Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing Fabales

E. Grill, W. Gekeler, E.-L. Winnacker* and H.H. Zenk

Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstr. 29, D-8000 München 2 and **Genzentrum der Universität München, Am Kloperspitz, D-8033 Martinsried, FRG*

Received 27 June 1986

Exposure of several species of the order Fabales to Cd^{2+} results in the formation of metal chelating peptides of the general structure (γ-Glu-Cys)$_n$-β-Ala (n = 2–7). They are assumed to be formed from homo-glutathione and are termed homo-phytochelatins, as they are homologous to the recently discovered phytochelatins. These peptides are induced by a number of metals such as Cd^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, AsO_4^{2-} and others. They are assumed to detoxify poisonous heavy metals and to be involved in metal homeostasis.

Homo-glutathione *Heavy metal* *Detoxification* *Homo-phytochelatin*

1. INTRODUCTION

Phytochelatins (PCs) are peptides consisting of L-glutamic acid, L-cysteine and a carboxy-terminal glycine. These compounds, occurring in plants [1] and some fungi [2,3], possess the general structure (γ-Glu-Cys)$_n$-Gly (n=2–11) and are capable of chelating heavy metal ions. There is evidence that these compounds originate from glutathione by addition of γ-glutamyl-cysteine units to the starter molecule glutathione, resulting in linear peptides of up to 23 amino acids in length (n=11), the largest PC so far observed [4]. While screening the plant kingdom for the occurrence of PC synthesis, we noticed that plants from the order Fabales, especially the tribe Phaseoleae, when exposed to $Cd(NO_3)_2$, induced a series of compounds similar, but not identical to phytochelatins. Here we report on the amino acid sequence, structure and taxonomic distribution of these compounds. These peptides are homologous to the hitherto known phytochelatins but contain β-alanine instead of glycine and were named homo-phytochelatins. Up to now these peptides have been identified only in species of the order Fabales.

2. MATERIALS AND METHODS

2.1. *Growth of organisms*

Seedlings of *Glycine max* (soybean) grown for 3 days in continuous light were exposed for 4 days to 20 μM $Cd(NO_3)_2$ in Hoagland's solution [5] with strong (0.5 l/min) aeration. The roots (60 g fresh wt) were frozen in liquid N_2, ground to a fine powder and processed as described in [1,4] to yield 20 mg of purified Cd complex. Other plant species were also grown from seeds under the above mentioned conditions. Cell suspension cultures of *Phaseolus vulgaris, P. aureus* and *P. multifloris* were cultivated as described [1].

2.2. *Analytical methods*

Cd analyses were carried out by atomic absorption spectroscopy (Perkin-Elmer, flame mode). Semi-preparative HPLC separations were conducted according to [1,3] using a Nucleosil C-18 column (16×250 mm) and a linear gradient of 0–20% acetonitrile/H_2O in 0.05% phosphoric acid at a flow rate of 7.5 ml/min and detection at 220 nm. Analytical HPLC was performed under the reported conditions, but the sulfhydryl containing compounds were detected at 410 nm after

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

a post column derivatisation using 75 μM 5,5'-dithiobis-(2-nitrobenzoic acid) in 50 mM potassium phosphate buffer, pH 7.6 at a flow rate of 2 ml/min [4].

Performic acid oxidation and amino acid analysis were performed as described [1] using a Kontron model Liquimat III amino acid analyser.

The TLC analysis of amino acids was carried out bidirectionally, firstly, by electrophoresis as mentioned below and secondly, by chromatography using *n*-propanol/water, 70:30 as a solvent. Peptides were dinitrophenylated [1] and partially hydrolyzed (6 N HCl; 18 min; 95°C). Subsequently the resulting products were separated by two-dimensional chromatography (first direction: electrophoresis for 1 h, 20 $V \cdot cm^{-1}$ with solvent water/acetic acid/formic acid, 822:150:28; second direction: TLC, silica gel F 60, precoated plastic sheets (Merck, FRG), with solvent *n*-propanol/water/25% (w/w) ammonia, 40:10:15). Cysteine was oxidized by L-amino acid oxidase and glutamic acid was incubated with L-glutamic acid decarboxylase as prescribed by the supplier (Sigma, Munich). DCI-MS was conducted using a Finnigan MAT 44S quadrupole instrument.

