Postharvest Biology and Technology 136 (2018) 132–138

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Control of green and blue mold and sour rot in citrus fruits by the cationic antimicrobial peptide PAF56

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ARTICLE INFO

Keywords:
Peptide PAF56
Citrus fruit
Control of diseases
Mode of action

ABSTRACT

The ability of the short cationic antimicrobial peptide PAF56 (amino acid sequence GHRKKWFW) to control postharvest infectious diseases of citrus fruits was evaluated. PAF56 inhibited the growth of *Penicillium digitatum*, *Penicillium italicum* and *Geotrichum candidum*. The minimum inhibitory concentrations (MICs) of PAF56 for the control of *P. digitatum*, *P. italicum* and *G. candidum* were 8, 16, and 8 μM, respectively. The conidia of *P. digitatum*, *P. italicum* and *G. candidum* were also highly sensitive to PAF56. The peptide PAF56 could effectively control green mold, blue mold and sour rot on inoculated citrus fruits. Fluorescence microscopy with the fluorescent stains SYTOX Green (SG) and Calcofluor White (CFW) was used to characterize the modes of action and interaction of PAF56 with the mycelia of *P. digitatum*, *P. italicum* and *G. candidum*. SG and CFW signals indicated that PAF56 could inhibit the formation of spores and change the cell membrane permeability and cell wall structure. A range of concentrations of PAF56 (from 16 to 128 μM) showed no hemolysis or toxicity to human red blood cells (erythrocytes).

1. Introduction

Fruits and vegetables play an important role in people's daily lives, but their quality and market value are seriously affected by decay. Traditional chemical antibiotics do not only raise concerns about food challenges but also cause environmental pollution and resistance development by pathogens (Schirra et al., 2011). The application of chemical fungicides to control postharvest diseases of fruits and vegetables is becoming more and more restricted. Control of postharvest diseases with alternative methods, such as antagonistic microorganisms (Parafati et al., 2015), chitosan oligosaccharides and antimicrobial peptides (AMPs), has attracted attention (Janisiewicz and Korsten, 2002). AMPs are gene-encoded, ribosomally synthesized polypeptides that are widely present in many organisms. AMPs have antibacterial, antifungal, antiviral and other biological functions. They are an important part of the non-specific immune function of the organism. Most of them are cationic and amphipathic, although they differ in length, sequence and structure. With the development of research on AMPs, due to their short duration of action against microbes and low toxicity to mammalian and plant cells, they are expected to be excellent substitutes for antibiotics and fungicides. AMPs have great potential applications in medicine, agriculture, the food industry, and animal husbandry (Jenssen et al., 2006; Keymanesh et al., 2009; Ciociola et al., 2016). Antimicrobial peptides such as BP15, 60 MsrA1 and MSI-99 have been proven to be effective in controlling fruit infectious diseases (Osusky et al., 2000; Chakrabarti et al., 2003).

Cationic antimicrobial peptides (CAMPs) represent one major group of AMPs, and CAMP usually display amphipathic properties. A combinatorial approach on a D-amino acid hexapeptide library was used to identify a group of cationic AMP (so-called PAFs), with antimicrobial activity against certain filamentous plant pathogens (López-García et al., 2002). PAF26 (amino acid sequence RKKWFW) is a synthetic de novo-designed hexapeptide, which has a similar sequence to other AMPs of natural and synthetic origin. PAF26 was previously identified against the fungal plant pathogen *Penicillium digitatum in vitro*, and PAF26 can effectively control green mold on citrus fruits (López-García et al., 2003; Muñoz et al., 2007). It has been shown that PAF26 could enter cells of *P. digitatum* and lead to cell death, but it is not lytic or cytotoxic to human cells (Muñoz et al., 2006; López-García et al., 2007). PAF26 can also be modified to design new sequences with improved antimicrobial and therapeutic properties. PAF56 (amino acid sequence GHRKKWFW) is derived from PAF26 with an extension of one glycine and one histidine residue (GH) at the N-terminus. Some fungi that have been demonstrated to be sensitive to PAF56 include the

Abbreviations: SG, SYTOX Green; CFW, Calcofluor White; MIC, the minimum inhibitory concentration; AMP, antimicrobial peptide

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https://doi.org/10.1016/j.postharvbio.2017.10.015
Received 13 July 2017; Received in revised form 24 September 2017; Accepted 31 October 2017

phytopathogens *P. digitatum* and *Fusarium oxysporum*. PAF56 could also inhibit the growth of the Gram-negative bacterium *Escherichia coli in vitro* (López-García et al., 2015).

Chongqing is an important origin of citrus in China (Wang et al., 2015). Green mold and blue mold caused by *P. digitatum* and *P. italicum* are important postharvest infectious diseases in citrus. These are the most economically important postharvest diseases of citrus in all production areas (Droby et al., 2002). Sour rot caused by *Geotrichum candidum* (syn. *Geotrichum citri-aurantii*) in the later stages of storage, which cannot be inhibited by the currently registered fungicides imazalil and thiabendazole (Ismail and Zhang, 2004). Thus, a safe, comprehensive and effective method to control citrus infectious disease is very desirable (Romanazzi et al., 2017). Up to now, there are few studies on the application of antimicrobial peptides to postharvest diseases and little effective strategy can control the three main postharvest diseases of citrus fruits. For PAF56, we know little about whether it can be applied to the control of citrus fruit diseases, except that it inhibits the growth of *P. digitatum in vitro*. In the present study, the effects of PAF56 on growth of *P. digitatum, P. italicum* and *G. candidum in vitro* and green mold, blue mold and sour rot in postharvest citrus fruit were determined. Meanwhile, the mode of action (cell membrane permeability) of PAF56 was investigated.

