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Characterization of Siam Tulip (*Curcuma alismatifolia*) Cystatin (*CaCPI*)*

Ruangwit Porruan[1] and Somboon Anuntalabhochai[1,2*]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Biotechnology Unit, University of Phayao, Muang, Phayao 56000, Thailand

**Corresponding author. E-mail: soanu.1@gmail.com*

ABSTRACT

Cystatin is a cysteine protease inhibitor that can regulate the proteolytic process of cysteine proteases by binding to the active site of those target enzymes. Plant cystatin has several reported roles, including in: protein turnover during development; programmed cell death; and plant defense mechanisms against insects, nematodes and phytopathogenic fungi. In this experiment, the CaCPI gene was isolated from the cDNA library of the Siam tulip (Curcuma alismatifolia cv. Chiang Mai Pink). Its full length cDNA sequence is 601 base pair, containing 372 base pair in an open reading frame encoding 123 amino acids and 32 and 197 base pair in the 5'and 3' untranslated regions, respectively. The deduced amino acid sequence consists of a putative N-terminal secretory signal peptide of 22 amino acids and an estimated molecular mass for the mature protein of 11.239 kDa. The CaCPI protein contains all of the highly conserved blocks, including Gly^{31}-Gly^{32}, the reactive site motif QXVXG (Q^{76} $V^{77}V^{78}A^{79}G^{80}$), P^{106}-W^{107}, and the LGRFAVDQHN block that are common among plant cystatins. The CaCPI gene was cloned into a pDEST17 expression vector and then transformed into the Escherichia coli strain BL21-Star for recombinant CaCPI protein production. After induction with 1 mM IPTG, the cell lysate of E. coli carrying pDEST17-CaCPI generated a CaCPI protein about 12 Kda in size on SDS-PAGE. This cell lysate inhibited papain activity.

Keywords: Cystatin, Cysteine protease inhibitor, Papain, Siam tulip, *Curcuma alismatifolia*

INTRODUCTION

Protease inhibitors are proteins that inhibit enzymes in the proteolytic process. They can be classified into superfamilies according to their specificity as follows: serine-protease, cysteine-protease, aspartate-protease and metallo-protease inhibitors (Terra and Ferreira, 1994). The Cysteine protease inhibitors, or Cystatins superfamily, regulate the proteolytic process of papain-like cysteine protease by

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binding to the active site of the target enzyme. Three common conserved motifs are important for protease inhibition activity (Bode et al., 1988; Machleidt et al., 1989; Stubbs et al., 1990). The first motif is the reactive site motif QXVXG, which physically interacts with the active site. The second motif contains the PW residues, which are located in the C-terminal part of the protein. The last motif is a glycine residue near the N-terminal part of the inhibitor.

Cystatins exist in a wide range of species from microbes to plants and animals. In plants, several roles have been attributed to cystatins, including the control of endogenous cysteine protease in physiological and developmental processes. These include, for example: development of stem and leaves (Rivard et al., 2007), protein turnover during seed maturation and germination (Fernandes et al., 1991; Kumar et al., 1999; Martinez et al., 2005b) and programmed cell death (Belenghi et al., 2003). Several lines of evidence suggest that plant cystatins are responsive to abiotic stresses such as drought, salt, abscisic acid and cold treatment, helping plants better tolerate stresses (Gaddour et al., 2001; Van der Vyver et al., 2003; Diop et al., 2004; Massonneau et al., 2005; Christova et al., 2006). In addition, plant cystatins have a significant role in plant defense mechanisms, where they have been shown to inhibit the activity of digestive cysteine proteases of herbivorous arthropods, field slugs and parasitic nematodes (Zhao et al., 1996; Walker et al., 1999; Visal-Shah et al., 2001; Arai et al., 2002). Moreover, some plant cystatins show detrimental effects against pathogenic fungi (Pernas et al., 1999; Soares-Costa et al., 2002; Martinez et al., 2003; Martinez et al., 2005a; Yang and Yeh, 2005; Christova et al., 2006)

Siam tulip is a popular ornamental plant exported from Thailand. Its export value has increased steadily every year, reaching THB 30-40 million in 2010, with expectations of as much as THB 200 million in the near future (Polsingha, 2010). We have constructed a cDNA library prepared from flower tissue of the Siam tulip (*Curcuma alismatifolia*) for use as a genetic source for improving new Siam tulip varieties. After sequencing, the CPI homolog gene was found in this cDNA library. Because cystatin function in Siam tulip has never before been characterized and reported, we have studied the expression of *CaCPI* and characterized its function.

