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Isolation and Characterization of Phytochelatin Synthase in Rice Seedlings

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ABSTRACT

Rice plants were treated with 50 μM copper sulfate to induce the synthesis of phytochelatins by means of a series of enzymatic reactions, including that of photochelatin synthase. Phytochelatin synthase extracted from 3-week-old rice seedlings was purified through a series of steps including precipitation with ice-chilled acetone, QAE A-50 anion exchange column, Amicon XM-50 ultrafiltration and Polybuffer Exchange (PBE) 94 chromatofocusing. This enzyme had a molecular mass of about 100 kDa with an isoelectric point of 4.0. The temperature and pH optima of this enzyme were 55˚C and pH 7.5, respectively. The enzyme was thermal tolerable and unstable under refrigeration at 4 or –20˚C. Cadmium was the most effective stimulator, followed by lead, copper, silver, cobalt and other divalent cations. Calcium and magnesium had no effect.

Key words: cadmium, copper, enzyme kinetics, enzyme purification, phytochelatin synthase, *Oryza sativa*

I. Introduction

Detoxification mechanisms of plants in response to heavy metals have been widely studied (Rauser, 1990; Steffens, 1990), especially where heavy metals in contaminated soil could be removed by plants through the processes of bioremediation (Baker *et al*., 1994). Plants produce metal-binding peptides under heavy metal stresses. The predominant class of such molecules produced in plants, algae and some fungi are the small cysteine-rich peptides, referred to as phytochelatins (Grill *et al*., 1985); these molecules are synthesized from glutathione via metal-dependent enzymatic pathways (Rauser, 1990; Steffens, 1990). In contrast to metallothioneins in animals, phytochelatins are induced by heavy metals that contain the unique sequences of $(\gamma\text{-Glu-Cys})_n$-Gly, where *n* may range from 2 to 11 (Rauser, 1995). Because of the implication of phytochelatins in heavy metal detoxification, chelation of heavy metals by the newly synthesized phytochelatins and activation of phytochelatin synthase have received a wide attention (Howe and Merchant, 1992).

Three biosynthetic pathways of phytochelaltin in plants have been suggested: (1) synthesis of phytochelatins by repetitive addition of γ-glutamylcysteine to glutathione, where the source of γ-glutamylcysteine is synthesized *de novo*; (2) synthetic pathway similar to (1), however with γ-glutamylcysteine coming from the degradation of glutathione via carboxypeptidase; and (3) synthesis by means of head-to-tail coupling of glutathione with an elimination of glycine at each step (Steffens, 1990). In a cell-free system of fission yeast, however, only two biosynthetic pathways of phytochelatins were postulated (Hayashi *et al*., 1991). Although the main pathway for synthesis remains unknown, phytochelatins have been shown to be synthesized from glutathione by phytochelatin synthase isolated from tobacco cultured cells using H^3-glycine-labelled glutathione as a precursor (Grill *et al*., 1989).

In order to better understand the synthesis of phytochelatins, attempts were made in this study to purify phytochelatin synthase from copper ion-treated rice seedlings.

II. Materials and Methods

1. Plant Materials

Rice (*Oryza sativa* Tainung 67) seeds were sterilized in 2% hypochlorite for 20 min. After being thoroughly washed, the seeds were immersed in water at 37˚C for four days, transferred to Hoagland's solution with or without 50 μM copper sulfate for 10 days and then hydroponically cultured in double-layer plastic pans in a growth chamber with 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and a 14 h/10 h light/dark light period. Both control and copper-

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treated seedlings were frozen in liquid nitrogen immediately after harvesting and stored at –20˚C .

2. Sulfhydryl Assays

The total sulfhydryl groups in tissues were determined using the method of Ellman *et al*. (1961) with minor modification. Frozen tissues (0.4 g) were ground with a mill and then stirred in a solution containing 1.2 ml of 6.7% sulfosalicylic acid at 4˚C for 10 min before centrifugation at 13,000× g for 20 min. The reaction mixture containing 1 ml of reaction product was measured for optical absorbance at 412 nm. Glutathione was used as a standard.

