The Catalytic Mechanism of Phytochelatin Synthase

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Abstract

Phytochelatin synthase (PCS, EC 2.3.2.15) is a key enzyme for heavy-metal detoxification in plants and other organisms. PCS, a dipeptidyltransferase, catalyzes the synthesis of glutathione-derived peptides (called phytochelatins or PCs) that bind heavy-metal ions. It is generally believed that the active site region is located in the more conserved N-terminal portion of PCS, as yet unidentified roles have been clearly proposed for the C-terminal region. Recently, structure information and mutagenic analyses of recombinant PCS have been revealing the molecular mechanisms underlying PC synthesis, however, a conclusive model has not been established. To gain insight into the catalytic model of PCS, we have conducted a limited proteolysis analysis of the recombinant protein from *Arabidopsis* (AtPCS1). In the present study, we find when the substrate GSH is available, it can protect some AtPCS fragments from proteinase K digestion. In conjunction with peptide microsequencing, it indicates that some major fragments are available, including three domains of 27, 25, 15 kDa. It is hypothesized that two acylations are required per catalytic cycle. PCS undergoes acylation at two sites during catalysis, albeit with different ligand requirements, and acylation at at least one of these sites appears to be necessary for net PC synthesis. The acyl-enzyme intermediate formed at the first site Cys-56 locates in the 25 kDa and the predicted second acylation site might locate in the 27 or 15 kDa. We proposed that PCS is a bisubstrate enzyme and has two substrate binding sites, in which the donor molecule is the GSH and the acceptor is another GSH molecule or a PC*n* molecule. Two binding sites have individually different substrate affinity. According to the result of AtPCS motif scan by SeqWeb, we find that AtPCS has many casein kinase II and PKC phospho-sites. Without GSH, AtPCS can be phosphorylated by CKII, and this reaction enhanced by Cd. With GSH, the pattern disappeared and suggested the phosphorylation sites might neighbored on the substrate binding sites. Substrates binding cover the phosphor-sites against the effect of CKII. Calf intestine alkaline phosphatase (CIAP) can not remove the phosphate group from the AtPCS. On the other hand, AtPCS, pretreated with CKII and ATP, appears a higher PCs formation activity. These results enable us to propose a prediction that the PCS might have two substrate binding sites with different substrate affinity, and the activity could be regulated through phosphorylation by CKII.

Results

Proteinase K treatment and domain mapping of recombinant AtPCS

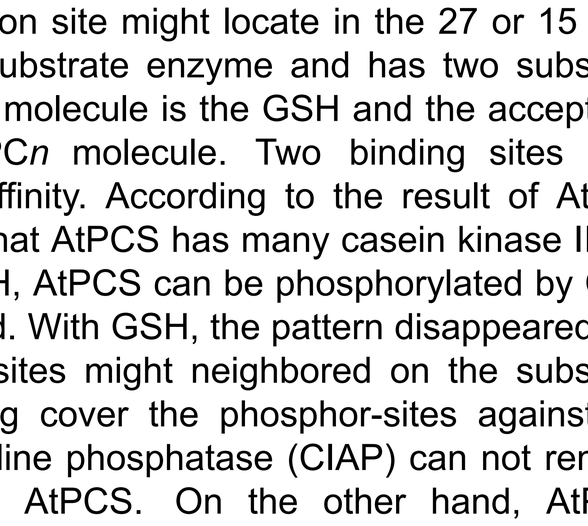

Fig. 1. Coomassie Brilliant Blue-stained SDS PAGE gel of the AtPCS generated proteolytic fragments.
AtPCS was digested at 4˚C with proteinase K at an enzyme to protease concentration ratio of 580:1. At the indicated time points, the digestion terminated by the addition of PMSF and 7μg of total protein was loaded per lane. M, prestained protein ladder (sizes given in kDa).

