Cloning, functional expression and characterization of a phytocystatin gene from jelly fig (Ficus awkeotsang Makino) achenes

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ABSTRACT. A cDNA clone encoding a phytocystatin was isolated and identified from about 300 expressed sequence tag (EST) clones in maturing jelly fig (Ficus awkeotsang Makino) achenes. This clone, named FaCYS, consists of 582 bp encoding 114 amino acids with a putative signal peptide. The predicted mature protein contains no cysteine and has a molecular mass of 10.8 kDa with an isoelectric point (pI) of 9.7. FaCYS constructed in nonfusion and fusion vectors were overexpressed in Escherichia coli as nonfusion and his-tagged recombinants, respectively. Both recombinants were found in the soluble fractions of the cell extracts. The purified nonfusion and his-tagged FaCYS exhibited papain inhibitory activity with similar Ki values of 2.7 × 10⁻⁷ M and 2.4 × 10⁻⁷ M, respectively. In addition, his-tagged recombinant proteins showed inhibitory activity toward human cathepsin B, cathepsin L and ficin with Ki values of 5.6 × 10⁻⁷ M, 3.0 × 10⁻⁸ M and 2.0 × 10⁻⁷ M, but no inhibitory activity against stem bromelain. It was tolerant at a wide range of pH values and thermally stable up to 50°C for 30 min. Furthermore, his-tagged FaCYS could arrest the fungal growth of Glomerella cingulata and Sclerotium rofsii.

Keywords: Cystatin; Cysteine protease inhibitor; Jelly fig achene; Phytocystatin.

INTRODUCTION

Cystatins, the natural inhibitors of papain-like cysteine proteases, have been found extensively throughout the animal and plant kingdoms. They reversibly act as pseudo-substrates to enter the active-site cleft of proteases and inhibit proteolytic activity. Three conservative regions were found in the cystatins of animal and plant origin, a G residue near the N-terminus, a central QXVXG motif, and a W residue near the C-terminus. These three conserved regions could form a tripartite wedge interacting with the cysteine protease to cause the inhibition (Turk et al., 1997; Nagata et al., 2000). Plant cystatins, namely phytocystatins particularly possess a unique conserved sequence, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N at the N-terminus of α₁-helix. In addition, the typical structure element of phytocystatins include four β sheets linked to an α-helix in the N-terminal region and two hairpin loops (Arai et al., 2002; Fabienne et al., 2002).

Phylogenetic analyses of known phytocystatins show that two groups are mainly present in plant kingdoms, which have the molecular weight of ∼11 kDa for group I, and ∼23 kDa for group II. Basically, group I shares high homology with the N-terminal region of group II. Group II contains an additional C-terminal extension of ∼10 kDa, which may have physiological importance (Martinez et al., 2007). Plant multicystatins, comprised eight tandem cystatin domains and a high molecular weight of ∼85 kDa, were also found in several plant species, including potato and tomato (Waldron et al., 1993; Girard et al., 2007). They showed inhibitory activity against papain. Following trypsin digestion, the obtained intact potato cystatin domains also showed inhibitory activity (Nissen et al., 2009). Cystatins play important roles in a variety of biological and pathological processes by regulating endogenous cysteine protease activities. The physiological roles of phytocystatins are diverse and complicated. They have been proposed to intervene in germination, seed maturation, storage protein turnover and programmed cell death (Solomon et al., 1999; Corr-Menguy et al., 2002; Martinez et al., 2009). They may protect plants from environmental
stimuli in response to biotic and abiotic stresses (Gaddour et al., 2001; Zhang et al., 2008). Some of them act as defense proteins when invaded by pathogens whose cysteine proteases play a role in cell replication, or when attacked by pests whose cysteine proteases serve as digestive enzymes in the gut (Gutierrez-Campos et al., 1999; Arai et al., 2002). Moreover, due to their broad specificity of proteases, phytocystatins are targets of interest for biotechnological applications, as stabilizers in food processing, for anti-cancer, anti-viral and chemotherapy of leishmaniasis (Aoki et al., 1995; Shyu et al., 2004; Gianotti et al., 2008; Ordóñez-Gutiérrez et al., 2009).