3. RESULTS

3.1. *Identification of the metal-binding peptides*

Exposure of the roots of growing soybean plants to Cd^{2+} and subsequent analysis of the crude extract by gel filtration [1] demonstrated that 82% of the Cd^{2+} found in the plant tissue was associated with inducible, low molecular mass material. This heavy-metal-binding fraction was subjected to HPLC analysis and displayed a partition pattern similar to that of phytochelatins. Besides homoglutathione three major sulfhydryl rich compounds were detected, having a slightly retarded retention time as compared to the usual phytochelatins (fig.1A). The Cd complex was purified and the individual peptides were isolated by semi-preparative HPLC. Automated amino acid analysis of the hydrolyzed peptide revealed that these peptides are composed only of glutamic acid, cysteine and a nonprotein amino acid. The latter was isolated by preparative TLC and subjected to DCI-MS. The following mass peaks were recorded: 90($M+H^+$, 100%); 116($M+C_2H_3^+$, 46%); 128($M+C_3H_3^+$, 17%); 146($M+C_4H_9^+$, 23%) *m/z*. Co-chromatography by TLC and the elution pattern of the amino acid analysis proved this compound to be β-alanine. Incubation of glutamic acid and cysteine with L-glutamic acid decarboxylase and L-amino acid oxidase, respectively, established an L-configuration for these amino acids. The amino acid composition of the three peptides isolated from *Glycine max* was analysed quantitatively (fig.1A). The following Glu:Cys:β-Ala ratios were obtained: peak 2, 1.97:2.09:0.94; peak 3, 3.09:3.0:0.91; peak 4, 3.96:3.86:1.18. Hydrazinolysis of the peptides in each case yielded free β-alanine. Dinitrophenylation of peptide 2 and subsequent acid hydrolysis yielded exactly 1 mol dinitrophenylated glutamic acid per mol peptide. Treatment of the *S*-benzylated peptide with γ-glutamyl transferase [6] yielded 1 mol glutamic acid per mol peptide. The residual peptide was subjected to a modified Edman degradation [7] and yielded *S*-benzylated cys-

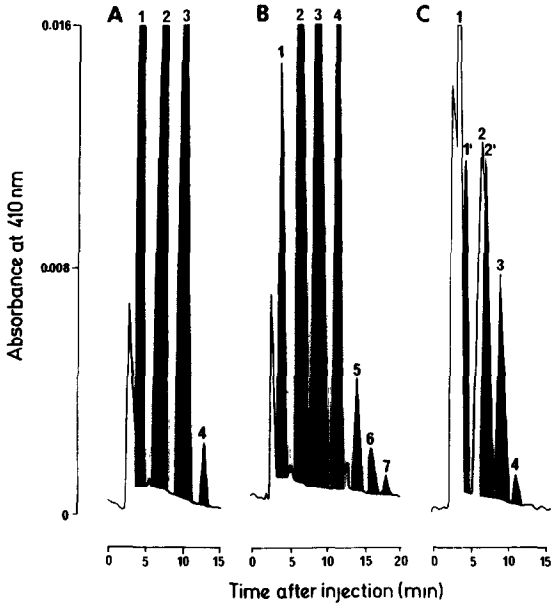

Fig.1. HPLC chromatogram of sulfhydryl group containing material from Cd^{2+} exposed plants. (A) *G. max*, (B) *P. aureus* (cell culture), (C) *L. odoratus*. Numbers signify the index *n* in $(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-Ala}$. n = 1, homo-glutathione; n = 2–7, homo-phytochelatins with varying (γ-Glu-Cys) units. Double peaks in C represent: 1 and 1', GSH and h-GSH; 2 and 2', PC_2 and h-PC_2. Non-metal exposed plants contain neither h-PC nor PC.

teine. By cyclic repetition of the γ-glutamyl transferase and Edman degradation treatments, the complete sequence of the peptide (peak 2) could be elucidated.

