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Effects of chitosan and oligochitosan on growth of two fungal pathogens and physiological properties in pear fruit

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ABSTRACT

The differences between chitosan (350 kDa) and oligochitosan (6 kDa) in inhibitory effect on phytopathogenic fungi and on decay control were investigated. Both chitosan and oligochitosan strongly inhibited spore germination and mycelial growth of *Alternaria kikuchiana* Tanaka and *Physalospora piricola* Nose. Relatively, chitosan and oligochitosan showed more obvious inhibitory effect on mycelial growth than spore germination. Although oligochitosan had better inhibitory effects on fungal pathogenicity *in vitro*, chitosan was more effective on disease control in pear fruit stored at 25 °C. When treated with oligochitosan, pear fruit increased the activities of chitinase (CHI) and β-1,3-glucanase. Differently, chitosan treatment significantly increased peroxidase (POD) activity of pear fruit. These results suggested that chitosan and oligochitosan triggered different mechanism for pathogenicity inhibition and disease control.

1. Introduction

Postharvest diseases caused by pathogenic fungi result in major losses of fruits and vegetables, and synthetic chemical fungicides are the primary means to application at present (Spadaro & Gullino, 2004). However, synthetic chemical fungicides are potentially harmful on human health and the emergence of pathogens which are resistant to these chemicals (Holmes & Eckert, 1999). Moreover, public concern over the indiscriminate use of synthetic fungicides has been growing. Thus, it is significant to develop new alternatives for disease control (Tian, 2006).

Chitosan, together with its derivatives, has been reported as a promising alternative to control postharvest diseases. Different from other synthetic chemical fungicides, chitosan is a natural polysaccharide with a chemical structure of poly β-(1 → 4)*N*-acetyl-D-glucosamine (Bautista-Baños et al., 2006). Chitosan and oligochitosan have broad-spectrum antibacterial activities (Jeon, Park, & Kim, 2001; Liu et al., 2006; No, Park, Lee, & Meyers, 2002; Zheng & Zhu, 2003). In addition, chitosan and oligochitosan as fungicides are effective in inhibiting spore germination, germ tube elongation and mycelial growth of fungal phytopathogens, such as *Alternaria solani* (Xu, Zhao, Han, & Du, 2007), *Botrytis cinerea* (Chien & Chou, 2006; El Ghaouth, Arul, Grenier, & Asselin, 1992; Liu, Tian, Meng, & Xu, 2007), *Fusarium* (Eweis, Elkholy, & Elsabee, 2006; Xu et al., 2007), *Rhizopus stolonifer* (El Ghaouth et al., 1992; Hernández-Lauzardo et al., 2008), *Penicillium* (Chien & Chou, 2006; Liu et al., 2007), *Phytophthora capsici* (Xu et al., 2007) and *Sclerotium rolfsii* (Eweis et al., 2006). Furthermore, chitosan and oligochitosan also act as an exogenous elicitor for fruits and vegetables, and thus protects hosts from further fungal infection. It has been reported that chitosan could boost the activity of defense-related enzymes or pertinent substance in *Arabidopsis* (Cabrera, Messiaen, Cambier, & Van Cutsem, 2006), tomato fruit (Liu et al., 2007), orange, strawberry and raspberry fruit (Fajardo, McCollum, McDonald, & Mayer, 1998; Zhang & Quantick, 1998). Oligochitosan has also shown effective to elicit production of hydrogen peroxide (Li et al., 2009; Lin, Hu, Zhang, Rogers, & Cai, 2005), to increase activities of phenylalanine ammonialyase (PAL) and POD (Vander, Varum, Domard, Eddine, & Moerschbacher, 1998), and to up-regulate gene expression of β-1,3-glucanase and chitinase (Lin et al., 2005). Thanks to their antifungal activity and resistance induction, chitosan and its derivatives have been successfully applied to extend the storage life of fruits and vegetables (Chien & Chou, 2006; El Ghaouth et al., 1992; Liu et al., 2007).