Jelly fig (Ficus awkeotsang Makino), a member of the family Moraceae, is a unique woody vine growing in the mountain areas of Taiwan. A water extract of the pericarpial portion of jelly fig achenes has been utilized to produce jelly curd, a beverage popular in local markets for around two hundred years. To extend the application of jelly fig achenes, several achene proteins have been identified and characterized (Wang and Tzen, 2005; Chua et al., 2008). In this study, a cDNA clone encoding a putative phytocystatin, FaCYS was isolated and identified from in-house generated EST clones in maturing, FaCYS was isolated and identified from in-house generated EST sequencing and sequence analyses. Moreover, due to their broad specificity of proteases, phytocystatins are targets of interest for biotechnological applications, as stabilizers in food processing, for anti-cancer, anti-viral and chemotherapy of leishmaniasis (Aoki et al., 1995; Shyu et al., 2004; Gianotti et al., 2008; Ordóñez-Gutiérrez et al., 2009).

MATERIALS AND METHODS

Plant materials

Mature and fresh maturing jelly fig (Ficus awkeotsang Makino) achenes were purchased from local growers. Maturing achenes, that had been pollinated approximately 30 days before, were used for mRNA extraction and cDNA library construction.

RNA isolation, cDNA library construction, in-house generated EST sequencing and sequence analyses

Total RNA was prepared by grinding 2 g maturing achenes in liquid nitrogen using the phenol/SDS method (Wilkins and Smart, 1996). The mRNA was purified from total RNA using oligo-dT magnetic beads (Dylan Biotech, Oslo, Norway). cDNA was synthesized with 5 µg of mRNA using a Stratagene ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). It was size fractioned into two parts of 0.5-1.0 kb and 1.0-2.0 kb (Chua et al., 2008). Two ZAP cDNA libraries were subsequently constructed with the two recovered DNA fractions. Mass excision was performed to convert the phage libraries into bacterial pBluescript libraries.

Bacterial libraries were sprayed and grown on LB plates with antibiotic ampicillin. Colonies were randomly selected and recombinant cDNA plasmids were prepared by sonication in a 10 mM phosphate buffer (pH 8.0). They were fractionated into soluble and precipitated fractions by centrifugation at 10,000 × g, then subjected to SDS/PAGE and western blot analyses.

Using a Miniprep Purification kit (Protech, Taipei, Taiwan) prior to EST sequencing by the dideoxy chain-termination method with a BigDye terminator cycle sequencing kit and an ABI 377 DNA sequencer (Perkin-Elmer, CT, USA). Approximately 200 and 100 EST clones derived from the two cDNA libraries were sequenced and analyzed with the Blast program (http://www.ncbi.nlm.nih.gov/blast/) at the National Center for Biotechnology Information in the USA (Altschul et al., 1997; Zhang and Madden, 1997). A cDNA clone named FaCYS encoding a putative phytocystatin was subsequently isolated and identified. The N-terminal signal peptide was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) in the World Wide Web Prediction Server Center for Biological Sequence Analysis (Emanuelsson et al., 2007). Sequence alignment was executed by the CLUSTAL W program at Pôle Bioinformatique Lyonnais (PBIL) (Lyons, France) (Thompson et al., 1994).

