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Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product

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The newly discovered enzyme γ-glutamylcysteine dipeptidyl transpeptidase was purified to apparent homogeneity from cell suspension cultures of *Silene cucubalus*. The enzyme catalyzes, in the presence of heavy metal ions, the formation of metal chelating peptides, the phytochelatins, from glutathione. The stoichiometry of the transfer reaction for the first phytochelatin member was determined to be: 2 (γ-Glu-Cys)-Gly → $(\gamma\text{-Glu-Cys})_2$-Gly + Gly. The enzyme is self regulated in that the reaction products, the phytochelatins, chelate the enzyme activating metal, thus terminating the enzymatic reaction. The higher order phytochelatins have a higher relative complexing affinity than the lower ones, as judged from their ability to terminate the enzymatic reaction.

Phytochelatin biosynthesis; Glutathione metabolism; Heavy metal ion activation; Glutamylcysteine, γ-; Dipeptidyl transpeptidase; Phytochelatin synthase; (*Silene cucubalus*)

1. INTRODUCTION

Phytochelatins are small, cysteine-rich peptides capable of binding heavy metal ions such as Cd^{2+}, Ag^{+}, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, etc. via thiolate coordination. The general structure of this set of peptides is $(\gamma\text{-Glu-Cys})_n$-Gly (n = 2-11) [1,2]. They are present as a consequence of heavy metal challenge in select fungi [3-5], but occur in the whole plant kingdom from algae [6] to orchids [7], with the exception of the order *Fabales*, which may contain in addition or solely the homophytochelatins $(\gamma\text{-Glu-Cys})_n$-β-Ala (n = 2-7) [7,8]. No information was available yet as to the resolution of the system, whether the phytochlatin synthesizing protein(s) is induced by the metals or whether it is constitutive in the plants [9]. Recently a new enzyme has been discovered and partly 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 $(\gamma\text{-Glu-Cys})_n$-Gly oligomers, thus synthesizing the metal-binding peptides, the phytochelatins [10]. The enzyme was named γ-glutamylcysteine dipeptidyl transpeptidase and given the trivial name phytochelatin synthase. The reaction catalyzed by this enzyme is: $(\gamma\text{-Glu-Cys})_n\text{-Gly} + (\gamma\text{-Glu-Cys})_n\text{-Gly} \rightarrow (\gamma\text{-Glu-Cys})_{n+1}\text{-Gly} + (\gamma\text{-Glu-Cys})_{n-1}\text{-Gly}$, where n = 1,2,3, etc.

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In the present communication the purification of phytochelatin synthase to apparent homogeneity is reported. Furthermore, the stoichiometry of the reaction catalyzed by this enzyme is given. Evidence is presented, that the enzyme is self-regulated in that the product of the reaction, the phytochelatins, chelates the enzyme-activating metal, thus terminating the enzymatic reaction.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[2-^{3}H-Gly]-glutathione was supplied by NEN and all other chemicals were of the highest purity available from Sigma, Merck or Boehringer Mannheim. Monobromobimane was from Calbiochem and *S*-monobromobimane-[2-^{3}H-Gly]-glutathione was synthesized as described [11].

2.2. *Enzyme purification*

Cell suspension cultures of *S. cucubalus* were grown as described [2]. 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 used for the enzyme purification. The transpeptidase was isolated exactly as previously given [10], involving the steps listed in table 1. Enzyme purity was assayed by SDS-PAGE.

2.3. *Enzyme assays*

(a) Radioactive test: in a total volume of 100 μl the incubation mixture contained 200 mM Tris-HCl buffer pH 8.0, 10 mM mercaptoethanol, 0.1 mM *S*-monobromobimane-[2-^{3}H-Gly]-glutathione (5000 cpm), 0.1 mM $Cd(NO_3)_2$ and differing amounts of phytochelatin synthase. The mixture was incubated at 35°C and terminated at different times by the addition of 20 μl of a glycine solution (100 mM) followed by 80 μl of a neutralized suspension of activated charcoal (10% w/v) in distilled water. The suspension was agitated for 1 min

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and the charcoal was removed by centrifugation (all Eppendorf systems). The radioactivity of a 150-μl aliquot was measured by scintillation counting (5 ml Quickszint 2000, Zinsser). Control values were obtained by using heat-denatured enzyme, or omitting the enzyme or the metal. For the reaction balance, the labeled monobromobimane derivative was replaced by [2-^{3}H-Gly]-glutathione. The radioactivity released in both cases was directly proportional to the amount of glycine released by the enzymatic reaction.

