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ENZYMIC SYNTHESIS OF PHYTOCHELATINS IN GRAM QUANTITIES*

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Key Word Index—*Silene vulgaris*; Caryophyllaceae; phytochelatin peptides; phytochelatin synthase.

Abstract—A procedure for the concentration of the enzyme phytochelatin synthase from a crude homogenate of *Silene vulgaris* suspension cells is described. This enzyme catalyzes the formation of the heavy metal complexing phytochelatins from glutathione. Optimization of the incubation parameters (glutathione and Cd^{2+} concentration, pH, temperature, time, antimicrobial agents) resulted in conditions that yielded 35 g of phytochelatin peptides $(\gamma\text{-glu-cys})_n\text{-gly}$ (n = 2–6) per liter incubation mixture during a 55 day period. The time-space yield was 633 mg $PC_n \times l^{-1} \times day^{-1}$. The isolation of total and individual phytochelatin peptides by semipreparative HPLC is described.

INTRODUCTION

Phytochelatins (PCs) are the principal heavy metal-detoxifying compounds of plants [1–3]. They are small, cysteine-rich peptides of the general structure $(\gamma\text{-glu-cys})_n\text{-gly}$ (n = 2–11), capable of binding heavy metal ions, i.e. elements with a specific weight of higher than $5\ g \times cm^{-3}$, by thiolate coordination [4]. It has clearly been demonstrated that glutathione (GSH) is the substrate for the enzyme catalyzing the formation of PCs in the presence of select heavy metal ions [5]. This constitutive enzyme, a γ-glutamylcysteine dipeptidyl transpeptidase, was named phytochelatin synthase and catalyzes the following reaction: $(\gamma\text{-glu-cys})_n\text{-gly} + (\gamma\text{-glu-cys})_n\text{-gly} \rightarrow (\gamma\text{-glu-cys})_{n+1}\text{-gly} + (\gamma\text{-glu-cys})_{n-1}\text{-gly}$, where n = 1, 2, 3 etc. Besides PCs, in selected plant families certain *iso*-forms of these peptides exist, that contain terminally located different amino acids, replacing glycine by β-alanine [5], serine [6] or glutamic acid [7]. These forms were called *iso*-phytochelatins (*iso*-PCs) because of their same function in heavy metal detoxification [3].

In order to have available standards for biochemical, physiological and ecological research and to render possible pharmacological experiments with this class of compounds as well as to investigate more closely the binding reactions between PCs and heavy metal, it was desirable to explore the possibility of generating PCs of different chain length by an enzymic process on a preparative scale. Although chemical synthesis for PCs is available [8–10], this process is lengthy and expensive. The present study shows that by means of the PC synthase obtained from a *Silene vulgaris* cell culture [11], which fortuitously contains the highest amount of this transpeptidase among several cell culture species tested, allowed the enzymic generation of PCs with 2 to 6 $(\gamma\text{-glu-cys})$-units in a range of $35\ g \times l^{-1}$.

RESULTS AND DISCUSSION

A vigorously growing strain of *S. vulgaris* was used throughout this work. After 7 days of cultivation the cells (late linear phase) were shock frozen with liquid nitrogen and could then be stored at −20° for several months. After extraction, the PC synthase activity was in the range of 25 nkat per kg fresh weight. Other storage methods yielded much inferior enzyme activities (< 20%). Deep frozen cells (2 kg) were thawed and extracted by stirring at pH 8.5 in the presence of mercaptoethanol which is necessary to retain the enzyme activity. The resulting brei was centrifuged, the supernatant filtered and $(NH_4)_2SO_4$ added to a final concentration of

*Dedicated to Prof. H. Nöth, München, on the occasion of his 70th birthday.

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15%. This extract was applied to a Phenylsepharose column which absorbed the protein. After washing the column, the enzyme was eluted with 10% ethylene glycol and the eluate fractionated. The fractions containing the enzyme were dialyzed overnight and this crude enzyme preparation was used for the synthesis of PC peptides. Compared to the initial crude cell extract, the enzyme yield was about 50% with an enrichment factor of *ca* 1.75 and a 13-fold concentration of the volume. In this manner, the protein fraction containing the PC synthase activity was freed of interfering low-Mr compounds.

When, using a spindle press and a filter battery, the enzyme activity yield was about 60%, but this procedure was only used for the preparation of larger amounts (several kg of cells) of enzyme.