3. Enzyme Purification

Frozen shoot tissues (500 g) were ground into a powder with a mill, homogenized in a Polytron with 1500 ml of 0.3 M Tris buffer (pH 8.0), and then centrifuged at 10,000× g for 15 min to remove the debris. The supernatant was mixed with an equal volume of chilled acetone and centrifuged at 10,000× g for 15 min to produce a pellet. The pellet was resuspended in 100 ml of cold 0.3 M Tris buffer (pH 8.0) and centrifuged at 10,000× g for 10 min. The supernatant (100 ml) was partially purified through the following steps: QAE A-50 column (2.5 × 40 cm, Pharmacia, Uppsala-1, Sweden) equilibrated by 10 mM Tris buffer (pH 8.0) and then eluted using a linear 0.05 – 1 M KCl gradient, ultrafiltrated with Amicon XM-50 (Amicon, Denvers, MA, U.S.A.) to remove proteins of smaller than 50 kDa, in molecular mass, and then concentrated in a Speedvac (Savant, Farmingdale, NY, U.S.A.) until the sample volume was reduced to 50 ml. The enzyme preparation was dialyzed against distilled water (1000 ml) for 3 – 4 h, lyophilized and redissolved in 5 ml of 25 mM imidazole-HCl buffer (pH 7.4) before elution was performed with Polybuffer (PB) 74 (Pharmacia) in a chromatofocusing column (0.9 × 30 cm) containing Polybuffer Exchanger (PBE) 94 (Pharmacia). Finally, the enzyme preparation was eluted through a AcA 44 Ultragel (IBF, Villeneuve-la-Garene, France) column (1.6 × 100 cm, Pharmacia), and the fractions containing the enzyme activity were pooled and concentrated with Amicon XM-50.

4. Enzyme assays

A. Radioactive Labelling

The enzyme activity was assayed using the method of Loeffler *et al*. (1989). The reaction mixture containing 30 μl of enzyme extract, 10 μl of 0.1 M ^{3}H-glycine labelled glutathione (50,000 cpm), 10 μl of 0.1 mM $Cd(NO_3)_2$ and 50 μl of 0.3 M Tris buffer (pH 8.0) in a 500 μl Eppendorf tube (Eppendorf, Westbury, NY, U.S.A.) was incubated at 37˚C for 90 min. Meanwhile, 10 μl of 0.1 M cold glutathione in the reaction mixture was used as the control for the labelling experiment. The reaction was terminated by adding 20 μl of 0.1 M glycine solution, followed by 80 μl of 10% activated charcoal in distilled water (W/V). After it was vortexed for 30 sec, the mixture was centrifuged at 10,000× g for 10 min. The supernatant (100 μl) was transferred into a vial containing 2.5 ml Fluoran-safe, and the radioactivity was measured using a Beckman LS 1801 scintillation counter (Beckman, Fullerton, MA, U.S.A.).

B. HPLC Separation

The products produced by the enzyme reaction were determined using the procedure described by Grill *et al*. (1989) with some modifications. The reaction mixture containing 30 μl of enzyme extract, 10 μl of 1 mM glutathione, 10 μl of 0.1 mM $Cd(NO_3)_2$ and 50 μl of 0.3 M Tris buffer (pH 8.0) in a 500 μl Eppendorf tube was incubated at 37˚C for 90 min. After incubation, the reaction was terminated by adding 150 μl of 2 M NaOH, vortexed for 30 sec and allowed to stand for 10 min; subsequently, 32 μl of 3.6 N HCI was added, and the mixture was vortexed for 30 sec and centrifuged at 10,000× g for 10 min. The supernatant was saved and quantitated using a Cl8 RP-HPLC column (Walters, Pat. No. 086344, Milford, MA, U.S.A.) eluted with a 0 – 60% acetonitrile gradient, and the active fractions were measured at 214 nm.

C. Enzyme Characterization

Three buffers were used to determine the pH optima at pH values ranging from 4.5 to 10. These buffers were citrate buffer (pH 4.5 – 6.5), Tris buffer (pH 7.0 – 8.5) and sodium glycine buffer (pH 9 – 10). The effects of different mineral ions on the enzyme activity were studied with their final ion concentration at 10 μM. Cadmium and copper concentrations were determined using flame atomic absorption spectroscopy (Hitachi Z6100, Tokyo, Japan).

D. Protein Content

The protein contents in plant tissue extract were detected by a Hitachi 3200 spectrophotometer (Hitachi) using the method described by Bradford (1976).

III. Results

1. Content of Sulfhydryl Groups

As shown in Table 1, the total amount of sulfhydryl

groups in the rice seedlings increased in the first 12 h under treatment with 50 μM copper sulfate. However, as the duration of treatment was prolonged, it decreased in the plants. In contrast, the amount of sulfhydryl groups did not increase in the control plants during the first 12 h. This indicated that the synthesis of phytochelatin and other sulfur-containing compounds occurred in the cadmium-treated seedlings during this period.

