A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Antifungal characterization of cathepsin L propeptide-like cysteine protease inhibitor against food spoilage yeasts

식품 부패 효모에 대한 카텝신 L 프로펩타이드 유사 cysteine protease 저해제의 항진균 특성 규명

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석사학위논문

Antifungal characterization of cathepsin L propeptide-like cysteine protease inhibitor against food spoilage yeasts

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이 논문을 석사학위 논문으로 제출함

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농 생 명 공 학 부

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Abstract

Recently, as risks have emerged related to the use of chemical antifungal food preservatives, the food industry has demonstrated a need for a novel antifungal agent. Several plant-derived peptides that inhibit proteases and fungal growth have been reported, but their antifungal properties have not been fully characterized. This study investigated the antifungal activity of SnuCalCpI15, a novel, cysteine protease propeptide from the tropical plant *Calotropis procera* R. Br.; this material may replace chemical preservatives that inhibit yeast growth in foods with high sugar content and low acidity. Yeast spoilage of such foods is a major problem. The effects of SnuCalCpI15 on the morphology of food spoilage yeasts were also explored. First, the inhibitory activities of the propeptide were evaluated with respect to three cysteine proteases and two serine proteases. SnuCalCpI15 specifically inhibited only papain-like cysteine proteases, including papain itself. The IC_{50} values were 32.19±23.90 nM, 8.42±1.74 μM, and 48.07±8.17 μM against 0.05 nM cathepsin L, 1.41 μM papain, and 1.60 μM bromelain, respectively. In contrast, SnuCalCpI15 did not inhibit serine proteases from bovine pancreas. The antifungal activities of SnuCalCpI15 against four food spoilage yeasts (*Candida albicans, Pichia anomala, Saccharomyces cerevisiae,* and

Rhodotorula mucilaginosa) were evaluated using the broth micro-dilution method and a time-kill assay. SnuCalCpI15 exhibited concentration-dependent growth inhibition of all four yeast strains. For *C. albicans* and *S. cerevisiae*, the MICs were 3,000±90.47 and 3,000±65.28 ppm, respectively. The MICs for *P. anomala* and *R. mucilaginosa* were 4,000±120.45 and 4,000±87.05 ppm, respectively. FITC-labeling of SnuCalCpI15 revealed that the propeptide initially bound to yeast cell surfaces, then entered the cells. Changes in cell membrane permeability were visualized using the Live/Dead cell viability assay and propidium iodide uptake. SnuCalCpI15 altered yeast cell membrane permeability. Morphological yeast cell changes were investigated via transmission electron microscopy; SnuCalCpI15 significantly enhanced cell wall thickness. These results suggest that SnuCalCpI15, a specific cathepsin L-like propeptide that inhibited papain-like cysteine proteases, is a potent alternative to chemical antifungal food preservatives to improve the shelf life and quality of foods vulnerable to yeast spoilage.

Keywords: propeptide, enzyme inhibitor, cysteine protease, food spoilage yeast, antifungal activity, growth inhibitory activity, cell wall

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Fig. 5. Locations of FITC-labeled SnuCalCpI15 on or in yeast cells. (A) *C. albicans*, (B) *P. anomala*, (C) *S. cerevisiae*, and (D) *R. mucilaginosa* cells were incubated with labeled propeptide for 10 min, 1 h, and 3 h. The final concentration of FITC-labeled SnuCalCpI15 was 900 ppm. Cells were visualized by confocal microscopy. Scale bar: 10 μm (*C. albicans*), 2 μm (*P. anomala*), 6 μm (*S. cerevisiae*), and 3 μm (*R. mucilaginosa*).

Fig. 6. Fluorescence micrographs of (A) *C. albicans*, (B) *P. anomala*, (C) *S. cerevisiae*, and (D) *R. mucilaginosa* after treatment with 1×MICs of SnuCalCpI15 for 3 h. Negative control: PBS (pH 7.4). Living cells stain green and dead cells stain orange and red. Scale bar: 25 μm (Control, 1×MIC of *C. albicans*), 8 μm (1×MIC of *P. anomala*, *S. cerevisiae*, and *R. mucilaginosa*).

Fig. 7. Membrane permeabilities of the four yeasts after treatment with 0.5×MICs and 1×MICs of SnuCalCpI15, as determined by PI uptake assay. All experiments were performed in triplicate; bars indicate standard deviations. Asterisks indicate significant differences between control and treated cells (*$p < 0.05$, Duncan's multiple range test).

Fig. 8. Transmission electron microscopy (TEM) exploring cellular damage caused by SnuCalCpI15. Yeast cells were grown in RPMI 1640 medium with and without SnuCalCpI15 at 1×MIC values for 48 h, then fixed and thin-sectioned for TEM. Scale bars: 500 nm.

1. Introduction

Many fungi pose direct or potential risks to humans and cause unexpected food spoilage. Certain yeast species are resistant to preservatives, and may degrade the preservatives; thus, they pose significant threats to the food industry (Casas, de Ancos, Valderrama, Cano, & Peinado, 2004; Loureiro, 2003; Querol & Fleet, 2006). The economic costs may constitute millions (possibly billions) of euro annually (Querol et al., 2006). Thus, new antifungals are required. Antifungals from plants, oceanic species, and microorganisms have been widely investigated (Vandeputte, Ferrari, & Coste, 2011).

Notably, several plant-derived peptides that inhibit proteases and exhibit antifungal properties have been described. Plants are exposed to many invasive fungi and have evolved to mount defenses that include proteins and peptides with antifungal activity (Selitrennikoff, 2001). There are 13 classes of antifungal proteins, which include inhibitors of serine proteases (*e.g.*, trypsin and chymotrypsin) and cysteine proteases (Paiva, Pontual, Coelho, & Napoleão, 2013; Selitrennikoff, 2001). However, the mechanism of antifungal activity remains unclear, as do the methods used by proteins that act intracellularly to reach their targets (Theis & Stahl, 2004). Moreover, most

protease inhibitors with antifungal activities inhibit trypsin-like enzymes; few studies have explored cysteine protease inhibitors (Melo et al., 2019).

A novel cysteine protease inhibitor with high-level heat resistance was previously identified in the tropical medicinal plant *Calotropis procera* R. Br. and named SnuCalCpI (Kwon et al., 2018); the inhibitor comprises the inhibitory domain (a propeptide) of a cysteine protease zymogen and belongs to the I29 family of the MEROPS database. The propeptide exhibits high-level thermal and pH stability; it selectively inhibits cysteine proteases (Kwon et al., 2018). The propeptide is derived from the N-terminal domain of the zymogen and inhibits the enzyme by blocking the active site, with particular affinity for the mature enzymatic domain (Carmona et al., 1996; Taylor et al., 1995). Thus, the propeptide would be expected to inhibit the homologous enzyme in a highly specific manner; accordingly, SnuCalCpI15 might exhibit antifungal activity.

Yeasts that spoil food and drink pose problems for food technologists; these yeasts cause considerable economic losses (Thomas, 1993) that remain underreported because of commercial confidentiality/embarrassment (Loureiro & Querol, 1999). A variety of yeast strains spoil low-pH high-sugar foods, such as soft drinks, fruit juices, and jams (Battey, Duffy, & Schaffner, 2002; Loureiro, 2000; Wareing & Davenport, 2004). Despite sterilization of

food, yeasts persist in dry environments, entering/contaminating food by various means during manufacturing (Hernández et al., 2018; Rico-Munoz & dos Santos, 2019). Given the health problems caused by chemical preservatives, foods lacking such preservatives are in demand. Therefore, non-chemical preservatives are needed to inhibit yeast spoilage of low-pH high-sugar foods.

The present study investigated the antifungal activities of the natural propeptide protease inhibitor SnuCalCpI against food spoilage yeasts. Inhibition of cysteine proteases was evaluated using the cathepsin L inhibition assay. The selective inhibitory activity of SnuCalCpI15 was evaluated by means of a general protease inhibition assay, using azocasein as a substrate. Finally, the antifungal activity of SnuCalCpI15 were determined as MIC and its effects on the yeasts were characterized with FITC-labeling, Live/Dead cell staining, propidium iodide uptake assay and transmission electron microscopy (TEM).

2. Materials and Methods

2.1. Materials

Escherichia coli BL21 (DE3) was used to express recombinant SnuCalCpI03 and SnuCalCpI15; *E. coli* Rosetta (DE3) was used to express SnuCalCpI17. All *E. coli* strains were grown in Luria-Bertani (LB) medium at 37℃.

Human recombinant cathepsin L (EC 3.4.22.15) was purchased from BioVision Inc. (Milpitas, CA, USA). Papain (EC 3.4.22.2) from papaya latex, bromelain (EC 3.4.22.32) from pineapple stem, trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) from bovine pancreas, and azocasein were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of the finest quality available.

The yeasts *Candida albicans* (KCTC 7678), *Pichia anomala* (KCTC 7295), and *Rhodotorula mucilaginosa* (KCTC 7829) were obtained from the Korean Collection for Type Cultures (KCTC). *Saccharomyces cerevisiae* (KACC 30008) was obtained from the Korean Agricultural Culture Collection (KACC).

2.2. Expression and purification of SnuCalCpIs

2.2.1. Expression of SnuCalCpIs

Recombinant *E. coli* cells were grown in 5-mL aliquots of LB medium with 50 μg/mL kanamycin at 37℃, with shaking overnight. Each overnight culture (2 mL) was inoculated into 200 mL of LB medium containing 50 μg/mL kanamycin; the inoculate thus constituted 1% of the total volume. When preparing SnuCalCpI17, chloramphenicol was added at a final concentration of 34 μg/mL. Growth proceeded at 37℃ with shaking until the optical density at 600 nm (OD_{600}) reached approximately 0.6-0.8. To induce recombinant gene expression, isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 0.5 mM for SnuCalCpI03 and SnuCalCpI15 preparation, and at a final concentration of 0.1 mM for SnuCalCpI17 preparation. The cultures were then incubated at 18℃ for 16 h.

2.2.2. Purification of SnuCalCpIs

Cells were harvested by centrifugation at 4,000×g for 20 min at 4℃. The cell pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.0) with 300 mM NaCl and 10 mM imidazole, then disrupted via sonication on ice. Sonication featured 400 cycles of 1 s pulses and 1 s pauses at 30% power. The

lysates were centrifuged at 10,000×g for 20 min; soluble recombinant SnuCalCpI proteins with C-terminal 6xHis tags were purified on affinity Ni-NTA columns (Qiagen, Hilden, Germany). Ni-NTA slurry was packed into econo-columns and washed with lysis buffer [50 mM Tris-HCl (pH 7.0) with 300 mM NaCl and 10 mM imidazole]. Supernatants of bacterial lysates were loaded onto the resin and shaken slowly for 1 h at 4°C. Unbound proteins were washed away using wash buffer [50 mM Tris-HCl (pH 7.0) with 300 mM NaCl and 20 mM imidazole]; recombinant SnuCalCpI proteins were eluted with elution buffer [50 mM Tris-HCl (pH 7.0) with 300 mM NaCl and 250 mM imidazole].

Ultrafiltration was used to eliminate imidazole (which exhibits antifungal activity) from the eluants and to concentrate recombinant SnuCalCpI proteins. Each eluant was concentrated by centrifugation at 4,000×g for 30 min through a 10-kDa molecular weight-cutoff Amicon filter (Millipore Co., Burlington, MA, USA), then subjected to buffer exchange with phosphate-buffered saline (PBS, pH 7.4). The purified protein was analyzed via sodium dodecyl sulphate-polyacrylamide gel electrophoresis to verify purity and molecular weight. Protein concentrations were determined using the Bradford method (Kruger, 2009) with bovine serum albumin as the standard.

2.3. Protease inhibitory activities of SnuCalCpIs

2.3.1. Cathepsin L inhibition assay

The cathepsin L (BioVision Inc.) inhibition assay was performed as described below. Twenty-microliter aliquots of SnuCalCpI solutions at various concentrations were mixed with 20 μL 5 nM cathepsin L and 60 μL 0.1 M sodium phosphate buffer (pH 6.0) with 2 mM ethylenediaminetetraacetic acid and 2 mM dithiothreitol. After the aliquots had been preincubated at 37℃ for 5 min, reactions were initiated by addition of 0.1 mL 4.8 μM Z-Phe-Arg-7-amino-4-methylcoumarin (Z-Phe-Arg-AMC) hydrochloride (from a 1 mM stock solution in dimethyl sulfoxide). Cathepsin L activity was detected by fluorescence of 7-amino-4-methylcoumarin (AMC) on excitation at 355 nm (emission: 460 nm) in respective wells of a 96-well microplates. The relative residual activity of cathepsin L was calculated using the following equation:

$$\text{Relative residual activity (\%)} = \frac{T^*}{T} \times 100$$

where T and T^* are the relative fluorescence units in the absence and presence of inhibitors after 20 min of reaction. The half-maximal inhibitory concentration (IC_{50}) was determined from the curve fitted by SigmaPlot ver. 12.5 software (Systat Software Co., San Jose, CA, USA).

