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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 *K*i 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 *K*i 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).

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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 expressed sequence tag (EST) clones in maturing jelly fig (*Ficus awkeotsang* Makino) achenes. Based on sequence alignment, FaCYS was classified as group I phytocystatin. The recombinant FaCYS was functionally over-expressed in *Escherichia coli*, purified and characterized as a thermal and pH-stable protein. Its antifungal activity was further examined.

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 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'-TCCATATGCGTCCTCACTGCCTCCTCG-3'; and JF-CYS3, 5'-CCGCTCGAGCTAATTGACAAGCGTAAA-3') were designed and synthesized. Primer JF-CYS5 had an *Nde*I recognition sequence; and primer JF-CYS3 had an *Xho*I 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 *Nde*I and *Xho*I 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 resins (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_3PO_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_2O_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 mM EDTA, and 2 mM 2-mercaptoethanol at 37°C for 5 min. Subsequently, the proteolytic reaction was initiated by adding 0.1 ml of 1 mM BANA at 37°C for 10 min, and terminated by adding 0.5 ml of 2% HCl/ethanol. The color was developed followed by the addition of 0.5 ml of 0.06% *p*-dimethylaminocinnamaldehyde (Sigma-Aldrich, MO, USA) in ethanol 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, $[(A\text{-}A^*)/A] \times 100\%$, 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 *K*i 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 × the amount of FaCYS (μg)] /[10 μg × (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-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 *K*i 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 30-80ºC 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 brassicae*, *Fusarium oxysporum*, *Glomerella cingulata*, *Phellinus noxius*, *Rhizoctonia solani*

and *Sclerotium rofsii*. These fungi were cultured in potato dextrose agar (PDA) for 1-2 weeks. The sclerotinia of *S. rofsii* was inoculated in 1/2 PDA plate with or without FaCYS at 24°C for 24 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 10^4 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 in maturing jelly fig achenes. The nucleotide sequence of *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), wine grape (XP_002274494), kiwifruit (KCPI1, Q6TPK4; AAR92224; AAR92225) (Rassam and Laing, 2004), Chinese cabbage (ADK13077), castor bean (XP_002531171), and black cottonwood (XP_002307893). FaCYS showed about 50-60% similarity to these phytocystatins. For example, it shares 53% identity with grapefruit CystL-1 in 108 a.a., 59% identity with putative phytocystatin from wine grapes (XP_002274494) in 108 a.a, and 56-58% identity with phytocystatin from kiwifruit (KCPI1, Q6TPK4; AAR92224; AAR92225) in 87 a.a.. In addition, FaCYS shares about 30% identity to OC-I and OC-II, also group I rice phytocystatins (AAA33903, CAA40860) (Abe et al., 1987; Kondo et al., 1991). OC-I was the first cloned and well studied phytocystatin to show homology with animal cystatins.

After sequence alignment with the phytocystatin homologs and a chicken egg-white cystatin, FaCYS showed the typical inhibitory motifs, a G residue near the N-terminus, a central QXVXG and a W residue near the C-terminus (Figure 1). These three conserved regions could form a tripartite wedge and interact with the cysteine protease to cause inhibition. The conserved glycine residue was for the optimal orientation of its N-terminal domain toward cysteine protease. The signature motif QXVXG and the conserved W residue were localized on the first and second hairpin loops, which may interact directly with the active site of target cysteine protease. FaCYS also contains a unique consensus sequence of phytocystatins at its N-terminal proximity, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N, only at the third position where D replaces [RKE]. Phytocystatins from kiwifruit (KCPI1, AAR9224, and AAR9225) and from grapefruit (CystL-1) showed that Q replaces [RKE] (Rassam and Laing, 2004; Shatters et al., 2004).

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 Ni^{2+}-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).