(b) By HPLC: this previously published procedure [10] was followed both for the quantitation of individual phytochelatins as well as glutathione.

2.4. *Analysis of substrates and reaction products*

In addition to the HPLC assay for glutathione, this tripeptide was quantitated according to [12]. Enzymatically liberated glycine was analyzed using an automated amino acid analyzer (Biotronic LC 6001). (γ-Glu-Cys)$_2$-Gly and (γ-Glu-Cys)$_3$-Gly which were formed enzymatically were sequenced after oxidation by performic acid and subsequent dinitrophenylation [2].

3. RESULTS AND DISCUSSION

3.1. *Detection and assay of the enzyme*

Previous experiments with cell suspension cultures of *Rauvolfia serpentina* demonstrated that phytochelatin biosynthesis was dependent on the biosynthesis of glutathione and on the presence of heavy metal ions [2]. Attempts to detect biosynthesis of the heavy-metal-binding peptides in extracts of *R. serpentina* failed, but instead a rapid degradation of exogenously supplied glutathione and phytochelatin was observed, irrespective of the presence of heavy metal ions, thus masking any possible phytochelatin formation. A screening program involving about forty different cell suspension cultures cultivated in the presence and absence of cadmium ions revealed that cell free extracts of *S. cucubalus* lacked these interfering activities. In fact, extracts of this metal-tolerant plant species were able to synthesize di- and tri-(γ-glutamylcysteinyl)-glycine (fig.1A). Phytochelatin synthesis did not require magnesium-ATP, glycine or (γ-glutamylcysteine)$_2$. Omission of cadmium ions (fig.1B) or of glutathione (endogenous glutathione was removed by gel filtration) resulted in no detectable synthesis of phytochelatin. However, phytochelatin synthesis was recovered by the addition of both components. The in vitro synthesized peptides were purified by HPLC and unequivocally identified as (γ-Glu-Cys)$_n$-Gly (n = 2 and 3) by amino acid analysis and chromatographic analysis of partially hydrolyzed, dinitrophenylated peptide derivatives [1,2]. The enzyme is obviously a strictly metal dependent γ-glutamylcysteine dipeptidyl transpeptidase.

Purification of the phytochelatin synthase required a faster assay for activity than by HPLC separation. Since the reaction appeared to proceed with the loss of one glycine from glutathione for each transferred γ-glutamylcysteinyl dipeptide, commercially available [2-^{3}H-glycine]-glutathione was used for assay. Labeled substrate and phytochelatin separated from [^{3}H-Gly]glycine by binding of the sulfhydryl-containing