2.3.2. Protease inhibition assay of SnuCalCpI15

The inhibitory activities of SnuCalCpI15 against cysteine proteases (papain and bromelain) and serine proteases (trypsin and chymotrypsin) were evaluated using the azocasein assay (Reichard, Buttner, Eiffert, Staib, & Ruchel, 1990) with a slight modification. For cysteine protease inhibition assays, azocasein (Sigma-Aldrich) was dissolved at 28 g/L in 50 mM Tris-HCl buffer (pH 7.0) with 30 mM cysteine and 10 mM ethylenediaminetetraacetic acid. Enzyme solutions were prepared in the same buffer [1.41 μM papain (Sigma-Aldrich) and 1.60 μM bromelain (Sigma-Aldrich)]. For serine protease inhibition assays, azocasein was dissolved at 28 g/L in 0.50%(w/v) sodium bicarbonate buffer ($NaHCO_3$), pH 8.3. Enzyme solutions were prepared in the same buffer [1.63 μM trypsin (Sigma-Aldrich) and 0.74 μM chymotrypsin (Sigma-Aldrich)]. Samples with various concentrations of SnuCalCpI15 (0.5 mL) were preincubated in a water bath at 37℃ with 0.5 mL of each enzyme solution. After 30 min, reactions were initiated by addition of 1 mL of 1.2 mM azocasein dissolved in the appropriate buffer; the mixtures were then incubated for 60 min at 37℃. Aliquots (50 μL) were removed at 10-min intervals, mixed with 200-μL aliquots of 5%(v/v) trichloroacetic acid to stop the reaction, and then vortexed vigorously. Subsequently, each solution was centrifuged at 4,000×g for 10 min (Micro-12, Hanil Scientific Inc., Gimpo,

Korea) and 100 μL of the supernatant was added to 100 μL 0.5 N NaOH. Absorbance was recorded at 440 nm. Residual protease activity was calculated using the following equation:

$$\text{Relative residual activity (\%)} = \frac{T^*}{T} \times 100$$

where *T* is the absorbance at 440 nm in the absence of SnuCalCpI15 and *T** is the absorbance in the presence of SnuCalCpI15. The IC_{50} was determined from the curve fitted by SigmaPlot ver. 12.5 software (Systat Software Co.).

2.4. Antifungal susceptibility testing

2.4.1. Determination of minimum inhibitory concentrations (MICs)

Four food spoilage yeasts (*C. albicans* KCTC 7678, *P. anomala* KCTC 7295, *S. cerevisiae* KACC 30008, and *R. mucilaginosa* KCTC 7829) were used in this study. With the exception of *S. cerevisiae*, the yeasts were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M 3-[*N*-morpholino]propanesulfonic acid (Sigma-Aldrich). *S. cerevisiae* was cultured in RPMI 1640 medium with 2%(w/v) glucose. All yeasts were incubated at 25℃ with shaking at 220 rpm for 24 h.

MICs were determined using the broth micro-dilution method described in the Clinical and Laboratory Standards Institute document M27-A3, with a slight modification (Bertout, Dunyach, Drakulovski, Reynes, & Mallie, 2011). Yeast cell suspensions prepared in RPMI 1640 medium were diluted in the same broth to a cell concentration of 2.2×10^3 colony forming units (CFU)/mL, based on absorbance at 530 nm. SnuCalCpI15 solutions at various concentrations were prepared by dilution of SnuCalCpI15 in PBS (pH 7.4). One hundred-microliter aliquots of samples were added to respective wells of a 96-well plate (Costar 3596, Corning Inc., Corning, NY, USA); each well then received 100 μL of cell suspension in RPMI 1640 medium to yield a final cell

concentration of 1.1×10^3 CFU/mL. The pH in each well was 7.2±0.1. The MICs were the lowest concentrations of SnuCalCpI15 that eliminated turbidity, compared to inhibitor-free control wells, during incubation for 72 h at 25℃. Cell growth was determined by measurement of absorbance at 530 nm using a UV/Vis microplate spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Waltham, MA, USA).

2.4.2. Time-kill assay

The four yeasts were cultured in RPMI 1640 medium at 25℃ for 24 h with shaking at 220 rpm. Cell suspensions were then diluted with the same broth to adjust the cell concentration to 2.2×10^5 CFU/mL, based on absorbance at 530 nm. One hundred-microliter aliquots of SnuCalCpI15 solutions at concentrations of 1×MIC and 2×MIC for *C. albicans, P. anomala, S. cerevisiae,* and *R. mucilaginosa* were mixed with 100-μL aliquots of cell suspensions; the mixtures (1.1×10^5 CFU/mL) were incubated at 25℃ with shaking at 130 rpm. During incubation, aliquots were removed at 12 h intervals and serially diluted 10-fold with PBS. Fifty-microliter aliquots of diluted samples were spread on Potato Dextrose Agar and viable cells were counted after incubation at 25℃ for 48 h. The limit of detection was 1.3 log CFU/mL.

2.5. FITC labeling

To visualize SnuCalCpI15 on or in yeast cells, a FluoroTag FITC Conjugation Kit (Sigma-Aldrich) was used to tag the protein. FITC is the most widely used fluorescent reagent; the fluorophore exhibits high quantum efficiency. FITC reacts with the free amino groups of proteins to form stable conjugates; it has an absorption maximum at 495 nm and an emission maximum at 525 nm.

SnuCalCpI15 samples were conjugated with FITC, in accordance with the manufacturer's protocol. Briefly, one vial of FITC (F7250) was reconstituted in 2 mL 0.1 M carbonate-bicarbonate buffer and vortexed until all FITC had been dissolved. Then, the FITC solution was diluted with an equal volume of 0.1 M carbonate-bicarbonate buffer. One milliliter of 5 mg/mL SnuCalCpI15 was added to a reaction vial and 250 μL of the FITC solution was added with stirring. The vial was completely covered with aluminum foil and incubated for 2 h at room temperature with gentle stirring. Then, the FITC-labeled protein was isolated using a Sephadex G-25M column (B4783) and eluted with PBS. The fluorescein/protein molar ratio was determined.

To prepare yeast cell suspensions, *C. albicans, P. anomala, S. cerevisiae,* and *R. mucilaginosa* were cultured in Yeast Molt broth overnight at 25℃.

Yeast Molt broth was used in place of RPMI 1640 to prevent interactions between free amino acids in RPMI 1640 and FITC. The cell suspensions were then diluted to 1.0×10^7 CFU/mL in the same broth, based on absorbance at 600 nm. One hundred-microliter aliquots of cell suspensions were mixed with 100-μL aliquots of labeled SnuCalCpI15; the mixtures were incubated in the dark at 25℃ for 10 min, 1 h, and 3 h. After incubation, cells were visualized with the aid of a confocal laser scanning microscope (SP8 X, Leica, Wetzlar, Germany).

2.6. Cell membrane permeabilization

2.6.1. Live/Dead yeast cell staining

To explore the effects of SnuCalCpI15 on cell membrane permeability, the LIVE/DEAD® *Funga*Light Yeast Viability Kit (Invitrogen, Carlsbad, CA, USA) was used. *C. albicans, P. anomala, S. cerevisiae,* and *R. mucilaginosa* were cultured in RPMI 1640 medium overnight at 25℃; cells were collected by centrifugation at 10,000×g at 4℃ for 3 min. All samples were resuspended in 1-mL aliquots of 0.9%(w/v) NaCl; the suspensions were diluted to 1.0×10^7 CFU/mL in the same solution. One hundred-microliter aliquots of cell suspensions were mixed with 100-μL aliquots of SnuCalCpI15 dissolved in PBS at 1×MIC for each yeast strain, then incubated for 3 h at 25℃. For staining, 1 μL of 3.34 mM SYTO 9 and 1 μL 20 mM PI were added to each yeast suspension; the mixtures were vortexed gently and incubated at room temperature in the dark for 15–30 min. For fluorescence microscopy, 5 μL of each stained yeast suspension was trapped between a slide and an 18-mm square coverslip, then observed using a confocal laser scanning microscope.

2.6.2. Propidium iodide uptake assay

The propidium iodide (PI) uptake assay was used to explore changes in yeast cell membrane permeability (Cheng, Du, Zhu, Guo, & He, 2016). *C. albicans, P. anomala, S. cerevisiae,* and *R. mucilaginosa* were cultured in RPMI 1640 overnight at 25℃. Cells were harvested by centrifugation at 10,000×g at 4℃ for 10 min and resuspended in PBS (pH 7.4) to a final cell density of 1.0×10^7 CFU/mL, based on absorbance at 600 nm. One hundred-microliter aliquots of SnuCalCpI15 solutions were added to respective wells of a 96-well plate (Costar 3610, Corning Inc.) at a final concentration of 1×MIC for each yeast strain; 100-μL aliquots of cell suspensions were then added. After cells had been incubated at 25℃ for 3 h with shaking at 120 rpm, PI was added to a final concentration of 20 μM; the suspensions were then incubated at 25℃ for 15 min in the dark. The turbidity (OD_{600}) of each suspension was measured using a UV/Vis microplate spectrophotometer (Multiskan GO); the fluorescence intensity (excitation, 535 nm; emission, 617 nm) was assessed with the aid of a spectrofluorophotometer (Spectramax M2e, Molecular Devices, San Jose, CA, USA). The fluorescence intensity was normalized to the cell density (OD_{600}) and PI uptake was expressed as relative fluorescence units in the presence and absence of SnuCalCpI15.

2.7. Energy-filtered transmission electron microscopy

Changes in yeast cell morphology were evaluated via TEM. *C. albicans, P. anomala, S. cerevisiae,* and *R. mucilaginosa* were cultured in RPMI 1640; the suspensions were diluted with the same broth to cell concentrations of 2.2×10^5 CFU/mL. One hundred-microliter aliquots of SnuCalCpI15 at concentrations of 1×MIC for each yeast strain were mixed with 100-μL aliquots of the suspensions; the mixtures were then incubated at 25℃ with shaking at 220 rpm for 48 h.

The cells were harvested, resuspended in 1 mL of Kamovsky fixative solution, and pre-fixed overnight at 4°C. The pellets were washed three times with 0.05 M sodium cacodylate buffer. Then, 1%(w/v) osmium tetroxide solution was added and cells were incubated at 4℃. After 2 h, the samples were washed three times with distilled water, resuspended in 1 mL 0.5%(w/v) uranyl acetate in distilled water, and incubated overnight at 4℃. After pellets had been stained, they were washed three times with distilled water; they were then subjected to ethanol-mediated stepwise dehydration for 10 min/step in 30%, 50%, 70%, 80%, 90%, and 100% ethanol. Subsequently, the cells were collected and embedded in Spurr resin. After cells had been polymerized overnight at 70°C, the samples were sectioned at 70 nm with a microtome (EM UC7, Leica) and examined using an electron microscope (Talos L120C, FEI,

Hillsboro, OR, USA).

2.8. Statistical analysis

Analysis of variance was performed using SPSS Statistics, version 25 (IBM Corp. Armonk, NY, USA) and the data are presented as means (±standard deviations) of the measurements of triplicate experiments. Differences between means were compared using Duncan's multiple range test. A p-value < 0.05 was considered to indicate statistical significance.

3. Results and Discussion

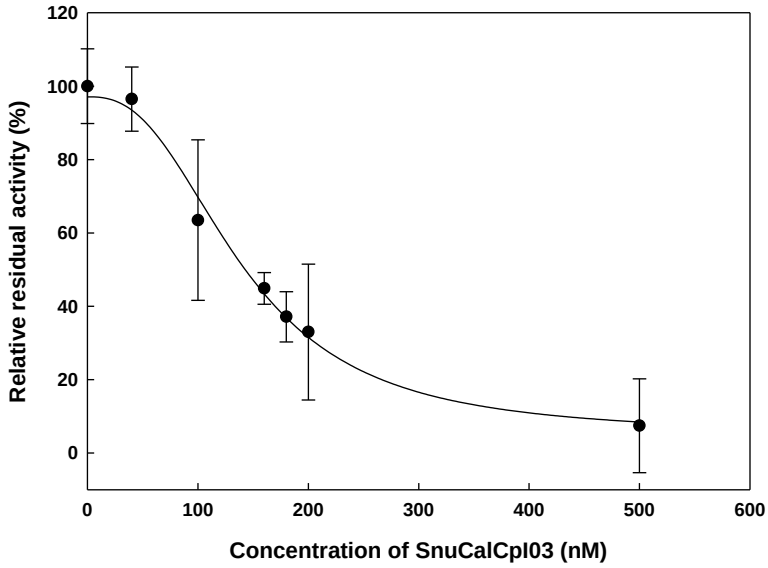
3.1. Protease inhibition by SnuCalCpIs

3.1.1. Cathepsin L inhibition

An enzyme inhibition assay using human cathepsin L was performed to evaluate the inhibitory activities of SnuCalCpI03, SnuCalCpI15, and SnuCalCpI17. Cathepsin L cleaves next to Phe and Arg of Z-Phe-Arg-AMC to release fluorescent AMC (Kwon et al., 2018), which is detected using a fluorophotometer after 20 min of hydrolysis.

