Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells

E. Grill, E.-L. Winnacker* and M.H. Zenk

Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-8000 München 2 and **Genzentrum der Universität München, D-8033 Martinsried, FRG*

Received 16 December 1985

The family of heavy metal-sequestering peptides with the formula $(\gamma\text{-Glu-Cys})_n\text{Gly}$; $n = 2$–8), the phytochelatins (PCs), are not restricted to higher plants. In *Schizosaccharomyces pombe* the occurrence of two of these peptides has previously been reported. Reinvestigation of the peptide pattern of the heavy metal-exposed fission yeast led to the discovery of five additional peptides, a pattern which now corresponds qualitatively exactly to the one in higher plants. From the kinetics of induction we propose that PCs are synthesized by consumption of glutathione or its biosynthetic precursor.

Phytochelatin *Heavy metal-binding peptide* *Induction kinetics* *Glutathione*

1. INTRODUCTION

Phytochelatins (PCs) are small sulfur-rich peptides that bind heavy metals [1,2]. They are composed of only 3 amino acids, namely L-cysteine, L-glutamic acid and glycine. Glutamic acid is linked to each cysteine by a γ-peptide linkage. Therefore the PCs cannot be regarded as primary gene products. The general structure of this set of peptides is $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n = 2$–7). PCs are induced upon exposure of plants to heavy metals such as Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Pb^{2+}, Zn^{2+} and are found in all plant species thus far analysed belonging to the Angiospermae and Gymnospermae. The hitherto assumed metallothioneins [3] do not exist in higher plants [1]. In fungi, however, metallothioneins do occur and are thought to be involved in the detoxification of heavy metals and in zinc homeostasis [4]. In the yeast *Saccharomyces cerevisiae* the copper-binding metallothionein was studied and the gene cloned [5]. No phytochelatins exist in *S. cerevisiae* (unpublished). Surprisingly, 2 peptides were recently isolated which had the structures of $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n = 2$ and 3), from the fission yeast, *Schizosaccharomyces pombe*, after exposure of this organism to Cd^{2+} [6]. They were named cadystin A and B and their structures were derived after several revisions [6].

Here we report the occurrence of a whole set of heavy metal-complexing peptides in *S. pombe* which were known previously from higher plants. In addition to cadystins A and B, corresponding to our phytochelatins $n = 2$ and 3, we were able to detect 5 additional homologous peptides with chain lengths ranging from $n = 4$ to 8. A qualitative comparison of the PC induction pattern between the fission yeast and higher plants showed that both display the same spectrum of PCs. The kinetics of induction of the different PCs in the yeast and plant support the suggestion [1] that the PCs are synthesized by elongation of the peptide with one (γ-Glu-Cys) unit at the expense of and possibly starting from glutathione. In contradiction to the name given for the cadystins [6], several metals aside from Cd^{2+} are able to induce these heavy metal-chelating peptides in the fission yeast.

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

2. MATERIALS AND METHODS

2.1. *Growth of organisms*

S. pombe L 972 (h^-) was cultivated in medium containing 2% tryptone, 2% glucose and 1% yeast extract with aeration at 30°C. Unless otherwise indicated, PC synthesis was induced by administering $Cd(NO_3)_2$ to a final concentration of 1 mM to a logarithmic growing culture. For preparative scale a 20 l fermentation was employed and the yeast was harvested by centrifugation after 24 h of Cd^{2+} exposure. Cell suspension cultures of *Rosa canina* were cultivated as reported [1].

2.2. *Isolation of chelating peptides*

Yeast cells (100 g) suspended in 50 ml of 0.1 M Tris-HCl (pH 8.0) were homogenized with glass beads (∅ 0.18 mm) in a cell grinder (Vibrogen, Bachofer, FRG). The extract was centrifuged (9000 × *g*, 30 min), the supernatant diluted with deionized water to 0.5 l, and the PC complex purified as in [1]. To sequence the individual peptides, fractionation was achieved on a preparative scale by HPLC (column 16 × 250 mm, Nucleosil C-18, Macherey and Nagel, FRG) with a linear gradient of 0–20% acetonitrile/H_2O in 0.05% phosphorous acid in 40 min at a flow rate of 7.5 ml/min and detection at 220 nm.