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 *K*i 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 *K*i values to those of reported

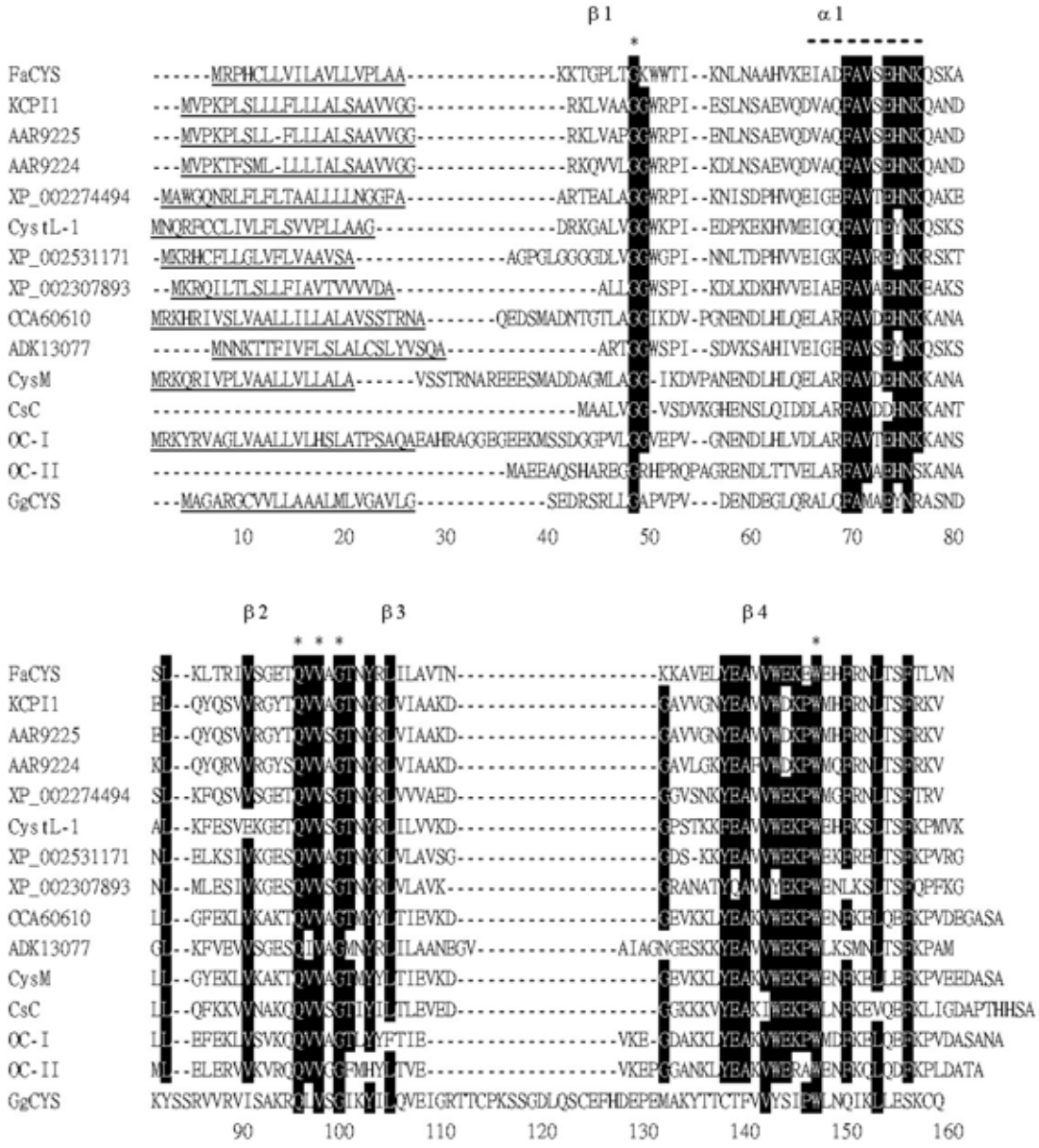

Figure 1. Protein sequence alignment of FaCYS (accession number, GU084175) with cystatins from kiwifruit (KCPI1, Q6TPK4; AAR9224; AAR9225), grapefruit (CystL-1, AAG38521), wine grape (XP_002274494), castor bean (XP_002531171), black cottonwood (XP_002307893), maize (CCA60610), Chinese cabbage (ADK13077), Job's tear (CysM, AB037156), chestnut (CsC, CAA11899), rice (OC-I, AAA33903; OC-II, CAA40860), and chicken (GgCYS, P01038). Short names of those reported cystatins were indicated before accession number. Signal peptides for the aligned sequences were indicated by under lines. Broken lines in the sequences represent gaps introduced for maximum sequence similarity. Five conserved amino acid residues, including a G residue near the N-terminus, a central [QXVXG] motif, and a W residue near the C-terminus, were marked by asterisks. The unique conserved amino acid sequence, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N, in the phytocystatin subfamily was marked by the upper broken line on the top of the sequences. Amino acid residues that were identical in at least eleven out of the fifteen sequences were highlighted. The location of secondary structures, including a α helix and four β sheets, were indicated.

