyme (Wolf et al., 1996). GS-conjugate conversion occurred at pH 5. The acidic pH and the vacuolar localization of the enzyme argues for an enzymatic activity distinct from PCS.

PCS has a clear preference for bimane-GS compared to GSH. The hydrolysis rate of GS-conjugate by AtPCS1 was more than twofold higher than transpeptidation of GSH to PCs under identical $Cd^{2+}$ and bimane-GS/GSH concentrations. The finding is also deducible from the enzymatic conversions catalyzed by PCS from *Silene* (Fig. 1) taking into account that the $K_m$-values for the *Silene* enzyme is 1.5 mM for bimane-GS and 7.5 mM for GSH while $v_{max}$ is only 2.5 fold higher for the turnover of GSH into PCs (Grill et al., 1989). An intriguing aspect is the generation of *S*-substituted PCs by PCS in the presence of *S*-alkylated GSH such as methyl-GS and hexyl-GS (Vatamaniuk et al., 2000; Oven et al., 2002). Although we observed in the presence of hexyl-GS correspondingly modified PC peptides (data not shown), GS-conjugates of nitrobenzene, benzene, uracil, acetamido-fluorescein or bimane were selectively converted into the EC derivative. The prevention of the EC polymerization in these cases could reflect steric hindrance of the more bulky group of the cyclic xenobiotics compared to the linear aliphatic *S*-substituted moiety, which may either prevent transfer of the *S*-substituted EC onto the acceptor peptide or block its binding (Fig. 6). As a likely consequence, the PCS-bound EC derivative is transferred onto water as an alternative acceptor molecule. The overall reaction is hydrolysis of the cysteinyl-glycine bond. The transfer of the dipeptidyl-group could either occur inter-molecularly between two PCS proteins or intramolecularly between the donor site (D) onto the acceptor site (A) (Fig. 6). Protein–protein interaction analyses employing the yeast two-hybrid system support an intramolecular mechanism (Schmöger and Grill, unpublished results).

A critical requirement for catalysis is the presence of ions such as $Cd^{2+}$, $Zn^{2+}$ or $Cu^{+/2+}$. No activity was detectable with affinity-purified AtPCS1 in the absence of these ions, strongly indicating the requirement of metal-mediated activation of the enzyme. The heavy metal ions have a high affinity for sulfhydryl groups and could interact with the abundant cysteine residues of PCS. The valence state of activating copper ions is unknown. Under the reducing environment of the

Table 2
Glutathione-*S*-conjugate catabolism by purified AtPCS1

| Compound | EC-conjugate (μMol) | Yield (%) |
|---|---|---|
| Bimane-GS | 0.29 | 100±3 |
| Benzyl-GS | 0.29 | 100±3 |
| Nitrobenzyl-GS | 0.28 | 98±3 |
| Phenylbenzyl-GS | 0.13 | 46±4 |
| Acetamido-fluorescein-GS | 0.10 | 36±3 |
| Uracil-GS | 0.08 | 28±3 |

cytosol and the assay conditions partial reduction of divalent $Cu^{2+}$ to $Cu^{+}$ takes place. It is likely that both forms of copper ion are sufficient for activation of PCS since divalent and monovalent heavy metal ions such as $Ag^{+}$ are able to activate the enzyme (Grill et al., 1989; Vatamaniuk et al., 2000; Oven et al., 2002). Interestingly, the partially purified enzyme from *S. cucubalus* cell cultures was active even in the absence of exogenously provided $Cd^{2+}$. Administration of the $Cd^{2+}$ resulted in a limited increase of PCS activity up to 3-fold which is much lower than the more than 500-fold enhancement observed with the purified AtPCS1. It seems that the *S. cucubalus* enzyme has been purified together with activating metal ions, while during affinity purification of AtPCS1 by $Co^{2+}$-interaction chromatography enzyme-bound metal ions were removed. The question arises whether in vivo, irrespective of heavy metal stress imposed, PCS is active or whether the *Silene* enzyme has been artefactually activated during the extraction process either by release of heavy metal ions such as $Zn^{2+}$ and $Cu^{+/2+}$ from metalloenzymes or by destruction of cellular compartments.