2.3. *Analytical procedure*

The PC content of plant and yeast cells was directly determined in crude extracts by HPLC analysis. Plant cells (0.40 g fresh wt) and sedimented yeast cells from 1–3 ml culture were suspended in 0.4 ml degassed water and lysed by addition of 0.1 ml of 2.5 N NaOH containing 1 mg $NaBH_4$ per ml. Plants were frozen in liquid nitrogen, homogenized with a mortar and pestle and the homogenate further sonified. The extract was acidified with 0.1 ml of 3.5 N HCl, cooled in ice for 10 min and after centrifugation the supernatant subjected to HPLC (4.6 × 250 mm, conditions as above except flow rate 2 ml/min and a gradient of 20 min). Purified PC was analysed in the same manner. The sequence of the purified peptides PC n = 2–8 was deduced as in [1].

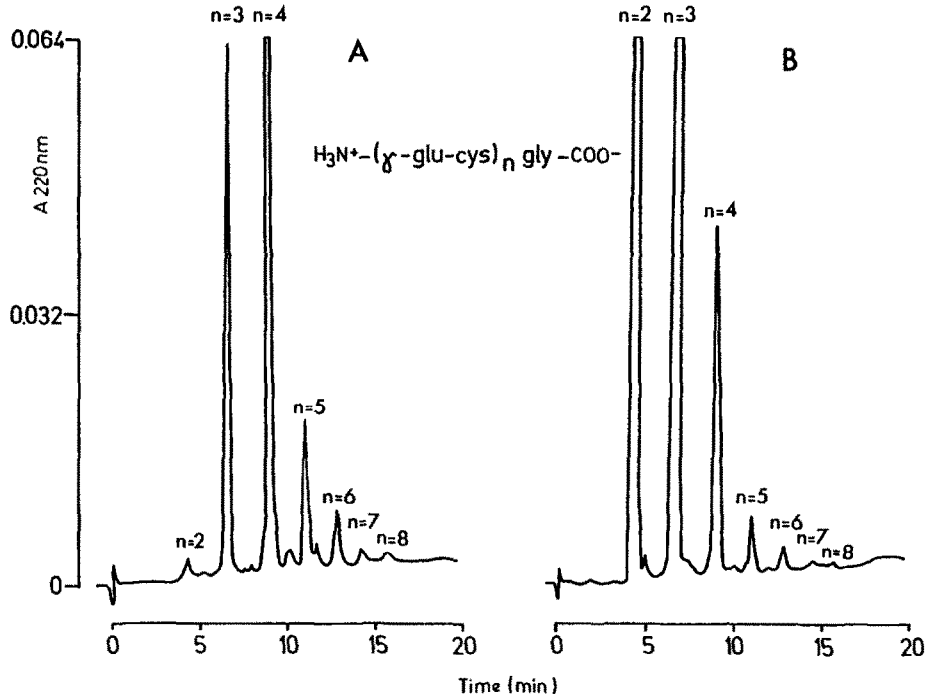

Fig.1. HPLC elution profile of purified phytochelatin complex from the yeast *S. pombe* (B) and the plant *R. canina* (A).

3. RESULTS

3.1. *Comparison of induction pattern*

When plant suspension cells or differentiated plants were exposed to solutions of Cd^{2+}, PCs were instantaneously induced [1,2] and a qualitative PC pattern was observed which seemed to be identical in all higher plants which have been investigated so far. The qualitative pattern of the different classes of PC molecules, however, depended on the time of exposure and the concentration of the metal. When suspension cells of *R. canina* were exposed to 200 μM $Cd(NO_3)_2$, harvested after 4 days, the PC purified and subjected to HPLC separation, an elution profile emerged which is shown in fig.1A. In this case, a PC species containing 4 (γ-Glu-Cys) pairs (PC_4) was the dominant peptide as observed in [1], followed by $PC_{3,5,6,7,8}$, respectively. PC_2, which differs from glutathione only by one additional (γ-Glu-Cys) unit, was present only in small amounts under these experimental conditions, as were the higher members of the PC family. A similar experiment conducted with the fission yeast *S. pombe* where cells were exposed to 1 mM $Cd(NO_3)_2$, harvested 24 h later and subjected to the same type of analysis gave the profile shown in fig.1B. The qualitative pattern of PC induction was the same; however, the dominant PCs were PC_2 in addtion to PC_3 observed earlier [6]. $PC_{4,5,6,7,8}$ were observed here for the first time to occur in a fungal organism. Isolation and purification of the peptides by preparative HPLC and subsequent sequencing established beyond doubt the occurrence of the higher members of the PC family in this organism as well.