group I phytocystatins such as from sugarcane (*Ki* of CaneCPI-2, 2×10^{-7} M), Job's tears (*Ki* of CysM, 1.9×10^{-7} M) and rice (*Ki* of OC-II, 8.3×10^{-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 3.0×10^{-8} M), CsC from chestnut (*Ki* of 2.9×10^{-8} M), and KCPI1 from kiwifruit (*Ki* of 1.6×10^{-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-

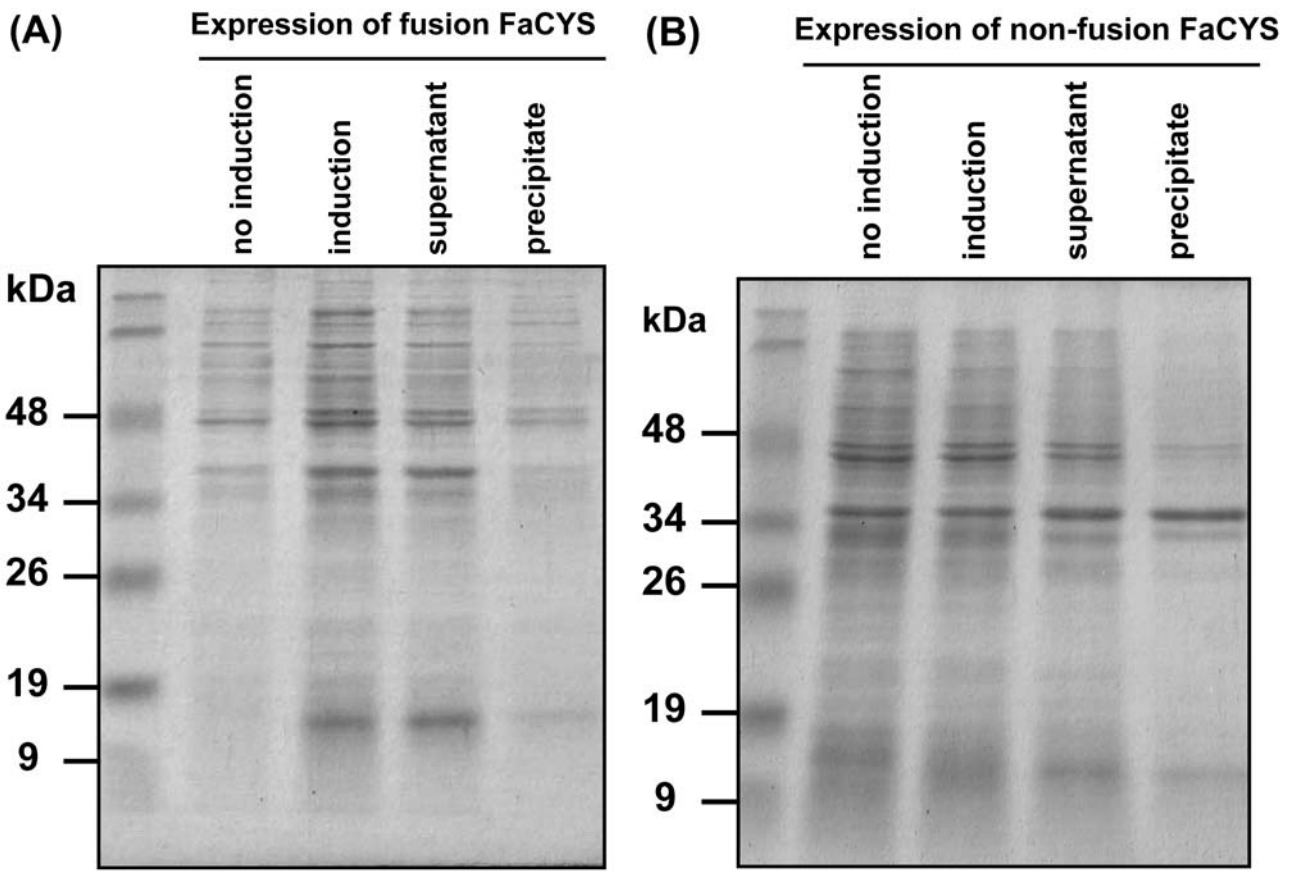

Figure 2. SDS/PAGE analysis of fusion and non-fusion FaCYS over-expressed in *E. coli*. (A) Total proteins were extracted from *E. coli* cells with or without IPTG induction, and fractionated into supernatant and precipitate; (B) Total proteins were extracted from *E. coli* cells with or without IPTG induction, and fractionated into supernatant and precipitate.

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 5.6×10^{-7} M, 3.0×10^{-8} M and 2.0×10^{-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 8.0×10^{-9}, 1.0×10^{-12}, 3.2×10^{-11}, and 1.5×10^{-10} M (Rassam and Laing, 2004); while *K*i of CsC against human cathepsin B and ficin were 4.7×10^{-7} and 6.5×10^{-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 inhibition 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.

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 exam 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).

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 phytopathgenic fungi, including *Fusarium oxysporum*, *Glomerella cingulata*,