MATERIALS AND METHODS

Cloning of *CaCPI* gene from a cDNA library

Bract samples of Siam tulip were prepared for mRNA isolation using TRIzol reagent (Invitrogen). The CreatorTM SMART™ cDNA Library Construction Kit (Clontech) was used to generate the cDNA library according to the manufacturer's instructions. DNA sequence analysis of the double-strand recombinant plasmids was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977) incorporating an autosequencer (ABI). The *CaCPI* cDNA was identified directly from the sequenced EST clones by using blastx against the data in the current non-redundant protein database of GenBank (http://www.ncbi.nlm.nih.gov). The signal peptide was analyzed by SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP).

Sequence alignment and phylogenetic analysis

The CaCPI protein sequence was aligned to other plant Cystatins retrieved from GenBank using ClustalW2 from the EMBL-EBI database (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Other previously characterized plant cystatins were compared to the Siam tulip sequences.

Expression of a recombinant Siam tulip cystatin in *E. coli*

A *CaCPI* fragment without a secretory signal sequence was amplified from the pDNR-CaCPI vector and cloned into the entry vector pENTR-3C (Invitrogen, USA). Then the CaCPI gene was transferred to the Gateway *E. coli* expression vector pDEST17 with the 6xHis tag using the clonase reaction forming pDEST17-CaCPI. pDEST17-CaCPI was then transformed into *E. coli* BL21 (DE3) cells by electroporation. Cells containing the pDEST17-CaCPI construct were grown at 37°C in Luria broth liquid medium until a D600 of 0.5-1 was reached. The recombinant CaCPI expression was induced by the addition of 1 mM isopropyl-beta-d-thiogalactopyra-noside and cultured at 21°C overnight.

Cell lysate preparation and SDS-PAGE analysis

After IPTG induction, *E. coli* BL21 cells with and without pDEST17-CaCPI were collected by centrifugation at 6,000 rpm for 5 min. Cell pellet were then washed and resuspended in 0.1 M phosphate buffer. Cell lysate was prepared by sonication. Total protein was quantified using the Bradford reagent, ready-to-use (Fermentas, USA) using BSA as the standard and was analyzed on SDS-PAGE.

Papain inhibitory activity assay

The proteinase inhibition assay by CaCPI was determined using BANA (N_{α}-Benzoyl-DL-arginine β-naphthylamide hydrochloride) as a substrate according to the previously described method (Pernas et al., 1998) with some modification. Briefly, total protein from cell lysate (0-250 mg) was pre-incubated with 5 µM papain (Sigma, USA) in activation buffer (100 mM Sodium phosphate, pH 6.5, 10 mM EDTA, 10 mM 2-mercaptoethanol) at 37°C for 10 min. The reaction was started by the addition of 100 µl of 200 µM of BANA as a substrate. The reaction mixture was incubated at room temperature for 20 min and 300 µl of 2% HCl in ethanol (w/v) was added to stop the reaction. The chromophore was then developed by the addition of 300 µl of 0.06% p-dimethylaminocinnamaldehyde in ethanol followed by incubation at room temperature for 15 min and a subsequent measurement of the A_{540}.

RESULTS

Sequence analysis

The cDNA sequence of the *CaCPI* gene was 601 base pairs in length, including 32 base pairs of the 5' untranslated region (UTR) and 197 base pairs of the 3' UTR. An open reading frame of 372 base pairs in length was found, which encoded a deduced amino acid sequence of 123 residues (Figure 1).

Signal peptide analysis, using the SignalP software, showed that the encoded CaCPI protein had a putative secretory signal of 22 amino acids at the N-terminal end. The predicted molecular masses of the precursor and the mature protein were 13.545, and 11.239 kDa, respectively (http://expasy.org/tools/peptide-mass. html). A CDD search (Marchler-Bauer et al., 2007) revealed that the deduced CaCPI protein sequence was identitical to the conserved cystatin-like domain (CY domain; cd00042). CaCPI contained all of the highly conserved blocks that are essential for cysteine proteinase activity. Such conserved regions included the Gly-Gly, the reactive site motif QXVXG (QVVAG), the P-W, and the highly conserved [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N block (LGRFAVDQ HN) that is common among plant cystatins (Figure 1) (Abe and Arai, 1991; Arai et al., 1991; Kondo et al., 1990; Margis et al., 1998; Chu et al., 2011).