2. Enzyme Purification and Characterization

Phytochelatin synthase required heavy metal ions for activity, with cadmium ion being most effective, and the amount of activity was proportional to the time of incubation and the amounts of enzyme extracts added (data not shown). The activity of phytochelatin synthase could be confirmed by the synthesis of $(EC)_2$-G and $(EC)_3$-G, and the retention times were 5.7 min and 8.1 min in a RP-HPLC, respectively, in the presence of cadmium as compared with the control (Fig. 1).

Phytochelatin synthase is apparently a soluble enzyme, compartmentalized primarily in the cytoplasm. As shown in Table 2, the yields of phytochelatin synthase from rice seedlings were relatively low (0.9%). A 102-fold increment in the specific activity of phytochelatin synthase was obtained following purification with acetone precipitation, anion ion-exchange, ultrafiltration and chromatofocusing chromatographies. The purified enzyme had a specific activity of 493 unit/mg (1 unit = 1,000 cpm/h released). The molecular mass and the isoelectric point of phytochelatin synthase were 100 kDa based on the results of gel filtration and 4.0 based on chromatofocusing results, respectively (data not shown).

Phytochelatin synthase showed an optimal pH of around 7.5, and the activity was more sensitive toward the alkaline side than the acidic side (Fig. 2). The effects of various ions on the activity of purified phytochelatin synthase are shown in Table 3. The enzyme required certain cations for activity to occur with cadmium being most effective, followed by lead, copper, manganese, cobalt and other divalent cations. Calcium and magnesium had no effect on the activity. The purified enzyme was also

Table 1. Effect of Copper Treatment on the Changes of Sulfhydryl Groups in Rice Seedlings

Treatment	Duration of treatment (hours)				
	0	6	12	24	48
	10^3 μmole/g fresh weight[a]				
Control	538 ± 8	518 ± 2	542 ± 1	506 ± 18	450 ± 6
50 μM $CuSO_4$	557 ± 11	635 ± 5	678 ± 5	599 ± 3	548 ± 4

[a] Measured in triplicate

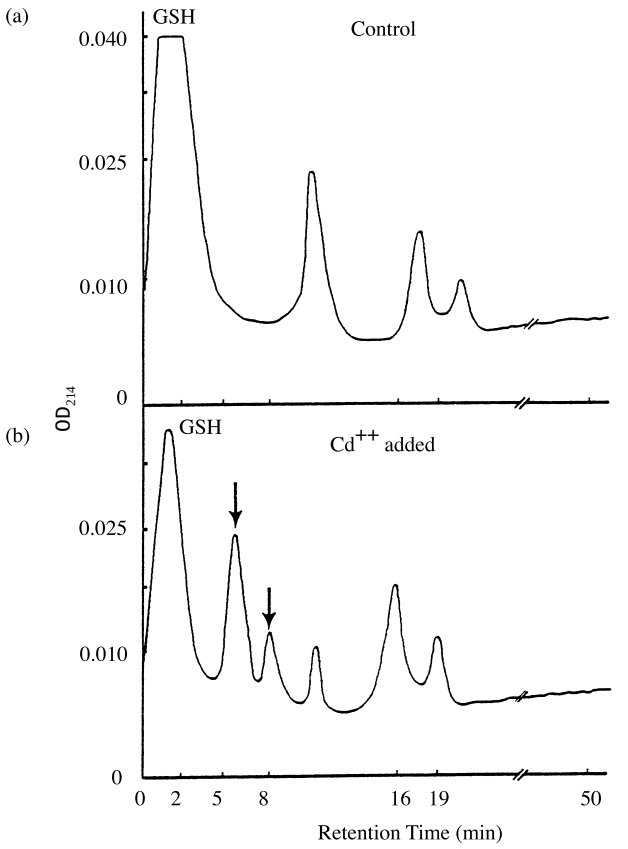

Fig. 1. HPLC profile of the reaction products synthesized by means of phytochelatin synthase. (a) Incubation medium (0.1 ml) containing 10 μl of 1 mM GSH, 60 μl of 0.05 M Tris buffer (pH 8.0) and 30 μl of crude enzyme extract in the absence of cadmium. (b) Incubation medium (0.1 ml) containing 10 μl of 1 mM GSH, 10 μl of 0.02 M Cd^{++}, 50 μl of 0.05 M Tris buffer (pH 8.0) and 30 μl of crude enzyme extract. Arrows at the retention times 5.7 min and 8.1 min indicate $(EC)_2$-G and $(EC)_3$-G, respectively.

