ORIGINAL ARTICLE

In Vitro Antibacterial Activity of Cysteine Protease Inhibitor from Kiwifruit (*Actinidia deliciosa*)

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Abstract The need for replacing traditional pesticides with alternative agents for the management of agricultural pathogens is rising worldwide. In this study, a cysteine proteinase inhibitor (CPI), 11 kDa in size, was purified from green kiwifruit to homogeneity. We examined the growth inhibition of three plant pathogenic Gram-negative bacterial strains by kiwi CPI and attempted to elucidate the potential mechanism of the growth inhibition. CPI influenced the growth of phytopathogenic bacteria *Agrobacterium tumefaciens* (76.2 % growth inhibition using 15 μM CPI), *Burkholderia cepacia* (75.6 % growth inhibition) and, to a lesser extent, *Erwinia carotovora* (44.4 % growth inhibition) by inhibiting proteinases that are excreted by these bacteria. Identification and characterization of natural plant defense molecules is the first step toward creation of improved methods for pest control based on naturally occurring molecules.

Keywords Cysteine protease inhibitor · Kiwifruit cystatin · Phytocystatin · Antibacterial · Plant natural resistance

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Introduction

Pesticides have made a great contribution to the quick and effective management of plant diseases and microbial contaminations in agricultural production worldwide. However, in recent years a large number of synthetic pesticides have been banned in the Western world due to their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain, and extension of their power to destroy both useful and harmful pests [1]. Furthermore, many pathogenic microorganisms have acquired resistance to synthetic pesticides [2] which seriously hinders the management of diseases of crops and agriculture products. Considering the deleterious effects of synthetic pesticides on life, there is an urgent need for alternative agents for the management of agricultural pathogens [3].

In the course of evolution, plants have developed protective mechanisms that allow them to successfully resist different kinds of unfavorable conditions including insects and phytopathogenic microorganisms [4, 5]. These proteins include hydrolase inhibitors, lectins, ribosome-inactivating proteins, enzymes, etc., all of which can, to some extent, create a protective barrier in the early stages of infection. Proteinase inhibitors can also exhibit a protective role, since both insect pests and pathogenic microorganisms use proteolytic enzymes for penetration into the host plant tissues.

Inhibitors of cysteine proteases are named cystatins. They are found in both animals [6, 7] and plants [8–10]. Most plant cystatins (phytocystatins) are small proteins from 10 to 16 kDa in size, containing no disulfide bonds, and showing sequence homology with the family 2 of animal cystatins, which includes the egg cystatin. The exact role of phytocystatins is not determined but it has

been suggested that they could act as regulators of proteolysis during seed maturation and germination [11] and/or contribute to plant defense by inhibiting exogenous proteases from insect pests and nematodes, phytopathogenic fungi and bacteria [12, 13]. It has been reported that transgenic plants overexpressing cystatins show enhanced resistance against insect [14] and nematodes [15]. Pernas et al. [16] demonstrated that chestnut cystatin inhibited the growth of phytopathogenic fungi *Botrytis cinerea*, *Colletotrichum graminicola*, and *Septoria nodorum*, but not that of the phytopathogenic bacteria *Erwinia chrysanthemi* and *Clavibacter michiganensis*.

Cysteine proteinase inhibitor (CPI) from green kiwifruit is synthesized as a pre-protein of 116 amino acids, with the first 26 amino acids representing a signal sequence which is cleaved off from the mature protein [17, 18]. It is an 11 kDa glycoprotein (pI 6.9) which binds Con A lectin, mannose-specific rBanLec and fucose-specific *Aleuria aurantia* lectin [17].

Green kiwifruit, *Actinidia deliciosa*, is very popular worldwide because of its high content of vitamins C, K, and E and its low caloric value [19]. However, its growth can be affected by various pathogens. A major disease of the kiwifruit vine is crown gall caused by *Agrobacterium tumefaciens*. This pathogen has a wide host range [20] and causes significant financial loss in commercial nurseries and orchards. *Burkholderia cepacia* can cause plant diseases such as onion skin rot, but was also found to affect humans, especially individuals suffering from cystic fibrosis. *Erwinia carotovora* is a plant pathogenic bacterium with a wide host range (carrot, potato, tomato, leafy greens, squash and other cucurbits) and is able to cause disease in almost any plant tissue it invades [21].