Cd^{2+} was used throughout this investigation since it is by far the strongest activator of the enzyme [3]. To optimize the yield of PCs from GSH in the presence of Cd^{2+}, experiments were conducted using *ca* 100 ml incubation mixtures and individual PCs were analysed after HPLC separation [12]. First, the GSH concentration was optimized. As shown in Fig. 1, a 6 mM GSH concentration saturates the reaction under standard conditions at 0.8 mM $Cd(NO_3)_2$. The conversion rate of GSH to PC at that concentration was measured over a 5 day period. Reducing the GSH concentration to 3 mM resulted in almost 60% conversion and this concentration was finally adopted.

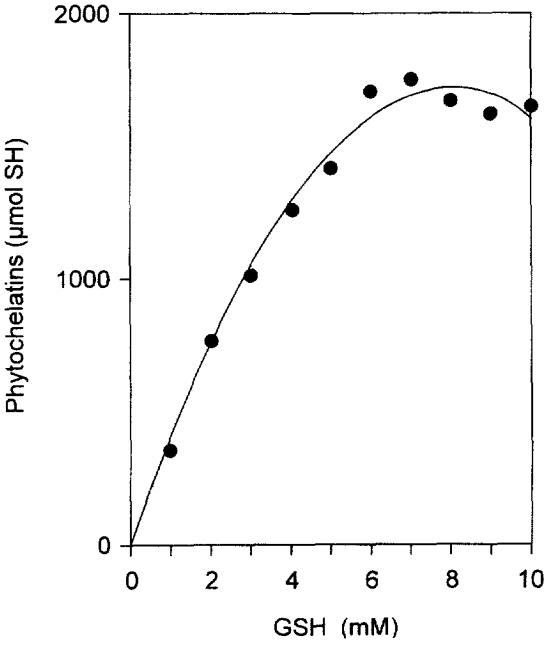

Fig. 1. Enzymic synthesis of phytochelatins in the presence of different glutathione concentrations. The substrates, 1–10 mM GSH and 0.8 mM $Cd(NO_3)_2$, were added daily to the incubation mixture consisting of 1800 pkat PC synthase, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, and 0.02% NaN_3, pH 8.0, in 115 ml. Incubation was for 5 days at 25°.

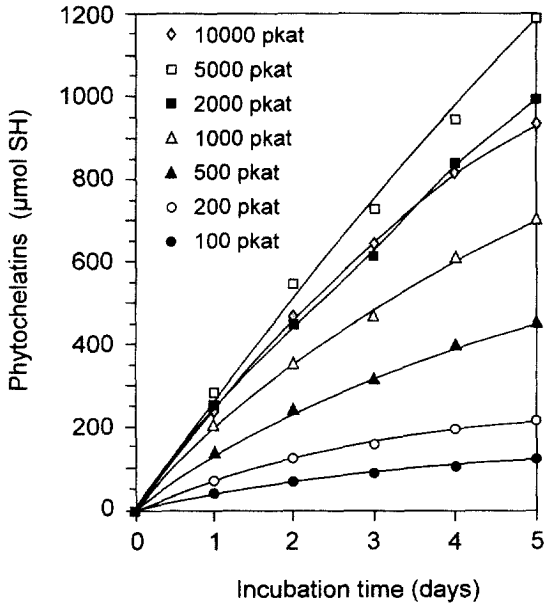

Fig. 2. Enzymic synthesis of phytochelatins in the presence of various amounts of PC synthase at a constant GSH concentration (1 mM). The incubation mixture contained, in addition, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, 0.02% NaN_3, pH 8.0, in 115 ml. Incubation was for 5 days at 25°.

The next parameter to be varied was the enzyme concentration. Figure 2 clearly shows that 5 nkat of enzyme in 115 ml volume resulted in the highest formation of PCs while doubling the enzyme concentration to 10 nkat decreased the yield. Interestingly, increasing the GSH concentration to 6 mM and adding an optimal 5 nkat to this preparation yielded about 10% less PC, therefore, 3 mM GSH was used for the peptide synthesis.

