Functional analysis of phytochelatin synthase from Arabidopsis thaliana and its expression in Escherichia coli and Saccharomyces cerevisiae

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Abstract

Plants, like other organisms, have adaptive mechanisms whereby they are able to respond to both nutrient deficiencies and toxicities. Phytochelatins (PCs) play an essential role in heavy-metal detoxification in plants, fungi and worms. PCs chelate heavy metals and then PC-metal complexes are translocated across the tonoplast and sequestered in vacuoles. PCs are synthesized from glutathione by the enzyme PC synthase (PCS). Comparison of the deduced amino acid sequences of PCS suggests that the C-terminal domain may be important for activation of the enzyme. We established the method for purification of PCS from Arabidopsis thaliana to perform enzymatic characterization. Moreover, the PCS gene was expressed in E. coli and S. cerevisiae to enhance tolerance to toxicity of cadmium ion. The obtained results implied that the some regions of the PCS may serve as regulatory region through interaction with cadmium ion and/or oxygen related compounds. Moreover, PCS expression dramatically enhanced cadmium ion tolerance of different organisms. Based on our findings, functional mechanism for PCS activation was hypothesized.

Keywords: Metal-binding peptides; Phytochelatins; Phytochelatin synthase; Cadmium; Oxidation-stress

1. Introduction

Some plants can hyperaccumulate metal ions that are toxic to virtually all other organisms at low dosages [1–3]. Plants, like all living organisms, have evolved a suite of mechanisms that control and respond to the uptake and accumulation of both essential and nonessential heavy metals. These mechanisms include the chelation [4] and sequestration of heavy metals by particular ligands. Phytochelatins (PCs) are a family of enzymatically synthesized cysteine-rich peptides. PCs form a family of structures with increasing repetitions of the γ-Glu–Cys dipeptide followed by a terminal Gly; (γ-Glu–Cys)n–Gly, where n is generally in the range of 2–11. PCs have been identified in a wide variety of plant species and in some microorganisms [5,6]. PCs are structurally related to glutathione (GSH; γ-Glu–Cys–Gly), and numerous physiological, biochemical and genetic studies have confirmed that GSH is the substrate for PC biosynthesis. Moreover, PCs may have higher antioxidant activity than a very well-known antioxidant, GSH [7–9].

PCs are synthesized post-translationally in the presence of metal ions; PC synthase (PCS; γ-glutamylcysteine dipeptidyl transferases, EC 2.3.2.15) catalyze the net transfer of a γ-Glu–Cys unit from one GSH molecule to another [γ-Glu–Cys–Gly + γ-Glu–Cys–Gly → (γ-Glu–Cys)2–Gly + Gly], or to a previously synthesized PC molecule [(γ-Glu–Cys)n–Gly + γ-Glu–Cys–Gly → (γ-Glu–Cys)n+1–Gly + Gly] to generate polymers containing 2–11 γ-Glu–Cys repeats [10]. A comparison of the deduced amino acid sequences shows that the N-terminal catalytic domains are very similar, whereas the C-terminal domains show little apparent conservation of amino acid sequences [6,11]. The C-terminal domain may play a role for regulation of the enzymatic activity [12]. This study aims to establish the method for purification and to perform enzymatic characterization of the PCS from...
Arabidopsis thaliana. Moreover, the PCS gene was expressed in E. coli and S. cerevisiae to enhance tolerance to toxicity of cadmium ion. Because signal transduction cascades are hypothesized to have recruited metal homeostasis factors, a better understanding of plant metal handling might also lead to new insights into other fundamental aspects of life physiology. In addition to the main function of heavy-metal detoxification, PCs may have a function as an antioxidant. Therefore the elucidation of regulatory mechanism of PC synthesis is very important both in bioremediation and stress control.

2. Materials and methods

2.1. E. coli and S. cerevisiae cultures, transformation and growth assays

The E. coli strains BL21 (DE3) and the S. cerevisiae strains INVSc1 were used in this study. E. coli cells were grown in LB or NZCYM. S. cerevisiae cells were grown in SD(Ura(−)), SG(Ura(−)) or YPG. The cell culture was monitored by Jasco spectrophotometer model V-550 (Japan Spectroscopic Co., Tokyo, Japan). Cell growth in different concentrations of Cd2+ was determined by measuring the optical density at 600 nm.