The two minor peptides were individually dinitrophenylated and oxidized with performic acid. Partial acid hydrolysates of the modified peptides were separated by two dimensional TLC revealing, respectively, a set of 6 and 8 partially hydrolyzed products. Quantitative analysis of these peptides afforded the expected dinitrophenylated peptide fragments.

From these experiments it can be concluded that the chain length pattern of these compounds is identical to the one observed in the phytochelatin series. The only difference, however, resides in the carboxy-terminus where β-alanine substitutes for glycine. The amino acid sequence and composition of the soja-derived metal-binding peptides is given in the general formula depicted in fig.2.

The term homo-phytochelatin (h-PC) is applied to this set of compounds containing β-alanine, as compared to the normal glycine containing phytochelatins (PCs), which represent the principal heavy-metal-binding peptides in the plant kingdom with the exception of the order Fabales [1].

3.2. *Distribution of homo-phytochelatins in higher plants*

Seedlings or cell suspension cultures of the order Fabales were exposed to 20 μM $Cd(NO_3)_2$ for 4 days, and the exclusive formation of h-PCs was observed in the following plants: *Phaseolus vulgaris, P. coccineus, P. aureus, P. lunatus, P. multifloris, Canavalia ensiformis, Cajanus cajan, Dolichos lablab, G. max, G. clandestina, Erythrina crista-galli, E. melanacantha, E. coralloides, Rhynchosia phaseoloides.* As an example fig.1B illustrates that the exposure of *P. aureus* to $Cd(NO_3)_2$ induces h-PCs with up to seven (γ-Glu-Cys) units (h-PC_7), as seen from the HPLC pattern

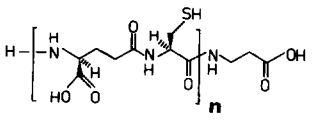

Fig.2. General formula for homo-phytochelatins. γ-Glu-Cys units from $n = 2$ to 7 were detected in plants of the order Fabales. $n = 1$, homo-glutathione.

of the peptide fraction. The M_r of the largest h-PC thus far observed is 1715.

h-PCs are the heavy metal chelators in all the homo-glutathione containing plant species up to now analysed. The members with increasing molecular mass are present in decreasing concentration as previously observed for the PC series [3,4].

The synthesis of h-PCs is induced in these plants by heavy metal ions such as AsO_4^{2-}, Cd^{2+}, Ga^{3+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Ag^+, Sb^{3+}. No induction of h-PC was recorded with Al^{3+}, B^{3+}, Ba^{2+}, Ca^{2+}, Ce^{3+}, Cs^+, K^+, Mg^{2+}, Mn^{2+}.

h-GSH has previously been discovered and characterized in *P. aureus* [8,9]. It is also present in mung bean [10] and has been suggested to occur in *G. max, P. limensis, P. vulgaris* and *Trifolium repens* [11]. The absence of glutathione (GSH) from the above mentioned Phaseoleae plants was verified by HPLC analysis. Instead of glutathione its β-alanine containing homologue, homo-glutathione (h-GSH; fig.2; $n = 1$) was isolated from each member of these plants and unequivocally identified.

Exposure of plants of the tribes Ononideae, Trifolieae, Vicieae, Astragaleae, and Loteae, all of the order Fabales, to Cd^{2+} revealed a peptide pattern which is exemplified by *Lathyrus ochrus* depicted in fig.1C. Clearly, double peaks could be perceived signifying the presence of both GSH and h-GSH (peak 1) as well as for PC_2 (peak 2). Separate analysis confirmed the assumption that in these plants both GSH and its β-alanine containing homologue (h-GSH) co-occur. This pattern is also applicable to the metal induced chelating peptides. Both sets of PC as well as h-PC are induced by Cd^{2+} in these plants. The following species of the order Fabales so far analysed revealed this mixed pattern: *Astragalus lusitanicus, A. cicer, A. gummifer, Cicer arietinum, Coronilla varia, Galega officinalis, L. ochrus, L. odoratus, L. silvester, Lens culinaris, Lotus ornithopodioides, Melilotus alba, Ononis natrix, Trifolium pratense, T. incarnatum, T. badium, T. subterraneum, T. lupinaster, T. pannonicum, Trigonella coerulea, T. foenum-graecum.*