Table 2. Purification of Phytochelatin Synthase from Rice Seedlings

Purification Step	Volume (ml)	Total protein (mg)	Total activity (unit)[a]	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	1540	2170	11525	5.3	100	1
Acetone precipitation	100	399	7545	18.9	65.4	3.6
Anion exchange chromatography	200	95	3185	33.5	27.6	6.3
Ultrafiltration	50	35	2455	70.5	21.3	13.4
Chromatofocusing	20	0.2	98.5	492.5	0.9	102.9

[a] One unit of enzyme activity is represented by 1,000 cpm/h released.

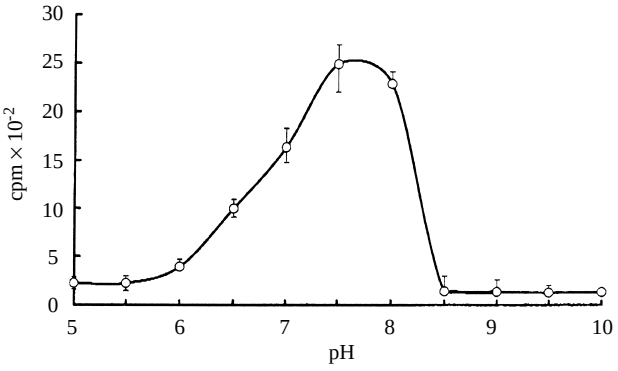

Fig. 2. Effect of pH on the activity of phytochelatin synthase.

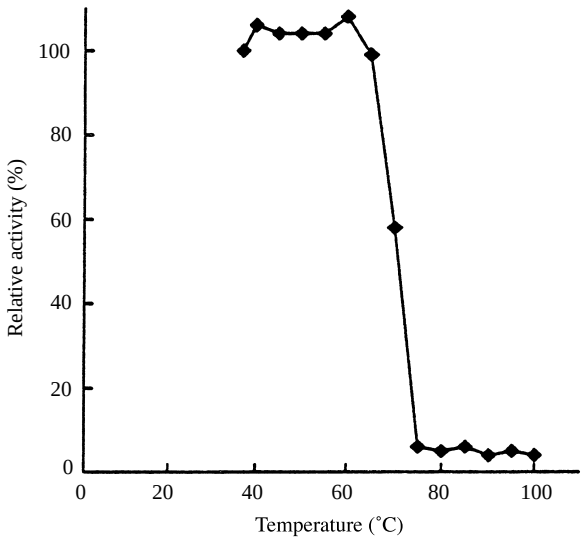

Fig. 3. Effect of temperature on the activity of phytochelatin synthase. The incubation temperature ranged from 30 to 100˚C at 5˚C intervals, and the duration of incubation was 10 min in a water bath.

slightly stimulated by the presence of silver ion.

3. Thermal Tolerance of Enzyme

The phytochelatin synthase activity increased slowly from 30˚C to 60˚C, then decreased sharply when the temperature was elevated above 60˚C and completely stopped at 75˚C (Fig. 3). The enzyme activity remained high at 55˚C for 20 min, and 90% of the activity was retained at 60˚C for 20 min. However, the purified enzyme was unstable at 4˚C and the activity stopped completely within 10 days. A similar observation was made when phytochelatin synthase was stored at –20˚C.

IV. Discussion

Copper ion induces the synthesis of sulfhydryl-rich

Table3. Effects of Various Mineral Ions on the Activity of Phytochelatin Synthase

Ions	Relative activity[a] cpm (%)
Tris (l0 mM)	876 (45)
Ag^{+}	1193 (62)
Ca^{2+}	887 (46)
Cd^{2+}	1914 (100)
Cu^{2+}	1171 (61)
Fe^{2+}	1556 (81)
Mg^{2+}	902 (47)
Mn^{2+}	815 (43)
Ni^{2+}	1258 (66)
Pb^{2+}	1582 (83)
Zn^{2+}	933 (48)

Notes: Concentrations of tested ions are all 10 μM in 10 mM Tris buffer, and tested salts are AgCl, $CaC1_2$, $MgC1_2$, $CuS0_4$, $CdCl_2$, $FeSO_4$, $NiC1_2$, $MnSO_4$, $PbSO_4$ and $ZnSO_4$.

[a] Measured in five replicates.

compounds in rice seedlings. This observation is consistent with the finding that sulfhydryl-containing glutathione and phytochelatins could be enhanced by the addition of cadmium ion to the nutrient solution (Scheller *et al*., 1987).