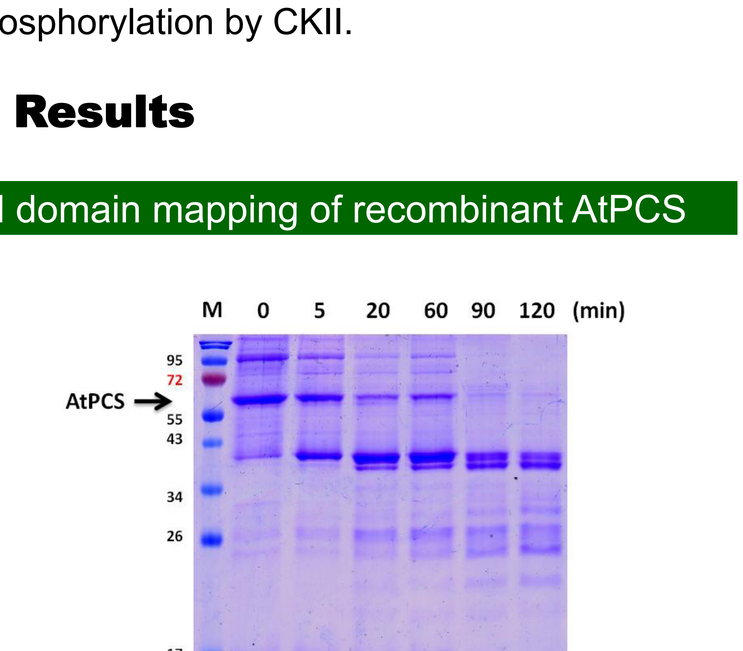

Fig. 2. Amino acid sequence of the N-terminals of the fragements generated from AtPCS by limited proteolysis with proteinase K.
The fragments were separated by SDS PAGE and blotted on to a PVDF membrane and sequenced by Edman degradation. X indicated that the sequence could not been identified.

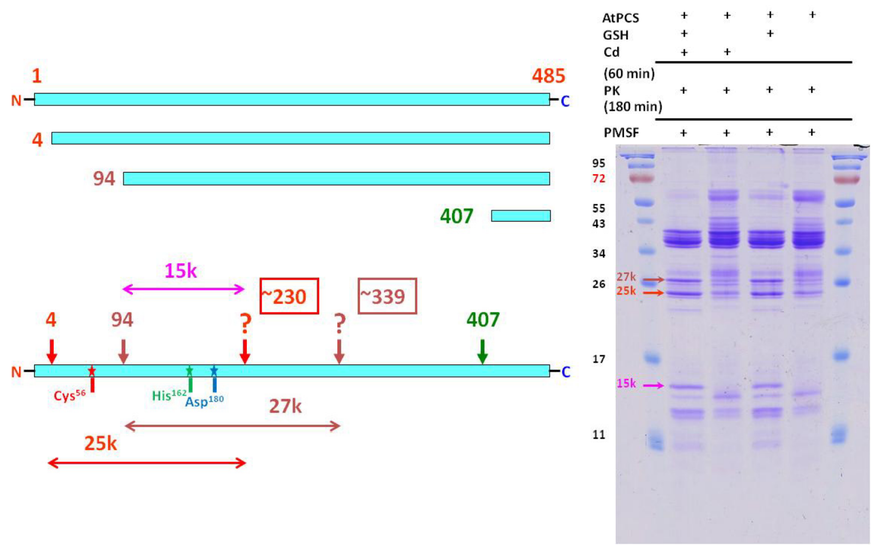

Fig. 3. Domain organization of AtPCS as judged by limited proteolysis.
Three major proteolytic fragments of AtPCS resulting from limited proteolysis with proteinase K were shown according to their individual locations. Major proteinase K cutting sites representing putative interdomain junctions of AtPCS were indicated as denoted by amino acid number in the sequence of AtPCS. The sizes of the three putative individual domains was indicated. Star donates the catalytic triad residues that were conserved in all known phytochelatin synthases.

Model for catalytic mechanism of eukaryotic phytochelatin synthase

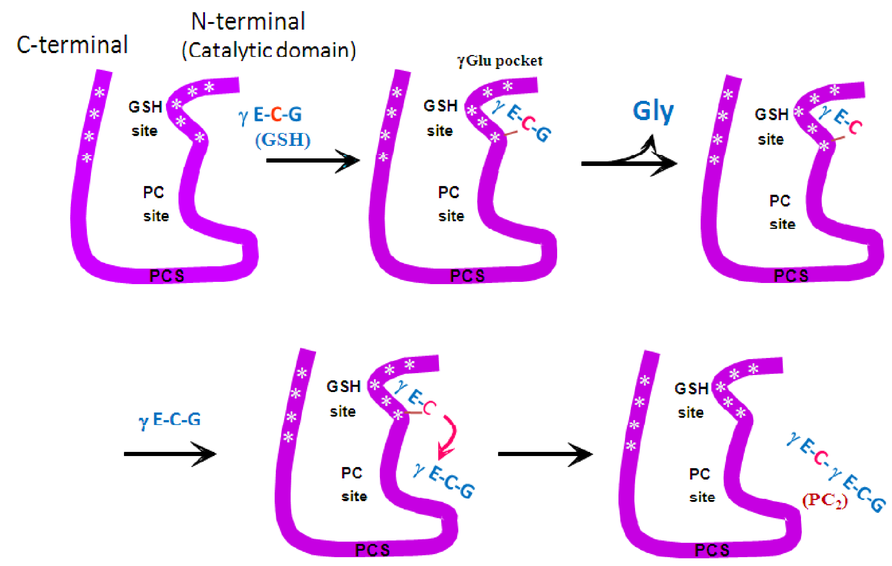

Fig. 4. A diagram showing a model of mechanism synthesizing PC_2.
It is inferred that there are two active sites on PCS, one is GSH binding site and the other is PC binding site. The GSH binding site has higher affinity with GSH and cuts off Gly from the GSH to leave γ-EC covalently bonded to PCS. The PC binding site can bind either GSH or PC*n* and accepts γ-EC transferred from the GSH binding site.