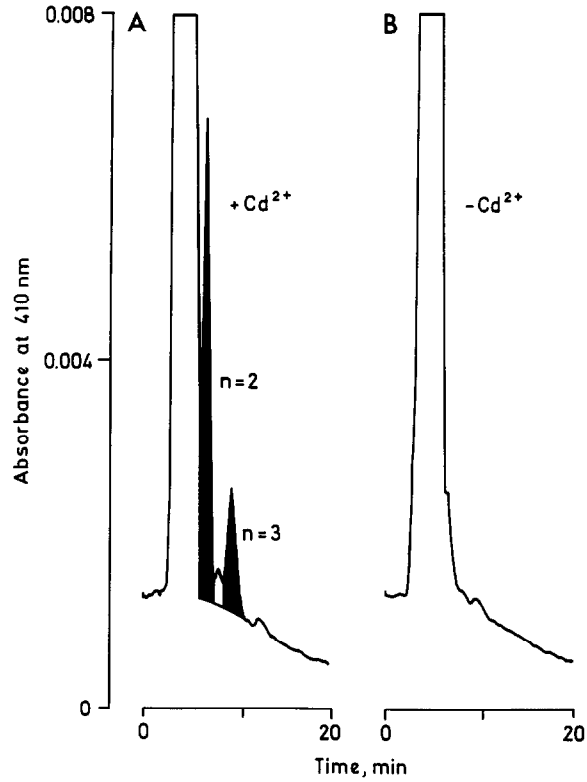

Fig.1. HPLC analysis of phytochelatin formation in crude extracts of *S. cucubalus* containing 1 mM glutathione in the (A) presence and (B) absence of 0.1 mM cadmium ions. Detection was specific for sulfhydryl groups. In this experiment, mercaptoethanol was omitted. The unshaded dominant peak represents untransformed glutathione; the black marked peaks correspond to the individual phytochelatin peptides. Number of γ-glutamylcysteine units per molecule is indicated by n.

peptides to mercurial agarose [13]. Another faster assay was developed based on the observation that the enzyme also uses *S*-monobromobimane-[2-^{3}H-glycine]-glutathione as substrate liberating labeled glycine during the reaction. Excess substrate and other sulfhydryl-containing material was simply absorbed onto charcoal and removed by centrifugation. Purified enzyme preparations could also be assayed by replacing the labeled *S*-monobromobimane derivative by [2-^{3}H-glycine]-glutathione. The recovery rate for labeled glycine in the charcoal supernatant was in all cases 95%.

3.2. *Enzyme isolation*

Following the detailed procedure [10], the enzyme was purified to apparent homogeneity as summarized in table 1. The purified enzyme showed a specific activity of 450–500 pkat/mg protein and was about 160-fold enriched compared to the crude extract. The purification factor was probably underestimated since in the final purification step, on the Mono Q column, the enzyme was more efficiently purified as judged by SDS-PAGE analysis than as reflected by the decrease in

Table 1

Summary of purification of γ-glutamylcysteine dipeptidyl transpeptidase from 1 kg (fresh weight) of *Silene cucubalus* suspension cells

Purification step	Volume (ml)	Protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Yield of activity (%)	Purification (-fold)
Crude cell extract	1550	1240	3720	3.0	100	1
Phenylsepharose	98	451	2300	5.1	62	1.7
Hydroxylapatite	28.5	140	1358	9.7	37	3.2
Ultrogel AcA 34	68	95	1809	19	49	6.3
QAE sepharose	14	16.6	632	38	17	12.7
Phenylsuperose	6.6	2.0	135	67.7	3.6	22.6
Zn-chelating sepharose*	3.2	0.13	65	500	1.7	167
Mono Q	2.4	0.014	6.5	463	0.2	154

*Fast Flow. Activity was measured by employing the radioactive assay

specific activity. The single band obtained from the silver-stained SDS-polyacrylamide gel (fig.2) indicates that the synthase was apparently free of major contaminating proteins.

Growth of the same strain of cells in the absence or in the presence of $Cd(NO_3)_2$ ions (10, 50, 100 and 500 μM) for a period of eight days had no influence on the enzyme level measured within the cells. In both cases, cells that were exposed or cells that were not exposed to the heavy metal showed an average total enzyme activity of 3.3 nkat/l medium and a specific activity of 4.2 pkat/mg protein. Under the conditions tested, a metal-induced increase in the level of the transpeptidase protein was not observed in the suspension culture cells.

3.3. *Stoichiometry of the transpeptidase reaction*

In order to determine the stoichiometry of the enzyme reaction, the transpeptidase (0.56 pkat) was incubated under standard conditions for 60 min at 35°C. Enzyme activity and incubation time were carefully chosen so that only (γ-Glu-Cys)$_2$-Gly was formed at the expense of glutathione during this period, after which substrate and products were quantitated. In a typical experiment applying these conditions, 1.95 nmol (by HPLC) or 2.25 nmol (by enzymatic assay [12]) of glutathione disappeared, 1.0 nmol (γ-Glu-Cys)$_2$-Gly (by HPLC) was formed and 0.95 nmol (by amino acid analysis) or 1.05 nmol (by radioactive assay) of liberated glycine were determined. The stoichiometry of the reaction is therefore:

2 (γ-Glu-Cys)-Gly → (γ-Glu-Cys)$_2$-Gly + Gly.

