Carbohydrate Polymers 192 (2018) 95–103

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Fungicidal effect of chitosan via inducing membrane disturbance against *Ceratocystis fimbriata*

Ke Xing[a,*], Yun Xing[b], Yuanfang Liu[a], Yu Zhang[a], Xiaoqiang Shen[c], Xiaoyan Li[d], Xiangmin Miao[a], Zhaozhong Feng[a], Xue Peng[a], Sheng Qin[e,*]

[a] School of Life Science, Jiangsu Normal University, Xuzhou 221116, Jiangsu, PR China
[b] College of Chemistry and Chemical Engineering, Anyang Normal University, Anyang 455000, Henan, PR China
[c] Wanbang Biopharmaceutical Technology Company, Xuzhou 221004, Jiangsu, PR China
[d] College of Life Sciences, Northeast Forestry University, Harbin 150040, Heilongjiang, PR China
[e] The Key Laboratory of Biotechnology for Medicinal Plant of Jiangsu Province, Jiangsu Normal University, Xuzhou 221116, Jiangsu, PR China

ARTICLE INFO

Chemical compounds studied in this article:
Chitosan (PubChem CID: 71853)
Acetic acid (PubChem CID: 176)
Fluorescein diacetate (PubChem CID: 65047)
Propidium iodide (PubChem CID: 104981)
Fluorescein isothiocyanate (PubChem CID: 18730)

Keywords:
Ceratocystis fimbriata
Sweet potato
Chitosan
Antimicrobial
Plasma membrane
Pathogenic fungi

ABSTRACT

In this study, the fungicidal effects and detailed action of chitosan against *Ceratocystis fimbriata* were evaluated. The results demonstrated that chitosan exhibited strong antifungal activity that restricted the mycelium extension and changed the hyphal morphology. Fluorescein diacetate (FDA) and propidium iodide (PI) double-staining directly visualized decreased cell viability in response to chitosan treatment. Investigation of the PI influx showed that chitosan induced irreversible cell membrane damage. The efflux of potassium ions from the cytosol into the extracellular matrix demonstrated that chitosan induced the leakage of intracellular components. Massive intracellular bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] accumulation indicated the dissipation of membrane potential. Furthermore, chitosan clearly decreased the activity of H^+/K^+ ATPase. Fluorescence microscopy revealed that the fluorescence distribution and intensity of fluorescein isothiocyanate (FITC) changed as the incubation time increased. These results indicate that chitosan exerts a fungicidal effect via its ability to disturb fungal membranes.

1. Introduction

Sweet potato is the fourth largest grain crop in China, which is the largest producer of sweet potato in the world. As a perfect food and medicine resource, sweet potato is abundant of dietary fiber, antioxidants, vitamins and minerals. However, *Ceratocystis fimbriata* is a plant pathogenic fungus that attacks sweet potato and a wide variety of annual and perennial plants (Engelbrecht & Harrington, 2005). *C. fimbriata* infection results in black rot disease, while also preventing consumption of sweet potato by causing them to become bitter and toxic. Therefore, *C. fimbriata* is an important constraint of sweet potato production in China. Although application of chemical pesticides is an effective measure for control *C. fimbriata* infection, it also causes a series of problems, such as environmental pollution, toxic residues in agricultural products, threatening human health and inducing pathogen resistance, all of which seriously hamper the sustainable development of agriculture. To overcome black rot of sweet potato and other plant diseases, more effective and environment-friendly antifungal agents must be developed.