In preliminary experiments, it was found that the ratio of 4 GSH to 1 Cd^{2+} was optimal for the enzymic conversion to PCs. This 4: 1 ratio was kept constant but the concentration of GSH was varied in the given volume. Careful analysis showed that under these conditions the increase of GSH to 12 mM could increase the yield of PCs about one third. However, the chain length of PCs was strongly influenced. While in the presence of 3 mM GSH and 0.8 mM Cd^{2+} the desired variety of PC_{2-5} and some PC_6 was produced (Fig. 3), 12 mM GSH and 3 mM Cd^{2+} yielded exclusively PC_2 (85%) and PC_3 (15%). In order to allow the production of the higher chain length peptides PC_{4-6}, 3 mM GSH and 0.8 mM $Cd(NO_3)_2$ were used for subsequent incubations [the anions Cl^-, SO_4^{2-}, CH_3COO^- had no influence on the enzyme reaction].

To determine the optimal pH and temperature for the reaction, these parameters were varied over an incubation period of 10 days. (Fig. 4A) and (Fig. 4B) demonstrate the pH and temperature range of the enzyme with a pH optimum of 8.0 and

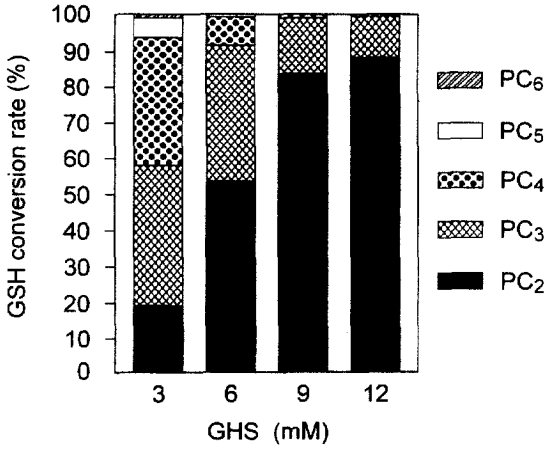

Fig. 3. Biosynthesis of individual phytochelatin species in the presence of different GSH concentrations at a constant GSH: Cd^{2+} ratio of 4: 1. The incubation mixture further consisted of 5000 pkat PC synthase, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, 0.02% NaN_3, pH 8.0, in 115 ml. Incubation was for 5 days at 25°.

an optimal temperature of 20–25° for the formation of PC species. Analysis of the chain lengths showed that pH 7.0 favoured PC_2 formation (65%) while pH 8.0 resulted in the desired variety of PCs (PC_2: 35%; PC_3: 47%; PC_4: 17%; PC_5: 1%). Slightly more alkaline conditions (pH 8.5) yielded less PC_4 (10%) and again more PC_2 (43%). It is noteworthy to mention that the pH value of the incubation mixture did not change during the reaction, therefore, buffers were not necessary. The aqueous solution was adjusted to the desired pH with NaOH. The finally adopted incubation mixture consisted of 3 mM GSH, 0.8 mM $Cd(NO_3)_2$, 0.01% NaN_3, 50–150 nkat phytochelatin synthase at pH 8.0 in a final volume of 1 liter at 25°.

During preliminary experiments it had been observed that the enzyme is quite stable at room temperature. On prolonged incubation, however, the content of the flask became turbid. Obviously, anaerobic bacteria had developed releasing sulphur-containing volatile compounds thus causing a rapid inactivation of the enzyme reaction. To prevent microbial growth, different bacteriostatic agents were tested, taking care not to interfere with the work-up of the peptides formed. NaN_3 was found to be the most appropriate agent. At 0.001 to 0.005%, this compound had little effect on the conversion of GSH to PCs. For an efficient control of the microbial growth, however, 0.01% NaN_3 had to be used and a penalty of about 10% less PC yield had to be taken into account (using 100 nkat $\times l^{-1}$ of PC synthase). Further increase to 0.5–1% NaN_3 completely inactivated the enzyme reaction.

The kinetic study under optimized conditions shown in Fig. 5 demonstrates a linear formation of total PCs for 55 days. After that time, 35 g $\times l^{-1}$ PC_n were synthesized. It should be stressed that for the achievement of this yield 3 mM GSH and 0.8 mM Cd^{2+} had to be added every day. Furthermore, every 10 days NaN_3 was added in sufficient amounts to give a 0.01% solution for the inhibition of microbial growth.