Bacterial over-expression of recombinant phytocystatin

According to the FaCYS open reading frame, two primers (JF-CYS5, 5’-TCCATATGCGTCCTCACTGC-CTCCTCG-3′; and JF-CYS3, 5’-CCGCTCAGCTAAT-TGACAAAGCTTAAAA-3′) were designed and synthesized. Primer JF-CYS5 had an Ndel recognition sequence; and primer JF-CYS3 had an Xhol recognition sequence and the stop codon. Using two primers, JF-CYS5 and JF-CYS3, the 0.35 kb FaCYS DNA fragment was amplified by PCR reaction (30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s). The amplified DNA fragment was ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) then transformed into E. coli DH5α. The nucleotide sequence of the insert was confirmed by sequencing. The insert DNA fragment was digested with Ndel and Xhol and then constructed into the 6xhis-tag fusion expression vector, pET28a, or the nonfusion expression vector, pET29a (Novagen, Madison, WI, USA). Recombinant protein over-expression in E. coli BL21(DE3) was induced after adding 1 mM isopropyl thio-β-D-galactoside (IPTG) under the T7 RNA polymerase/promoter system. After induction for 4 h at 37°C, the E. coli cells were harvested and lysed by sonication in a 10 mM phosphate buffer (pH 8.0). They were fractionated into soluble and precipitated fractions by centrifugation at 10,000 × g, then subjected to SDS/PAGE and western blot analyses.

Recombinant phytocystatin affinity purification

To enable his-tagged recombinant protein purification, the extract was incubated overnight with TALON resin (Clontech, Palo Alto, CA, USA) at 4°C under gentle agitation. The resin was equilibrated and washed with appropriate buffers according to the manufacturer’s instructions. His-tagged FaCYS were eluted with a buffer containing 150 mM imidazole, and underwent dialysis with a 10 mM phosphate buffer (pH 8.0). For nonfusion recombinant phytocystatin purification, papain was used...
as a ligand enzyme to prepare an affinity column as in our previous work (Shyu et al., 2004). Papain was coupled to CNBr-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden) at a concentration of 5 mg/ml of gel according to the manufacturer’s instructions. The nonfusion recombinant FaCYS from the soluble fraction of the E. coli cell lysate was incubated overnight with papain coupled resin at 4°C under gentle agitation. The affiliated proteins were eluted with 50 mM K$_2$PO$_4$ at pH 11.5 containing 0.5 M NaCl and 10% (v/v) glycerol. The eluate was adjusted to pH 7.4 with a 5 M sodium formate buffer at pH 3.0, then underwent dialysis with a 10 mM phosphate buffer (pH 8.0). The eluate was further applied to a cation-exchange column (HiTrap SP FF column, GE Healthcare, Uppsala, Sweden) previously equilibrated with a 50 mM phosphate buffer at pH 7.5. The flowthrough fraction of the HiTrap SP column was collected for SDS/PAGE analyses and the purified nonfusion protein purified to apparent homogeneity was obtained. The protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as standard.

**Antibody preparation and western blot analysis**

The recombinant his-tagged phytocystatin overexpressed in E. coli was first purified, resolved by SDS/PAGE, then eluted from the gel with Electro-Eluter, according to its instruction manual (Bio-Rad, Hercules, CA, USA). Antibodies against the recombinant his-tag fusion FaCYS were raised in chickens, and chick egg immunoglobulins were collected for immunoassays (Polson, 1990). Proteins resolved by SDS/PAGE in these immunoassays were transferred onto a nitrocellulose membrane (Pall, Ann Arbor, MI, USA) in a Trans-Blot system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. The membrane was subjected to immunodetection by using secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), then incubated for color development with 4-chloro-1-naphthol (Sigma) containing H$_2$O$_2$.

**Protease activity assay and protease inhibitory measurement**

A protease inhibitory assay was performed using papain or ficin as the target enzyme and N-benzoyl-L-arginine-2-naphthylamide (BANA) (BANA, papain and ficin were purchased from Sigma-Aldrich, MO, USA) as the substrate (Abe et al., 1994). Various amounts of FaCYS were incubated with 10 µg of papain (2.9 U/mg) or 30 µg of ficin (0.21 U/mg) in a 250-µl assay solution containing 0.1 M sodium phosphate at pH 6.1, and 2 mM EDTA, at 37°C for 10 min. The proteolytic reaction was carried out by adding 250 µl of 20 µM Z-Phe-Arg-MCA at 37°C for 10 min, and terminated after addition of 500 µl of stop buffer, pH 4.3 containing 30 mM sodium acetate and 70 mM acetic acid. The liberated product, 7-amino-4-methyl-coumarin, was estimated by a spectrofluorometer (Beckman coulter Inc., Fullerton, USA) at an excitation of 360 nm and an emission of 465 nm. Protease residual activity (%) and the Ki value was then determined according to the equation described above.