Although chitosan and its derivatives have been considered as versatile biopolymers in agriculture applications, their potential application as antimicrobial compounds, elicitors or preservatories of decay remains to be further explored (Bautista-Baños et al., 2006; Yang, Meng, Liu, & Tian, 2009). Little information is known about comparison of inhibitory effect of chitosan and oligochitosan

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on fungal pathogenicity and disease control at present. *Alternaria kikuchiana* Tanaka and *Physalospora piricola* Nose are two kinds of fungal pathogens of pear fruit in storage. Using these two fungi as subject, this study aimed at the difference between chitosan and oligochitosan (1) as fungicides by comparison their half maximal inhibitory concentration (IC_{50}) on spore germination, germ tube elongation and mycelial growth; (2) as elicitor by comparison the inductive effect on the activities of related enzymes of host; (3) as preservatories on decay control of pear fruit.

2. Materials and methods

2.1. Chitosan and oligochitosan

Chitosan with 90% deacetylation and viscosity average molecular weight of 350 kDa dissolved at 25 g/L in 1% HCl by stirring at room temperature, and the original solution was then diluted to a series of 0.1, 0.3, 0.5, 0.7, 1.0, 2.0 and 5.0 g/L, and the pH value of each solution was adjusted to 5.5 with NaOH. Oligochitosan with average molecular weight 6 kDa was dissolved in sterile distilled water at 25 g/L, and the original solution was then diluted to a series of the concentrations as the same as chitosan at pH 5.5.

2.2. Pathogen inoculums

A. kikuchiana Tanaka was isolated from infected pear fruit showing typical black spot symptom, then incubated in potato dextrose agar (PDA) medium at 25 °C. According to Koch's postulates, the pear fruit was infected with this microorganism, and the typical black spot symptom appeared again. *P. piricola* Nose isolated from pear fruit was provided from Chinese Academy of Forestry and cultivated in potato sucrose agar (PSA) medium. To stimulate the spore generation, the cultivation was incubated under black-light lamp when the hypha developed to the edge of dish and followed to be strickled. Fungal spores were obtained by flooding the cultures with sterile distilled water containing 0.05% (v/v) Tween-80. Spore suspensions were filtered through four layers of sterilized cheesecloth to remove adhering mycelia. The spore concentration was adjusted with the aid of a hemocytometer prior to use.

2.3. Measurements of spore germination and germ tube elongation

The effects of chitosan and oligochitosan on spore germination and germ tube elongation of *A. kikuchiana* and *P. piricola* were assayed according to Liu et al. (2007). Fifty microlitre aliquots of both conidial suspensions at 1×10^6 spores mL^{-1} were plated evenly on Petri dishes (60 mm in diameter) with 10 ml solid PDA or PSA medium, which contained different concentrations (0, 0. 1, 0. 3, 0. 5, 0. 7, 1.0, 2.0 and 5.0 g/L) of chitosan and oligochitosan, and the Petri dishes incubated at 25 °C for 6 h. More than 200 spores of each pathogen were measured for germination spore and germ tube length per treatment within each replicate. Then the inhibitory rate of spore germination and germ tube length and their IC_{50} was determined. Each treatment contained three replicates and the experiment was repeated twice.

2.4. Measurements of mycelial growth

The mycelial disks (7 mm in diameter) from two-week-old cultures of the fungi were placed in the centre of Petri dishes (60 mm in diameter) with 10 ml solid PDA or PSA medium containing different concentrations of chitosan or oligochitosan (0, 0. 1, 0. 3, 0. 5, 0. 7, 1.0 and 5.0 g/L), then incubated at 25 °C. The mycelial growth was determined by measuring colony diameter at 3 days after inoculation, and the inhibitory rate of fungal growth and hence the IC_{50} was calculated. Each treatment contained three replicates and the experiment was repeated twice.