The rationale behind our investigation was to assess the role of CPI in the natural resistance mechanisms of kiwifruit. Identification and characterization of natural plant defenses is the first step towards improving our knowledge on pest control with biologically occurring molecules and thus decreasing the need for and ultimately the use of chemical spraying. In this study, we examined the in vitro growth inhibition of three Gram-negative bacterial strains by kiwi CPI and attempted to elucidate the mechanism of growth inhibition.

Materials and Methods

Purification of Cysteine Proteinase Inhibitor From Kiwifruit and MS Analysis

CPI was purified according to a previously published procedure by Popovic et al. [17]. After extraction and centrifugation, actinidin was precipitated by heating (60 °C for 10 min). Proteins were precipitated using ammonium sulphate to saturation of 100 % and, after re-suspension in deionized water, CPI was purified on papain affinity column. Homogeneity of purified CPI was assessed by SDS-PAGE. Purified CPI was analyzed by Autoflex (Bruker Daltonik GmbH, Bremen, Germany) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Samples deposited on gold coated target were desorbed and ionized by a 337 nm nitrogen laser which operated at 3 Hz repetition rate, using an accelerating voltage of 20 kV. The matrix was sinapic acid in 30 % acetonitrile with 0.1 % TFA. Spectra were acquired in positive-ion linear mode.

Antisera Production

Antibodies against purified phytocystatin (*Actinidia deliciosa*) were raised in rabbits according to Harboe and Ingild [22]. Animals were injected with 0.5 mL of an emulsion (1:2, v:v) of the CPI protein solution (1 mg/mL) in complete Freund's adjuvant. Bleeding was performed 50 days following the first immunization and every 2 weeks thereafter. The serum was partially purified using ammonium sulfate fractionation (50 % saturation). Antisera specificity was checked in Western blot (data not shown).

Bacterial Growth Inhibition Assay

Bacterial strains from the laboratory collection, *Agrobacterium tumefaciens* LBA 4404, *Burkholderia cepacia* ATCC 2159, and *Erwinia carotovora* were obtained from Dr. Tanja Beric (Faculty of Biology, University of Belgrade). Bacteria were grown on Luria agar (LA) plates at 28 °C for 20 h. Overnight cultures were prepared in Luria broth (LB) media by inoculation with a single colony from agar plates and incubated at 28 °C for 12 h. Overnight cultures were diluted with fresh LB media to approximately 10^4 colony-forming units (CFU) and incubated at 28 °C for 18 h in the presence of varying concentrations of CPI (0, 1.87, 3.75, 7.5 or 15 μM). For each concentration, three replicates were used. The mean growth values were obtained and then converted into the inhibition percentage of growth in relation to the control treatment by using the formula $MGI\,(\%) = ((dc - dt)/dc) \times 100$, where dc and dt represent OD_{620} in control and treated wells, respectively. The experiments were conducted twice.

Detection and Quantification of CPI in Growth Medium

Following a growth period (20 h) the growth media (with and without CPI) was separated from cells by centrifugation (5,000×*g*, 10 min). The supernatant (20 μL) was

subsequently applied to a nitrocellulose membrane using 96-well dot blot hybridization manifold (VWR, Vienna, Austria). The membrane was blocked using 30 mM TBS (Tris buffer saline) pH 7.4, containing 1 % w/v BSA and 0.5 % v/v Tween 20 (Serva, Heidelberg, Germany) for 1 h at RT. For visualization, the membrane was incubated with rabbit polyclonal anti-CPI antisera (dilution 1:50,000) for 2 h at RT. Visualization of bound antibodies was done using alkaline phosphatase-labeled anti-rabbit IgG antibodies (dilution 1:20,000, Millipore, Billerica, USA) and BCIP/NBT solution. Visualized dot blots were dried between two sheets of filter paper and scanned using a Lexmark X 2350 desktop scanner (Lexmark). It was assumed that BCIP/NBT visualization was proportional to the amount of CPI, and dot intensities were quantified using GelPro Analyzer 3.1 (Media Cybernetics). For each concentration a positive control (dilutions of CPI in sterile LB) was considered as the maximal amount of detected CPI (100 %) and intensities for each dot were expressed as a relation to the control dot by using the formula, MGI (%) = ((dc − dt)/dc) × 100, where dc and dt represent dot intensity in control and treated sample for each CPI concentration, respectively.