3.4. *Regulation of the enzyme by the reaction products*

Incubation of enzyme under standard conditions but in the absence of metal ions led to absolutely no formation of the phytochelatin peptides. Addition of Cd^{2+} to the incubation mixture instantaneously activated the enzyme (fig.3), (γ-Glu-Cys)$_2$-Gly was formed and after a lag period, the peptide containing three (γ-Glu-Cys) units was also synthesized. The formation of both peptides came to a halt, when all of the metal ions were complexed yielding a phytochelatin-sulfhydryl: metal ratio of about 2:1. This ratio was also determined for the complex isolated from metal-exposed plant cells [1]. A second addition of Cd^{2+} to the incubation mixture with terminated phytochelatin formation resulted in an additional synthesis of the penta- and heptapeptide, and after a lag, also nonapeptide (fig.3). The reaction under these conditions was continuous for 160 min, but prolonged incubation led also to unspecific oxidations upsetting the reaction balance. This experiment indicated, however, that phytochelatin synthesis was terminated when all the free metal ions necessary for transpeptidase activation were complexed by the (γ-Glu-Cys)$_n$-Gly peptides formed by the action of the enzyme.

In further support of this view, metal-free phytochelatins (100 μg), in the same peptide composition as they

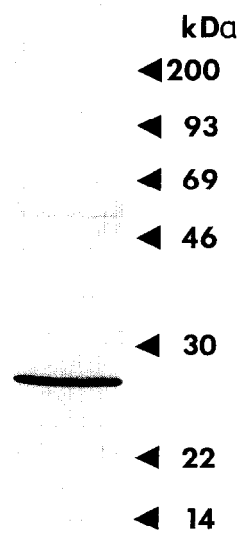

Fig.2. SDS-Polyacrylamide gel (11%) electrophoretic analysis of purified pytochelatin synthase visualized by silver stain. Position of molecular mass standards (in kDa) are indicated by arrowheads.

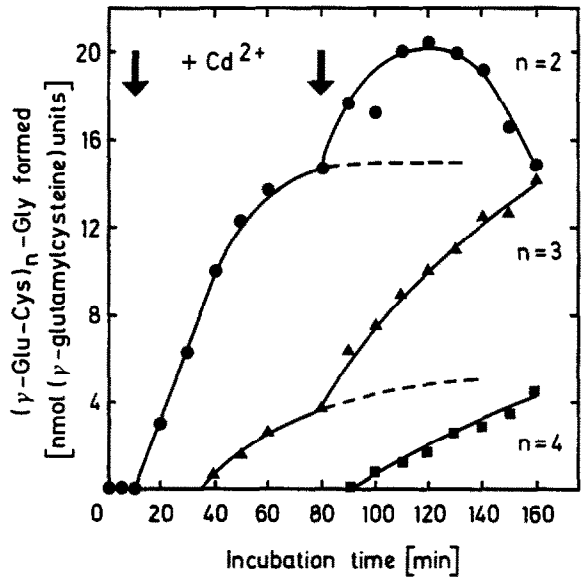

Fig.3. Formation of phytochelatins from glutathione with a purified enzyme preparation (2.4 pkat) of *S. cucubalus* (HPLC enzyme assay). Arrows indicate subsequent addition of 0.1 mM $Cd(NO_3)_2$ to the standard reaction mixture. Numbers of γ-glutamylcysteine units per phytochelatin molecule are indicated by n.

were isolated from metal-exposed *Rauvolfia* cells [2], were added to a metal-activated enzyme preparation synthesizing phytochelatin peptides. As shown in fig.4A, the addition of an uncomplexed phytochelatin mixture led to an immediate termination of the peptide synthesis. The same effect could be attained with the addition of metal-complexing Na-EDTA (8 μg). Both experiments indicate that phytochelatin synthase is activated by heavy metal ions, which are associated with the enzyme. The activating metal ions will successively be complexed by the product of enzyme catalysis, the phytochelatins. The enzymatic reaction is terminated when the enzyme-activating metals are completely removed by complexation with the phytochelatin peptide and are therefore inaccessible to the dipeptidyl transpeptidase. This system represents a self-regulating loop. The concentration of heavy metal ions and indirectly phytochelatin peptides govern the activity of phytochelatin synthase. The enzyme substrate, glutathione, is ubiquitously present in plant cells in millimolar concentrations and therefore should not limit the reaction.