2.5. Effects of chitosan or oligochitosan on postharvest diseases of pear fruit

Pear (*Pyrus pyrifolia* L. cv. Xuehua) fruit were harvested at the mature stage, from an orchard in the Haidian district, Beijing and transported to our laboratory immediately. All fruit, selected based on the size and the absence of physical injuries or disease infection, were disinfected in solution of 2% (v/v) sodium hypochlorite for 2 min, then rinsed with tap water and dried in air. After dried, pear fruit were wounded (3 mm deep and 3 mm wide) with a sterile nail at the equator. Then 15 μL conidial suspension of *A. kikuchiana* or *P. piricola* at 1×10^4 spores mL^{-1} was pipetted to each wound. After 2 h, 15 μL chitosan or oligochitosan at 0.1, 0.5, 1.0, 1.5, 5.0 and 10 g/L, along with sterile distilled water as the control, were added respectively into a specific wound. Treated fruit were put in 200 mm × 130 mm × 50 mm plastic boxes with sterile water to maintain a high relative humidity (about 95%) and stored at 25 °C. Disease incidence of pear fruit caused by *A. kikuchiana* or *P. piricola* was determined at different time intervals after inoculation and hence the IC_{50} of disease incidence was determined. Each treatment contained three replicates with 10 fruits per replicate and the experiment was repeated twice.

2.6. Determination of defense-related enzymes in pear fruit

For enzyme assays, pear fruit were wounded, and 15 μL of 1.5 g/L chitosan or oligochitosan was added to each wound as described above, and fruits inoculated with sterile water in the wound served as control. After treatment, the fruits were stored at 25 °C. Flesh samples surrounding the wounds of 10 fruits were taken at 0, 1, 2 and 3 days. Each treatment contained three replicates and the experiment was repeated twice.

PPO and POD were extracted and assayed by the method of Meng, Tian, Li, and Liu (2008), with some modifications. Tissue samples (10 g) of each treatment were homogenized with 25 mL of 100 mmol/L sodium phosphate buffer (pH 6.4) containing 0.5 g of polyvinyl polypyrrolidone (PVPP) and ground at 4 °C. The homogenate was centrifuged at 15,000*g* for 30 min at 4 °C and the supernatant was used for the enzyme assay. Specific activity of enzymes was expressed as U mg^{-1} protein, where one unit was defined as ΔOD_{398} min^{-1} mg^{-1} protein for PPO and $0.01\Delta OD_{460}$ min^{-1} mg^{-1} protein for POD.

For CHI and β-1,3-glucanase activity assay, fresh sample of pear fruit (10 g) was mixed with 20 mL of sodium acetate buffer (50 mmol/L, pH 5.0) and ground thoroughly at 4 °C. The homogenate was centrifuged as above. Activity of β-1,3-glucanase was determined, with laminarin as substrate, following the method described by Yao and Tian (2005). Chitinase was measured according to the method of Wirth and Wolf (1990) with chitin as substrate. Reducing sugars as reaction production were measured spectrophotometrically at 550 or 500 nm using a UV-160 Spectrophotometer (Shimadzu, Japan), respectively. Specific activity of enzymes was expressed as U mg^{-1} protein, where one unit was defined as 1 μmol h^{-1} mg^{-1} protein for CHI and β-1,3-glucanase.

Protein content was determined according to Bradford (1976) with bovine serum albumin (Sigma Chemicals Co., St. Louis, USA) as standard.

2.7. Statistical analysis

All statistical analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA.

Mean separations were performed by Duncan's multiple range tests. Differences at $P < 0.05$ were considered to be significant.

3. Results

3.1. Effects of chitosan or oligochitosan on spore germination and germ tube elongation

As shown in Fig. 1, spore germination and germ tube elongation of *A. kikuchiana* and *P. piricola* were significantly inhibited by chitosan or oligochitosan with concentration-dependent mode ($P < 0.05$). Both chitosan and oligochitosan at 5.0 g/L inhibited completely spore germination of the two fungi after 6 h inoculation. The IC_{50} of chitosan on the spore germination of *A. kikuchiana* and *P. piricola* was 1.85 and 1.81 g/L, respectively (Table 1). Correspondingly, the IC_{50} of oligochitosan was 1.51 and 1.61 g/L. Thus, compare to chitosan, oligochitosan was more effective on inhibition spore germination.