2.2. Purification of PCS

PCS gene was obtained as reported previously (JB paper). E. coli BL21 (DE3) carrying pET-8c-PCS was cultivated at 37 °C for 12 h in 5 ml LB with Ampicillin. The culture was transferred to 250 ml of LB, cultivated further for 12 h, and added 1 mM isopropylthiogalactoside (IPTG). After 4 h culture, the cells were harvested by centrifugation and suspended in 50 ml of 10 mM Tris–HCl buffer (pH 8.0). The cells were disrupted by sonication using SONIFEAR model 450 (BRANSON Co.), and insoluble materials were collected by centrifugation. The solution obtained was applied to a DEAE-TOYOPEARL column (TOSOH Co., Tokyo, Japan) equilibrated with 10 mM Tris–HCl buffer (pH 7.5). Finally, protein was eluted with a linear 0–500 mM NaCl gradient. The fractions of the major activity were pooled and applied to a HitrapSP column (Amersham-Pharmacia Biotech) that had been equilibrated with 10 mM phosphate buffer (pH 7.5). Finally, protein was eluted with a linear 0–0.5 M NaCl gradient in buffer. The fractions of the major activity were pooled and applied to a HitrapQ sepharose column (Amersham-Pharmacia Biotech) that had been equilibrated with 10 mM Tris–HCl buffer (pH 8.0) on the automated liquid chromatography system AKTA explorer 10 (Amersham-Pharmacia Biotech). The protein was eluted with a 0–0.52 M NaCl gradient in buffer. Fractions were collected and assayed for enzyme activity.

2.3. Measurement of phytochelatin synthase activity

PCS activity was assayed according to the method reported by Grill et al. (1989) in reaction media containing crude enzyme fraction (50 μg protein), 0–20 mM GSH, 10 mM 2-mercaptoethanol, 200 mM Tris–HCl buffer (pH 8.0) and 0–0.75 mM CdCl2 at 37 °C for 30–180 min. The extracted sample was neutralized by the addition of 3.6 N HCl to stop the reaction. The postcolumn derivatization method involving DTNB (5,5′-dithiobis-2-nitrobenzoic acid) was used for sensitive detection of PCs. The clear supernatant was subjected to reversed-phase high-performance liquid chromatography (HPLC) [column: Waters Symmetry 300 C18 5um 4.6 × 150 mm HPLC Column; solvent system: A: 0.02% TFA, 5.0 mM octanesulfonic acid, B: 30% acetonitrile, 0.02% TFA; gradient 13–100% B in 30 min; flow rate: 1.0 ml/min], and the eluted sample was continuously mixed with the thiol-reactive solution (10% acetonitrile, 75 μM DTNB, 100 mM potassium phosphate buffer (pH 8.0)) to detect the PC and GSH at 412 nm using Jasco HPLC UV-2075 Plus (Japan Spectroscopic Co., Tokyo, Japan).

3. Results and discussion

3.1. Expression of the PCS gene in E. coli and S. cerevisiae

Fig. 1 shows enhancement of tolerance to cadmium ion by PCS expression in different organisms, E. coli
and *S. cerevisiae*. In the presence of Cd$^{2+}$, growth rate of *E. coli* cells with PCS gene was significantly higher than that of control cells without PCS gene (Fig. 1A) indicating that PCS was properly expressed and functioned in *E. coli*. Moreover, tolerance of *S. cerevisiae* cells carrying PCS gene (0.1 mM Cd$^{2+}$) was also improved when compared with the case of control cells without PCS gene (pYES2) (Fig. 1B). PCs in cells of *E. coli* and *S. cerevisiae* carrying PCS gene could be detected by the method explained above (data not shown). Therefore, it was shown that PCS gene from *A. thaliana* could catalyze PC synthesis and enhance tolerance to Cd$^{2+}$ even in *E. coli* and *S. cerevisiae*.