**Thermal and pH stability test**

To examine its thermal stability, the purified recombinant FaCYS was incubated at 30-80°C for 30 min in a 10 mM phosphate buffer, pH 7.5; while one protein sample was placed on ice as the control. After incubation at room temperature for another 30 min, the recombinant protein was subjected to papain inhibitory activity measurement as described above. Furthermore, the protein was incubated at 50°C and 60°C for 0-30 min prior to determine papain inhibitory activity (%). To examine its pH stability, the recombinant FaCYS was in a 50 mM sodium citrate buffer at pH 2.0-4.0, 50 mM sodium acetate buffer at pH 4.0-6.0, 50 mM sodium phosphate at pH 6.0-8.0 and 50 mM Tris-HCl at pH 8.0-10.0. Papain inhibitory activity was determined after incubation at 37°C for 30 min.

**Antifungal activity assay**

The purified recombinant FaCYS was applied to examine the growth inhibition of phytopathogenic fungi including Alternaria brassicace, Fusarium oxysporum, Glomerella cingulata, Phellinus noxius, Rhizoctonia solani for 15 min, and the absorbance of the mixture was measured at 540 nm. The reaction without any protease and FaCYS was used as the zero control. The protease inhibitory activity (%) was calculated by the equation, \[(A - A')/A \times 100\%\], and protease residual activity (%) was calculated by the equation, \[(A'/A) \times 100\%\], where A and A' represented the OD$_{540}$ in the absence and presence of FaCYS. The Ki value was determined by plots (1/v versus [I]) (Dixon, 1953), where v represented protease residual activity and [I] was the concentration of FaCYS. Moreover, molar ratio of [FaCYS]/[papain] was determined by \[ [23 \times \text{the amount of FaCYS (µg)}] / [10 µg \times \text{(molecular mass of recombinant FaCYS)}] \] according to 10 µg of papain used in the assay and the molecular mass, ~23 kDa (for papain), ~10.9 kDa (for nonfusion FaCYS) and ~11.7 kDa (for fusion FaCYS).

When using another protease, cathepsin B, cathepsin L or bromelain as target enzyme, Z-Phe-Arg-MCA (Peptide Institute, Osaka, Japan) was used as substrate (Rassam and Laing, 2004). Various amounts of recombinant FaCYS were incubated with 2.6 µg of cathepsin B (200 U/mg) (Calbiochem, Darmstadt, Germany), 20 ng of cathepsin L (6000 U/mg) (BioVision, CA, USA) or 20 µg of bromelain (2.4-3 FIP U/mg) (Acros Organics, NJ, USA) in a 250-µl assay solution containing 0.1 M sodium phosphate at pH 6.1, and 2 mM EDTA, at 37°C for 10 min. The proteolytic reaction was carried out by adding 250 µl of 20 µM Z-Phe-Arg-MCA at 37°C for 10 min, and terminated after addition of 500 µl of stop buffer, pH 4.3 containing 30 mM sodium acetate and 70 mM acetic acid. The liberated product, 7-aminomethylcoumarin, was estimated by a spectrophotometer (Beckman coulter Inc., Fullerton, USA) at an excitation of 360 nm and an emission of 465 nm. Protease residual activity (%) and the Ki value was then determined according to the equation described above.
and Sclerotium rofsii. These fungi were cultured in potato dextrose agar (PDA) for 1-2 weeks. The sclerotinias of S. rofsii was inoculated in 1/2 PDA plate with or without FaCYS at 24°C for 4 h. The hyphal growth inhibition was observed and recorded under a light microscope.