3.2. Effects of chitosan or oligochitosan on mycelial growth

Chitosan and oligochitosan significantly inhibited mycelial growth of *A. kikuchiana* and *P. piricola* ($P < 0.05$) (Fig. 2). As the same as spore germination, the inhibitory effect of chitosan and oligochitosan on mycelial growth was also concentration-dependent mode. After 3 d, 5.0 g/L chitosan or oligochitosan completely inhibited mycelial growth of two fungi. The IC_{50} of chitosan and oligochitosan on mycelial growth of *A. kikuchiana* was 1.72 and 1.46 g/L, while the IC_{50} on mycelia growth of *P. piricola* was 0.40 and 0.35 g/L, respectively (Table 1). Therefore, oligochitosan generally showed better inhibitory effect than chitosan, especially on *P. piricola*.

3.3. Effects of chitosan or oligochitosan on postharvest diseases of pear fruit

The symptom of black spot disease caused by *A. kikuchiana* appeared in control and treated pear fruit after 72 h inoculation (Table 2), while lesion diameter developed slowly (Fig. 3). Differently, slight disease symptom caused by *P. piricola* was observed only in control and oligochitosan-treated fruits at 72 h, thus disease incidences and lesion diameter were mainly investigated at 96 and 120 h (Table 2, Fig. 3). Treatments with chitosan and oligochitosan reduced the disease incidence caused by *A. kikuchiana* and *P. piricola* and inhibited the lesion expansion of two fungi in pear fruit (Table 2, Fig. 3). Moreover, the disease control effects of chitosan and oligochitosan were concentration-dependent and weakened over inoculated time. The IC_{50} of chitosan on disease incidence caused by *A. kikuchiana* was 1.32 g/L after inoculation 72 h, 7.94 g/L after inoculation 96 h and 10.7 g/L after inoculation 120 h, meanwhile the IC_{50} of oligochitosan was 1.75 g/L after inoculation 72 h and 10 g/L after inoculation 96 h (Table 3). The black spot disease already appeared on all oligochitosan-treated pear fruit after inoculation 120 h. Relatively, the IC_{50} of chitosan and oligochitosan on disease incidence caused by *P. piricola* was respectively, 1.57 and 7.47 g/L after inoculation 96 h, while 2.05 and 8.79 g/L after inoculation 120 h (Table 3). Therefore, compare to oligochitosan, chitosan was more effective on disease control, especially to that caused by *P. piricola*.

3.4. Effects of chitosan or oligochitosan on induction of defense-related enzymes in pear fruit

Peroxidase and polyphenol oxidase were two kinds of redox enzymes. The activities of PPO and POD in flesh around wound of pear fruit were raised first and then decreased from 24 h (Fig. 4A and B). However, compare to oligochitosan, chitosan inoculation significantly enhanced the activities of POD and PPO in flesh around wound of pear fruit, especially to POD (Fig. 4A and B). Chitinase and β-1,3-glucanase are two kinds of pathogenesis-related protein. The CHI and β-1,3-glucanase activities in flesh around wound of control pear fruit increased with storage time, while chitosan and oligochitosan inoculation obviously enhanced the activities of both enzymes (Fig. 4C and D). Furthermore, oligochitosan inoculation was more effective than chitosan, for example, the activities of CHI and β-1,3-glucanase in oligochitosan-treated fruit after 48 h were almost 1.5-fold to those in control fruit.