3.2. *The induction of PC formation in fission yeast*

To gain information concerning the sequence of formation of peptides with different chain lengths (whether they are degradation products of a high-M_r precursor peptide, are formed de novo, and require a peptide starter such as glutathione) a kinetic experiment was performed. The fission yeast cells were grown in nutrient broth and after an A value of 1.6 was reached (7 h), $Cd(NO_3)_2$ was added. Compared to control cells, those cells which were exposed to the heavy metal showed a slight depression in growth rate. While the control cells did not show any PC content, a rapid synthesis of PC_2 without any noticeable lag phase, followed with some delay by PC_3 and PC_4, was observed as shown in fig.2A. This pattern of induction rules out the degradation of a high-M_r peptide into smaller units. In a second type of experiment the *S. pombe* cells were exposed to different concentrations of Cd^{2+} ranging from 0 to 4 mM. The cells were harvested after 48 h of exposure to the heavy metal and analysed for PC. In this experiment the level of glutathione was also monitored simultaneously with the PCs. As shown in fig.2B, there was a drastic decrease in GSH content in *S. pombe* cells with increasing heavy metal concentration indicating consumption of glutathione or its precursor for PC synthesis. This phenomenon has already been observed with plant cells, where within 3 h after Cd^{2+} exposure the glutathione content dropped almost to 40% of the initial concentration accompanied by a concomitant increase of (γ-Glu-Cys) units incorporated into PC [7]. In addition, a potent inhibitor of glutathione biosynthesis, buthionine sulfoximine [11], was administered at a concentration (500 μM) which did not affect growth of plant cells for 24 h. Subsequent exposure to 200 μM Cd^{2+} showed PC induction was almost completely abolished (< 10%) when compared to non-inhibited cells [7].

As shown in fig.2B, PC_2 and PC_3 were the dominant PC peptides at all concentrations of Cd^{2+}. Between 1 and 2 mM Cd^{2+}, both PC_3 and PC_4 decreased in concentraion while PC_2 still increased. It should be noted that the data were plotted as molar concentration of PC. If the plot were based upon the number of (γ-Glu-Cys) units incorporated into a PC species, PC_2–PC_5 would increase by a factor of 2–5, respectively. The pattern of the induced PC seems to be also dependent on the growth phase of the organism. As depicted in table 1, if Cd^{2+} (1 mM final concentration) was added very early in the growth phase (A_{660} 0.5) of *S. pombe*, with exposure of 24 h, only PC_2 (90 mol%) and a small amount of PC_3 (10 mol%) were formed; the higher members of the PC family were absent. However, if the heavy metal was added at a late stage of the growth phase of this yeast (A_{660} 4.5), exposed and analysed in the same way, the cells produced all of the known PCs with PC_3 being the dominant (65 mol%) peptide. From these experiments (fig.2A,B, table 1) it can be concluded