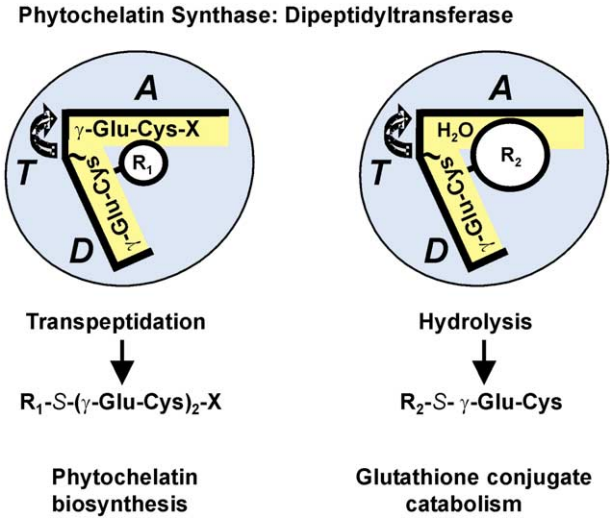


Fig. 6. Model of phytochelatin synthase-mediated formation of phytochelatins and glutamylcysteinyl-*S*-conjugates. Bound GSH and GS-conjugates at the donor site (*D*) of PCS are processed via cleavage of the peptide bond of the carboxylterminal glycine residue. The peptide bond energy (~) is preserved at the transpeptidation site (*T*) of PCS. Transfer of the resulting EC-moiety, indicated by an open arrow, onto an acceptor molecule such as GSH or $PC_n$, (γ-Glu-Cys-X), yields $PC_2$ and elongated $PC_{n+1}$, [$(\gamma\text{-Glu-Cys})_2$-X], respectively. Modifications of the sulfhydryl group of the donor dipeptide seem to be decisive whether transpeptidation or transfer onto a water molecule, that is hydrolysis, occurs. Bulky sulfur-linked residues ($R_2$) such as cyclic hydrocarbons with functional groups mediate efficient hydrolysis to the respective EC product by preventing transpeptidation due to steric interference. Dipeptides containing either metal ions or linear hydrocarbons such as methyl and hexyl-residues ($R_1$) linked to the sulfur group are preferentially processed via transpeptidation onto an acceptor molecule bound to the acceptor site (A) of PCS which leads to the generation of PC molecules or their S-modified derivatives. PCS is a metalloenzyme that is catalytically activated by heavy metal ions such as $Zn^{2+}$ and $Cu^{+/2+}$.

It is likely, that in vivo PCS is a $Zn^{2+}$ and / or $Cu^{+/2+}$ containing metalloenzyme. The increased affinity of AtPCS1 for $Cu^{+/2+}$ and its higher activation by $Cu^{+/2+}$ compared to $Zn^{2+}$ favour a copper-PCS complex. In keeping with this hypothesis, cell culture media that provide both ions at low μM concentrations are sufficient to trigger the formation of PCs (Grill et al., 1988). In addition, differently isolated AtPCS1 did not require exogenous metal ions for functionality, and revealed only a marginal twofold activation of PCS by supplied $Cd^{2+}$ (Vatamaniuk et al., 2000). The PCS isolated by Vatamaniuk et al. (2000) was analyzed, however, in the absence of reducing agents which resulted in a 100-fold lowered specific activity of PCS (Oven et al., 2002). Consequently, mechanistic insights of substrate-enzyme interactions using such inactive (oxidized) PCS fractions have to be interpreted with caution as must the statement concerning a low affinity of PCS for $Cd^{2+}$ (Vatamaniuk et al., 2000). In fact, $Cu^{+/2+}$, $Cd^{2+}$, and $Zn^{2+}$ binding by PCS (protein concentration below 0.1 pM) is rather efficient taking into account that 3 μM, 15 μM, and 50 μM ion concentrations, respectively, half-maximally stimulated PCS (Fig. 5) in the presence of more than 9 orders of magnitude higher concentration of the reducing agent dithiothreitol (1 mM). At these metal ion concentrations the ratio of $Cu^{+/2+}$, $Cd^{2+}$, and $Zn^{2+}$ to dithiothreitol equals approximately 1:300, 1:60, and 1:20, respectively. Maximal AtPCS1 activity was observed at 40 μM $Cu^{+/2+}$, 30 μM $Cd^{2+}$, and 100 μM $Zn^{2+}$. Thus, the activation-mediating binding site(s) of the dipeptidyltransferase appear to be saturated at a 10- to 30-fold molar excess of dithiothreitol to metal ions. Hence, PCS more efficiently complexes these heavy metal ions than the dimercapto compound by at least an order of magnitude. Dithiothreitol as a dithiolic reductant is a strong heavy metal binding molecule similar to dimercaptopropanol that is used as a therapeutic agent for heavy metal detoxification (Domingo, 1995). The high affinity of PCS for $Cu^{+/2+}$ is striking. The more efficient hydrolysis of GS-conjugate by $Cu^{+/2+}$-activated versus $Cd^{2+}$-activated PCS is at variance to the metal-stimulated PC synthesis. PC formation is more efficient in the presence of $Cd^{2+}$ by a factor of 2–5 compared to $Cu^{+/2+}$ (Grill et al., 1989; Vatamaniuk et al.,