Inhibition of Cysteine Protease Activity in Cells and Fermentation Fluid

Strains of bacteria *A. tumefaciens* and *B. cepacia* were grown in LB media for 18 h. Following the growth period, cells were separated from the media by centrifugation ($5,000 \times g$, 15 min). Media in which the cells were grown (fermentation fluid) was collected and stored at −20 °C until further use. For obtaining cell lysates, cells were disrupted by three cycles of freeze and thaw, which was followed by sonication in an ice bath using Branson Sonifier 150 (Branson Ultrasonic Corp., 20 kHz frequency, with 9.5 W output, 20 min). After sonication, cell lysates were clarified by centrifugation ($13,000 \times g$, 15 min). Inhibition of enzymatic activity in fermentation fluids and cell lysates was assessed using a caseinolytic assay [23]. In brief, 25 μL of the cell lysates or fermentation fluids were pre-incubated for 10 min with varying concentrations of CPI (1.25, 2.5, 5 or 10 mM) and mixed with 1 % (w/v) casein, 300 mM potassium phosphate, 12 mM L-cysteine, 12 mM ethylenediaminetetraacetic acid (EDTA), and 12 mM sodium hydroxide, pH 7. After 20 min of incubation at 37 °C, up to 5 % of trichloroacetic acid was added into the reaction mixture, and following 1 h incubation at 37 °C, the mixture was centrifuged ($13,000 \times g$, 10 min) and A_{280} was measured in the supernatants. One unit is defined as the amount of enzyme that produces ΔA_{280} of 1.0 per minute at 37 °C, when measuring TCA-soluble products of casein hydrolysis. Results are expressed as percentage of specific activity with

various concentrations of CPI compared to the sample incubated in the absence of inhibitory reagent.

Statistical Analyses

GraphPad Prism version 5.03 for Windows (San Diego, California, USA) was used for all calculations. Analysis of variance was performed at the significance level of $P \leq 0.05$ using Kruskal–Wallis test followed by Dunn post-test. When appropriate, means were separated by using Tukey's test ($P \leq 0.05$). The data from two independent experiments were analyzed separately but were not significantly different ($P > 0.05$). The EC_{50} value (concentration causing 50 % reduction in bacterial growth) was calculated using BioDataFit 1.02 at http://www.changbioscience. com/stat/ec50.html.

## Results

Purification of CPI and MS Analysis

Following separation on the papain affinity column, the CPI preparation provided homogenous protein species according to SDS-PAGE (data not shown). Experimentally determined molecular mass of the CPI was 10,908 and 11,070 Da as was previously desribed [17, 18]. Purified CPI, identified by MALDI-TOF MS analysis, was used for further analysis.
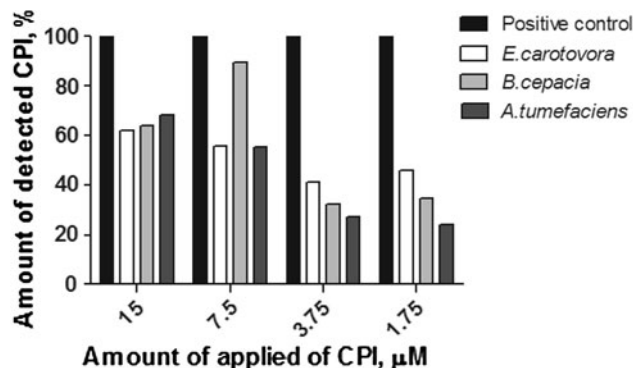
Bacterial Growth Inhibition Assay

In order to examine antibacterial activity of CPI approximately 10^4 CFU of *A. tumefaciens*, *B. cepacia*, and *E. carotovora* were grown in LB the presence of varying concentrations of CPI (0, 1.87, 3.75, 7.5 or 15 μM) compared to the growth of the control culture in the absence of CPI. The maximal inhibition of cell growth was achieved using 15 μM CPI. Relative growth for *A. tumefaciens* in the presence of 15 μM CPI was 23.8 %, for *B. cepacia* 22.4 %, and for *E. carotovora* 55.5 %, as shown in Fig. 1.