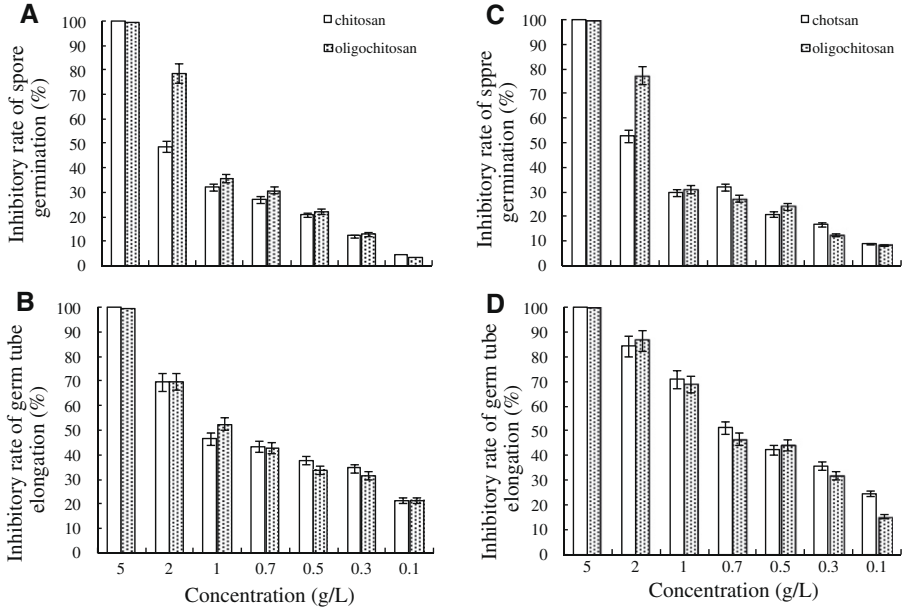

Fig. 1. Effects of chitosan or oligochitosan on inhibitory rate of spore germination (A and C) and germ tube elongation (B and D) of *A. kikuchiana* (A and B) or *P. piricola* (C and D) after 6 h inoculation.

Table 1
IC_{50} of chitosan or oligochitosan on development of *A. kikuchiana* and *P. piricola*.

Species	Chitosan (g/L)			Oligochitosan (g/L)		
	Spore germination	Germ tube elongation	Mycelium growth	Spore germination	Germ tube elongation	Mycelium growth
Alternaria kikuchiana	1.85	0.93	1.72	1.51	0.95	1.46
Physalospora piricola	1.81	0.63	0.40	1.61	0.78	0.35

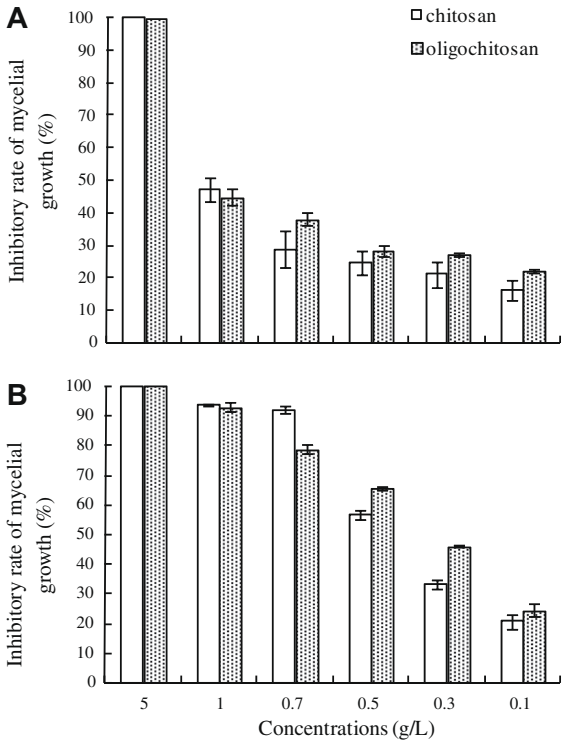

Fig. 2. Effects of chitosan or oligochitosan on inhibitory rate of mycelial growth of *A. kikuchiana* (A) or *P. piricola* (B) after 3 d inoculation.