All SnuCalCpIs inhibited recombinant human cathepsin L (Fig. 1). Non-linear curve fitting revealed that the IC_{50} values of SnuCalCpI03, SnuCalCpI15, and SnuCalCpI17 against 0.05 nM cathepsin L were 140.28±13.44 nM, 32.19±23.90 nM, and 5.87±0.41 nM, respectively. Although SnuCalCpI15 and SnuCalCpI17 effectively inhibited cathepsin L, subsequent experiments were performed using only SnuCalCpI15, given its greater solubility (data not shown). The results showed that SnuCalCpI, propeptide of plant-derived cysteine protease, inhibited a mammalian cysteine protease, specifically human cathepsin L.

A


Relative residual activity (%)
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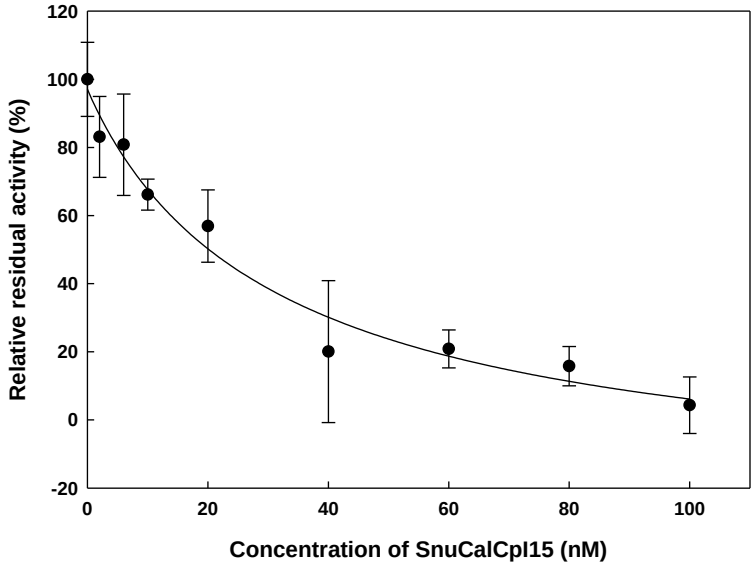
0
100
200
300
400
500
600
Concentration of SnuCalCpI03 (nM)

B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60
40
20
0
-20
0
20
40
60
80
100
Concentration of SnuCalCpI15 (nM)

C

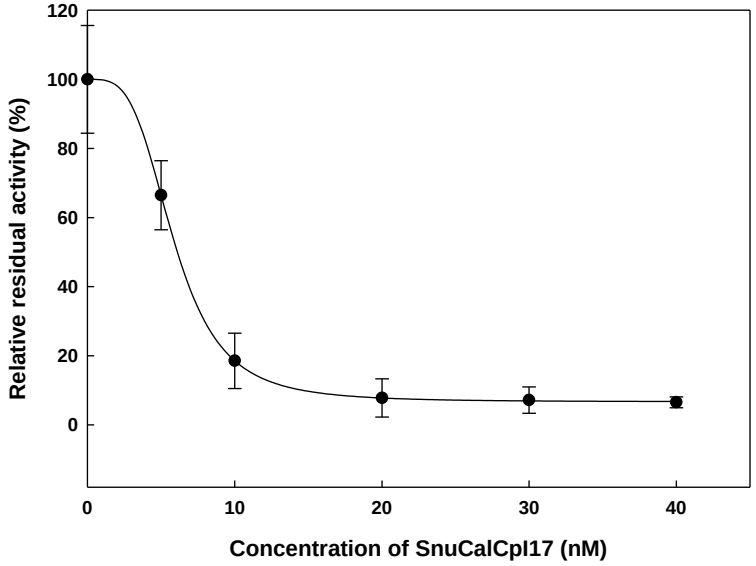


Fig. 1. Inhibitory activities of (A) SnuCalCpI03, (B) SnuCalCpI15, and (C) SnuCalCpI17 against human cathepsin L.

3.1.2. Specificity of protease inhibition of SnuCalCpI15

Protease inhibitors have been divided into 48 families based on similarities in amino acid sequences. Of these, 31 have been assigned to 26 clans based on their three-dimensional structures (Rawlings, Tolle, & Barrett, 2004; Shamsi, Parveen, & Fatima, 2016). SnuCalCpI15 is a member of the I29 family of the MEROPS peptidase database; the mature enzyme is a papain-like cysteine protease (clan CA, family C1) (Kwon et al., 2018). Although enzymes in the same family exhibit similar amino acid sequences, the inhibitory activities of propeptides vary greatly depending on the proteases from which they are derived (Carmona et al., 1996). However, plant-derived propeptides are relatively less selective for plant enzymes, compared with the human and animal-derived cathepsin L propeptides (Taylor et al., 1995). Therefore, to explore the selectivity of the protease inhibitory capacity of SnuCalCpI15, a protease inhibition assay was performed using azocasein as a substrate. Papain and bromelain were chosen as two representative cysteine proteases; trypsin and chymotrypsin were chosen as two representative serine proteases. Trypsin and chymotrypsin were tested because many features of the active sites are shared with cysteine proteases, including the nucleophiles and certain bases (Powers, Asgian, Ekici, & James, 2002). Moreover, both serine cysteine protease inhibitors have been reported to exhibit antimicrobial

activity.

SnuCalCpI15 inhibited the cysteine proteases, but not the serine proteases (Fig. 2). The IC_{50} of SnuCalCpI15 for 1.41 μM papain was 8.416±1.744 μM, whereas the IC_{50} for 1.60 μM bromelain was 48.069±8.172 μM; thus, the IC_{50} was higher for bromelain than for papain (Table 1). Papain and bromelain are both clan CA family C1 cysteine peptidases (of the "papain family"); accordingly, they share common motifs and highly conserved amino acid sequences (Barrett & Rawlings, 1996). Based on the sequences of papain-like cysteine proteases, the papain family is divided into two subfamilies: cathepsin L- and cathepsin B-like proteases (Cygler & Mort, 1997). Notably, the sequence homology among plant cysteine proteases (74% at the amino acid level) is much higher than the homology between plants and mammals (25%); thus, the selectivity of plant propeptides for plant enzymes is weaker than their selectivity for mammalian cathepsins (Carmona et al., 1996). Because SnuCalCpI15 is a plant-derived propeptide, it exhibited relatively low inhibitory activity against human cathepsin L, compared to plant cysteine proteases (papain and bromelain). Thus, SnuCalCpI15 specifically inhibited papain-like cysteine proteases, including papain itself.

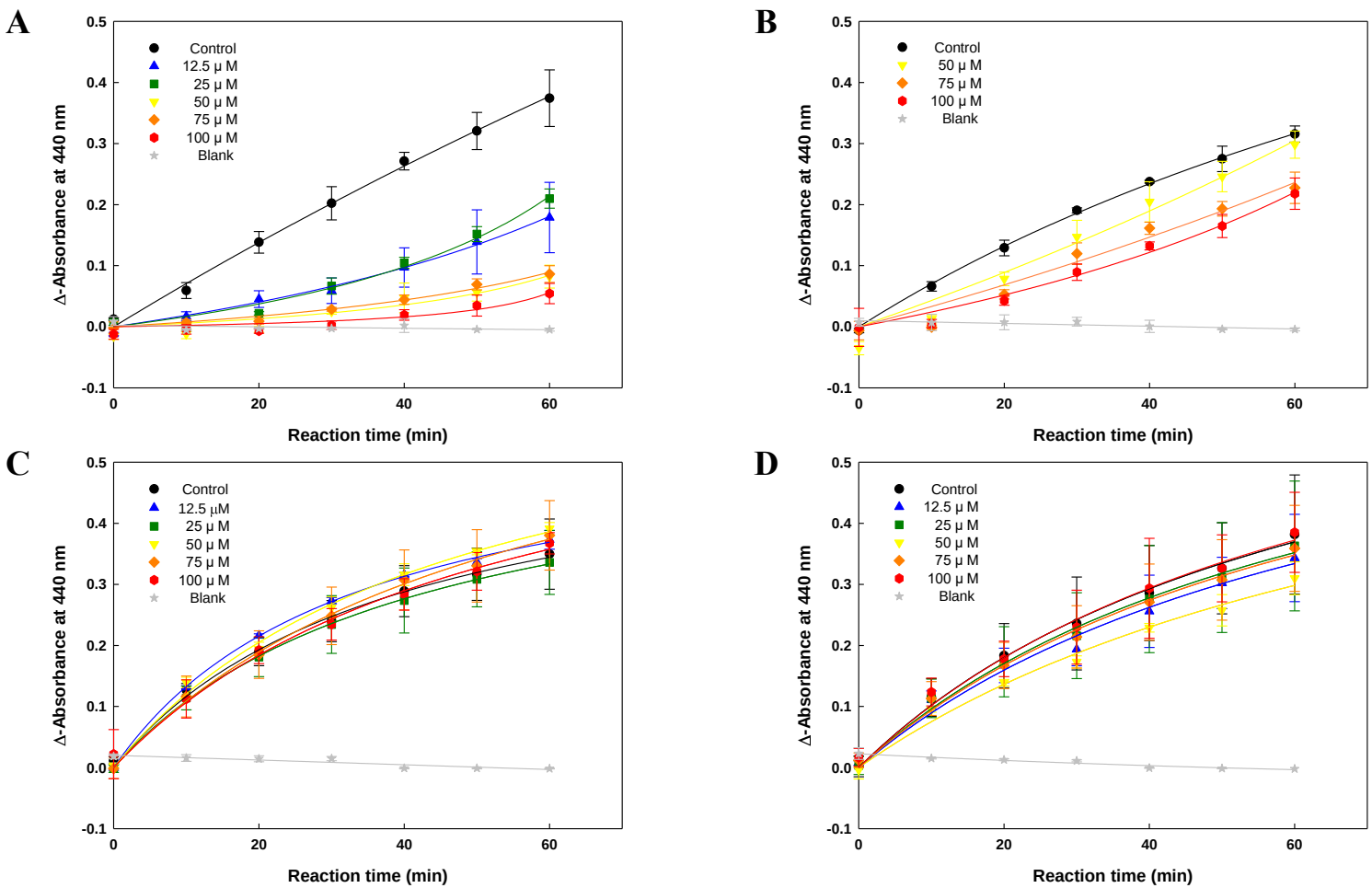


Fig. 2. Time courses of protease inhibition by SnuCalCpI15 of (A) papain, (B) bromelain, (C) trypsin, and (D) chymotrypsin acting on azocasein (600 μM). Reaction temperature 37°C; reaction time 1 h.

Table 1. Inhibitory activities of SnuCalCpI15 against various proteases

Protease	**Origin**	**IC_{50} (μM)**	**R^2**
Cysteine proteases			
Papain	Papaya latex	8.416±1.744	0.9631
Bromelain	Pineapple stem	48.069±8.172	0.9437
Serine proteases			
Trypsin	Bovine pancreas	ND*	
Chymotrypsin	Bovine pancreas	ND	

Means±standard deviations.

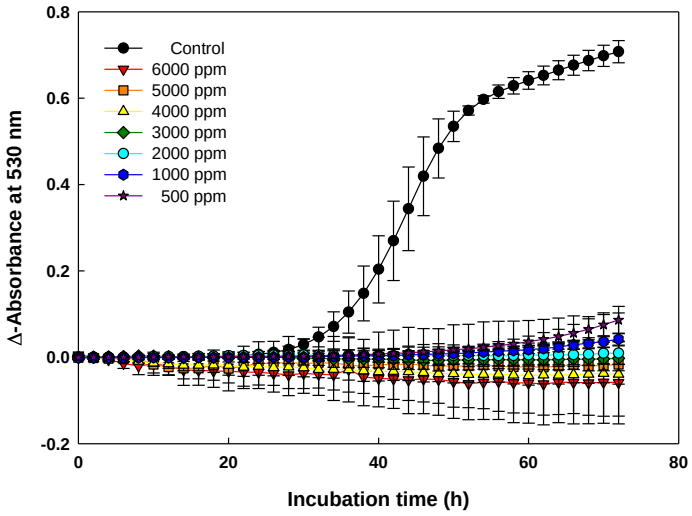
* ND: Not determined.