Detection and Quantification of CPI in Growth Medium

Apart from the sterile growth media and negative control (not shown), presence of CPI was clearly detected in the samples where concentration of added CPI was 1.87, 3.75, 7.5 or 15 μM, in all three bacterial strains (Fig. 2). In growth media of *A. tumefaciens* a median of 44 % of CPI was detected (Fig. 3). For *B. cepacia* and *E. carotovora* a median of 55 % and 51 % of CPI, respectively, was detected in growth media (Fig. 3). Obtained results
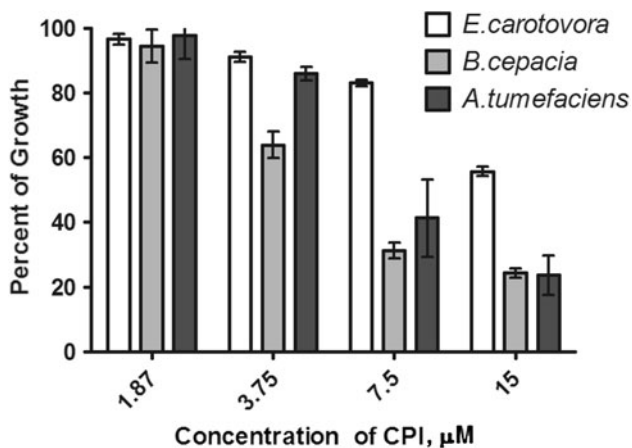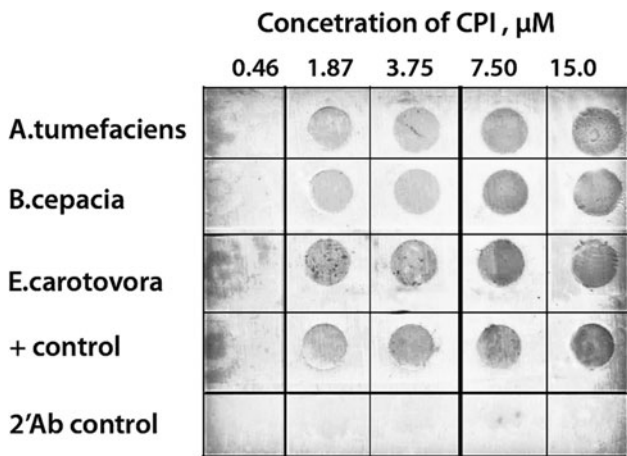
Fig. 1 Effects of CPI on the growth inhibition of *E. carotovora* (*white*), *B. cepacia* (*light grey*) and *A. tumefaciens* (*dark grey*). For the bioassay, 10^4 cells were incubated in the presence of increasing concentrations of inhibitor. The errors bars indicate standard deviations for triplicate experiments

Fig. 2 Detection of CPI by dot blot in the growth medium in which bacteria (*A. tumefaciens*, *B. cepacia* and *E. carotovora*) were grown. Positive controls consisted of dilutions of CPI in sterile LB; 2′Ab control—anti-rabbit AP-labeled IgG

therefore indicated that CPI was detectable in media in which bacterial cells were grown.