4. Discussion

Chitosan and its derivatives offer a great potential as natural biodegradable substances which have anti-microbial and eliciting activities (Bautista-Baños et al., 2006; Benhamou, 1996). In the present study, we demonstrated that chitosan and oligochitosan were effective in inhibiting spore germination and mycelial growth of *A. kikuchiana* and *P. piricola*. By the comparison of IC_{50} of chitosan and oligochitosan on spore germination, germ tube elongation and mycelial growth (Table 1), we discovered that two potential anti-microbial displayed different efficacy to two pathogens at different developmental stages. At the mycelial growth stage, both chitosan and oligochitosan appeared higher effective on two fungi than that at the spore germination stage (Table 1), most likely due to a stronger drug resistance of fungal spores. Moreover, the mycelial growth of *P. piricola* was more sensitive to chitosan and oligochitosan than that of *A. kikuchiana* (Table 1). When compared to chitosan, however, oligochitosan shows a stronger inhibition on the pathogenicity of *A. kikuchiana* and *P. piricola in vitro*. This result is consistent with the results reported by Liu et al. (2007), who suggested that the sensitivity of *B. cinerea* and *P. expansum* to chitosan might vary with different development stages. Furthermore, our results indicated that both chitosan and oligochitosan were effective in controlling postharvest diseases caused by *A. kikuchiana* and *P. piricola* in pear fruit stored at 25 °C (Table 2, Fig. 3). However, chitosan is relatively more effective than oligochitosan (Table 3), different from the result *in vitro* (Table 1). The local concentration reduction of oligochitosan in artificial wound of fruit, because of dilution due to its lower viscosity and higher diffusivity than chitosan, may result in relative weak control effect in pear fruit if only antifungal activity was taken into account.

In addition to antifungal activity, chitosan and oligochitosan also induce hosts to produce defense-related enzymes (Bautista-Baños et al., 2006; Lin et al., 2005) and increase antioxidative activity (Harish Prashanth, Dharmesh, Jagannatha Rao, & Tharanathan, 2007; Kim & Thomas, 2007). Chitosan treatments enhanced activities of chitinase and β-1,3-glucanase in orange and strawberry fruits (Bautista-Baños et al., 2006; Fajardo et al., 1998; Zhang & Quantick, 1998). The increase of activities of PPO and POD in chitosan-treated tomato or cucumber fruit resulted in enhancing resistance of host against *B. cinerea* (Ben-Shalom, Ardi, Pinto, Aki, & Fallik, 2003; Liu et al., 2007). Compared with chitosan, oligochitosan has been reported to be more effective at eliciting multiple plant defense responses (Xu et al., 2007). Such different effectiveness has been suggested to associate with their degrees of polymerization, acetylation and the plant species (Bautista-Baños et al., 2006; Cabrera et al., 2006). In this study, we found that the activities of PPO, POD, CHI and β-1,3-glucanase in chitosan- and

Table 2
Effect of chitosan and oligochitosan with different concentrations on decay incidence caused by *A. kikuchiana* and *P. piricola* in pear stored at 25 °C.

Treatment		*A. kikuchiana*			*P. piricola*	
		72 h	96 h	120 h	96 h	120 h
Control		100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 3.3a	100.0 ± 3.3a
Chitosan (g/L)	0.1	86.7 ± 3.3b	96.7 ± 3.3a	100.0 ± 0.0a	83.3 ± 3.3b	96.7 ± 3.3a
	0.5	73.3 ± 3.3d	86.7 ± 8.8abc	90.0 ± 5.8bc	70.0 ± 5.8cd	93.3 ± 3.3ab
	1	56.7 ± 3.3ef	73.3 ± 3.3cde	86. 7 ± 3.3cd	60.0 ± 3.3d	93.3 ± 3.3ab
	1.5	36.7 ± 3.3g	66.7 ± 3.3def	93.3 ± 3.3abc	36.7 ± 3.3ef	70.0 ± 3.3c
	5	6.7 ± 3.3hi	60.0 ± 5.8efg	86.7 ± 3.3cd	20.0 ± 5.8gh	66.7 ± 3.3c
	10	3.3 ± 3.3i	50.0 ± 0.0g	76.7 ± 3.3e	10.0 ± 3.3h	43.3 ± 3.3c
Oligochit osan (g/L)	0.1	83.3 ± 3.3bc	90.0 ± 0.0ab	100.0 ± 0.0a	80.0 ± 3.3bc	100.0 ± 3.3a
	0.5	76.7 ± 3.3cd	85.6 ± 2.9abc	96.7 ± 3.3a	76.7 ± 3.3bc	86.7 ± 3.3b
	1	63.3 ± 3.3e	88.9 ± 5.9abc	100.0 ± 0.0ab	63.3 ± 3.3d	86.7 ± 3.3b
	1.5	53.3 ± 3.3f	83.3 ± 3.3cde	93.3 ± 3.3abc	43.3 ± 3.3e	83.3 ± 3.3b
	5	10.0 ± 0.0h	54.4 ± 5.7g	80.0 ± 0.0de	30.0 ± 5.8fg	53.7 ± 6.7d
	10	3.3 ± 3.3i	45.6 ± 2.9g	80.0 ± 0.0de	16.7 ± 3.3h	50.0 ± 5.7d