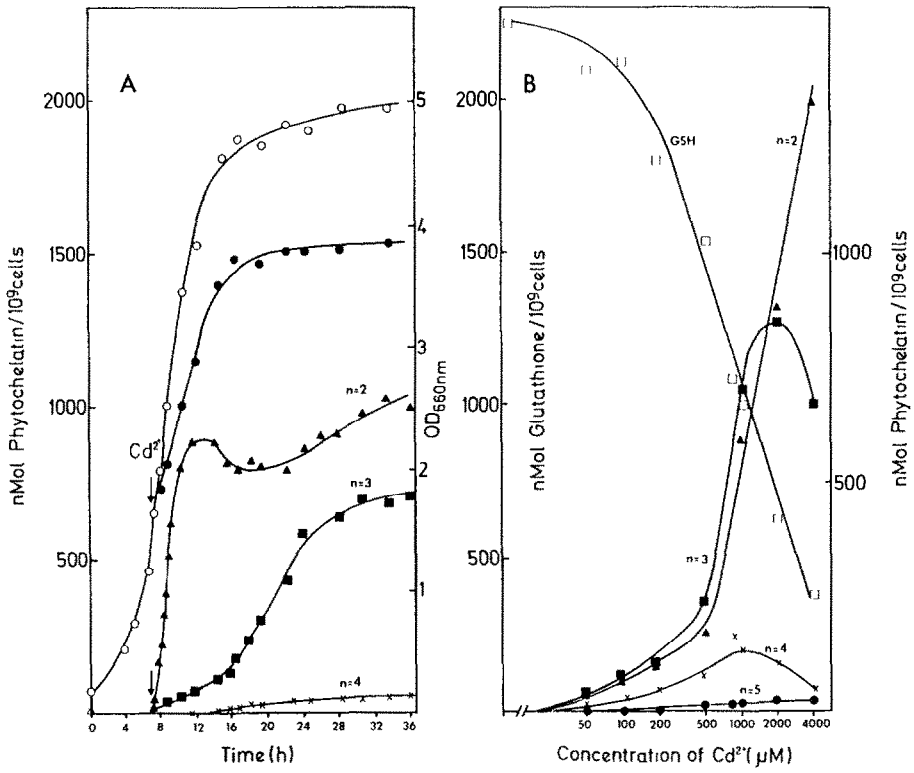

Fig.2. (A) Time course of phytochelatin induction and growth of control (○—○) and Cd^{2+}-exposed cells (●—●). Phytochelatin was absent in control cells. The PC species is indicated by the index number *n* (see fig.1). (B) Cd^{2+} concentration dependence of glutathione and phytochelatin content. Cells were analysed for PC content 48 h after Cd^{2+} exposure.

that 1–2 mM Cd^{2+} will maximally induce metabolism of the yeast cells towards PC synthesis and PC_2 seems to be the first product of this synthesis. Most probably the PCs are synthesized at the expense of glutathione and/or its precursor. However, the loss from the GSH pool observed (fig.2B) cannot compensate for all the PC synthesized. If PC synthesis uses GSH a drastic stimulation of GSH synthesis must be occurring in these cells to account for the amount of PC formed during metal induction.

3.3 *Different metals as inducers of PC*

In the case of *S. pombe*, Cd^{2+} was the sole heavy metal previously used to induce PCs [6]. We observed with higher plant cells that PCs were in-

Table 1
Dependence of phytochelatin pattern on growth phase

Growth phase	$A_{660\ nm}$		nmol PC/10^9 cells (mol% PC)						
	Cd addition	Harvest	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7	PC_8
Early logarithmic	0.50	1.76	835 (89.2)	96 (10.3)	5 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)
Stationary	4.5	4.6	170 (16.4)	674 (65.1)	175 (16.9)	12 (1.2)	3 (0.3)	1 (0.1)	0.5 (0.05)

Table 2
Induction of phytochelatins by heavy metal ions

Compound	Concentration (μM)	nmol PC/10^9 cells			
		PC_2	PC_3	PC_4	PC_5
$Cd(NO_3)_2$	200	148	303	46	11
$Bi(NO_3)_3$	200	80	24	10	1
Na_2HAsO_4	20	8.4	7.8	0	0
$CuSO_4$	200	7.0	5.2	0	0
$Pb(NO_3)_2$	2000	6.3	4.8	0	0
$Zn(NO_3)_2$	2000	5.6	4.3	0	0
$AgNO_3$	20	4.8	4.3	0	0
KNO_3	2000	0	0	0	0
Na_2SO_4	200	0	0	0	0
Control	0	0	0	0	0

duced by a surprisingly large number of metals [7], well exceeding the range of metals observed for metallothionein induction in mammals and fungi [4]. To test the ability of the fission yeast cells to respond towards various metal stimuli by PC formation, cells were exposed to about 20 metal cations (as their nitrate salts) and 3 anions, at different concentrations. As shown in table 2, Cd^{2+} proved to be the strongest inducer of PC in this yeast, followed by Bi^{2+}, AsO_4^{3-}, Cu^{2+}, Pb^{2+}, Zn^{2+}, and Ag^+. Since our yeast strain grew fully only in a complex organic medium some interactions of metals with the medium were observed. Therefore some metals not listed here may in the future also prove to be active in inducing PC synthesis. However, it is important to note that not only Cd^{2+} but also several metals or metal derivatives are able to induce clearly PC synthesis in the fission yeast.