2000; Oven et al., 2002). Consequently, the $Cu^{+/2+}$-PCS metalloenzyme preferentially catalyzes GS-conjugate metabolism.

Mechanistically, it is appealing that PCS requires metal-dependent activation to convert GSH into the PC peptides (Grill et al., 1989; Oven et al., 2002). The newly formed PCs compete for complexation of heavy-metal ions with PCS and, as a consequence, the enzyme is downregulated in its activity by the equilibrium between newly formed metal-free PCs and the metal-binding site(s) of PCS. A basic activity of PCS, however, has to be postulated for catabolism of GS-conjugates in vivo. In agreement with this idea is the observation that even in $Cu^{2+}$- and $Zn^{2+}$-free culture media small levels of PCs are present (Grill et al., 1988). Suppression of the futile conversion of GSH into PCs could be realized either by a very low residual or no transpeptidase activity of PCS for unconjugated GSH. The substrate preference of AtPCS1 for the metal-GSH complex versus GSH (Vatamaniuk et al., 2000) would support this view.

## 4. Conclusions

The analyses of PCS catalyzed reactions argue for two cellular functions of the enzyme: the formation of PC peptides as part of a heavy-metal detoxification system and, secondly, the degradation of GS-*S*-conjugates in the detoxification pathway of xenobiotics. Mechanistically, the former role is the result of γ-EC transpeptidation onto GSH or derivatives thereof while the catabolic function reflects transpeptidation of *S*-EC-adducts onto the acceptor molecule water. Thus, the dipeptidyltransferase seems to fulfil, beside the known function in heavy-metal detoxification, a second crucial role in GSH metabolism in plants and possibly in several other organisms expressing a functional PCS.

## 5. Experimental

### 5.1. Plant material and chemicals

The plant cell suspension of *Silene cucubalus* was cultured as described by Grill et al. (1989). All chemicals used were of p.A. grade or highest purity available from Fluka (Neu-Ulm), Sigma-Aldrich (Deisenhofen), Calbiochem and Merck (Darmstadt).

### 5.2. Enzyme assay for purification

The conversion of bimane-GS into bimane-EC was quantified by HPL chromatography on C18-reversed phase column (Prontosil 120 × 5 mm; Bischoff, Leonberg) as described by Newton and Fahey (1995). The assay was carried out as described below with 10 μM $Cd^{2+}$ unless otherwise stated. Bimane-PC was identified by increasing the level of organic solvent in the running buffer to 50% MeOH. PC formation was assayed by online derivatization of sulfhydryl groups with Ellman's reagent (Grill et al., 1987).

### 5.3. Enzyme isolation

The enzyme purification was carried out at 4 °C and buffer solutions were supplemented with β-mercaptoethanol (10 mM) unless otherwise stated. The cell-free extract of cell suspensions from *Silene cucubalus* (0.3 kg fresh wt., 16 g dry wt.) was concentrated by both DEAE-agarose chromatography and subsequent elution as described earlier for isolation of PCs (Grill et al., 1985). The protein fraction, referred to as concentrated protein extract, was adjusted to 8% (w/v) ammonium sulfate, centrifuged and the supernatant applied to phenyl sepharose Cl-4B column (3.0 × 15 cm; Pharmacia). Chromatography and elution was carried out as described (Grill et al., 1989). The active enzyme fraction (55 ml) was concentrated by ammoniumsulfate precipitation (50% w/v). The precipitate was collected (8000 × *g*, 15 min), dissolved in 4 ml buffer A (10 mM Tris–Cl pH 7.8, 10 mM NaCl) and chromatographed over a size exclusion column (2.5 × 109 cm; AcA 34-Ultragel, Serva) in buffer A. Active fractions (27.5 ml) were applied to a hydroxylapatite column (2 × 12.5 cm, Biorad) and eluted by a gradient of 0.2 M potassium phosphate buffer, pH 8.0, at a flow rate of 20 ml/h. The active eluate (59 ml) was concentrated and re-buffered into buffer B (10 mM Tris–Cl, pH 8.0, 1 mM mercaptoethanol) by ultrafiltration (UM2 membrane, Amicon) in order to subsequently bind the enzyme fraction (10 ml) onto an anion exchange column (QAE Fast Flow, 1.0 × 10 cm, Pharmacia). The enzyme was eluted by a gradient of 0.05 to 0.4 M KCl in buffer B at a flow rate of 60 ml/h and collected in a total volume of 6 ml.