Inhibition of Cysteine Protease Activity in Cells and Fermentation Fluid

To test whether CPI can inhibit cysteine protease activity in cells and in fermentation fluid, enzymatic activity was tested in caseinolytic assay. For *B. cepacia* and *A. tumefaciens*, inhibition of caseinolytic activity was observed in the fermentation fluid of both *A. tumefaciens* and *B. cepacia* while no inhibition was observed in the cell lysates (Table 1). *A. tumefaciens* seemed to be more affected, as was evident from its higher inhibition (53 %). *B. cepacia* was also affected, but

Fig. 3 Quantification of CPI in growth media in which bacteria *E. carotovora* (*white*), *B. cepacia* (*light grey*) and *A. tumefaciens* (*dark grey*) were grown. Positive control (*black*) consisted of dilutions of CPI in sterile LB

observed inhibition was lesser (30 %) then in case of *A. tumefaciens* (Table 1).

Discussion

In this study, in vitro antibacterial properties of CPI purified from green kiwifruit (*Actinidia deliciosa*, Liang Ferguson) were evaluated. The CPI protein was isolated from kiwifruit extract by ammonium sulphate precipitation and papain affinity chromatography. Homogeneity and identity of purified protein, assessed by SDS-PAGE and MALDI-TOF analysis respectively, confirmed that the isolated protein was a mature form of CPI (GenBank Q6TPK4).

The effective concentration of CPI needed for 50 % of in vitro growth inhibition (EC_{50}) for *A. tumefaciens* and *B. cepacia* was 7.01 and 3.79 μM, respectively. Inhibition toward *E. carotovora* growth was also detected, however as 15 μM concentration of CPI induced a growth inhibition of only 45 %, EC_{50} could not be calculated.

Densitometric analysis of dot blots clearly proves evidence that CPI remains in the media during bacterial growth. Presence of CPI in the growth media following the 18 h incubation period indicated that CPI does not seem to be prone to digestion by bacterial proteases. This observation could potentially shed light on the mechanisms of inhibition. As previously described, egg cystatin reveals inhibitory activity towards secretory cysteine proteases from fermentation media of different gram negative bacteria, and effectively inhibits their growth [24]. Detection of CPI in bacterial growth media by dot blot analysis suggests that it might inhibit bacterial growth by inhibiting extracellular cysteine proteases. Data observed in the inhibition assay confirmed this assumption, as CPI inhibited enzymes excreted in the fermentation fluid, whereas cell lysate proteases were unaffected.

Table 1 CPI inhibition of the caseinolytic activity of proteases in fermentation fluid and cell lysate

CPI concentration (µM)	% of inhibition of extracellular proteases		% of inhibition of cell lysate proteases	
	A. tumefaciens	B. cepacia	A. tumefaciens	B. cepacia
1.25	32.41	2.47	/	/
2.5	34.21	7.15	/	/
5	37.97	10.99	/	/
10	52.99	29.39	/	/

/—no inhibition observed

Although kiwifruit is abundant in the cysteine protease actinidin, the fact that the Ki value of CPI against actinidin is rather high (14 nM) when compared to that of papain (0.16 nM), indicates that CPI does not readily inhibit actinidin [18]. The amount of actinidin in kiwifruit, up to 400 mg per kg of fresh fruits [25], is more than 60 times higher compared to that of CPI (6 mg/kg of fresh fruit). Furthermore, since CPI is synthesized as a pre-protein with the first 26 amino acids representing a signal peptide that designates it to extracellular region, and actinidin, on the other hand, is an intracellular protein [26], it is unlikely that the main role of CPI is to inhibit and regulate actinidin activity during plant development.

The protective role of cysteine protease inhibitors has been clearly shown for phytopathogenic fungi and insects [14, 16, 27] but reports dealing with antibacterial activity of these proteins are rare. In vitro antibacterial properties that have been clearly demonstrated in this study against two important phytopathogenic bacteria, A. tumefaciens and B. cepacia, make CPI an important potential target for further investigation into its use as a non chemical pest control agent. Whether the use of CPI as an alternative spraying agent could be effective in vivo remains to be further investigated.

Conclusion

CPI from green kiwifruit exerts in vitro antibacterial activity against phytopathogenic *Agrobacterium tumefaciens* and *Burkholderia cepacia.* This work clearly demonstrates that CPI has a protective role in the kiwifruit which designates it as a potential target for the development as an alternative to pesticide for controlling plant diseases and maintaining food quality.

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