4. DISCUSSION

Plants of the tribe Phaseoleae chelate a number

of heavy metals exclusively through the formation of peptides which consist of a linear chain of γ-bound L-glutamic acid, L-cysteine and a carboxy-terminal β-alanine only. These compounds were termed homo-phytochelatins.

Because of the γ-glutamyl linkages and presence of β-alanine, for which no triplet code exists, the h-PCs are not synthesized via mRNA and therefore cannot be regarded as primary gene products. The exclusive formation of h-PCs in h-GSH containing Fabales and the co-occurrence of peptides containing either glycine or β-alanine at the carboxy-terminus within the same plant species are indicative that the biosynthesis of PCs or h-PCs occurs by the consumption of GSH or h-GSH. As previously suggested for GSH, the tripeptide and its homologue seem to be the substrates for PC synthesis [1,3,4]. The occurrence of GSH or h-GSH in plants would thus determine PC or h-PC formation.

Analysis of plants from the primitive algae to the orchids as to their abilities to chelate heavy metals revealed that algae, ferns, gymnospermae and angiospermae have developed the mechanism to sequester heavy metals by PC formation. Other mechanisms have not been identified [1]. The ubiquity of PC as the heavy metal binding principle in plants is only limited by the presence of its homologue h-PC in some higher plants of the order Fabales, reflecting the complete or partial substitution of GSH by h-GSH in these plants. The tribe Phaseoleae exclusively contains homoglutathione. Therefore, h-PCs most probably represent the Cd-binding components noticed earlier to occur in soybean [12] and *P. vulgaris* [13]. Other tribes of the Fabales seem to be transient in that they contain both GSH and h-GSH, a fact that is displayed by the ability of these plants to synthesize both PCs as well as h-PCs upon exposure to heavy metals. The function of the PC and h-PC peptide series is apparently assigned to detoxification and homeostasis of heavy metals in plants.

ACKNOWLEDGEMENTS

This study was supported by Bundesminister für Forschung und Technologie, Bonn, and Fonds der Chemischen Industrie. The excellent technical assistance of Miss Susanne Kunz is gratefully acknowledged.

REFERENCES

[1] Grill, E., Winnacker, E.-L. and Zenk, M.H. (1985) Science 230, 674–675.
[2] Kondo, N., Isobe, M., Imai, K. and Goto, T. (1985) Agric. Biol. Chem. 49, 71–83.
[3] Grill, E., Winnacker, E.-L. and Zenk, M.H. (1986) FEBS Lett. 197, 115–120.
[4] Grill, E., Winnacker, E.-L. and Zenk, M.H. (1986) submitted.
[5] Hoagland, D.R. and Snijder, W.C. (1933) Proc. Am. Soc. Hortic. Sci. 30, 288–296.
[6] Orlowski, M. and Meister, A. (1965) J. Biol. Chem. 240, 338–347.
[7] Chang, J.Y. (1981) Biochem. J. 199, 557–564.
[8] Carnegie, P.R. (1963) Biochem. J. 89, 459–471.
[9] Carnegie, P.R. (1963) Biochem. J. 89, 471–478.
[10] Magnicol, P.K. and Bergmann, L. (1984) Plant Sci. Lett. 36, 219–223.
[11] Price, C.A. (1957) Nature 180, 148–149.
[12] Casterline, J.L. and Barnett, N.M. (1982) Plant Physiol. 69, 1004–1007.
[13] Weigel, H.J. and Jäger, H.J. (1980) Plant Physiol. 65, 480–482.