AGCGATCGGAAAAGCGAATAAAGAGGAAAGGG
ATG GTT TCC TTG GCC GCG AAG ACG ATC CTC ATT TTC TCC GTC TTT
M V S L A A K T I L I F S V F
CTC TGC GTA ATA GCG ATA GCA GAT CCG ATC TCC ATG GCC AAC
L C V I A I A D P I S M A N
CTC GGA GGG ATC AGA GAC GTT GAG GGG GAG GCT GCC AAC GGC
L G G I R D V E G E A A N G
CTC GAG ATC GAG CAG CTA GGT CGC TTC GCC GTC GAC CAA CAC
L E I E Q L G R F A V D Q H
AAC AAG AAG GAG AAC GCT CTT CTG GAG TTT GCG AGG GTG ATC
N K K E N A L L E F A R V I
AAA GCG AGG GAG CAG GTG GTC GCT GGG ACT CTG CAC CAC TTG
K A R E Q V V A G T L H H L
ACC GTC GAG GTC ATT GAT GCG GGA AAG AAG AAG ACA TAC GAG
T V E V I D A G K K K T Y E
GCC AAG GTG TGG GTC AAG CCA TGG CTC AAC TTC AAG GAG CTT
A K V W V K P W L N F K E L
CAG GAG TTC AGT CAC GCT GGA GAC TCA TCC TAA
Q E F S H A G D S S **stop**
GACATCCCCTGCCATAAGCTAGGGGGTTGAAAGTGAAGACTTCGCCAA
GACTTTATATTGTGTGTCAATAAATGGAGTGATTGCATACTTTGAAAAC
TGATGTCTCTATGTTGTCTGTACTTTGGCCCTCAAACTACCAACTTATAT
AATTAACTAAGAAGTTTATCTACAAAAAAAAAAAAAAAAAAAAAAAAA
AAA

Figure 1. Nucleotide and deduced amino acid sequence of *CaCPI*.

Note: Signal peptide is underlined. The primary reactive site GG and the consensus sites of the secondary contact, QVVAG and PW are boxed. The unique conserved amino acid sequence, LGR-FAVDQHN, in the phyto-cystatin subfamily is highlighted.

The phylogenetic dendrogram was constructed by comparing the deduced sequences of CaCPI with other previously published plant cystatins (CPI). Interestingly, CaCPI was characterized as part of the group of CPI from strawberry (*Fragaria ananassa*), wheat (*Triticum aestivum*) and taro (*Colocasia esculenta*), which also has antifungal properties (Figure 2).

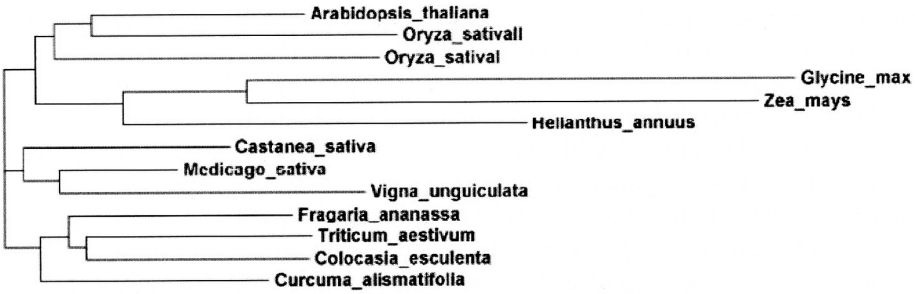

Figure 2. The phylogenetic dendrogram corresponding to the deduced CaCPI protein and 12 other plant cystatins.

SDS-PAGE Analysis of CaCPI

The SDS-PAGE results show that cell lysate from *E. coli* BL21(DE3) carrying pDEST17-CaCPI have an extra band at 12 kDa that is absent from the control *E. coli* BL21(DE3) cells (Figure 3). This band is the CaCPI protein and the peptide size is the same as the size predicted by the computer software.

However, uninduced *E. coli* BL21(DE3) carrying pDEST17-CaCPI can produce recombinant CaCPI too, but at relatively low intensity compared to induced cells (Figure 3).

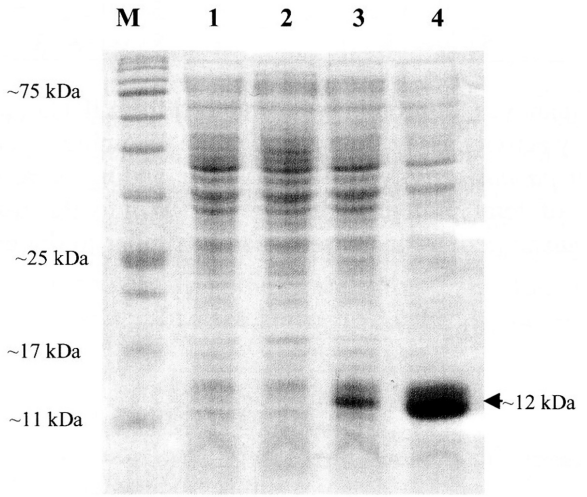

Figure 3. SDS-PAGE analysis of total protein of cell lysate from (1) control *E. coli* BL21 (2) control *E. coli* BL21 induced with 1mM IPTG (3) uninduced *E. coli* BL21carrying pDEST17-CaCPI (4) *E. coli* BL21 carrying pDEST17-CaCPI induced with the 1mM IPTG (M) protein marker.