Recombinant AtPCS1 containing a hexa-histidine tag was cloned and isolated by affinity interaction chromatography as described (Oven et al., 2002). The enzyme (1.5 μkat × $mg^{-1}$) was stored in buffer B supplemented with 20% glycerol and 1% bovine serum albumine at −20 °C.

### 5.4. Glutathione derivatization

Glutathione and PC derivatives were generated according to a previously published procedure (Martinoia et al., 1993) with minor modifications. Briefly, the substrate (10 mM sulfhydryl groups) was incubated with 10 mM sulfhydryl-modifying reagent (monobromobimane, benzylbromid, jodouracil, jodoacetamido-fluorescein, nitrobenzylbromid, and phenylbenzylbromid) in buffer

solution containing 1 μM EDTA and 100 mM $(NH_4)CO_3$, pH 8.0. After reaction at 35 °C for 1 h the incubation mixture was dried over a stream of nitrogen and the residue resuspended in aqueous solution containing 5 mM Tris–Cl, pH 8.2. Derivatization of GSH with jodouracil and jodoacetamido-fluorescein yielded approximately 40% product. The products were preparatively separated by HPLC prior to incubation with PCS (conditions as mentioned below).

### 5.5. Phytochelatin synthase characterization

AtPCS1 incubations for activity measurement were performed with approximately 4.4 pkat enzyme in a total volume of 30 μl at 35 °C for 20 min. Substrate concentration and buffer condition were, unless otherwise stated, 1 mM bimane-GS in 100 mM Tris–Cl, pH 8.2, 1 mM DTT, and 30 μM $Cd(NO_3)_2$. All experiments were performed in triplicate, except otherwise stated. For analysis of the metal ion-dependence of the reaction, 0.1 M $Cd(NO_3)_2$, $Zn(NO_3)_2$, and $CuSO_4$ were used to adjust the final concentration as given. The pH-dependence of AtPCS1 was determined in different buffers (100 mM): pH 5.6–6.9: potassium phosphate; pH 7.4–8.5: Tris–Cl; pH 8.9–9.7: glycine buffer. In all cases maximal turnover has been in the linear range of the reaction (less than 40% substrate conversion). The quantification of educts and products was carried out by HPLC analysis (Newton and Fahey, 1995). The sample was stopped by addition of 70 μl EtOH, kept on ice for 5 min, and subsequently cleared by centrifugation (14 000 × *g*, 5 min). The supernatant was 10-fold diluted with running buffer C (0.25% v/v acetic acid in water, pH 3.9) and 0.1 ml injected onto a C18 reverse-phase column. The column was developed at a flow rate of 1.2 ml × $min^{-1}$, first at isocratic condition (buffer C supplemented with 16% v/v methanol) for 8 min, then by a gradient of methanol up to 90% in buffer C (10 min). Detections of bimane-derived molecules was performed at 390 nm.

Analysis of turnover of different GS-derivatives was performed essentially as described above, however, substrate concentration and enzyme level were increased tenfold. Incubation time was extended to 60 min. The turnover rates of GS-benzyl, GS-nitrobenzyl, and GS-phenylbenzyl as well as the resulting EC products were analyzed by HPLC, similarly to GS-bimane, with an initial isocratic phase of 25% (50% in the case of GS-phenylbenzyl) methanol in buffer C (14 min) and a subsequent gradient of methanol up to 90%. The identity of the peak fractions was established by co-chromatography of correspondingly modified GSH and EC standards. The absorption of the eluent was measured at 275 nm for GS-nitrobenzyl and 240 nm for GS-benzyl and GS-phenylbenzyl. In case of GS-acetamido-fluorescein and GS-uracil, HPLC was performed at isocratic condition (13 min) using buffer C supplemented with 50 and 16% MeOH, respectively, and the substances were detected by their absorption at 450 and 270 nm, respectively. Separation of both GS-adducts and its des-glycine products have not been accomplished. Turnover was calculated by the molar ratio of glycine to glutamic acid employing combined collection of substrate and product after HPLC and subsequent amino acid analyses of the peptide derivatives as previously described (Grill et al., 1985).