4. DISCUSSION

As has been clearly demonstrated here, the family of heavy metal-sequestering peptides, the PCs, which range from M_r 559 ($n = 2$) to M_r 1931 ($n = 8$), are not restricted to higher plants. It is surprising that unlike the yeast *Saccharomyces* which follows the animal pattern of complexing heavy metals via genuine metallothionein, the fission yeast *S. pombe* uses a pathway hitherto only found in higher plants. PC_2 which we originally did not observe because of the addition of metal to late log or stationary phase plant cell cultures [1,2] is now clearly found both in plant suspension cells as well as in differentiated plants (fig.1A; and unpublished). Exposure of young meristematic tissue or of plant cell cultures during early growth phase to Cd^{2+} induced PC_2 as the dominant peptide as demonstrated for the fission yeast (table 1). Kinetic experiments on the rate of synthesis of the individual peptide monomers within *S. pombe* after exposure to Cd^{2+} demonstrated that most probably PC_2 is the first of the peptides formed and transfer of a (γ-Glu-Cys) unit directly or from one glutathione onto another should be feasible. PCs in *S. pombe* are not only induced by cadmium, but the peptide synthesis is also induced at least by Bi^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Ag^+ and AsO_4^{3-} as well. Also in this respect, *S. pombe* behaves like the higher plant system [1] rather than the phylogenetically more closely related *Sac. cerevisiae*. PCs are induced by Cd^{2+} and other metals, show a high capacity for Cd binding, and contain a high amount of cysteine residues. These properties, however, do not qualify them to be 'metallothionein'-like [9]. They may have a functional resemblance to metallothioneins, but the occurrence of only 3 different amino acids and the repetitive occurrence of the γ-glutamyl bond definitively distinguishes them completely metallothioneins. These peptides are a new class of natural compounds. For the peptides with 3–8 (γ-Glu-Cys) units we have proposed the name phytochelatins [1,2,8]; the members containing 2 and 3 units had the previously applied name cadystin [6]. Since both peptides are induced by other metals in addition to Cd^{2+} and since PC_2 appears to be the biosynthetic precursor of the phytochelatin family, and since their predominant occurrence is in the plant kingdom comprising more than 300 000 species, we propose the name phytochelatin for these members as well. The abbreviation will be PC and the index number will give the number of (γ-Glu-Cys) units per molecule. Up to now, 9 members of phytochelatins (PC_{2-10}) have been detected in higher plants [7].

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. Our thanks are due to Dr T.M. Kutchan for linguistic help in preparing this manuscript. We also thank Professor A. Meister for suggesting the use of buthionine sulfoximine.

REFERENCES

[1] Grill, E., Winnacker, E.-L. and Zenk, M.H. (1985) Science 230, 764–766.
[2] Grill, E., Zenk, M.H. and Winnacker, E.-L. (1985) Naturwissenschaften 72, 432–434.
[3] Rauser, W.E. (1984) J. Plant Physiol. 116, 253–260.
[4] Kägi, J.H.R. and Nordberg, M. (1978) Metallothionein, Birkhäuser, Basel.
[5] Butt, T.R., Sternberg, E., Herd, J. and Crooke, S.T. (1984) Gene 27, 23–33.
[6] Kondo, N., Isobe, M., Imai, K. and Goto, T. (1985) Agric. Biol. Chem. 49, 71–83.
[7] Grill, E., Winnacker, E.-L. and Zenk, M.H. (1986) in preparation.
[8] Grill, E. (1985) in: Metallothionein (Kägi, H.J.R. and Nordberg, M. eds) Birkhäuser, Basel.
[9] Murasugi, A., Wada, C. and Hayashi, Y. (1981) Biochem. Biophys. Res. Commun. 103, 1021–1028.
[10] Linsmaier, E.M. and Skoog, F. (1965) Physiol. Plant. 18, 100–127.
[11] Griffith, O.W. and Meister, A. (1979) J. Biol. Chem. 254, 7558–7560.