Figure 6 shows the kinetic investigation of individual PCs formed during the time course depicted in Fig. 5. Only 70 to 80% of the GSH present was converted to PCs. After 5 days of incubation, PC_4 was the most abundant PC (as originally noticed *in vivo* [13]) but after 55 days, PC_2 became the most prominent. It is also remarkable that a small amount (*ca.* 1%) of PC_6 was present during the first 20 days together

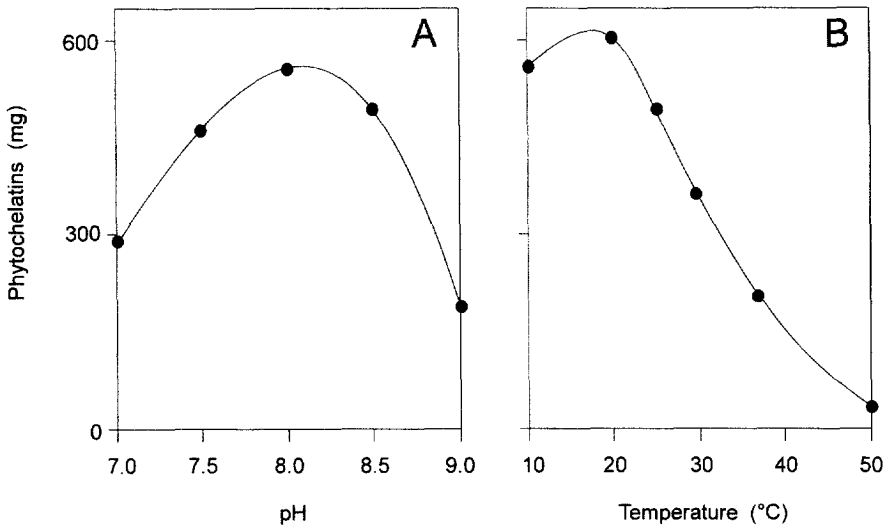

Fig. 4. The effect of pH (A) and temperature (B) on the enzymic synthesis of total PCs. The incubation mixture consisted of 5000 pkat PC synthase, 10 mM β-mercaptoethanol, 3 mM GSH, 0.8 mM $Cd(NO_3)_2$, and 0.02% NaN_3, pH 8.0, in 115 ml. Incubation was for 10 days at 25°.

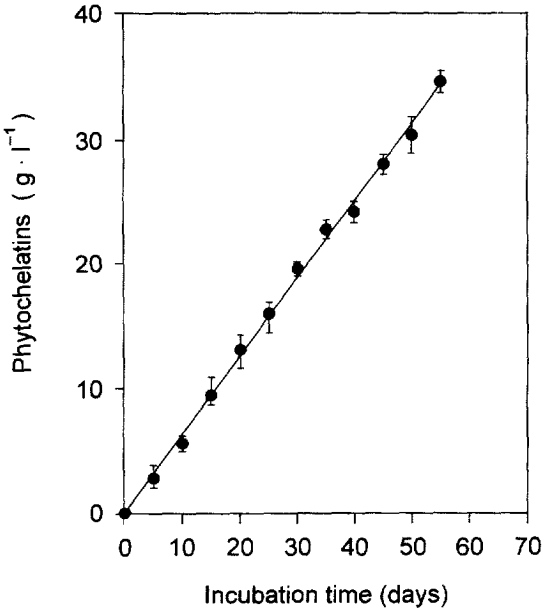

Fig. 5. Enzymic production of total phytochelatins under optimized conditions. The incubation mixture consisted of 150 nkat PC synthase, 10 mM β-mercaptoethanol, 3 mM GSH, and 0.8 mM $Cd(NO_3)_2$, pH 8.0, in 1120 ml. Every 10th day, 0.01% NaN_3 was added and the mixture incubated at 25°. The data shown are the average of three independent experiments.

with a more substantial amount of PC_5, but with time the concentration of these long chain PCs diminished.

In summary, with 150 nkat of PC synthase per liter of incubation mixture a yield of 35 g× l^{-1} total PCs was achieved during 55 days while *ca* 70% GSH was converted, which indicates a space-time yield of 633 mg $PC_n \times l^{-1} \times day^{-1}$.

The final goal was a simple isolation procedure for total PCs and also individual PC species. A preliminary, two-step separation method was developed to recover most of the untransformed GSH and to obtain a pool of total PCs. The incubation mixture containing all ingredients including the protein and 35–38 g of PCs in a volume of 1.1 l was added to a Dowex 1 × 2 column (acetate form). After washing the column with water, the bulk of GSH (for reuse) as well as Cd^{2+} was removed with acetic acid and the PCs subsequently eluted with HCl. The PCs-containing eluate was neutralized and freeze dried yielding an average of 90% PCs (from eight 1 l work-ups). About 10–15 g of freeze dried PCs were taken up in 100 ml dilute HCl and loaded onto a RP-18 Silica gel column. The column was washed with water to elute the salts and residual GSH. Subsequently, the PCs were eluted in one step containing 50% acetonitrile and recovered by evaporation of the solvent and freeze drying with a yield of 70%. The Cd^{2+} content of the PCs was less than 0.008%.