Phytochelatin synthase partially purified from *Silene cucubalus* suspension-cell cultures synthesized phytochelatins by using glutathione as a precursor (Grill *et al*., 1989). However, two pathways of phytochelatin biosynthesis were postulated in *Schizosacchromyces pombe* based on genetic analysis of their Cd-hypersensitive mutants (Hayashi *et al*., 1991). In the present study, the partially purified phytochelatin synthase of rice seedlings could synthesize phytochelatins in the presence of glutathione. This may indicate that glutathione is the major precursor for phytochelatin biosynthesis in higher plants under heavy metal stress. This was also proved by challenging *S. pombe* or tomato cell cultures with cadmium ion causing phytochelatin synthesis and rapidly reducing glutathione level (Grill *et al*., 1989; Scheller *et al*., 1987).

The purified phytochelatin synthase from rice only provoked the synthesis of phytochelatins, and their n number of phytochelatins was from 2 to 3. This is toward the lower side of n numbers within the phytochelatin family, $(\gamma\text{-Glu-Cys})_n$-Gly (Rauser, 1995). The tolerance of heavy metal toxicity in plants related to the higher *n* number of phytochelatin has been suggested (Steffens, 1990). To elucidate their relationships with the enzyme synthesizing capability or other mechanisms, further studies are needed.

Cadmium ion is known as the best activator for phytochelatin synthase (Grill *et al*., 1989; Hayashi *et al*., 1991), while the role of zinc ion in enhancing the enzyme activity of phytochelatin synthase remains unclear. German groups showed that the activating effect of zinc ion on the enzyme activity was distinct (Loeffler *et al*., 1989). In the present study, the effect of zinc ion was negligible

in activating the activity of this enzyme, which agrees with other reports (Reese and Wagner, 1987; Reese *et al*., 1988).

Although the addition of glycine in preservation buffers has been found to prevent a rapid loss of enzyme activity (Grill *et al*., 1989), temperature also plays an important role. Our findings indicated that the purified enzyme solution lost almost all its activity when stored at –20˚C for two weeks, but that most of the activity could be retained when it was kept at –70˚C (unpublished data). Similar observations were made for phosphoenol pyruvate carboxylase (Edwards and Walker, 1983).

Phytochelatins collected through HPLC separation have been customarily measured at 214 nm with UV spectrophotometry. Our previous studies showed that OD_{214} could be affected by both internal and external noises (Tsay and Chen, 1993). However, these noises could be minimized through the attachment of a post-column with HPLC to detect the reaction products of phytochelatins with Ellman's reagent at OD_{412} (Howe and Merchant, 1992).

Chromatofocusing with PBE 94 Polybuffer Exchange and elution using PB 74 buffer was useful for purifying acid phosphatase (Chen *et al*., 1992) and inorganic pyrophosphatase (Horng, 1992)[1]. This technique appears to be effective for purification of phytochelatin synthase as well. So far, only the Zn-chelating Sepharose column chromatographic method produces similar results (Loeffler *et al*., 1989). PBE 94 forms a pH gradient ranging from 4.0 to 7.0. Because of the strong buffering capacity of the eluting buffer, PB 74, it is difficult to adjust the enzyme solution to pH 7.5, the optimal pH for enzyme activity. However, this problem could be overcome by adding highly concentrated Tris buffer.

In the present study, a rapid method of enzyme purification through chill-acetone precipitation was able to overcome the instability of phytochelatin synthase under regular purification at 4˚C. The combination of various column chromatographic techniques was effective in increasing the specific activity of phytochelatin synthase up to 102 fold. Studies on the enzyme properties showed that this enzyme is thermal stable but has low stability at low temperatures; the optimal pH for this enzyme was found to be around 7.5, and its most effective activator was cadmium ion.

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Phytochelatin Synthase in Rice Seedling

水稻中植物螯合物合成酵素的純化與鑑定

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摘　要

三週大的水稻幼苗以50 μM 硫酸銅處理十天後，經一連串酵素作用而誘導植物螯合物的合成。銅處理的植株經液態氮凍化研磨、以緩衝液抽取得到之上清液經低溫丙酮進行劃分沈澱、QAE A-50 離子交換層析、Amicon XM-50 超過濾及 PBE 94 色層聚焦分析等步驟而得到純化植物螯合物合成酵素。該酵素的分子質量數約爲100 kDa 而等電點爲4.0 。其最適溫度及最適酸鹼度分別爲55℃ 和pH 7.5 。該酵素屬熱穩定性而在4 或−20℃ 的冷藏下都不穩定。在試驗的兩價陽離子中，鎘離子屬最佳的促進劑，銅、鉛、錳及鈷等離子次之，其他離子效用輕微，鈣與鎂離子則無作用。