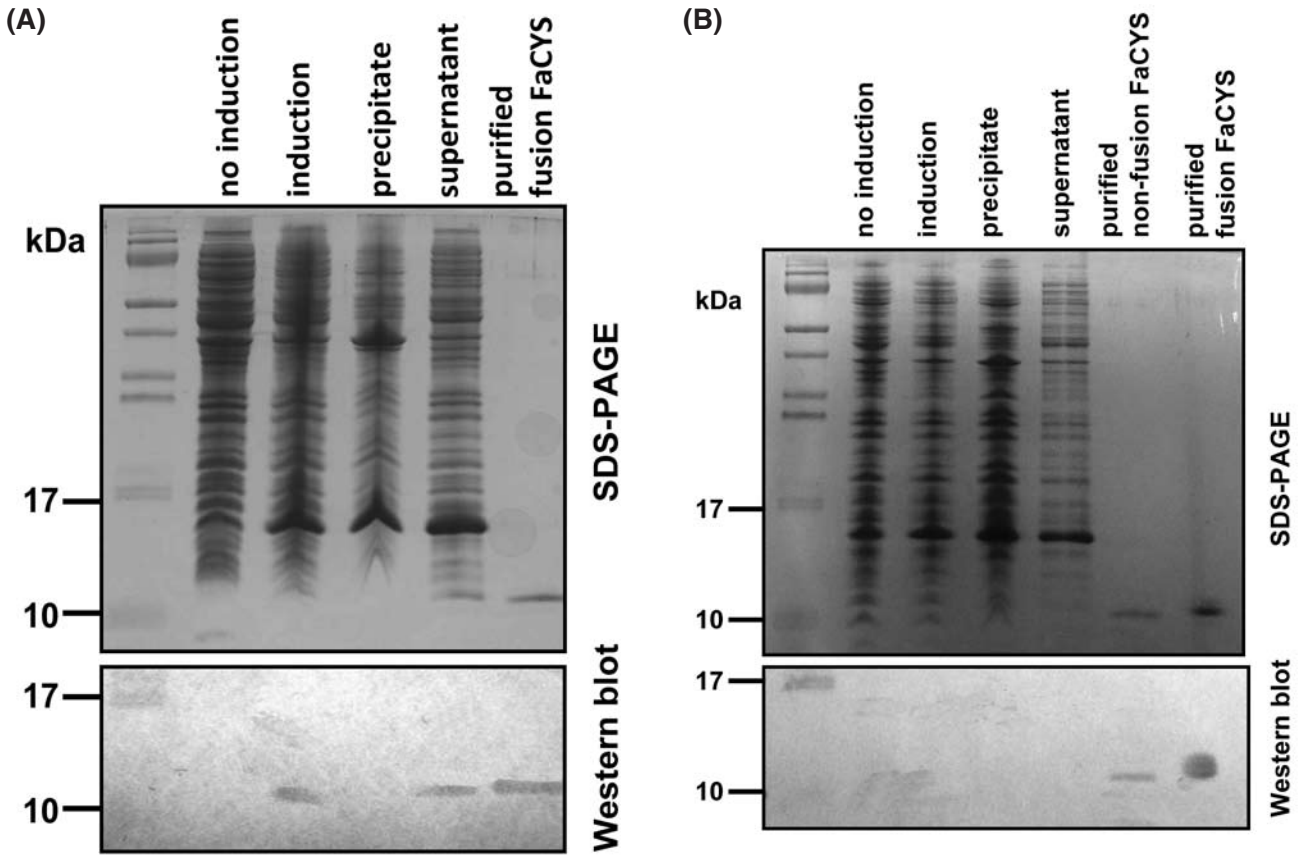

Figure 3. SDS/PAGE and western blotting analysis of the purified fusion and non-fusion FaCYS over-expressed in *E. coli*. (A) Total proteins were extracted from *E. coli* cells with or without IPTG induction, and fractionated into supernatant and precipitate. The recombinant his-tagged FaCYS was then purified from soluble proteins of the *E. coli* lysate by affinity column. These proteins were resolved by SDS/PAGE. A duplicate gel was transferred onto a nitrocellulose membrane and subjected to immunoblotting with antibodies against his-tagged FaCYS; (B) Total proteins were extracted from *E. coli* cells with or without IPTG induction, and fractionated into supernatant and precipitate. The nonfusion recombinant was then purified from soluble proteins of the *E. coli* lysate by papain-coupling affinity column and cation exchange column. These proteins were resolved by SDS/PAGE. A duplicate gel was transferred onto a nitrocellulose membrane and subjected to immunoblotting with antibodies against his-tagged FaCYS.