3.2. Antifungal activity of SnuCalCpI15

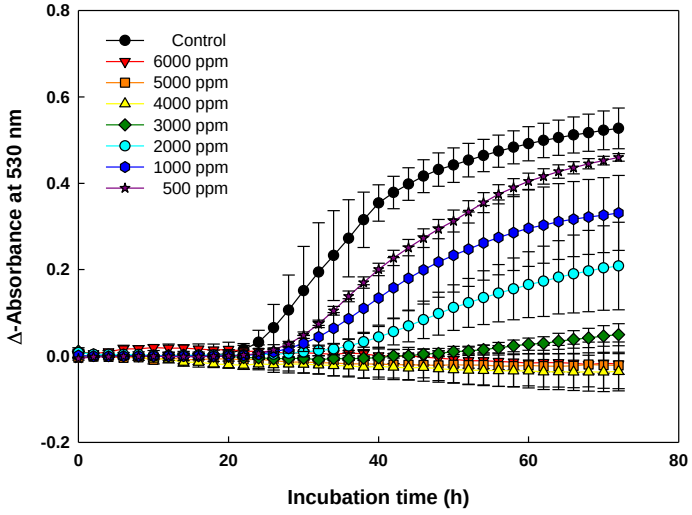
3.2.1. Minimum inhibitory concentrations (MICs)

The antifungal activities of SnuCalCpI15 against *C. albicans* KCTC 7678, *P. anomala* KCTC 7295, *S. cerevisiae* KACC 30008, and *R. mucilaginosa* KCTC 7829 were evaluated using the broth micro-dilution method. After cells had been treated with SnuCalCpI15 at various concentrations, cell growth curves were measured in terms of the optical density at 530 nm (Fig. 3). During the incubation, all negative controls (no propeptide) entered the stationary phase. SnuCalCpI15 inhibited yeast cell growth in a concentration-dependent manner. The MICs of SnuCalCpI15 for the four yeasts are shown in Table 2. For *C. albicans* and *S. cerevisiae*, the MICs were 3,000±90.47 and 3,000±65.28 ppm, respectively. The MICs for *P. anomala* and *R. mucilaginosa* were 4,000±120.45 and 4,000±87.05 ppm, respectively. However, minimum fungicidal concentration (MFC) was not determined in all yeasts. Thus, SnuCalCpI15 inhibits yeast growth, but does not kill the yeasts.

A


Control
6000 ppm
5000 ppm
4000 ppm
3000 ppm
2000 ppm
1000 ppm
500 ppm
Δ-Absorbance at 530 nm
Incubation time (h)
0.8
0.6
0.4
0.2
0.0
-0.2
0
20
40
60
80

B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Δ-Absorbance at 530 nm
Incubation time (h)
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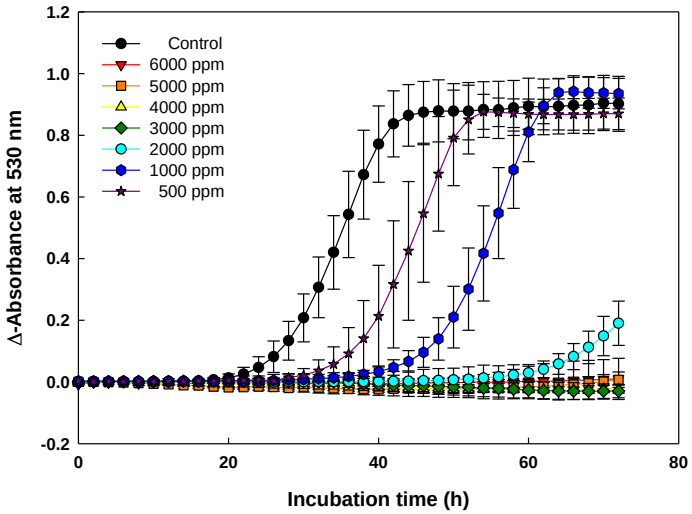
C



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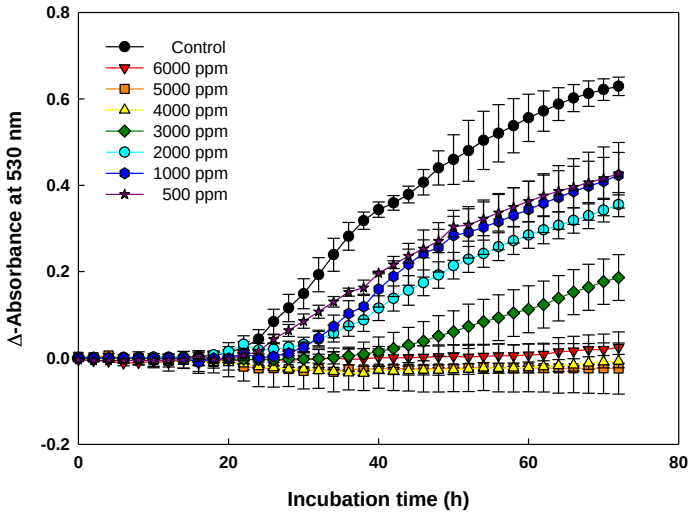


Fig. 3. Growth curves of (A) *C. albicans* KCTC 7678, (B) *P. anomala* KCTC 7295, (C) *S. cerevisiae* KACC 30008, and (D) *R. mucilaginosa* KCTC 7829 treated with SnuCalCpI15 at concentrations from 500 to 6,000 ppm. Data are means±standard deviations (n = 3).

Table 2. Minimum inhibitory concentrations (MICs) of SnuCalCpI15 for four food spoilage yeasts

Yeast strain	**MIC (ppm)**
C. albicans KCTC 7678	3,000±90.47
P. anomala KCTC 7295	4,000±120.45
S. cerevisiae KACC 30008	3,000±65.28
R. mucilaginosa KCTC 7829	4,000±87.05

All data are averages of triplicate determinations.

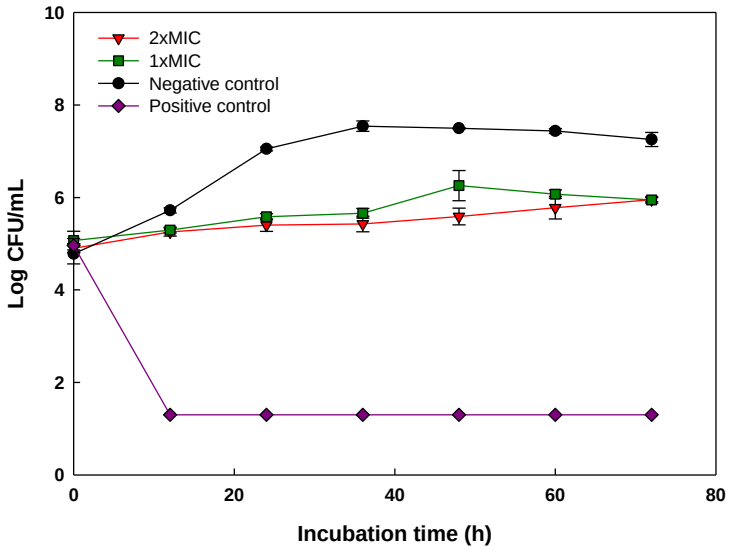
3.2.2. Yeast viability

SnuCalCpI15 at the 1×MIC values did not kill any yeast (data not shown). Thus, a time-kill assay was performed. All negative controls (no propeptide) grew to >2 log CFU/mL, compared to the initial concentration (Fig. 4). In contrast to the results of the broth micro-dilution experiment, all yeasts attained the stationary phase within 24 h because the initial inoculum was approximately 1.1×10^5 CFU/mL. All positive controls reached the limit of detection within 12 h. *C. albicans* and *S. cerevisiae* treated with SnuCalCpI15 at the 1×MIC and 2×MIC values grew by at least 1 log CFU/mL, compared to the initial inoculum (Fig. 4A and C). No difference between the 1×MIC and 2×MIC treatments was evident after 72 h of incubation. No *P. anomala* experimental group grew; the initial cell number was maintained (Fig. 4B). Only *R. mucilaginosa* treated with SnuCalCpI15 at 2×MIC exhibited a reduction in numbers (of 0.5 log CFU/mL) (Fig. 4D). This was the only fungicidal effect noted.

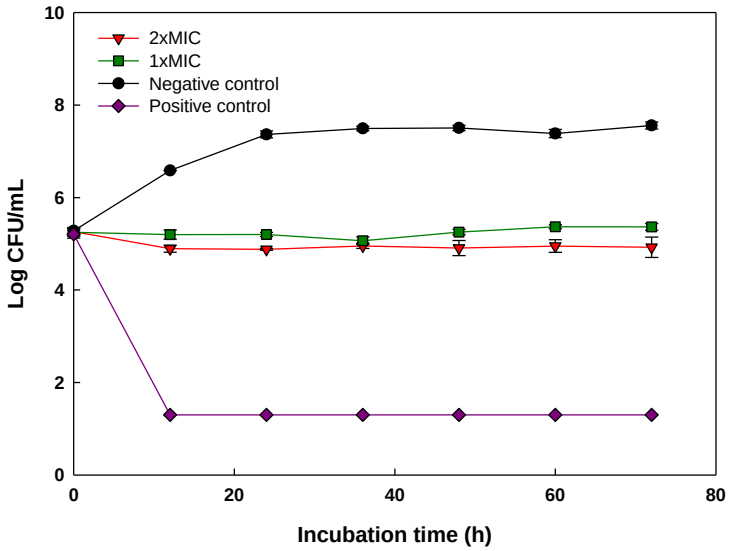
Although the mechanism of action of antifungal protease inhibitors remains unknown (Selitrennikoff, 2001), the time-kill assay verified that SnuCalCpI15 inhibited the growth of all four yeasts. The different MICs reflect differences in yeast strain susceptibilities. The results of the time-kill assay suggested that SnuCalCpI15 inhibits the growth of food spoilage yeasts, thus potentially

preventing food deterioration.

A


2xMIC
1xMIC
Negative control
Positive control
Log CFU/mL
Incubation time (h)
0
2
4
6
8
10
0
20
40
60
80

B


2xMIC
1xMIC
Negative control
Positive control
Log CFU/mL
Incubation time (h)
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60
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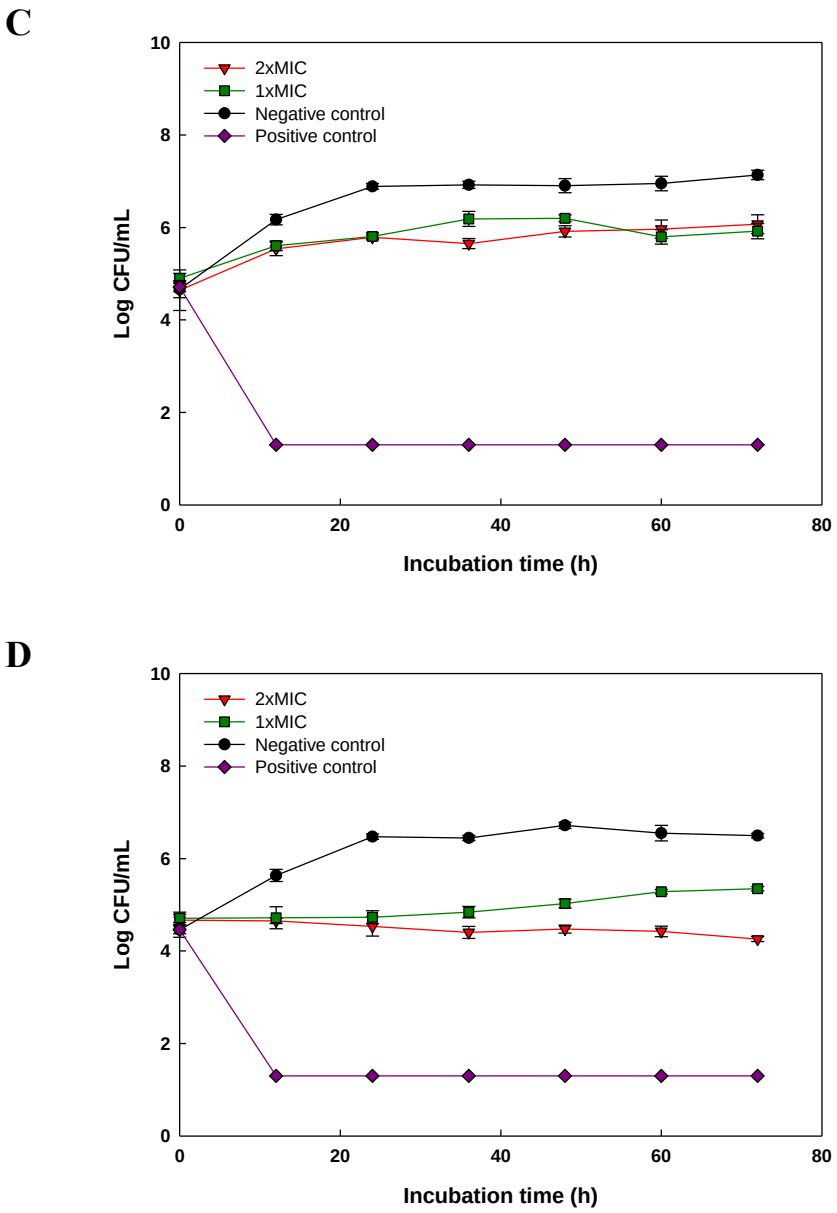
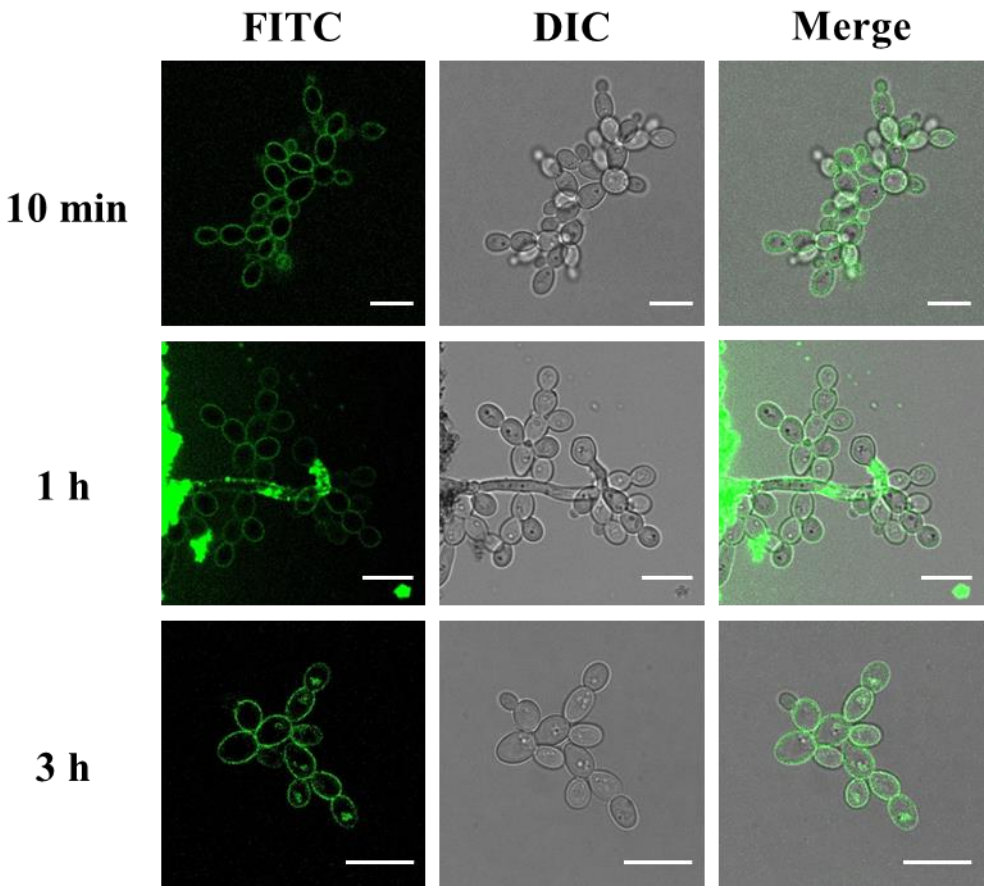


