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Inhibiting effects of epsilon-poly-lysine (ε-PL) on *Pencillium digitatum* and its involved mechanism

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ABSTRACT

As a natural antimicrobial food additive, εpsilon-poly-lysine (ε-PL) was reported to be effective in preventing food spoilage. However, few studies regarding the inhibitory effect of ε-PL against phytopathogenic fungi, especially post-harvest pathogens, were reported. In this study, the antifungal activity of ε-PL via membrane damage in *Penicillium digitatum* was investigated. The data showed that mycelial growth, spore germination rate, and germ tube length of *P. digitatum* were markedly inhibited by ε-PL. Moreover, ε-PL was also effective in reducing the lesion diameter in citrus fruit. Scanning electron microscopy showed that mycelial morphology was seriously damaged after ε-PL treatment. Electrical conductivity measurement and propidium iodide assays showed that *P. digitatum* lost plasma membrane integrity by ε-PL. The level of malondialdehyde demonstrated that ε-PL led to lipid peroxidation in the fungal pathogen. These results indicate that ε-PL may serve as a sustainable partial substitute for chemical pesticides.

1. Introduction

Citrus, originating in tropical and subtropical regions, is one of the most widely produced fruits globally (Talibi et al., 2014). Citrus fruit are rich in vitamin C, carotene, phosphorus, iron, and other nutrients. However, due to their high water content and nutrient composition, citrus fruit are vulnerable to microbial infection (Yang et al., 2010). The whole decay occurring in citrus fruit per year about 90% is caused by *Penicillium digitatum* during the period between harvest and storage (Macarisin et al., 2007). Post-harvest diseases caused by pathogens have been consistently managed by synthetic fungicides. However, some chemical fungicides may be hazardous to consumer health and to the environment. Thus, safe and promising strategies are required to prolong the storage life of post-harvest fruits and vegetables.

ε-Poly-lysine (ε-PL), a polycationic peptide, was first found as a secretion from *Streptomyces albulus* no.346, now designated *S. albulus* NBRC 14147 (Bo et al., 2014). Compared with synthetic fungicides, ε-PL possessing low toxicity is a natural generally regarded as safe (GRAS) antimicrobial (Ye et al., 2013). A number of studies have demonstrated that ε-PL was very effective in controlling foodborne bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Listeria monocytogenes* (Ye et al., 2013; Li et al., 2014b; Zhang et al., 2015). ε-PL was already approved by the Food and Drug Administration (FDA, USA) for preserving food in 2003, and has been widely used in Korea, Japan, and the U.S.A (Yamanaka and Hamano, 2010; Li et al., 2014b). China has also approved it as a variety of food additive in April 2014. ε-PL exhibits high solubility in water and thermal stability at high temperatures. In fact, an ε-PL solution was still stable after autoclaving at 120 °C for 20 min (Hiraki, 2000; Yoshida and Nagasawa, 2003). ε-PL also can serve as a lysine source because it can be decomposed into lysine without any side effects on the human body (Li et al., 2014a,b). Based on the merits of ε-PL, it has a variety of applications in medical and electronic products, in addition to the field of food preservation (Shukla et al., 2012; Shi et al., 2015).

The antimicrobial effects of ε-PL against bacterial pathogens have been extensively investigated. However, few studies on the effectiveness of ε-PL against fungal pathogens have been reported to date, especially those concerning post-harvest pathogens that infect fresh fruits. This study aimed to investigate the antifungal activity of ε-PL against *P. digitatum in vitro*. The control efficacy of ε-PL on green mold caused *in vivo* was also evaluated. Its influence on the membrane of *P. digitatum* was studied by assaying

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morphological changes, plasma membrane integrity, electrical conductivity, and lipid peroxidation.

2. Materials and methods

2.1. Pathogen culture

Pathogenic *P. digitatum* isolated from infected citrus fruit was maintained and periodically subcultured on potato dextrose agar (PDA). Spores were obtained from a 7-day-old culture at 28 °C. The suspension of spores in sterile distilled water was filtered using two layers of UV disinfected gauze to remove any adherent mycelia. A homogenous spore suspension was obtained by using a haemocytometer.

2.2. Fruit

Fresh citrus fruit (*Citrus reticulata* Blanco) without any wounds or infections were purchased at a local supermarket in the Changqing district of Jinan City, Shandong Province, China. Preparations prior to inoculation were performed as described by Askarne et al. (2012). Fruit of uniform size were surface disinfected by soaking in 2% (v/v) sodium hypochlorite for 2 min, then rinsed with tap water, and air dried. These fruit were used for further inoculation experiments.