Phellinus noxius, *Rhizoctonia solani* and *Sclerotium rofsii*. 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. rofsii* (Figure 6). *G. cingulata* spore germination was inhibited to 27% and the of *S. rofsii* 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. rofsii* (Yang and Yeh, 2005). Similarly, FaCYS showed a toxic effect on the mycelia growth of *S. rofsii*. Though the mechanism for phytocystatin antifungal activity is still unclear, *S. rofsii* appears sensitive to phytocystatins. It is known that *S. rofsii* 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

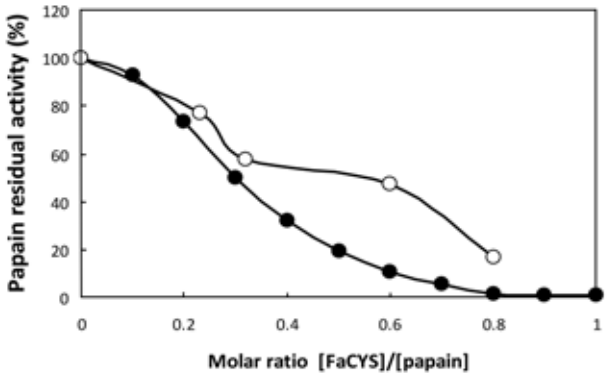

Figure 4. Inhibition of papain activity by the recombinant FaCYS. Ten microgram of papain was incubated with various amounts of the purified his-tagged and nonfusion FaCYS. The residual hydrolytic activity was determined.

length, N-terminal and C-terminal extension regions of tarocystatin were produced separately (Wang et al., 2008). Full length tarocystatin possess stronger papain inhibition