Fig. 4. Time-kill curves of (A) *C. albicans* KCTC 7678, (B) *P. anomala* KCTC 7295, (C) *S. cerevisiae* KACC 30008, and (D) *R. mucilaginosa* KCTC 7829 treated with the 1×MICs and 2×MICs of SnuCalCpI15. Data are means±standard deviations (n = 3).

3.3. Localization of SnuCalCpI15

To explore whether SnuCalCpI15 acted outside the yeast cell or in the cytoplasm, propeptide location was explored using FITC-labeled material. For *C. albicans*, FITC-labeled SnuCalCpI15 was observed on the cell surface after 10 min of incubation; it remained on the cell surface for 1 h. However, SnuCalCpI15 was detected inside cells after 3 h (Fig. 5A). Similar patterns were observed for *P. anomala* (Fig. 5B) and *S. cerevisiae* (Fig. 5C); however, SnuCalCpI15 entered these yeasts more rapidly. For *P. anomala*, the fluorescence intensity of internal FITC-labeled SnuCalCpI15 increased over time. Notably, the propeptides aggregated inside *S. cerevisiae* cells after 1 h of culture. Unlike the other yeasts, *R. mucilaginosa* did not internalize the propeptide by 3 h (Fig. 5D). Regardless of internalization, SnuCalCpI15 remained present on all cell surfaces. The entry time depended on the yeast strain, perhaps reflecting distinct interactions between the propeptide and the cell wall/membrane. SnuCalCpI15 accumulated both inside and outside yeast cells, and affected cell growth.

A



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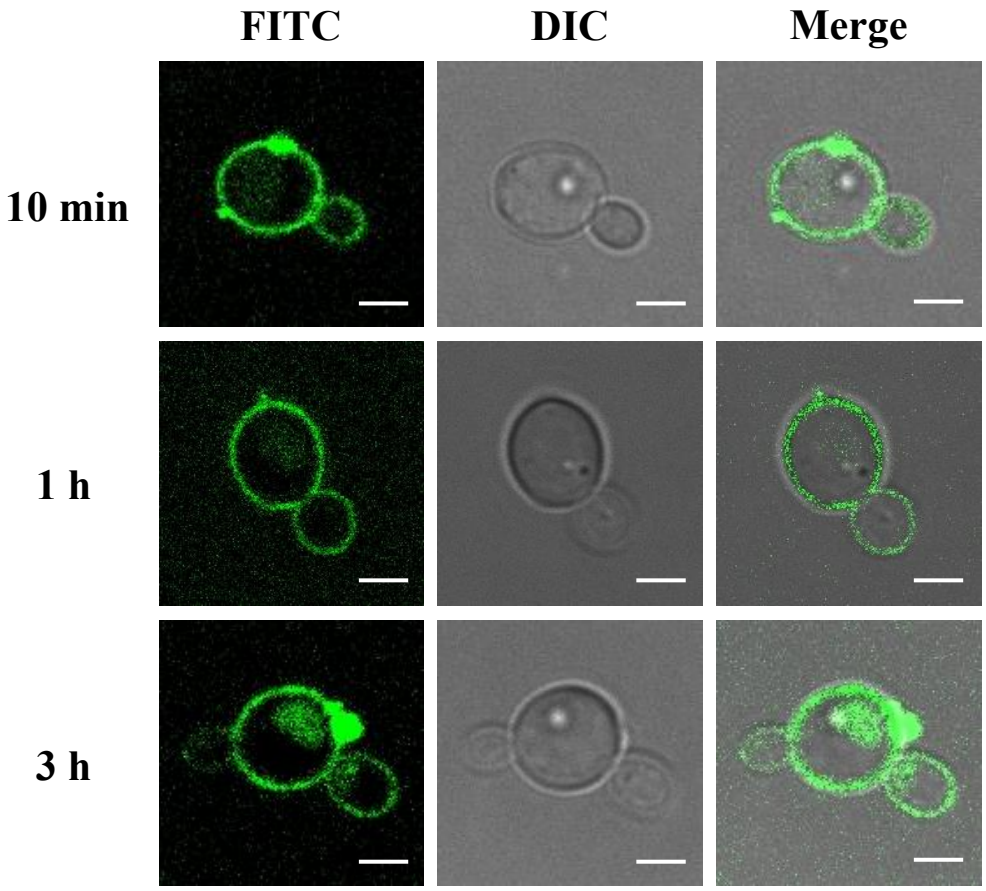

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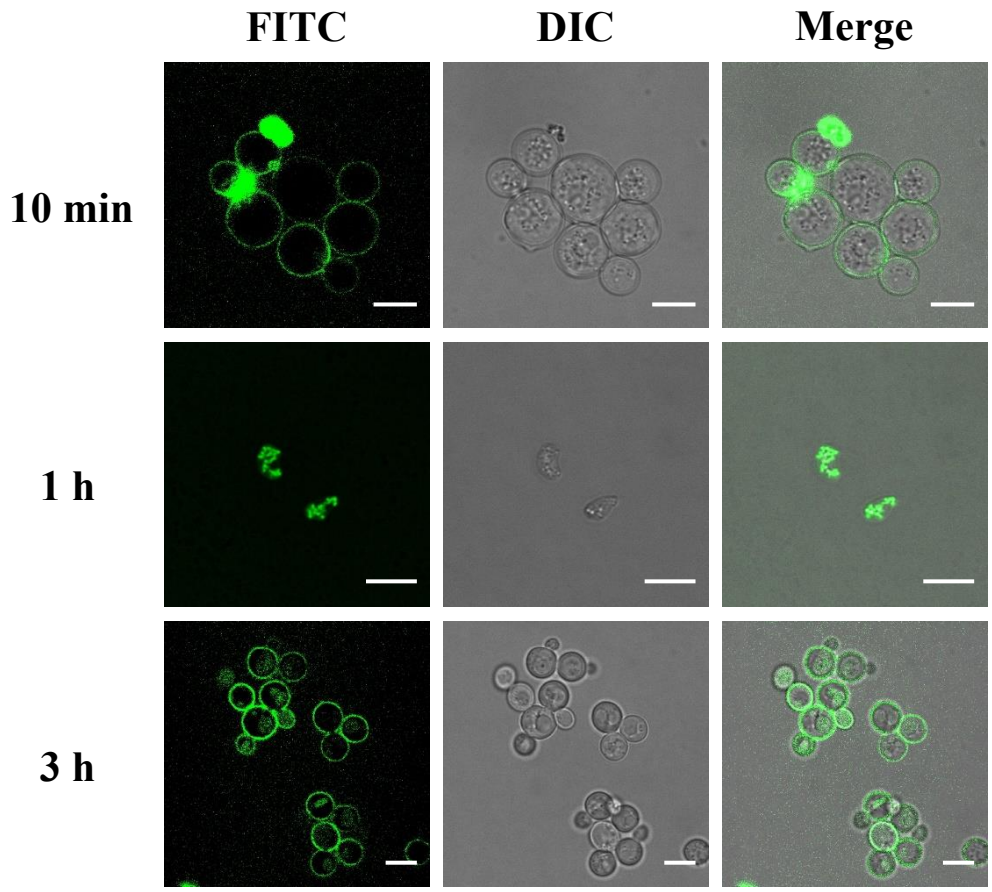


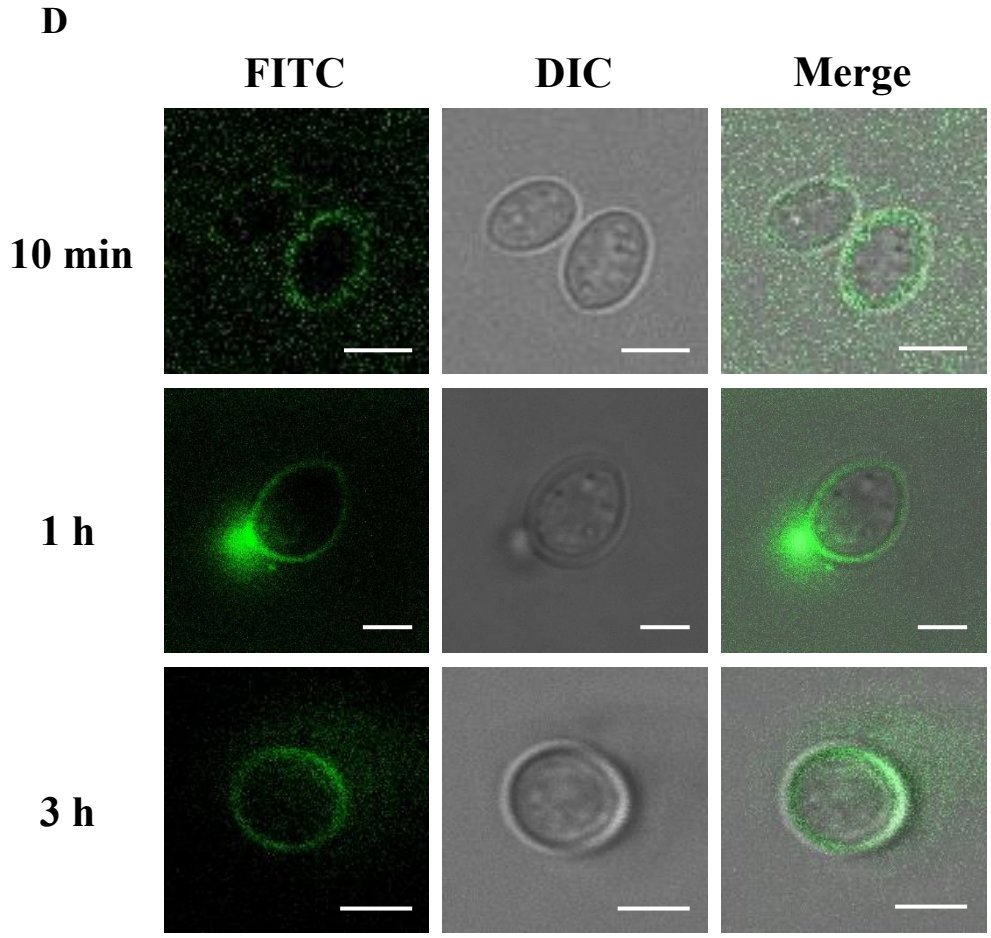


Fig. 5. Locations of FITC-labeled SnuCalCpI15 on or in yeast cells. (A) *C. albicans*, (B) *P. anomala*, (C) *S. cerevisiae*, and (D) *R. mucilaginosa* cells were incubated with labeled propeptide for 10 min, 1 h, and 3 h. The final concentration of FITC-labeled SnuCalCpI15 was 900 ppm. Cells were visualized by confocal microscopy. Scale bar: 10 μm (*C. albicans*), 2 μm (*P. anomala*), 6 μm (*S. cerevisiae*), and 3 μm (*R. mucilaginosa*).