3.2. Purification of PCS

The method for purification of PCS from recombinant proteins expressed in *E. coli* was established. As explained above, crude enzyme fraction was applied to a DEAE-TOYOPEARL column, HitrapSP column, and HitrapQ sepharose column. Purity of PCS was assayed by SDS-PAGE (8%) (Fig. 2A) and enzyme activity was analyzed by HPLC using a postcolumn derivatization method. In the case of purified PCS treated with 10 mM GSH and 0.5 mM CdCl$_2$, PC synthesis corresponding to PC$_2$–PC$_5$ could be clearly observed (Fig. 2B).

3.3. Characterization of PCS activity

PCS activity was compared at different conditions. When free GSH concentrations were adjusted to 0–20 mM, PCS reaction was saturated at higher than 15 mM. The length of synthesized PC was almost same (Fig. 3A). When the Cd$^{2+}$ concentrations were adjusted to 0–0.75 mM, as concentration became higher, the length of synthetic PC became longer (Fig. 3B). Without Cd$^{2+}$, even in the presence of GSH, PCs were not synthesized. Therefore, quantity and length of synthesized PCs were not dependent on the concentration of substrate but the concentration of Cd$^{2+}$. Moreover, amount of PC$_2$ and PC$_3$ decreased when longer PCs were synthesized. Therefore, it was suggested that shorter PCs were used as substrates for synthesis of longer PCs. The reaction time for largest amount of PC synthesis

![Fig. 2. Purification and activity measurement of PCS (A) SDS-PAGE (8%); M: low molecular weight marker (B) PCS activity analysis by HPLC.](image)

![Fig. 3. Activation of PCS in different conditions. (A) GSH concentration (0–20 mM); (B) Cd$^{2+}$ concentration (0–7.5 mM); (C) time (30–180 min); (D) preservation condition (–80, –20, 4, 25°C, added Cd$^{2+}$, N$_2$, substitution).](image)
was 60 min (Fig. 3C). There was no relation between PC length and the reaction time.

The best storage condition to maintain PCS activity was at −20 °C (Fig. 3D). When Cd\(^{2+}\) was added to PCS solution, PCS activity could be stably maintained during preservation. Furthermore, in the presence of oxygen, preservation of enzyme activity could be improved. Therefore, it was suggested that some region of the PCS may serve as regulatory region interacted by Cd\(^{2+}\) and/or oxygen.

3.4. Model for PCS activation

Based on the results explained above, amino acid sequence of C-terminal region of the PCS was precisely examined and homologous regions to functional domains of metallothionein and thioredoxin were found in the C-terminal domain of the PCS. Therefore, it was suggested that C-terminal region of the PCS may play an important role as regulatory region for PC synthesis. The functional mode of PCS activation was shown in Fig. 4. The arrangements of Cys residues, −Cys−Cys−Arg−Glu−Thr−Cys−Val−Lys−Cys−, is reminiscent of those found in metallothionein (−Cys−Cys− or −Cys−Xn−Cys− (n = 3−4)) [6,13] and thioredoxin (−Cys−X1−X2−Cys−) (where X represents another amino acid) [14]. Metallothioneins are cysteine-rich proteins with low molecular weight and known to transport and storage of essential metal ions and detoxification of nonessential ones. Thioredxins are small ubiquitous proteins which act as general protein disulfide reductases in living cells. We are now constructing mutant PCS with replacement of Cys with Ala in C-terminal region to study role of Cys residues in regulation of the PCS. Actually a mutant PCS without C-terminal region was constructed and shown that the mutant could synthesize PCs, although the wild-type enzyme could synthesize PCs more efficiently (data not shown). Therefore it is highly possible that the C-terminal region of PCS may be important for efficient PC synthesis. Characterization of mutant PCS with substitutions of Cys residues in C-terminal region shown in Fig. 4 is now in progress.

4. Conclusion

(1) Expression of PCS enhanced Cd\(^{2+}\) tolerance both in *E. coli* and *S. cerevisiae*. (2) The method for purification of recombinant PCS using *E. coli* carrying PCS gene from *A. thaliana* was established. (3) Conditions necessary for activation and preservation of PCS were examined. (4) The functional model for activation of PCS was hypothesized based on the amino acid sequence of the C-terminal region of the PCS.

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