For A. brassicae, F. oxysporum and G. cingulata, the spores of pathogens were suspended in 0.1% (w/v) glucose to a concentration of 10⁴ spores/mL. The fungal spore stock (approximately 500 spores) was cultured in the glucose solution with or without FaCYS at 24°C for 24 h. Inhibition of spore germination was examined under a light microscope. For P. noxius and R. solani, mycelium discs of 1 cm in diameter were placed at the center of PDA plates and incubated at 24°C. As the colonies reached 2.5 cm in diameter, 20 µl of PBS buffer, with or without FaCYS, were poured onto the discs (1 cm in diameter and four discs per plate). The plates were incubated at 24°C for 2-3 days. The experiment was carried out three separate times under the same conditions.

RESULTS AND DISCUSSION

Isolation and characterization of a cDNA clone coding for a putative jelly fig phytocystatin

A full-length cDNA fragment (FaCYS, Genbank accession numbers: GU084175) encoding a putative phytocystatin was obtained during sequencing and analyses of 300 EST clones of maturing jelly fig achenes. The nucleotide sequence in FaCYS consists of 582 bp including a 69-nucleotide 5'-untranslated region, an open reading frame of 345 nucleotides and a 168-nucleotide 3'-untranslated region. Its deduced protein sequence comprised 114 amino acids with a putative signal peptide (1-19 a.a.). The predicted mature protein contains no cysteine and had a molecular mass of 10.8 kDa with an isoelectric point (pI) of 9.7. According to the NCBI Blast search, FaCYS is closely related to the reported or putative group I phytocystatins from grapefruit (CystL-1, Genbank accession numbers: AAG38521) (Shatters et al., 2004). Moreover, no FaCYS was immunodetected either inside the seeds or in the pericarp of jelly fig achenes (data not shown). Furthermore, no FaCYS was found in the cell lysate soluble fractions of E. coli cell lysate soluble fractions (Figure 2A). Nonfusion FaCYS induction was not as clear as for his-tagged FaCYS (Figure 2B). Purified his-tagged FaCYS was obtained by Ni²⁺-containing affinity chromatography in a single step of purification; while nonfusion FaCYS was purified via a papain-coupled affinity column and a cation-exchange column. Both recombinant FaCYSs were purified to apparent homogeneity as revealed by SDS/PAGE (Figure 3A and 3B). Western blot analyses indicated that these purified recombinants could be recognized by antibodies raised against his-tagged FaCYS (Figure 3A and 3B); therefore, both recombinants were heterologously expressed in E. coli BL21(DE3).

Inhibitory activity of recombinant FaCYS toward papain and other cysteine proteases

The expression of recombinant his-tagged FaCYS was better than that of recombinant nonfusion FaCYS. The expression of his-tagged FaCYS with 1.2 mg/L yield was not as efficient as the previous work; in contrast, bacterial over-expression of pineapple phytocystatins reached 63.7 mg/L and 3.7 mg/L of yields by using pET28a and pET29a as expression vectors, respectively (Shyu et al., 2004). Moreover, no FaCYS was immunodetected either inside the seeds or in the pericarp of jelly fig achenes (data not shown). It is thus suggested that FaCYS is a minor protein in jelly fig achenes.

Inhibitory activity of recombinant FaCYS toward papain and other cysteine proteases

The nonfusion and his-tagged FaCYSs were bacterial-expressed, purified and then characterized. The purified recombinants showed inhibitory activity against papain in a dose-dependent manner with Ki value of 2.7 × 10⁻⁷ M for nonfusion FaCYS and 2.4 × 10⁻⁷ M for his-tagged FaCYS, respectively (Figure 4). FaCYS possessed papain inhibitory activity with similar Ki values to those of reported

Heterologously over-expression and western blot analysis of FaCYS

The cDNA fragments of FaCYS were constructed in nonfusion and his-tagged fusion expression vectors, pET28a and pET29a. In comparison with no IPTG induction, his-tagged FaCYS was induced after 4 hours of adding IPTG and was mostly found in E. coli cell lysate soluble fractions (Figure 2A). Nonfusion FaCYS induction was not as clear as for his-tagged FaCYS (Figure 2B). Purified his-tagged FaCYS was obtained by Ni²⁺-containing affinity chromatography in a single step of purification; while nonfusion FaCYS was purified via a papain-coupled affinity column and a cation-exchange column. Both recombinant FaCYSs were purified to apparent homogeneity as revealed by SDS/PAGE (Figure 3A and 3B). Western blot analyses indicated that these purified recombinants could be recognized by antibodies raised against his-tagged FaCYS (Figure 3A and 3B); therefore, both recombinants were heterologously expressed in E. coli BL21(DE3).