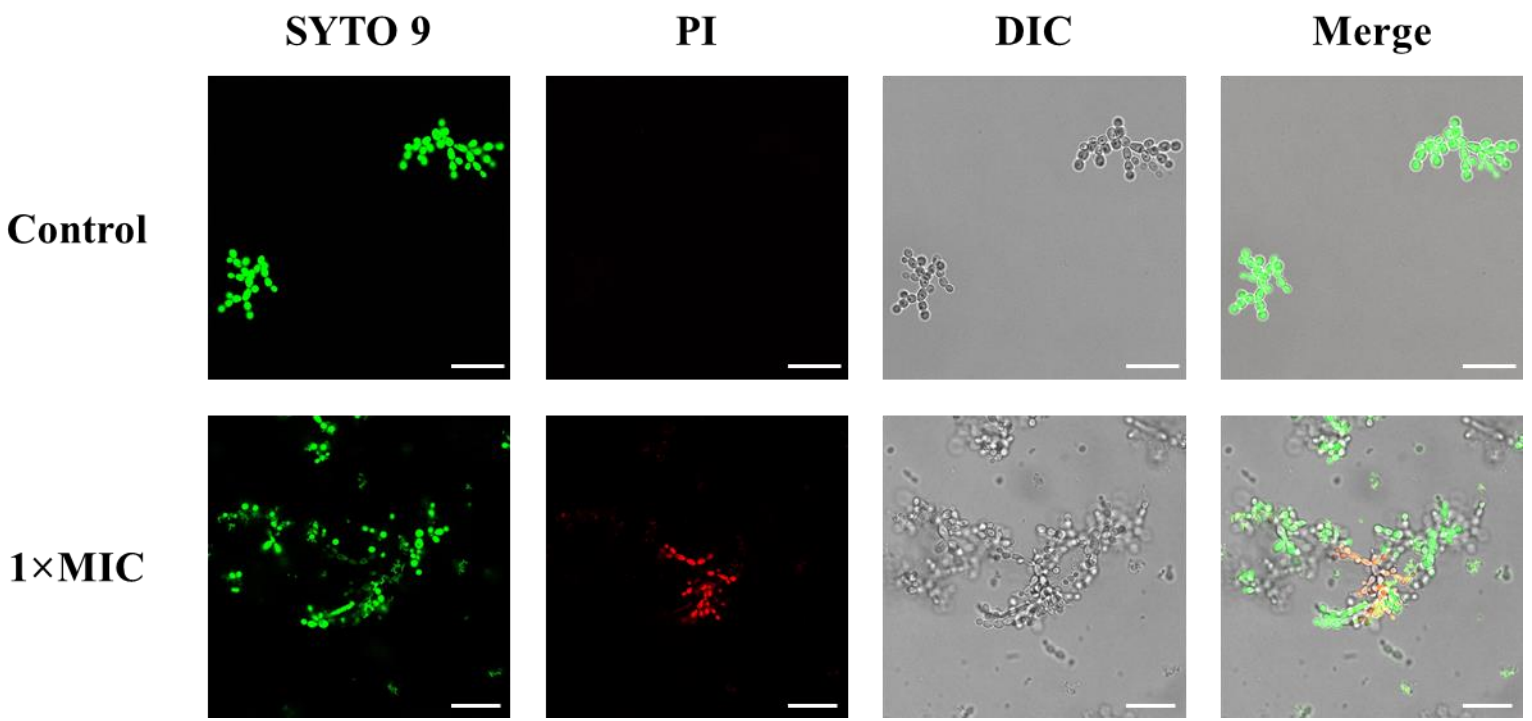
3.4. Membrane integrity

3.4.1. Live/Dead yeast cell staining

The effect of SnuCalCpI15 on yeast cell membrane permeability was visualized using a Live/Dead *Funga*Light Yeast Viability kit, which contains solutions of SYTO 9 and PI. Green fluorescent SYTO 9 stains all yeast cells after binding to nucleic acid. In contrast, red-fluorescent PI penetrates only yeast with damaged membranes, in which it binds to nucleic acids. Therefore, yeast cells with intact cell membranes exhibit fluorescent green and cells with damaged membranes exhibit fluorescent red.

The four yeast strains were treated with PBS and SnuCalCpI15 at the 1×MICs. After 3 h of incubation, cell suspensions were stained with both SYTO 9 and PI; fluorescent images were then obtained via confocal laser scanning microscopy. *C. albicans* not treated with SnuCalCpI15 fluoresced green from SYTO 9 stain. In contrast, treated *C. albicans* fluoresced both green and red from SYTO 9 and PI stain, respectively (Fig. 6A). The staining patterns were similar for *P. anomala* (Fig. 6B), *S. cerevisiae* (Fig. 6C), and *R. mucilaginosa* (Fig. 6D). Thus, the results from the fluorescence microscopy on stained yeast cells indicate that SnuCalCpI15 increased the cell membrane permeability.

A



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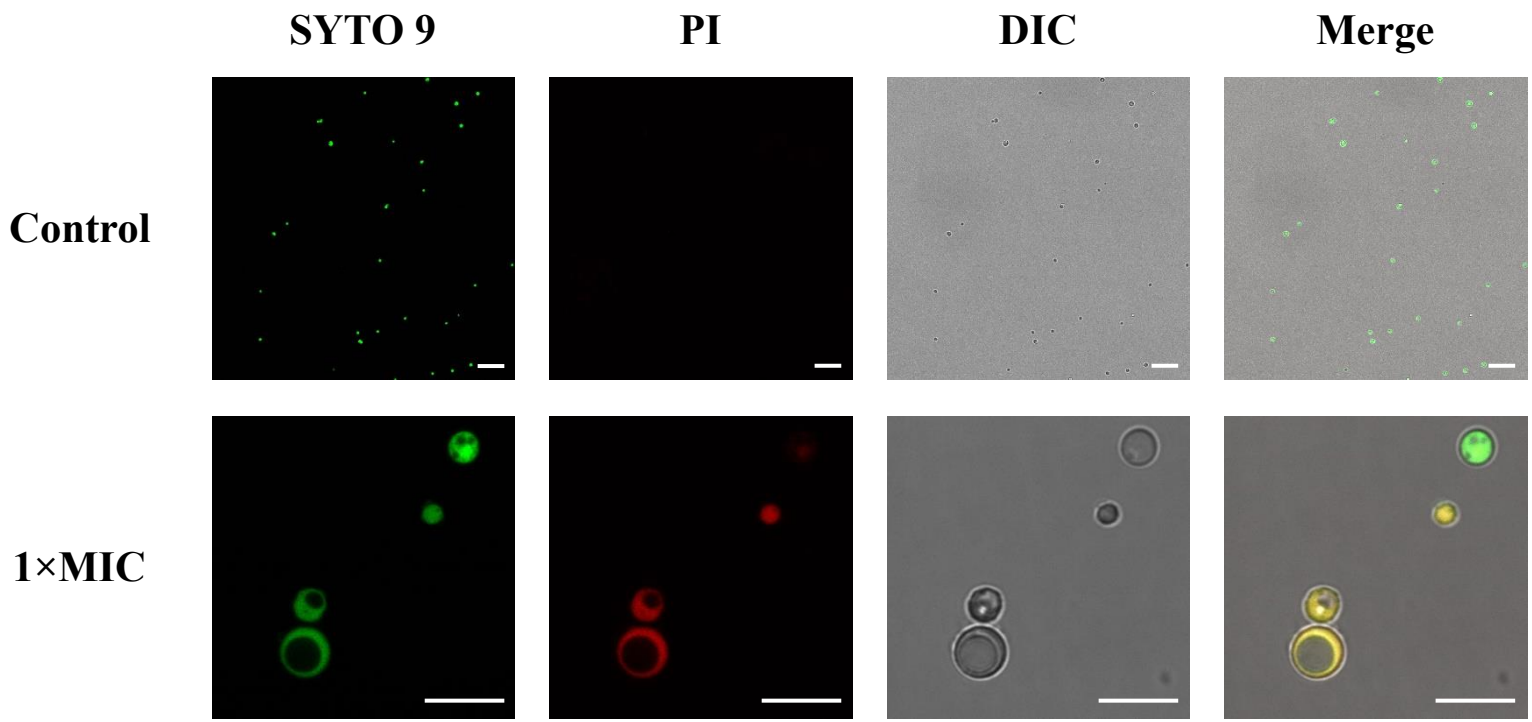

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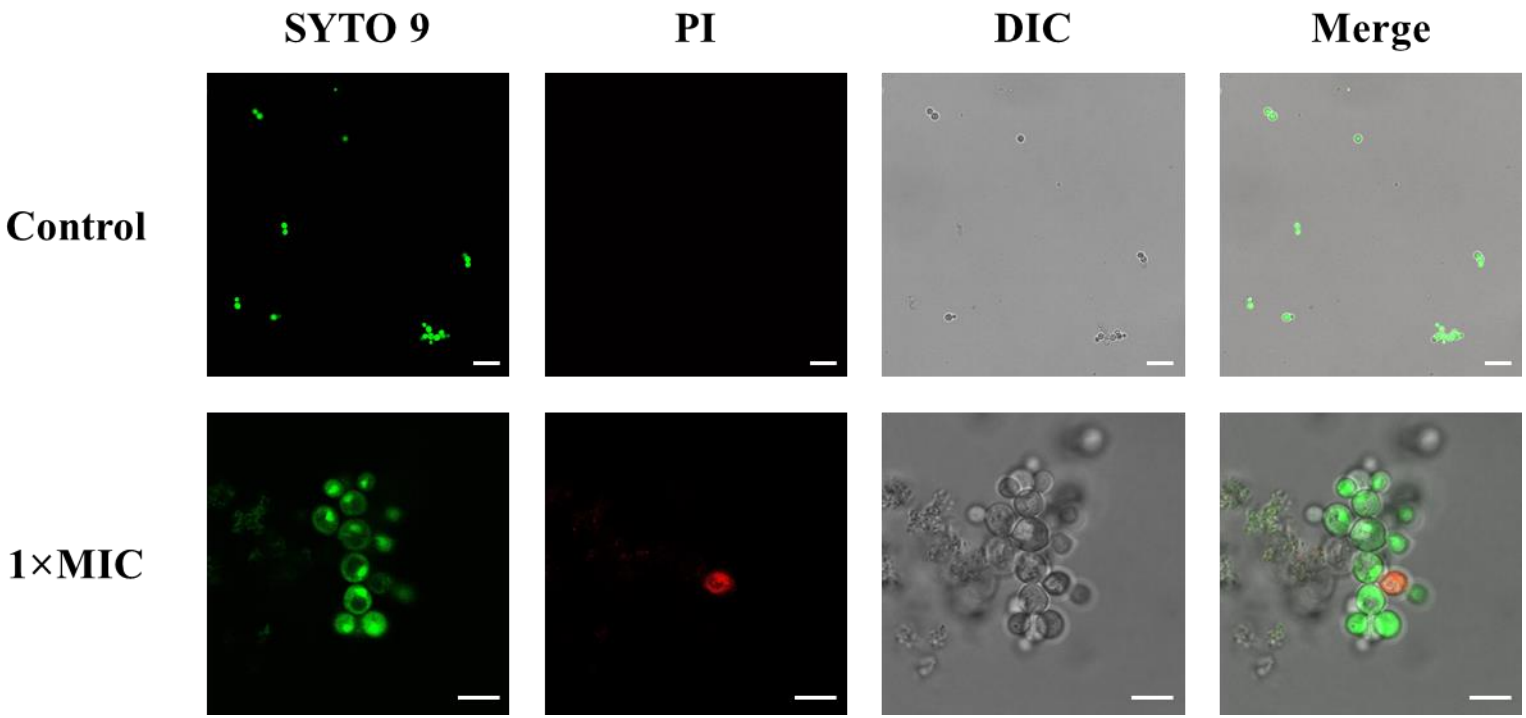