In order to separate the mixture of total PCs into the individual PC species with different chain length, 250 mg lots of the PC mixture were subjected to semipreparative HPLC on RP-18 or Nucleosil C-18 columns using a phosphoric acid [14]

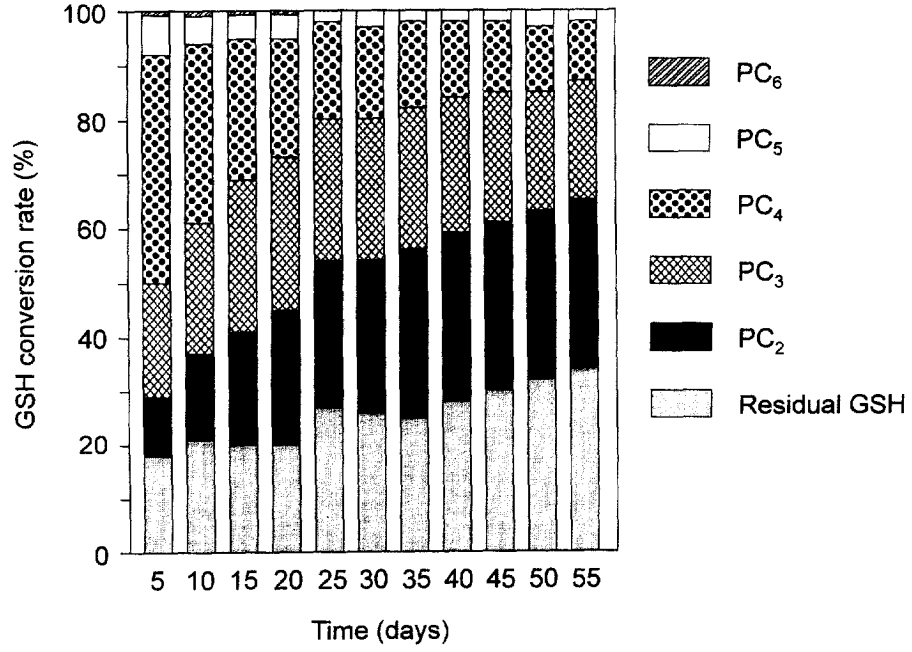

Fig. 6. Conversion rate of GSH into individual PCs as a function of incubation time under optimized conditions. The incubation mixture consisted of 150 nkat PC synthase, 3 mM GSH, and 0.8 mM $Cd(NO_3)_2$, pH 8.0, in 1 l. Every 10th day, 100 mg NaN_3 was added and the mixture incubated at 25°.

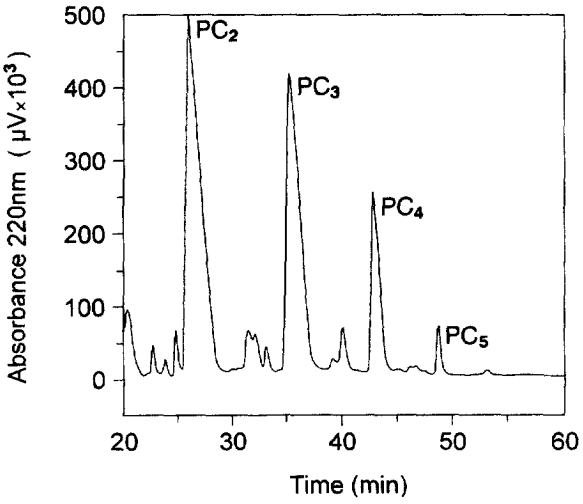

Fig. 7. Semipreparative HPLC separation of a mixture of prepurified total PCs (250 mg in 2 ml 2N HCl). The solution was applied to a Nucleosil 100 10C18 column (25 × 250 mm) at a flow rate of 210 ml min^{-1}. Elution of individual PCs was achieved using a CH_3CN-trifluoroacetic acid gradient according to Ref. [21]. The individual PCs were detected at 220 nm.

or trifluoroacetic acid [15] acetonitrile gradient. Figure 7 shows a typical HPLC profile recorded at 220 nm. The individual PCs with differing chain lengths were fractionated, the peptides isolated by lyophilisation and, after reduction with BH_4^-, rechromatographed by HPLC on a 30 mg scale. An aliquot of the PC_3 peak was then subjected to analytical HPLC using postcolumn Ellman's derivatisation [16] and proven to be free of major detectable impurities.