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group I phytocystatins such as from sugarcane (Ki of Can-
ecPI-2, $2 \times 10^{-7}$ M), Job’s tears (Ki of CysM, $1.9 \times 10^{-7}$ M) and rice (Ki of OC-II, $8.3 \times 10^{-7}$ M) (Kondo et al., 1990; Yoza et al., 2002; Gianotti et al., 2006). The Ki value of FaCYS against papain were 1-3 orders magnitude higher than other reported group I phytocystatins including OC-I from rice (Ki of $3.0 \times 10^{-8}$ M), CsC from chestnut (Ki of $2.9 \times 10^{-9}$ M), and KCPI1 from kiwifruit (Ki of $1.6 \times 10^{-10}$ M) (Kondo et al., 1990; Rassam and Laing, 2004). However, it should be noted that these experimental conditions were different.

Papain almost lost its activity when incubated with equimolar FaCYS (Figure 3). Since both recombinants showed similar Ki values, it suggested that the polyhisti-
dine tag did not structurally interfere with FaCYS activity. Besides, FaCYS showed inhibitory activity against human cathepsin B, cathepsin L and ficin with \( K_i \) values of \( 5.6 \times 10^{-7} \) M, \( 3.0 \times 10^{-8} \) M and \( 2.0 \times 10^{-7} \) M, but showed no inhibitory activity against pineapple stem bromelain (data not shown). Regarding other reported phytocystatins, \( K_i \) of KCPI1 to human cathepsin B, cathepsin L, ficin and bromelain were \( 8.0 \times 10^{-9} \), \( 1.0 \times 10^{-12} \), \( 3.2 \times 10^{-11} \), and \( 1.5 \times 10^{-10} \) M (Rassam and Laing, 2004); while \( K_i \) of CsC against human cathepsin B and ficin were \( 4.7 \times 10^{-7} \) and \( 6.5 \times 10^{-8} \) M (Pernas et al., 1998). A pineapple phytocystatin, AcCYS1 had a putative signal peptide and an extended unique AE-rich N-terminal trunk (NTT). NTT-containing AcCYS1 could inhibit bromelain, and was post-translationally removed during ripening (Neuteboom et al., 2009). Without NTT, AcCYS1 exhibited poor inhibitory activity against bromelain (Neuteboom et al., 2009; Shyu et al., 2004). KCPI1 exhibited good inhibition activity to papain and bromelain; in contrast, FaCYS showing inhibitory activity against papain was unable to inhibit the proteolytic activity by bromelain.

\[ \text{pH and thermal stability of the recombinant FaCYS} \]

To assess the thermal stability of FaCYS, the recombinant proteins were subjected to a heat treatment of 30-80°C for 30 min. FaCYS was shown to be thermally stable up to 50°C (Figure 5A). However, it rapidly lost almost 60% of its inhibitory activity when the treatment temperature was 60°C for 10 min (Figure 5B). To examine pH effect on FaCYS, the purified his-tagged FaCYS was incubated at various pH values (pH=2-10) for 2 h prior to measuring papain inhibitory activity. Different pH environments, including those strongly acidic or basic, had no apparent affect on FaCYS activities (data not shown). Despite the lack of intra-disulfide bonds to sustain protein structure, our results indicated that FaCYS was a pH- and thermo-stable protein. Although phytocystatins were known as a proteins without disulfide bonds, several phytocystatins were reportedly thermo-stable proteins, such as pineapple cystatin, stable up to 60°C for 30 min (Shyu et al., 2004), and rice OC-II, up to 65°C for 10 min (Ohtsubo et al., 2007).