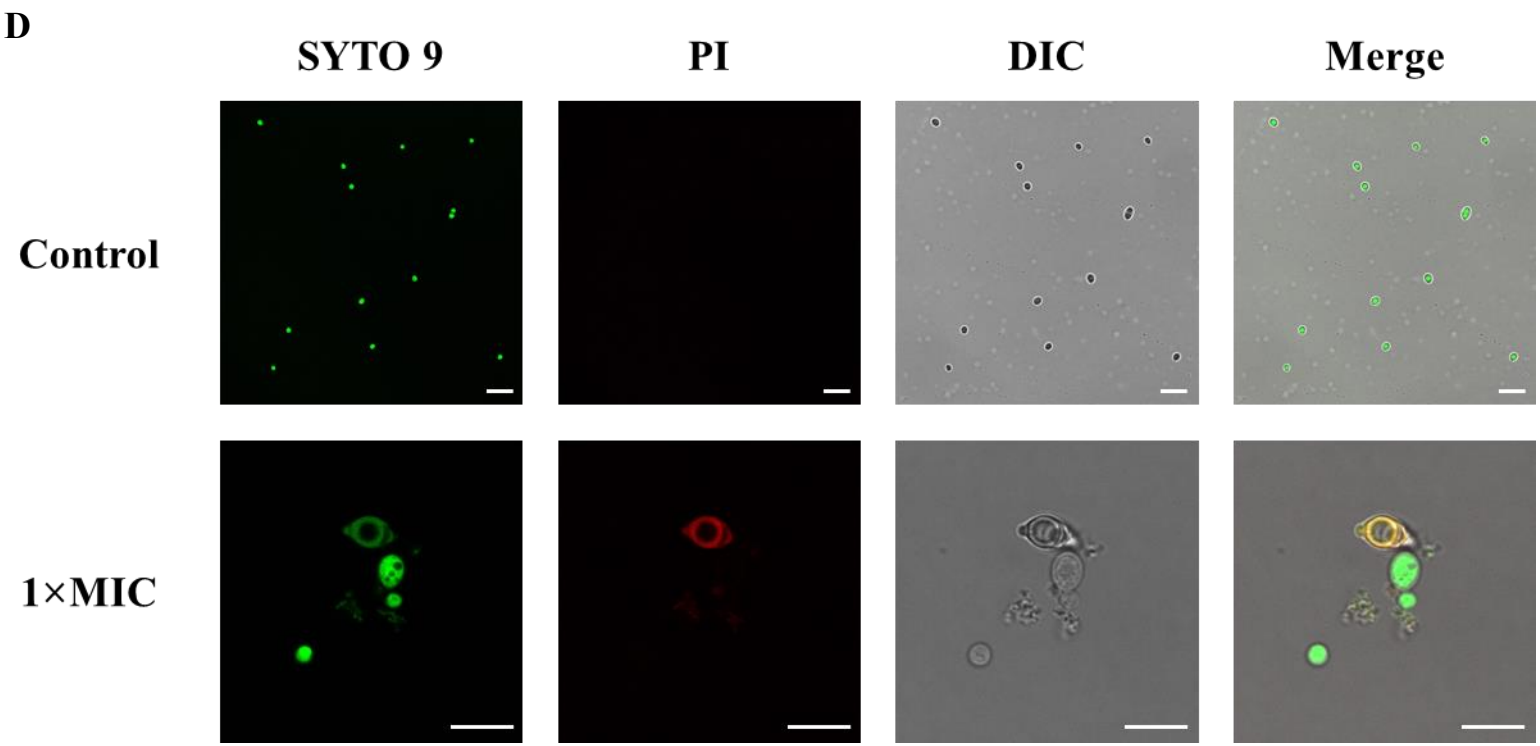

Fig. 6. Fluorescence micrographs of (A) *C. albicans*, (B) *P. anomala*, (C) *S. cerevisiae*, and (D) *R. mucilaginosa* after treatment with 1×MICs of SnuCalCpI15 for 3 h. Negative control: PBS (pH 7.4). Living cells stain green and dead cells stain orange and red. Scale bar: 25 μm (Control, 1×MIC of *C. albicans*), 8 μm (1×MIC of *P. anomala*, *S. cerevisiae*, and *R. mucilaginosa*).

3.4.2. Propidium iodide uptake assay

The PI uptake assay was used to quantify changes in the cytoplasmic membrane integrity of yeast cells induced by SnuCalCpI15. The concentration-dependent effects of SnuCalCpI15 were compared between the 0.5×MIC and 1×MIC values. After 3 h of incubation, cell suspensions were stained with PI and fluorescence intensity measured using a fluorescence spectrophotometer. The PI uptakes (Fig. 7) were normalized using the following formula:

$$\frac{F}{F_0} = \frac{\text{Fluorescence value after treatment}/OD_{600}}{\text{Fluorescence value for untreated control}/OD_{600}}$$

In the presence of SnuCalCpI15, yeasts stained with PI exhibited enhanced fluorescence intensities, compared to the negative controls (no propeptide). For *C. albicans* and *R. mucilaginosa*, the fluorescence intensity exhibited greater enhancement after SnuCalCpI15 addition to 1×MIC, relative to 0.5×MIC ($p < 0.05$); this was not observed for *P. anomala* or *R. mucilaginosa* ($p < 0.05$).

The results of the Live/Dead yeast cell staining and PI uptake assays suggested that the antifungal activity of SnuCalCpI15 is partly attributable to an effect on membrane permeability. It is unclear how peptides enter yeast

cells, but several hypotheses have been proposed (Vriens, Cammue, & Thevissen, 2014). Therefore, it is not known exactly how SnuCalCpI15 affects cell membrane permeability, and further research is needed based on this hypothesis.

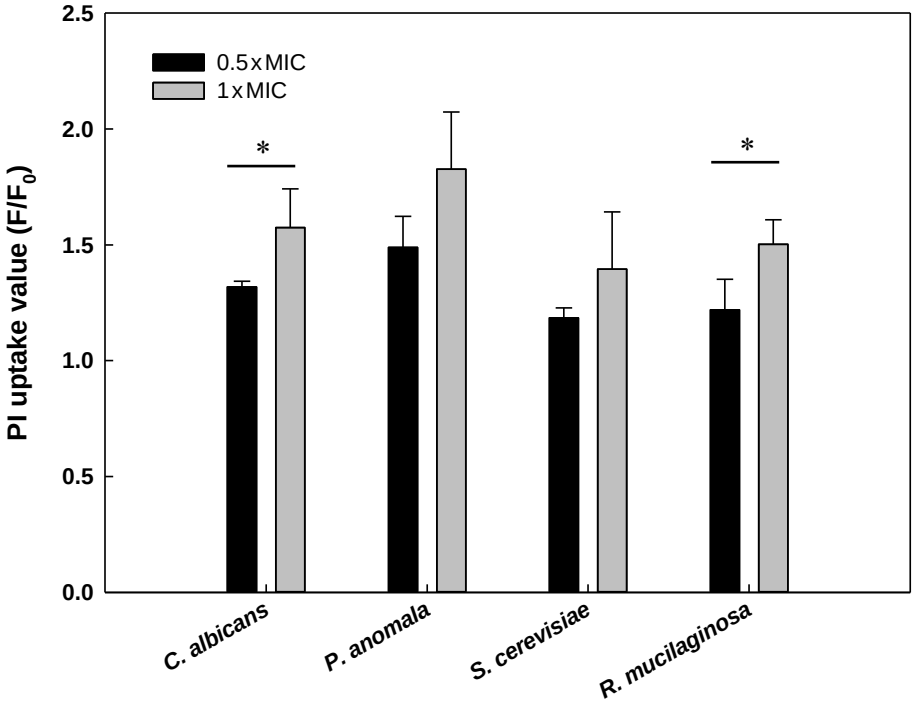

Fig. 7. Membrane permeabilities of the four yeasts after treatment with 0.5×MICs and 1×MICs of SnuCalCpI15, as determined by PI uptake assay. All experiments were performed in triplicate; bars indicate standard deviations. Asterisks indicate significant differences between control and treated cells (*$p < 0.05$, Duncan's multiple range test).

3.5. Morphological analysis

TEM was used to assess structural changes in yeasts after exposure to SnuCalCpI15. The four yeasts were treated with SnuCalCpI15 at the 1×MICs for 48 h. TEM revealed morphological changes (Fig. 8). Control (no propeptide) cells were well-organized and hosted typical cell organelles. In contrast, SnuCalCpI15 treatment of *C. albicans* significantly enhanced cell wall thickness and reduced the cytoplasmic region. *P. anomala* exhibited similar changes, although only the tip thickness was increased. However, the cell membrane of *P. anomala* became rougher than the control. The cell wall/membrane integrity of *S. cerevisiae* was also compromised. In *R. mucilaginosa*, the cell wall thickness was increased; notably, two layers were observed. These results suggested that SnuCalCpI15 damages the cell wall and membrane, characterized by changes in cell wall thickness.

Growing yeast cells continuously change shape via cell wall remodeling. Poorly coordinated cell wall expansion and assembly during growth may be fatal; wall thinning in regions of expansion may trigger lysis, unless this expansion is balanced by assembly of new wall material. However, the mechanism of coordination remains largely unknown.

The results of FITC-labeling and membrane permeability analysis indicated that SnuCalCpI15 penetrates cell membranes. Therefore, SnuCalCpI15 accumulation inside the cell impairs cell wall integrity. The cell wall is essential for yeast survival. The polysaccharidic structure of yeast cell wall allows cells to resist internal turgor pressure and protects against mechanical damage (Pérez, Cortés, Cansado, & Ribas, 2018). A cell wall integrity signal cascade is associated with the typical cell wall stress response (Pérez & Cansado, 2010). During yeast fission, the pathway is presumably activated at the cell surface by the putative transmembrane stress sensors Wsc1 and Mtl2, which may interact with the cell wall via long serine-/threonine-rich domains extending from the cell wall matrix (Cruz, Muñoz, Manjón, García, & Sanchez, 2013). Regardless of ongoing fission, Wsc1 and Mid2 serve as major plasma membrane sensors that detect cell wall damage or stress and trigger the cell wall integrity cascade (Levin, 2011; Straede & Heinisch, 2007); their signals activate the transcription of wall repair genes via the mitogen-activated protein kinase pathway (Arroyo, Bermejo, Garcia, & Rodriguez-Pena, 2009; Levin, 2011). Thus, cell wall strain may be monitored and cell wall synthesis may be adjusted accordingly, enabling coordinated growth and cell wall assembly (Davi et al., 2018). Therefore, when SnuCalCpI15 stresses a yeast, the cell wall integrity pathway is activated to repair the damage. Excessive signal

induction or damage to the cell wall integrity pathway would compromise cell wall integrity, leading to increased cell wall thickness and/or production of an uneven wall. Further studies are needed to investigate this hypothesis.

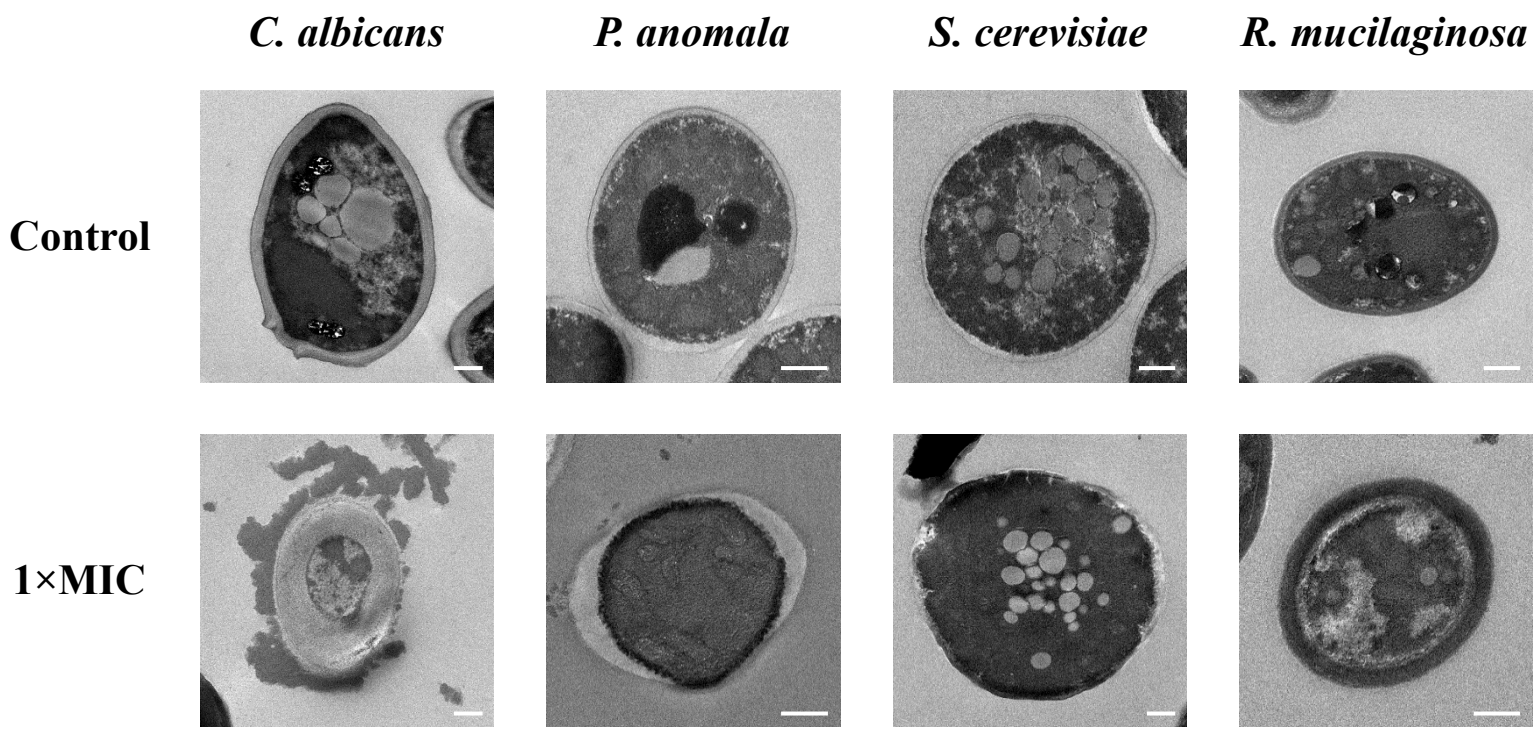

Fig. 8. Transmission electron microscopy (TEM) exploring cellular damage caused by SnuCalCpI15. Yeast cells were grown in RPMI 1640 medium with and without SnuCalCpI15 at 1×MIC values for 48 h, then fixed and thin-sectioned for TEM. Scale bars: 500 nm.