2. Materials and methods

2.1. Plant material

Navel oranges [*Citrus sinensis* (*L.*) *Osbeck*] were harvested from a local orchard (Beibei, Chongqing) during the commercial ripening period. All fruits were sorted based on size, color, shape and weight and were without physical injuries or infections. The fruit were surface-disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed with distilled water, and then air-dried at room temperature prior to use.

2.2. Pathogens

P. digitatum, *P. italicum* and *G. candidum* were isolated from decayed citrus fruits and identified by morphology and sequence of the internal transcribed spacer (ITS) rDNA region (Jeong et al., 2016). They were cultured on potato dextrose agar (PDA) plates for 7 d at 25 °C. Conidia were collected, filtered, and titrated with the aid of a hematocytometer and adjusted to the appropriate concentration.

2.3. Antifungal peptide

Peptide PAF56 (GHRKKWFW) was synthesized at > 90% purity from GenScript Corporation (Nanjing, China) by solid-phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Stock solutions of peptides were prepared at 1 mM in sterile ultrapure water and stored at −40 °C.

2.4. In vitro fungicidal activity

The peptide was tested for fungicidal activity against *P. digitatum*, *P. italicum* or *G. candidum*. Dose–response curves were assayed by using sterile 96-well plates essentially as described (López-García et al., 2002) with minor modifications. A volume of 180 µL of fungal conidia (1×10^4 conidia/ml) in 1/20 diluted potato dextrose broth (5% PDB) was mixed in each plate well with 20 µL of 10 × concentrated peptide solution from serial 2-fold dilutions. PAF56 was added to a final concentration of 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µM, respectively. Three replicates were prepared for each treatment. The samples were incubated at 25 °C. The growth of fungi was determined by OD_{600}. The data were measured at 48 h after mixing by a Multiskan Spectrum microplate spectrophotometer (BioTek Instruments, Inc., USA). The minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration that showed no growth.

Peptide fungicidal activity was also assayed by incubation of *P. digitatum*, *P. italicum* or *G. candidum* conidia as described (Muñoz et al., 2007) with minor modifications. Different concentrations of PAF56 (according to preliminary test, the PAF56 concentration was 16, 32, 64 µM for *P. digitatum* and *P. italicum,* and 4, 8, 16 µM for *G. candidum.*) were mixed with conidia (10^3 conidia/ml) in sterile distilled water for 16 h at 25 °C. Conidia in sterile distilled water were used as controls. Treatments were prepared in triplicate. Then, samples were plated on PDA plates to determine conidia viability by counting colony forming units (CFU). The percentage of conidia viability after exposure to PAF56 were compared with controls. Percent conidia viability (%) = (the number of viable conidia of the treatments/the number of viable conidia of the controls) × 100%.

2.5. Fruit decay test

Citrus fruit were wounded (4 mm deep and 3 mm wide) with a sterile nail at two sites around the equator. Inoculums contained 10^4 conidia/ml (*P. digitatum*, *P. italicum* or *G. candidum*) and peptide PAF56 at 64 µM in sterile water. There were two inoculation methods: conidia were mixed with PAF56 and either immediately inoculated or incubated for 16 h at 25 °C prior to inoculation. Citrus fruit were inoculated with conidia alone as the controls. For each treatment, three replicas (fifteen fruits per replica, two wounds per fruit) were prepared. All the fruit was stored at 25 °C and 90% RH. Observations were made daily after the diameters of the control lesions attained measurable sizes until the two lesions were about to intersect. The disease incidence (DI) on each infected orange was calculated as follows: DI (%) = (number of decayed wounds/number of total wounds) × 100%, and the lesion diameter (LD) was assessed by measuring the average diameter of the damaged area. Disease incidence and lesion diameter of infected wounds and mean values ± S.D. for each treatment were observed and subsequently calculated.

2.6. Fluorescence microscopy

Fluorescence microscopy and the fluorescent dyes SYTOX Green (SG) (Molecular Probes; Invitrogen Corp, Carlsbad, CA, USA) and Calcofluor White Stain (CFW) (Fluorescent Brightener 28; Sigma-Aldrich, St. Louis, MO, USA) were used to characterize the mode of action and interaction of PAF56 with the mycelia of *P. digitatum*, *P. italicum* and *G. candidum*. SG has two properties, namely, its > 500-fold fluorescence enhancement upon nucleic acid binding and its lack of penetration of living cells. Abnormal chitin accumulation could lead to fungal cell wall alterations. Calcofluor White Stain can bind specifically to chitin contained in the cell walls of fungi.

Aliquots of 450 µL of conidia (10^4 conidia/mL) were incubated in 1/20-diluted potato dextrose broth (5% PDB) in 1.5 mL light-safe microcentrifuge tubes for 48 h at 25 °C. Subsequently, 50 µL of PAF56 from 10 × stock solutions was added to reach final concentrations of 10 and 100 µM and allowed to grow for another 48 h. After this incubation time, the fungal suspensions were stained with two different fluorescent dyes: SYTOX Green and Calcofluor White as described (Puig et al., 2016) with minor modifications. Finally, the mycelium was washed and resuspended in 20% glycerol solution. The experiment consisted of three replicates of each treatment. The mycelium was visualized and photographed using an Eclipse TS100 epifluorescence microscope (Nikon Corporation, Japan), with DAPI and FITC filter sets.

2.7. Measurement of extracellular conductivity

The extracellular conductivity of *P. digitatum*, *P. italicum* and *G. candidum* spores were assayed using a DDS-307A conductivity meter (INESA, Shanghai, China). Ten mL PDB with 10^5 conidia/ml fungal suspensions was incubated in an environmental incubator shaker at