AtPCS is phosphorylated by CKII and this effect can regulate its catalytic activity

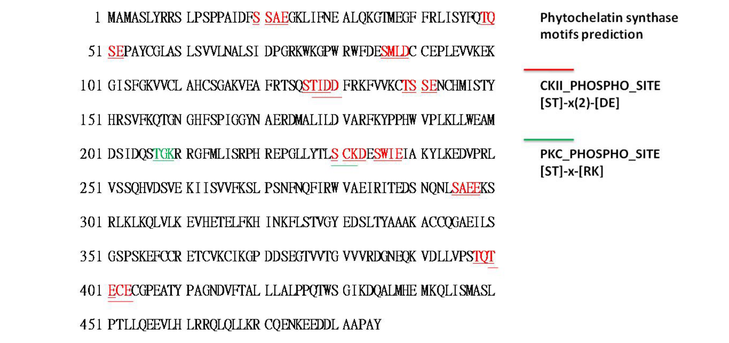

Fig. 5. AtPCS motifs prediction.
Using the program of SeqWeb to Look for AtPCS motifs by searching through proteins for the patterns defined in the PROSITE Dictionary of Protein Sites and Patterns. Red underline indicated CKII_phospho_site pattern ([ST]-x(2)-[DE]) and green underline indicated PKC_phospho_site pattern ([ST]-x-[RK]).

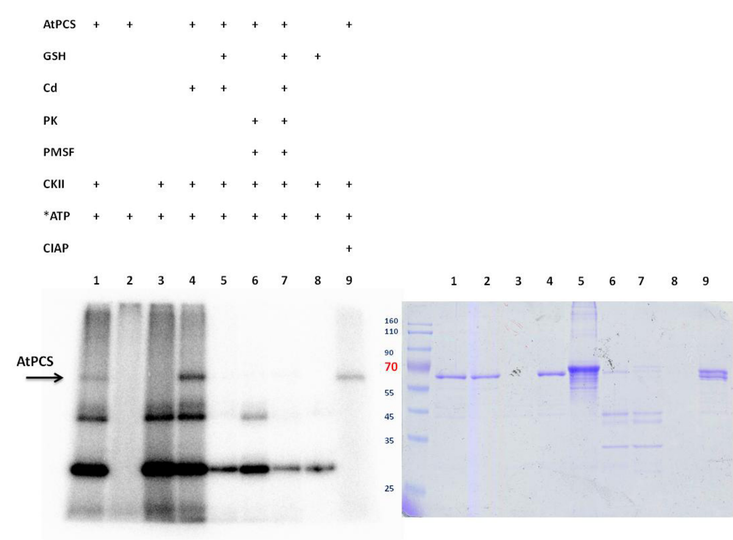

Fig. 6. AtPCS could be phosphorylated by Casein Kinase II (CKII).
The phosphorylation of AtPCS by the CKII were performed in the different treated reactions. AtPCS first incubated with or without substrates (GSH and Cd) for 30 min, and then treated with or without proteinase K for another 1h. The digestion terminated by the addition of PMSF. CKII and radioactive ATP was added to final reaction. Calf intestine alkaline phosphatase (CIAP) was added to remove the phosphate group in the lane 9. After SDS PAGE, radioactivity was exposed on a phospho-image plate and visualized on the autoradiogram.

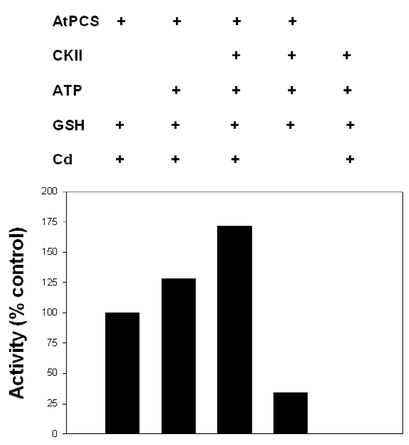

Fig. 7. Effect of phosphorylation by CKII on PCS activity.
The reaction conditions were as listed. AtPCS firstly incubated with or without CKII and ATP for 12h, and then another 3h incubation with or without GSH and Cd was processing subsequently at 37˚C. The values shown are percentage activities versus purified enzyme assayed in reaction media lacking CKII and ATP.