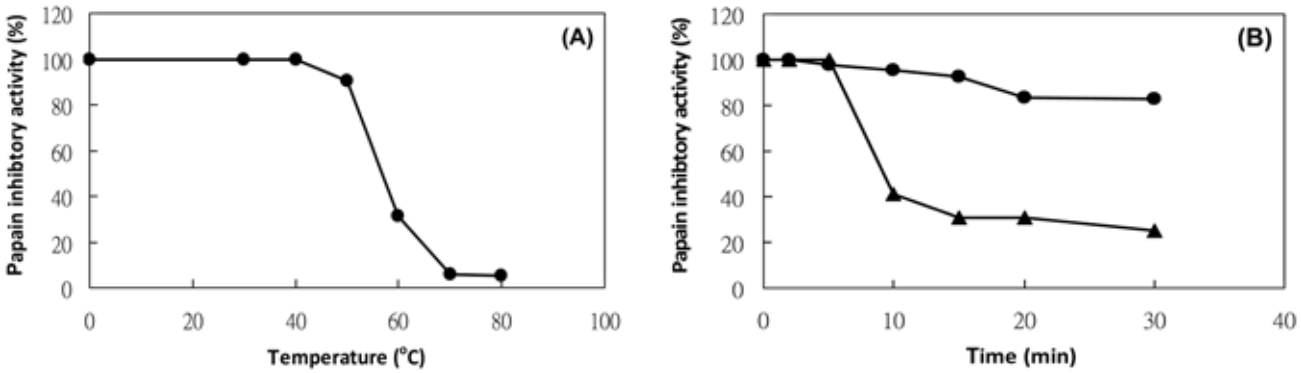

Figure 5. Thermal stability of his-tagged FaCYS. The purified recombinant FaCYSs were subjected to a heat treatment either under different temperatures for 30 min (A) or at 50°C and 60°C for different periods of time (B) prior to measure papain inhibitory activity.

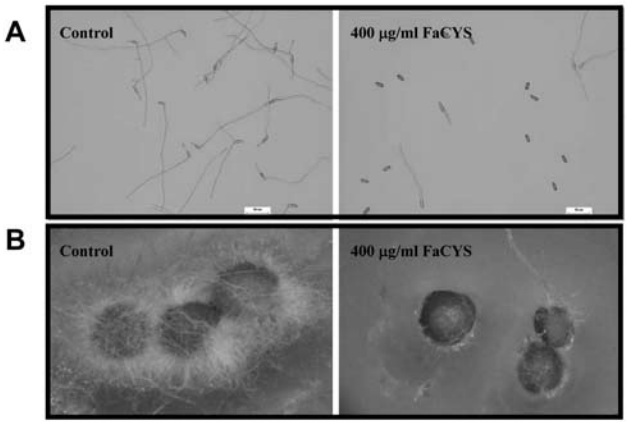

Figure 6. Growth inhibition of phytopathgenic fungi by his-tagged FaCYS. It was observed by microscopy that recombinant FaCYS inhibited the spore germination of *G. cingulata* and the mycelia growth of *S. rofsii* at a concentration of 400 μg/mL.

than those of the N-terminal region, while the C-terminal lacked inhibitory activity. By contrast, the N-terminal region of tarocystatin exhibited greater anti-fungal activity than the full length did, while the C-terminal part showed no antifungal activity. Wang et al. (2008) have suggested that the anti-fungal effects are not related to protease inhibitory activity.

CONCLUSION

Jelly fig is a native plant in Taiwan and the pericarpial portion of its achenes has been long used to produce jelly curd, a popular local beverage. We recently evaluated jelly fig achene proteins for further applications. *FaCYS* encoding a jelly fig phytocystatin was subsequently isolated and identified. This cDNA clone was bacterial-expressed, and the purified his-tagged recombinant protein was characterized as a pH and thermo-stable protein. We found that the recombinant protein showed anti-fungal activity, and exhibited inhibitory activity toward papain, cathepsin B and ficin, but no inhibitory activity against bromelain. FaCYS presents a minor protein in jelly fig achenes; and to our 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 序列經比對及演譯分析結果，預測爲植物性硫氫蛋白酶抑制蛋白 (phytocystatin)。此選殖株被命名爲 *FaCYS*，全長含有 582 bp，可轉譯得到 114 個胺基酸。經預測帶有訊息胜肽，成熟的 FaCYS 不含任何 cysteine，分子量爲 10.8 kDa，pI 值爲 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 *K*i 值極爲接近，分別爲 2.7×10^{-7} M 與 2.4×10^{-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 *K*i 值分別爲 5.6×10^{-7} M、3.0×10^{-8} M 和 2.0×10^{-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。