4. Conclusion

In this study, the antifungal activity of a cathepsin L-like propeptide against four food spoilage yeasts was investigated and its cysteine protease inhibitory activity was evaluated. The cysteine protease propeptide SnuCalCpI15 from *C. procera* R. Br. inhibited three cysteine proteases (primarily papain, as well as cathepsin L and bromelain). SnuCalCpI15 inhibited the growth of four food spoilage yeasts (*C. albicans*, *P. anomala*, *S. cerevisiae,* and *R. mucilaginosa*) that contaminate and cause unintended decay in low-pH high-sugar foods. The antifungal activity of SnuCalCpI15 may reflect interactions with the yeast cell surface, changes in yeast cell membrane permeability, and damage to the yeast cell wall. The detailed antifungal mechanism requires further investigation. The data suggest that SnuCalCpI15 is a potent alternative to chemical antifungal food preservatives that may improve the shelf life and quality of low-pH high-sugar foods vulnerable to yeast spoilage.

5. References

Arroyo, J., Bermejo, C., Garcia, R., & Rodriguez-Pena, J. M. (2009). Genomics in the detection of damage in microbial systems: cell wall stress in yeast. *Clinical Microbiology and Infection, 15 Suppl 1*, 44-46.

Barrett, A. J., & Rawlings, N. D. (1996). Families and clans of cysteine peptidases. *Perspectives in Drug Discovery and Design, 6*(1), 1-11.

Battey, A. S., Duffy, S., & Schaffner, D. W. (2002). Modeling yeast spoilage in cold-filled ready-to-drink beverages with *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii,* and *Candida lipolytica*. *Applied and Environmental Microbiology, 68*(4), 1901-1906.

Bertout, S., Dunyach, C., Drakulovski, P., Reynes, J., & Mallie, M. (2011). Comparison of the Sensititre YeastOne(R) dilution method with the Clinical Laboratory Standards Institute (CLSI) M27-A3 microbroth dilution reference method for determining MIC of eight antifungal agents on 102 yeast strains. *Pathologie Biologie, 59*(1), 48-51.

Carmona, E., Dufour, E., Plouffe, C., Takebe, S., Mason, P., Mort, J. S., & Menard, R. (1996). Potency and selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. *Biochemistry, 35*(25), 8149-8157.

Casas, E., de Ancos, B., Valderrama, M. J., Cano, P., & Peinado, J. M. (2004). Pentadiene production from potassium sorbate by osmotolerant yeasts. *International Journal of Food Microbiology, 94*(1), 93-96.

Cheng, Y., Du, Z., Zhu, H., Guo, X., & He, X. (2016). Protective effects of arginine on *Saccharomyces cerevisiae* against ethanol stress. *Scientific Reports, 6*(1), 31311.

Cruz, S., Muñoz, S., Manjón, E., García, P., & Sanchez, Y. (2013). The fission yeast cell wall stress sensor-like proteins Mtl2 and Wsc1 act by turning on the GTP ase Rho1p but act independently of the cell wall integrity pathway. *Microbiologyopen, 2*(5), 778-794.

Cygler, M., & Mort, J. S. (1997). Proregion structure of members of the papain superfamily. Mode of inhibition of enzymatic activity. *Biochimie, 79*(11), 645-652.

Davi, V., Tanimoto, H., Ershov, D., Haupt, A., De Belly, H., Le Borgne, R., Minc, N. (2018). Mechanosensation dynamically coordinates polar growth and cell wall assembly to promote cell survival. *Developmental Cell, 45*(2), 170-182 e177.

Hernández, A., Pérez-Nevado, F., Ruiz-Moyano, S., Serradilla, M., Villalobos, M., Martín, A., & Córdoba, M. (2018). Spoilage yeasts: what are the sources of contamination of foods and beverages? *International*

Journal of Food Microbiology, 286, 98-110.

Kruger, N. J. (2009). The Bradford method for protein quantitation. In J. M. Walker (Ed.), *The Protein Protocols Handbook* (pp. 17-24). Totowa, NJ: Humana Press.

Kwon, C. W., Yang, H., Yeo, S., Park, K. M., Jeong, A. J., Lee, K. W., Chang, P. S. (2018). Molecular cloning and anti-invasive activity of cathepsin L propeptide-like protein from *Calotropis procera* R. Br. against cancer cells. *Journal of Enzyme Inhibition and Medicinal Chemistry, 33*(1), 657-664.

Levin, D. E. (2011). Regulation of cell wall biogenesis in Saccharomyces cerevisiae: the cell wall integrity signaling pathway. *Genetics, 189*(4), 1145-1175.

Loureiro, V. (2000). Spoilage yeasts in foods and beverages: characterisation and ecology for improved diagnosis and control. *Food Research International, 33*(3-4), 247-256.

Loureiro, V. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology, 86*(1-2), 23-50.

Loureiro, V., & Querol, A. (1999). The prevalence and control of spoilage yeasts in foods and beverages. *Trends in Food Science & Technology, 10*(11), 356-365.

Melo, I. R. S., Dias, L. P., Araujo, N. M. S., Vasconcelos, I. M., Martins, T. F., de Morais, G. A., Oliveira, J. T. A. (2019). ClCPI, a cysteine protease inhibitor purified from *Cassia leiandra* seeds has antifungal activity against *Candida tropicalis* by inducing disruption of the cell surface. *International Journal of Biological Macromolecules, 133*, 1115-1124.

Paiva, P. M., Pontual, E. V., Coelho, L., & Napoleão, T. H. (2013). Protease inhibitors from plants: biotechnological insights with emphasis on their effects on microbial pathogens. *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education, 1*, 641-649.

Pérez, P., & Cansado, J. (2010). Cell integrity signaling and response to stress in fission yeast. *Current Protein and Peptide Science, 11*(8), 680-692.

Pérez, P., Cortés, J. C., Cansado, J., & Ribas, J. C. (2018). Fission yeast cell wall biosynthesis and cell integrity signalling. *The Cell Surface, 4*, 1-9.

Powers, J. C., Asgian, J. L., Ekici, O. D., & James, K. E. (2002). Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chemical reviews, 102*(12), 4639-4750.

Querol, A., & Fleet, G. H. (2006). Yeasts in food and beverages.

Rawlings, N. D., Tolle, D. P., & Barrett, A. J. (2004). Evolutionary families of

peptidase inhibitors. *Biochemical Journal, 378*(Pt 3), 705-716.

Reichard, U., Buttner, S., Eiffert, H., Staib, F., & Ruchel, R. (1990). Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *Journal of Medical Microbiology, 33*(4), 243-251.

Rico-Munoz, E., & dos Santos, J. L. P. (2019). The fungal problem in thermal processed beverages. *Current Opinion in Food Science, 29*, 80-87.

Selitrennikoff, C. P. (2001). Antifungal proteins. *Applied and Environmental Microbiology, 67*(7), 2883-2894.

Shamsi, T. N., Parveen, R., & Fatima, S. (2016). Characterization, biomedical and agricultural applications of protease inhibitors: a review. *International Journal of Biological Macromolecules, 91*, 1120-1133.

Straede, A., & Heinisch, J. J. (2007). Functional analyses of the extra- and intracellular domains of the yeast cell wall integrity sensors Mid2 and Wsc1. *Federation of European Biochemical Societies Letters, 581*(23), 4495-4500.

Taylor, M. A. J., Baker, K. C., Briggs, G. S., Connerton, I. F., Cummings, N. J., Pratt, K. A., Goodenough, P. W. (1995). Recombinant pro-regions from papain and papaya proteinase IV are selective high affinity inhibitors of the mature papaya enzymes. *Protein Engineering, Design*

and Selection, 8(1), 59-62.

Theis, T., & Stahl, U. (2004). Antifungal proteins: targets, mechanisms and prospective applications. *Cellular and Molecular Life Sciences, 61*(4), 437-455.

Thomas, D. S. (1993). Yeasts as spoilage organisms in beverages. In *The Yeasts* (pp. 517-561). Academic Press.

Vandeputte, P., Ferrari, S., & Coste, A. T. (2011). Antifungal resistance and new strategies to control fungal infections. *International Journal of Microbiology, 2012*.

Vriens, K., Cammue, B. P., & Thevissen, K. (2014). Antifungal plant defensins: mechanisms of action and production. *Molecules, 19*(8), 12280-12303.

Wareing, P., & Davenport, R. R. (2004). Microbiology of soft drinks and fruit juices. In *Chemistry and Technology of Soft Drinks and Fruit Juices* (pp. 279-299).

국문초록

화학적 항진균제 및 식품 보존제의 인체 위험성이 주목받음에 따라 새로운 생장 저해 전략을 가진 항진균 물질의 필요성이 대두되었다. 식물은 진균에 대한 자체 방어 기제로 여러가지 펩타이드를 분비하는데, 이 중에서 프로테이스 저해제들은 여러 항진균 활성이 보고되었음에도 불구하고 아직 그 메커니즘이 정확하게 밝혀지지 않았다. 본 논문에서는 열대 약용 식물로부터 얻어져 SnuCalCpI15라고 명명된 새로운 물질인 시스테인 프로테이스 프로펩타이드가 효소 저해제로서 가지는 특이성과 항진균 활성을 조사하고, 4가지 식품 부패 유발 효모에 대한 형태 변화 효과를 관찰하였다. 효소 저해 활성의 검증 및 특이적 저해 활성 확인을 위해서 시스테인 프로테이스 3종과 세린 프로테이스 2종에 대한 저해 효과를 비교한 결과, SnuCalCpI15는 특이적으로 파파인 유사 시스테인 프로테이스만을 저해하였으며, bovine pancreas 유래 세린 프로테이스는 저해하지 못하였다. 파파인과 브로멜라인에 대한 IC_{50}값은 각각 8.416±1.744 μM과 48.069±8.172 μM이었다. 식품 유래 효모에 대한 SnuCalCpI15의 항진균 효과를 확인하기 위하여 낮은 pH 및 고당도

식품에서 의도하지 않은 부패를 유발한다고 알려진 효모 4종 (*C. albicans* KCTC 7678, *P. anomala* KCTC 7295, *S. cerevisiae* KACC 30008, 및 *R. mucilaginosa* KCTC 7829)을 선발하였고, broth micro-dilution test와 time-kill assay를 통해 SnuCalCpI15 처리가 효모에 미치는 생장 저해 효과를 평가하였다. 그 결과, 진균 사멸 효과는 관찰되지 않았지만 농도 의존적 정균 효과가 관찰되었고, 생장 최소억제농도는 *C. albicans*, *S. cerevisiae*에 대하여 각각 3,000±90.47 ppm과 3,000±65.28 ppm, 그리고 *P. anomala*, *R. mucilaginosa*에 대해 각각 4,000±120.45 ppm과 4,000±87.05 ppm으로 결정되었다. 이러한 저해 효과의 원인을 추론하기 위해서, SnuCalCpI15에 형광물질인 FITC를 표지 한 후 효모에 처리함으로써 SnuCalCpI15의 위치를 추적한 결과 대부분의 단백질이 세포 표면에 위치해 있지만 배양 시간이 지남에 따라 점차 효모 내부로 진입한다는 것을 알 수 있었다. 이어서 SnuCalCpI15가 효모의 세포막 변화를 유발하는지 확인하기 위해, 살아있는 세포와 세포막 투과성이 변하여 사멸한 세포를 형광 염료인 SYTO 9과 PI를 이용하여 염색하였고 형광 현미경으로 관찰하여 세포막이 손상되었음을 확인하였다. 또한 PI uptake assay를 통해

세포막 투과성의 변화 정도를 정량 하였다. 마지막으로 세포의 전체적인 형태 변화 여부를 보기 위해 TEM을 이용하여 4가지 효모의 이미지를 획득한 결과, SnuCalCpI15 처리에 따라 효모 세포벽의 두께 증가, 형태 유실 및 종에 따라 세포막의 거친 정도 증가가 관찰되었다. 결과적으로, SnuCalCpI15는 세포막 투과성을 변화시켜 세포 내부로 들어가며, 내외부에 축적되어 세포벽과 세포막의 완전성에 타격을 준다는 것을 확인할 수 있었다. 세포벽의 완전성 결함은 효모 세포벽 합성을 관장하는 cell wall integrity 경로에 SnuCalCpI15가 영향을 준 것으로 예상되지만, 정확한 메커니즘 규명을 위해서는 추가 연구가 필요하다. 결론적으로, 파파인 유사 시스테인 프로테이스 저해활성을 가지는 식물 유래 프로펩타이드 SnuCalCpI15는 식품 부패 효모의 생장을 저해하므로, 효모에 의한 식품 부패를 억제하는 보존제로서의 기능을 수행할 수 있음을 시사한다.

주제어: 프로펩타이드, 효소 저해제, 시스테인 프로테이스, 식품 부패 효모, 항진균, 생장 저해, 세포벽

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