The fact that phytochelatin peptides with differing chain lengths are found in plants [2,7] suggests that the binding constants of the individual peptides for heavy metals may differ depending on the chain length. In order to test this hypothesis and to gain further insight into the regulatory mechanism of phytochelatin synthase, the enzyme reaction was interrupted by the addition of various concentrations of pure and metal-free $(\gamma\text{-Glu-Cys})_2\text{-Gly}$ or $(\gamma\text{-Glu-Cys})_7\text{-Gly}$.

Fig.4B gives the results showing that for 50% inactivation of the transpeptidase by metal complexation 150 nmol pentapeptide and only 14 nmol pentadecapeptide were necessary. Based on a complex ratio of 2 sulfhydryl groups to 1 metal atom, the binding affinity of the pentadecapeptide should be only approximately 3.5 times higher over that of the pentapeptide. This is a first indication that the higher order phytochelatins have a higher relative complexing affinity than the lower order ones.

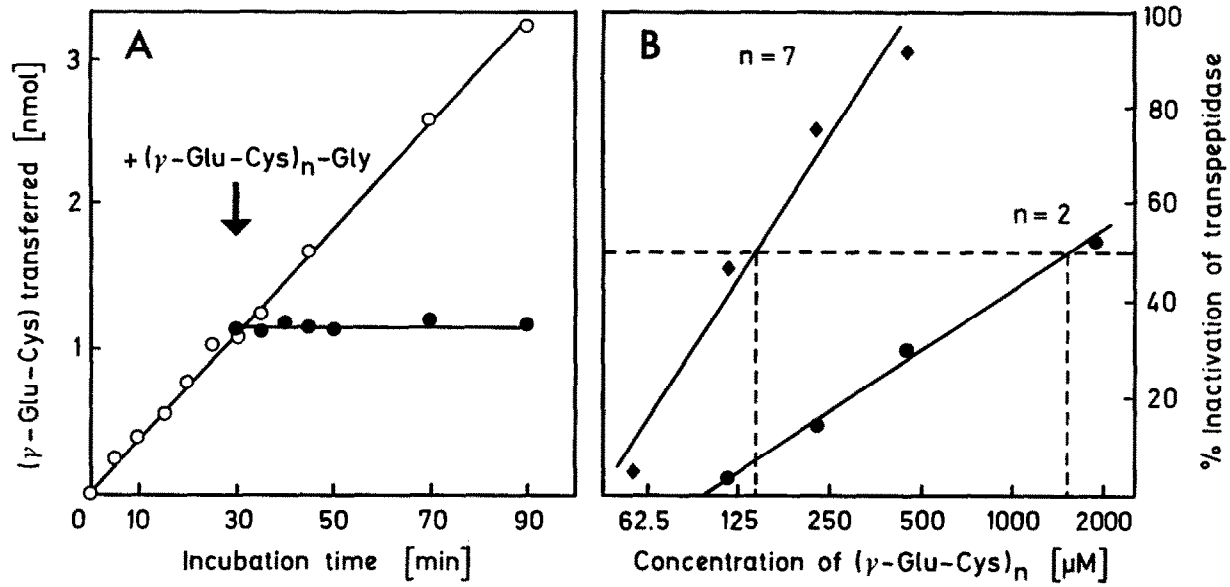

Fig.4. Termination of the transpeptidase reaction upon addition of metal-free phytochelatin. Radioactive enzyme assay with 0.1 mM *S*-monobromobimane-[2-^{3}H-Gly]-gluthatione and 0.61 pkat purified enzyme. (A) Immediate termination of metal-chelating peptide synthesis by addition of an uncomplexed phytochelatin mixture (100 μg). (B) Ability of metal-free $(\gamma\text{-Glu-Cys})_2\text{-Gly}$ and $(\gamma\text{-Glu-Cys})_7\text{-Gly}$ to inactivate phytochelatin synthase. 100% inactivation indicates that no further formation of phytochelatins could be observed 60 min after addition of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2 or n = 7).

The metal-complexing ability of plants through constitutive phytochelatin synthase is an efficient and well-regulated system which allows plants to cope with the heavy metal stress within certain physiological limits.

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