2.3. Measurement of mycelial growth

The inhibitory effect of ε-PL on the mycelial growth of *P. digitatum* was determined by the agar dilution method (Yao and Tian, 2005). ε-PL (powder, purity 99%, Zhejiang Silver Elephant Bioengineering Co., Ltd., Zhejiang province, China) was dissolved in sterile water. Different concentrations of ε-PL were mixed with PDA media in a proportion of 1: 9. Approximately 20 mL of PDA containing different concentrations of ε-PL (12.5, 25, 50, 100, and 200 mg L^{-1}), were respectively poured into 90 mm-diameter Petri dishes. The 7 mm-diameter mycelial disks taken from a 3-day-old culture of *P. digitatum* were placed in the centre of each Petri dish, and incubated at 28 °C. Mycelial growth was then assayed to measure the diameter of the colonies using the cross method after 7 days. The experiment was performed in triplicate.

2.4. Assay of spore germination and germ tube elongation

The effect of ε-PL on spore germination and germ tube elongation in *P. digitatum* was tested as described previously (Liu et al., 2010). The spore suspension (50 μL) containing 1×10^6 spores mL^{-1} was plated on Petri dishes (90 mm in diameter) containing 20 mL PDA with different concentrations (0, 12.5, 25, 50, 100, and 200 mg L^{-1}) of ε-PL. The Petri dishes were incubated at 28 °C. Spores germination and germ tube elongation were

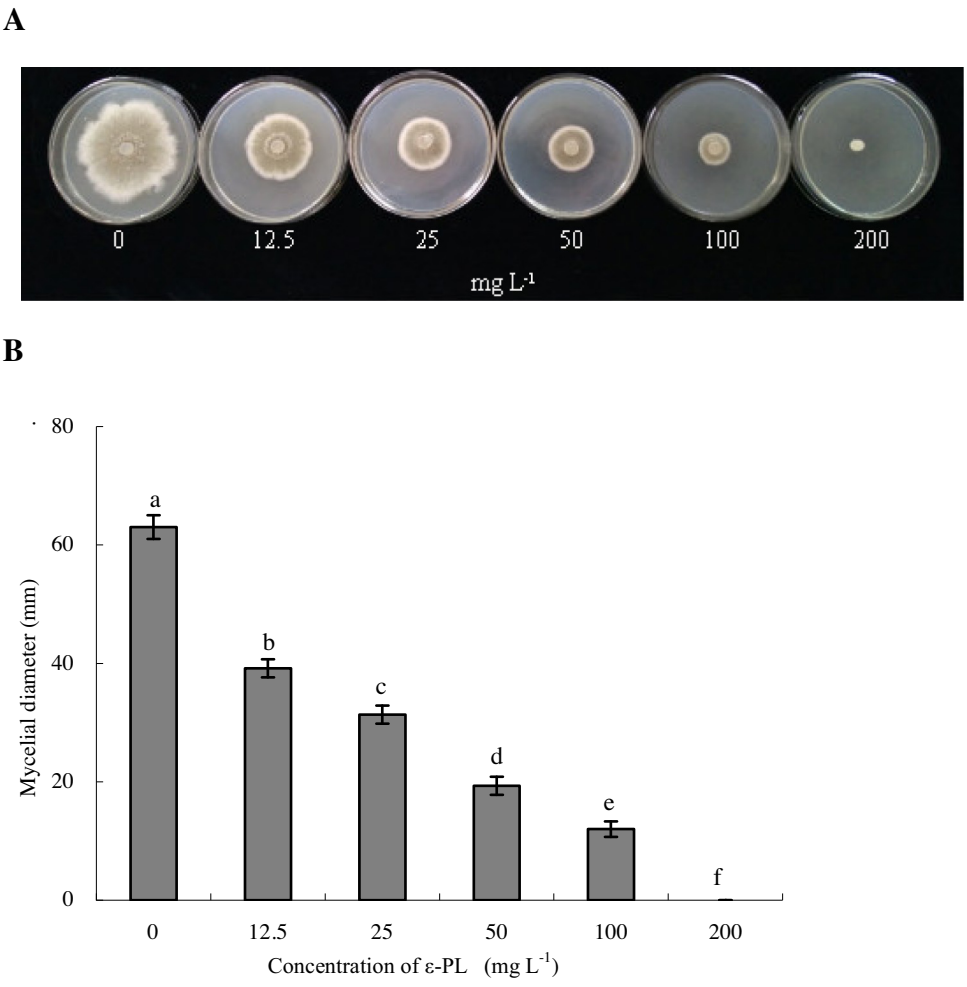

Fig 1. Antifungal activity of ε-PL on *P. digitatum*. (A) Colony diameter of *P.digitatum*, and (B) mycelial growth. The colony diameter of *P. digitatum* was measured after seven days of incubation at 28 °C.Vertical bars represent standard deviations of the means. Treatments followed by different letters are statistically different by the Duncan's multiple range ($P < 0.05$).