Chitosan is the partially deacetylated product of chitin, which is the major substance in the exoskeleton of crustaceans and the cell walls of fungi (Sanford, 2003). Chitosan is the most abundant polysaccharide in marine systems and the second largest renewable resource on the earth. Unlike other plant-based resources (such as cellulose and starch), the natural sources of chitosan are mainly from marine environments and do not compete for land or other human resources (Fernandez & Ingber, 2014). The widespread source, lower price and versatile biological activities of chitosan have led to increased attention by researchers. Because of its excellent biocompatibility (Duan, Liang, Cao, Wang, & Zhang, 2015; Peng et al., 2010), biodegradation (Sashiwa, Yajima, & Aiba, 2003), non-toxicity (Jena, Mohanty, Mallick, Jacob, & Sonawane, 2012) and antimicrobial activity (Sathiyabama & Parthasarathy, 2016; Xing et al., 2008, 2016), chitosan has been extensively used as carrier for drug delivery systems (Upadhyaya, Singh, Agarwal, & Tewari,

* Corresponding authors.
E-mail addresses: xingke@jsnu.edu.cn (K. Xing), xy7627@aliyun.com (Y. Xing), 18361297321@163.com (Y. Liu), 871309551@qq.com (Y. Zhang), 836528060@qq.com (X. Shen), xyli821187@163.com (X. Li), mxm0107@jsnu.edu.cn (X. Miao), fzz2012@jsnu.edu.cn (Z. Feng), pengxue@jsnu.edu.cn (X. Peng), shengqin@jsnu.edu.cn (S. Qin).

https://doi.org/10.1016/j.carbpol.2018.03.053
Received 14 November 2017; Received in revised form 13 February 2018; Accepted 16 March 2018
Available online 18 March 2018
0144-8617/

2014), medical dressing (Phaechamud, Yodkhum, Charoenteeraboon, & Tabata, 2015), water filtration systems (Abebe, Chen, & Sobsey, 2016), food preservation (Alnoman, Udompijitkul, & Sarker, 2017; Liu et al., 2016; Qiu et al., 2014), natural bio-control (Jobin, Couture, Goyer, Brzezinski, & Beaulieu, 2005) and as an elicitor (Hadwiger, 2013). In agriculture, chitosan not only inhibits spore germination and mycelium growth of pathogens (Palma-Guerrero, Jansson, Salinas, & Lopez-Llorca, 2008), but also induces increased plant resistance to abiotic and biotic stresses (Xing, Zhu, Peng, & Qin, 2015), such as wounding and pathogens infection. These advantages are incomparable to other chemical fertilizers and pesticides. Therefore, chitosan has great application potential to control plant diseases in agricultural operations (Mansilla et al., 2013).

As we discussed above, chitosan has wide-spectrum antimicrobial activity. However, the antimicrobial activity of chitosan maybe varied depending on different microbial species (Kong, Chen, Xing, & Park, 2010; Xing et al., 2016). No studies have been reported on the antifungal effect of chitosan against *C. fimbriata* up to now. Therefore, the evaluation of antifungal activity of chitosan against *C. fimbriata* is the premise and foundation for the application of chitosan on postharvest sweet potato. Furthermore, the exact antimicrobial mechanism of chitosan is still unknown. Several theories and hypotheses have been proposed to describe the antimicrobial mechanisms of chitosan, including electrostatic interactions, membrane damage mechanisms, chitosan-DNA/RNA interactions, metal chelation and deposition onto the microbial surface (Xing et al., 2015). These theories and hypotheses do not indicate antimicrobial action of chitosan, but instead suggest that this activity occurs via a dynamic interactive system. Previous studies (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2011; Xing, Chen, Kong, et al., 2009) have suggested that membrane damage is an early stage of necrosis. In such case, the cellular structure and functions of cell membrane would be affected, as well as the cell membrane permeability. However, previous studies mainly focused on whether the integrity of plasma membrane was destroyed, and ignored the concrete details during the membrane breaking process. Therefore, this study attempts to provide the direct evidence for the membrane disruption action of chitosan. Some researches (Choi & Lee, 2014; Vylkova, Sun, & Edgerton, 2007) reported that when a cationic compound existed in the surroundings, the cells would have an early reaction of K^+ efflux. The continuous efflux of K^+ would lead to the depolarization of the cell membranes, which finally resulted in the inactivation of K^+ dependent enzymes. We hypothesize that chitosan, the natural cationic polymer, could also cause such chain reactions.