\[ \text{Antifungal activity of recombinant FaCYS} \]

Phytocystatins have gained attention for protecting plants from herbivorous insect and fungal attack. The growth inhibition effect of purified recombinant FaCYS was tested on some severe tropical phytopathogenic fungi, including *Fusarium oxysporum*, *Glomerella cingulata*, *
Phellinus noxius, Rhizoctonia solani and Sclerotium rolfsii. The results showed no FaCYS inhibitory effect on *F. oxysporum*, *P. noxius* and *R. solani*, but an FaCYS inhibitory effect was observed on spore germination of *G. cingulata* and the mycelia growth of *S. rolfsii* (Figure 6). *G. cingulata* spore germination was inhibited to 27% and the of *S. rolfsii* mycelia growth was almost completely inhibited as the FaCYS concentration reached 400 μg/mL.

Various phytocystatins cause different growth inhibitions on fungi, and these inhibitions vary depending on fungal species. Recently, a tarocystatin from *Colocasia esculenta*, a group II phytocystatin, inhibited the mycelia growth of *S. rolfsii* (Yang and Yeh, 2005). Similarly, FaCYS showed a toxic effect on the mycelia growth of *S. rolfsii*. Though the mechanism for phytocystatin anti-fungal activity is still unclear, *S. rolfsii* appears sensitive to phytocystatins. It is known that *S. rolfsii* causes great damage (Southern Blight) to tomato, peanut and banana; while *G. cingulata* causes Anthracnose to mango and papaya. The inhibition effects of FaCYS provide potential biotechnological applications on these fruits. In addition, full length, N-terminal and C-terminal extension regions of tarocystatin were produced separately (Wang et al., 2008). Full length tarocystatin possess stronger papain inhibition...
knowledge, it is the first report of phytocystatin from the *Ficus* genus as well as the *Moraceae* family. However, the current work revealed that recombinant FaCYS in pET28a and pET29a expression was not efficient. Obtaining enough recombinant FaCYS for further detailed study and application of this unique protein is now our mission.

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愛玉瘦果植物性硫氫蛋白酶抑制蛋白基因的選殖、功能性表達與重組蛋白質特性分析

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由成熟愛玉瘦果的 cDNA 資料庫中分析約 300 個表現序列標籤 (EST clone)。其中一 cDNA 選殖株之 DNA 序列經比對及鑑譯分析結果，預測為植物性硫氫蛋白酶抑制蛋白 (phytocystatin)。此選殖株被命名為 FaCYS，全長含有 582 bp，可轉譯得到 114 個胺基酸。經預測具有訊息勝肽，成熟的 FaCYS 不含任何 cysteine，分子量為 10.8 kDa，pI 值為 9.7。將 FaCYS 分別選殖至非融合與融合 (his-tagged) 的表達載體中，將重組質體轉殖於大腸桿菌並表達得到可溶性的非融合與融合 FaCYS 重組蛋白質。純化後所得之非融合與 his-tagged 融合 FaCYS 重組蛋白皆具有 papain 之抑制活性，其 Ki 值極為接近，分別為 2.7 x 10⁻⁷ M 與 2.4 x 10⁻⁷ M。進一步分析發現 his-tagged FaCYS 對人類 cathepsin B、cathepsin L 及 ficin 亦具有抑制活性，其 Ki 值分別為 5.6 x 10⁻⁷ M、3.0 x 10⁻⁸ M 和 2.0 x 10⁻⁷ M；但對 bromelain 則無抑制活性。his-tagged FaCYS 在不同的 pH 環境中具有很好的耐受性，亦具有不錯的熱穩定性，於 50°C 下 30 分鐘後仍維持對 papain 的抑制活性。此外，此重組蛋白可抑制 Glomerella cingulata 及 Sclerotium rofsii 菌絲的生長。

關鍵詞：硫氫蛋白酶抑制蛋白；硫氫蛋白酶；愛玉瘦果；植物性硫氫蛋白酶抑制蛋白；重組蛋白。