Papain Inhibitory Activity

To test inhibition ability to papain cysteine protease activity of the recombinant CaCPI protein, 5 μM of papain were pre-incubated with various amounts of crude protein (0-250 μg) from cell lysate of control *E. coli* BL21(DE3) cells and IPTG-induced *E. coli* BL21 carrying pDEST17-CaCPI (Figure 4). The results show that the cell lysate control in the *E. coli* BL21(DE3) cells did not exhibit papain inhibition activity like the crude recombinant CaCPI protein from E. coli BL21(DE3) carrying pDEST17-CaCPI. At low concentrations of the BL21 control protein (0-50 μg), residual papain activity was not statistically different. But crude protein from IPTG-induced E. coli BL21(DE3) with pDEST17-CaCPI showed residual papain activity declined rapidly when added to the reaction mixture. At 100 μg of protein including CaCPI, residual papain activity was only 21.3% for a 78.7% inhibition level (Table 1).

Table 1. Residual papain activity of papain preincubation with protein from control *E. coli* BL21 or IPTG-induced E. coli BL21 carrying pDEST17-CaCPI.

Total protein (µg)	Residual papain activity (%)	
	Control BL21	pDEST17-CaCPI
0	100.00±5.60	100.00±2.47
25	100.00±7.15	66.40±17.47
50	103.16±7.11	34.78±8.33
100	115.02±7.21	21.34±1.19
150	118.18±1.37	33.60±5.97
200	126.88±11.86	58.50±6.53
250	151.78±15.51	83.00±5.93

The 12 kDa recombinant CaCPI protein was produced from IPTG induced E. coli cells. Papain inhibitory activity assay from E.coli crude recombinant protein showed an ability to inhibit papain activity. However, after adding more crude protein (more than 100 µg of total protein including CaCPI) into the reaction mixture, the percentage of papain activity increased steadily similar to the control BL21 protein (Figure 4).

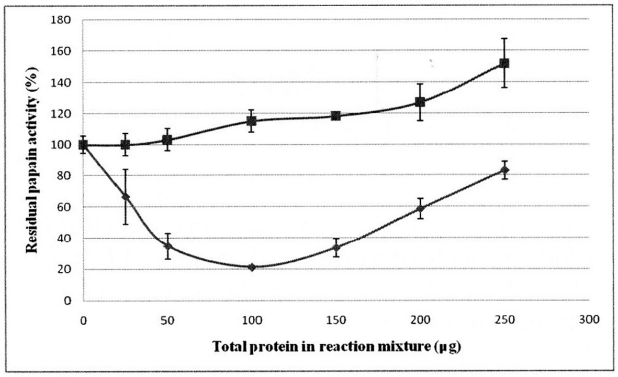

Figure 4. Percentage of residual papain activity of 5 µM papain with/without various amount of total protein from cell lysate of (■) control E. coli BL21 and (♦) IPTG-induced E. coli BL21carrying pDEST17-CaCPI.

DISCUSSION

CaCPI was characterized into group-1 phytocystatin, which was the same as oryzacystatin (OC)-1 from rice. Group-1 phytocystatins was the biggest group of phytocystatin found in plants. They contained only one cystatin domain of about 100 residues with a molecular size around 12-16 kDA and shared high homology with chicken egg white cystatin (Chu et al., 2011; Abe et al., 1987).

For amino acid sequence analysis, the Siam tulip cystatin CaCPI contained conserved motifs and catalytic active motifs that were observed in all previously reported plant CPI sequences. Phylogenetic dendrogram revealed that Siam tulip CaCPI was characterizing as part of the group of cystatin that included strawberry, wheat and taro (Figure 2). This group had antifungal properties. In addition, several reports have found antifungal activity in the *Curcum*a genus, including *Curcuma longa* (Niamsa and Sittiwet, 2009) *Curcuma zedoaria* (Shinobu-Mesquita Cristiane et al., 2011) and *Curcuma caesia* (Krishnaraj and Mathivanan, 2008), suggesting the Siam tulip CaCPI would also contain proteins responsible for antifungal activity.

The recombinant CaCPI protein produced from *E. coli* cells shows ability to inhibit papain activity (Figure 4). However at high concentration of the recombinant CaCPI protein (above 100 μg proteins), the papain activity apparently increases as well as in the control. It is possible that some proteins are released from bacterial cells during extraction and mixed in the crude recombinant protein. These proteins can absorb optical density at 540 nm such as chromophore, enzyme-substrate intermediates, which could result in an error when adding higher volumes of crude protein into the reaction mixture (Pernas et al., 1998)

In this study, we succeeded in isolating CaCPI from a Siam tulip cDNA library. It was found to have 123 amino acid residues and grouped with other plant cystatins with antifungal activity using the clustalW program. Recombinant CaCPI was produced from *E. coli* and had the ability to inhibit papain cysteine protease activity.

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