Endogenous complexing of heavy metals by PCs is a most efficient way to avoid heavy metal poisoning in plants [1–3, 17]. This mechanism seems to be distributed throughout the whole plant kingdom [12]. In this paper it has been shown that phytochelatin synthase, which is present in high amounts in the cell suspension culture of *Silene vulgaris*, is an excellent catalyst for the production of PCs in preparative amounts. Using 150 nkat × l^{-1} of PC synthase 35 g × l^{-1} of total PCs could be synthesized from GSH in the presence of Cd^{2+} over a period of 55 days. During this time, about 70% of the GSH substrate was converted. The space time yield was *ca* 630 mg total $PC_n \times l^{-1} \times day^{-1}$. The enzyme demand was 4.3 nkat × g^{-1} PC. The final yield of individual PCs under these conditions over 45 days was PC_2: 46%; PC_3: 33.2%; PC_4: 17.3%; PC_5: 3.3%; PC_6: 0.2%. The rate of PC formation under optimized conditions was linear up to 55 days. The presence and maintenance of 0.01% NaN_3 in the incubation mixture was absolutely essential since even under anaerobic conditions, microbial growth occurred that inactivated the reaction. PC synthesis ceased after about 55 days because microbial contamination could no longer be controlled. The microbes obviously overcame the bacteriostatic effect of N_3^-. If microbial activity could be controlled beyond this time of 55 days with an agent that does not interfere with the work-up of PCs, there is no doubt that the reaction would continue even longer. This observation demonstrates the extreme stability of this enzyme under these conditions, which is in stark contrast to its withstanding all attempts at purification to homogeneity. Up to now, PCs were obtained from plant suspension cells that were exposed to heavy metals. About 100 mg total PCs could be obtained from about 100 g (dry wt) cells. Laborious chemical synthesis [8–10] yields only 50–100 mg quantities of PCs. The enzymic method given here certainly represents the most efficient and inexpensive way to obtain these metal detoxifying compounds. This process is to our knowledge the most efficient peptide synthesis reported for a cell free, higher plant system and allows now the production of PCs for pharmacological, environmental and biochemical testing as well as standards for analytical purposes. The peptides of the PC type are nontoxic to mammals and only poorly immunogenic, most likely because of their structural similarity to GSH.

It should be stressed that we have never claimed that *S. vulgaris*, which is a heavy metal tolerant plant and usually grows on excessive heavy metal-containing soils, uses the PC system for that tolerance. *S. vulgaris* cell cultures were solely selected by us in a screening system involving about 100 different plant species in suspension culture from our departmental culture collection. The assumption [18] that we chose *S. vulgaris* because of its heavy metal tolerance is not valid. The *Silene* cell culture has proven to be the most stable, highest PC synthase containing culture that we have thus far found.

In our opinion, the enzymic method presented here for the synthesis of gram quantities of PCs will prove to be valuable in the preparation of these precious heavy metal-binding peptides for future research and application.

EXPERIMENTAL

Plant material

The suspension culture used was derived from *Silene vulgaris* (MOENCH) Garcke [= *S. cucubalus* WIB, = *S. inflata* (SAL.) SM.]. This strain is deposited at the German Collection of Microorganisms, division plant cell cultures, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, from where it can be obtained. The cells were grown in Fernbach flasks in the medium according to Ref. [19] on a gyratory shaker (100 rpm) at 23° in continuous light (650 lx) for 7 days. One flask (1.2 l) yielded *ca* 30 g of cells (dry wt). This strain has been cultivated and

used for more than 10 years and has had an absolutely reliable enzyme yield.