Based on above scientific problems and hypothesis, the antifungal effects of chitosan on *C. fimbriata* and the related physiologic responses were evaluated in the present study. The objectives of this work were (i) to evaluate the antifungal effect of chitosan against *C. fimbriata,* (ii) to determine whether the membrane structure was damaged and (iii) to investigate related physiologic responses during the membrane breaking process.

2. Materials and methods

2.1. Chemicals

Chitosan (degree of deacetylation 92%, molecular weight of 300 kDa, purchased from Zhaoqing Changlong Biotechnology Co. Ltd., China) was dissolved in 0.01% acetic acid to prepare the chitosan stock solution, then added to experimental solutions to obtain final concentrations of 0, 0.5, 1, 2 and 3 mg/mL. Lactophenol cotton blue was purchased from Qingdao Hopebio-Technology Co., Ltd., China. Fluorescein diacetate (FDA) was obtained from Shanghai Yisheng Biotechnology Co., Ltd., China. A H^+/K^+-ATPase assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Propidium iodide (PI), bis-(1, 3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], lysing enzyme and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Yatalase was purchased from TaKaRa Bio (Kyoto, Japan).

2.2. Fungal strains and cultivation

Ceratocystis fimbriata ACCC30008 was obtained from the Agricultural Culture Collection of China. *C. fimbriata* was maintained on potato dextrose agar (PDA: 200 g peeled potato, 20 g dextrose, 15 g agar powder and 1000 mL distilled water) slants at 4 °C, and activated on the PDA plates at 25 °C for 5 days before each microbiological assay.

2.3. Colony extension assay and mycelial morphology observation

The assay to measure the antifungal activity of chitosan against mycelial radial extension was performed as previously described (Xing et al., 2016) with minor modifications. Briefly, *C. fimbriata* mycelial discs with a diameter of 6 mm were inoculated in the center of PDA plates, amended with final concentrations of chitosan at 0, 0.5, 1, 2 and 3 mg/mL. After being incubated at 25 °C, the diameters of each colony were measured at 10 days (d) with a vernier scale. To observe the mycelial morphology of *C. fimbriata*, climbing coverslips were prepared according to our previously described method (Xing et al., 2017). Coverslips with attached mycelium were taken from PDA plates containing 1 mg/mL chitosan or not. After staining with lactophenol cotton blue, coverslips were observed by light microscopy (400×).

2.4. Live/dead staining assay

Double-labeling with FDA and PI was used to check cell viability. Chitosan was added to the *C. fimbriata* conidial suspension (10^6 spores/mL) at a final concentration of 1 mg/mL. Samples were removed at different time intervals (3, 6, 9 and 12 h). FDA (dissolved in DMSO) and PI (dissolved in ddH_2O) were added to the conidial suspension at a final concentration of 10 μg/mL and 5 μg/mL, respectively, then incubated in the dark. A BX63 fluorescence microscope (Olympus, Tokyo, Japan) was used to capture photographic images of stained spores. The excitation/emission wavelengths of FDA were 488/530 nm, and the excitation/emission wavelengths of PI were 493/636 nm.

2.5. PI influx assay

Fungal membrane permeabilization following treatment with chitosan was observed by PI influx assay. Conidial suspensions (10^6 spores/mL) were treated with chitosan at 25 °C for different incubation time (3, 6, 9 and 12 h). All samples were collected by centrifugation, then suspended again. Subsequently, PI was added to the conidial suspensions at a final concentration of 5 μg/mL. After incubation for 10 min at 4 °C, the PI influx of *C. fimbriata* was determined using a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The red fluorescence emitted by PI was detected at ~ 670 nm (FL3 channel). At least 10,000 events were collected for each sample. The data were collected and analyzed by BD Accuri C6 Software.

2.6. Leakage of potassium ions

The ability of chitosan to induce fungal membrane disturbances was determined by measuring the efflux of potassium ions from *C. fimbriata.* Conidial suspensions (10^6 spores/mL) were treated with chitosan at 25 °C for different length of time (3, 6, 9 and 12 h). All samples were then centrifuged at 8000 g for 5 min to remove cell debris. The potassium ion concentration in the supernatant was analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Opmina 8000, PE Co., Ltd., USA).