### 5.6. Mass spectrometry

The bimane-GS and its EC derivative produced by AtPCS1 were purified by HPLC. Collected fractions were dried over a stream of nitrogen and subjected to electrospray ionization mass spectrometry (ESI-MS) using a MAT LCQ instrument (Finnigan, San Jose, CA).

## Acknowledgements

The financial support of the Fonds der Chemischen Industrie is gratefully acknowledged. We would like to thank Dr. B. Cordes, Organische Chemie der Technischen Universität München, for ESI-MS analyses, Professor H.-M. Eichinger, Bayerische Hauptversuchsanstalt für Landwirtschaft, Weihenstephan, for amino acid analyses, and Dr. J. Greenwood for critical reading.

## References

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475–486.

Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. 53, 159–182.

Domingo, J.L., 1995. Prevention of chelating agents of metal-induced developmental toxicity. Repr. Tox. 9, 105–113.

Grill, E., Winnacker, E.-L., Zenk, M.H., 1985. Phytochelatins, the principal heavy metal-complexing peptides of higher plants. Science 230, 574–576.

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

Grill, E., Thumann, J., Winnacker, E.-L., Zenk, M.H., 1988. Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media. Plant Cell Rep. 7, 375–378.

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

Lamoureux, G.L., Rusness, D.G., 1986. Xenobiotic conjugation in higher plants. ACS Sym. Ser. 299, 62–105.

Lamoureux, G.L., Rusness, D.G., 1993. Glutathione in the metabolism and detoxification of xenobiotics in plants. In: de Kok, L.J., Stulen, I., Rennenberg, H., Brunhold, C., Rauser, W.E. (Eds.), Sul-

fur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, pp. 221–237.

Lamoureux, G.L., Rusness, D.G., Schroeder, P., Rennenberg, H., 1991. Diphenyl ether herbicide metabolism in a spruce cell suspension culture: the identification of two novel metabolites derived from a glutathione conjugate. Pest. Biochem. Physiol. 39, 291–301.

Marrs, K.A., 1996. The functions and regulation of glutathione *S*-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 127–157.

Martinoia, E., Massonneau, A., Frangne, N., 2000. Transport processes of solutes across the vacuolar membrane of higher plants. Plant Cell Physiol. 41, 1175–1186.

Martinoia, E., Grill, E., Tommasini, R., Kreuz, K., Amrhein, N., 1993. An ATP-dependent glutathione *S*-conjugate "export" pump in the vacuolar membrane of plants. Nature 364, 247–249.

Meister, A., 1995. Glutathione metabolism. Methods Enzymol. 251, 3–7.

Newton, G.L., Fahey, R.C., 1995. Determination of biothiols by bromobimane labeling and high performance liquid chromatography. Methods Enzymol. 251, 148–166.

Oven, M., Page, J.E., Zenk, M.H., Kutchan, T.M., 2002. Molecular characterization of the homo-phytochelatin synthase of soybean *Glycine max*: relation to phytochelatin synthase. J. Biol. Chem. 277, 4747–4754.

Schmöger, M.E., Oven, M., Grill, E., 2000. Detoxification of arsenic by phytochelatins in plants. Plant Physiol. 122, 793–801.

Steinkamp, R., Rennenberg, H., 1985. Degradation of glutathione in plant cells: evidence against the participation of a γ-glutamyltranspeptidase. Z. Naturforsch. 40c, 29–33.

Vatamaniuk, O.K., Mari, S., Lu, Y.P., Rea, P.A., 2000. Mechanism of heavy metal ion activation of phytochelatin (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. J. Biol. Chem. 275, 31451–31459.

Wolf, A.E., Dietz, K.-J., Schröder, P., 1996. Degradation of glutathione *S*-conjugates by a carboxypeptidase in the plant vacuole. FEBS Letters 384, 31–34.

Zenk, M.H., 1996. Heavy metal detoxification in higher plants—a review. Gene 179, 21–30.