Enzyme preparation

After 7 days of cultivation, the cells were suction filtered, immediately shock frozen with liquid N_2 and then stored at −20° for up to 13 months without loss of enzyme activity. Frozen cells (2 kg fr. wt) were mixed with 1.2 l of 30 mM Tris-HCl buffer, pH 8.5, containing 10 mM β-mercaptoethanol. The slurry was allowed to thaw under continuous stirring in a water bath (35°). The homogeneous brei was pressed through 4 layers of cheesecloth, the residue discarded and the filtrate centrifuged (8000× g, 30 min, 4°). To the supernatant was added solid $(NH_4)_2SO_4$ under constant magnetic stirring in an ice bath to a final concentration of 15%. The mixt. was centrifuged as above and the supernatant loaded onto a Phenylsepharose Cl-4B column (50 × 200 mm) previously equilibrated with 10 mM Tris-HCl containing 10 mM β-mercaptoethanol and 15% $(NH_4)_2SO_4$. After the protein solution was adsorbed, the column was washed with 1 l of equilibration buffer and the phytochelatin synthase eluted and fractionated (10 ml) with 1 l equilibration buffer containing 10% ethylene glycol. The frs containing the enzyme were pooled (*ca* 150 ml) and dialyzed (overnight, 4°) against buffer (10 mM Tris-HCl, pH 8.5, 10 mM β-mercaptoethanol, 5 l) to remove the ethylene glycol. From 2 kg cells usually 150 nkat total PC synthase was obtained.

Incubation

For preparative PC synthesis, the following mixt. was incubated in 1 l Erlenmeyer flasks: 10 mM mercaptoethanol, 3 mM GSH, 0.8 mM $Cd(NO_3)_2$, 0.01% NaN_3 adjusted to pH 8.0 (NaOH), H_2O and 150 nkat enzyme soln were added to yield 1120 ml. The flasks were tightly stoppered and incubated at 25°. Addition of buffer was unnecessary thus simplifying the work-up. The flasks were equipped with a stirring bar that was used daily when 344 mg GSH and 211 mg $Cd(NO_3)_2$ were freshly added to the flasks and dissolved. Every 10th day, 0.5 ml of a 20% NaN_3 solution was added. For optimization, the concentration of various ingredients were varied. Samples were taken daily and the PC content determined by HPLC.

Analytical procedures

PC synthase was determined by HPLC with a postcolumn Ellman's reaction according to Ref. [16]. GSH was determined by the above procedure as well as by a method that applies to GSH reductase [20]. Cd^{2+} was determined by atomic absorption spectroscopy (PE-1100B) using Merck standards and flame mode.

Isolation of peptides

After appropriate time intervals, the incubation mixt. was processed further; usually the Erlenmeyer flasks contained 30–35 g of total PCs. The incubation mixt. was transferred to a Dowex 1 × 2 column (500 g, acetate form, 10 × 10 cm) and the column washed with 2 l H_2O. Elution of excess GSH and most of the free Cd^{2+} was achieved with 2 l 0.75 N HoAc acid and the PCs were subsequently eluted with 0.5 N HCl. While eluting with HCl, a yellow band became visible, which moved slowly through the column and contained the PCs. This fr. (1 l) was neutralized (NaOH pellets) and freeze dried yielding a slightly yellow powder containing 90% of the original PCs (determined from 8 separate runs). An aliquot of this powder (15 g) was dissolved in 50 ml 3.6 M HCl and 50 ml H_2O was added. This mixt. was carefully applied to a Lichroprep RP-18 silicagel column (9 × 15 cm, 500 g, Merck) at a flow rate of 1 l × hr^{-1} at room temp. The column was washed with 1 l H_2O to remove first NaCl and Cd^{2+} and later residual GSH. When the GSH was exhaustively eluted, the water was changed to 50% MeCN in degassed H_2O and the PCs were eluted. The elution of both GSH and PCs was monitored by the Ellman's test [16]. MeCN was removed from the PC containing fr. (300 ml) *in vacuo* (< 40°) and then freeze dried (Cd^{2+} content less than 0.008%). An average yield of 6 independent runs was > 80% in the RP 18 step.

To separate the above mixt. into the individual PC species according to their chain length, the following method was used: 250 mg of the above powder was dissolved in 2 ml 2M HCl. This soln was applied to a Nucleosil 100 10C18 (25 × 250 mm) column (Macherey and Nagel) at a flow rate of 210 ml × min^{-1} using an MeCN-trifluoroacetic acid gradient according to Ref. [21]. The individual PCs were detected at 220 nm. The elution profile showed a clear separation of PC_2 (26 min), PC_3 (36 min), PC_4 (43 min) and PC_5 (48 min). The individual PCs could easily be fractionated, reapplied in 30 mg quantities to the semipreparative column, fractionated again and finally checked for impurities by analytical HPLC [16, 21].

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