Note: values followed by different letter are significantly different according to Duncan's multiple range test ($P < 0.05$).

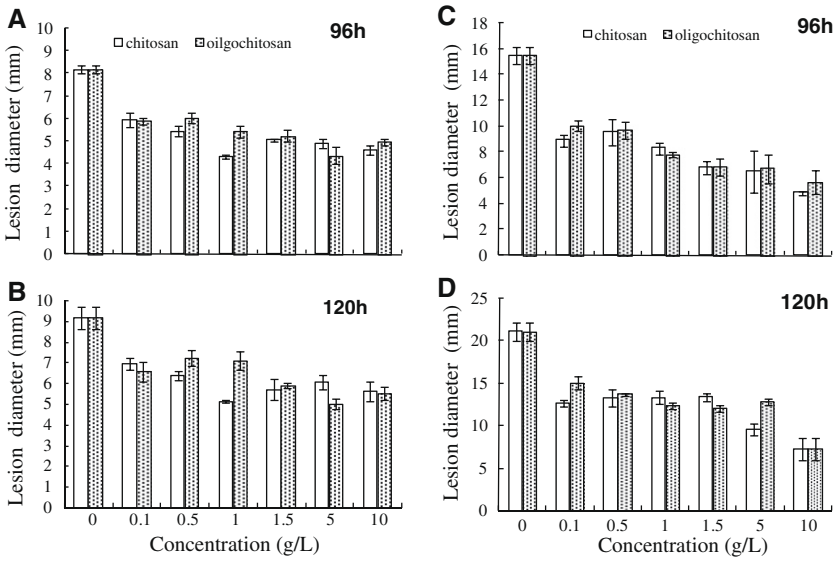

Fig. 3. Effects of chitosan or oligochitosan on lesion growth of pear fruit caused by *A. kikuchiana* (A and B) and *P. piricola* (C and D) at 96 and 120 h after inoculation.

Table 3
IC_{50} of chitosan or oligochitosan on disease incidence caused by *A. kikuchiana* and *P. piricola* in pear fruit.

Species	Chitosan (g/L)			Oligochitosan (g/L)		
	72 h	96 h	120 h	72 h	96 h	120 h
Alternaria kikuchiana	1.32	7.94	10.7	1.75	10	–
Physalospora piricola	–	1.57	7.47	–	2.05	8.79

oligochitosan-treated fruit increased significantly during storage (Fig. 4). Relatively, oligochitosan treatment significantly enhanced the activity of CHI and β-1,3-glucanase in pear fruit (Fig. 4C and D), while chitosan treatment significantly increased the POD activity (Fig. 4A). Recently, salicylic acid (SA) and antagonistic yeast have been found to play important roles in stimulating the activities of antioxidant enzymes during pathogenic infection (Xu, Qin, & Tian, 2008; Xu & Tian, 2008). These enzymes including POD, glutathione peroxidase (GPX) and catalase (CAT), have been involved in mechanisms of antagonistic yeast and SA against fungal decay in fruits by alleviation of carbonylated proteins caused by pathogenic infection (Xu & Tian, 2008; Xu et al., 2008). Therefore, the fact that activity increase of antioxidant enzymes, POD and PPO in pear fruit induced by chitosan might be beneficial for fruit against infection by the fungal pathogens. The detail reasons for the difference of effectiveness of chitosan and oligochitosan *in vitro* and *in vivo* are worth of further investigation.

5. Conclusion

The present study reported the different effects of chitosan and oligochitosan on developmental inhibition of *A. kikuchiana* and

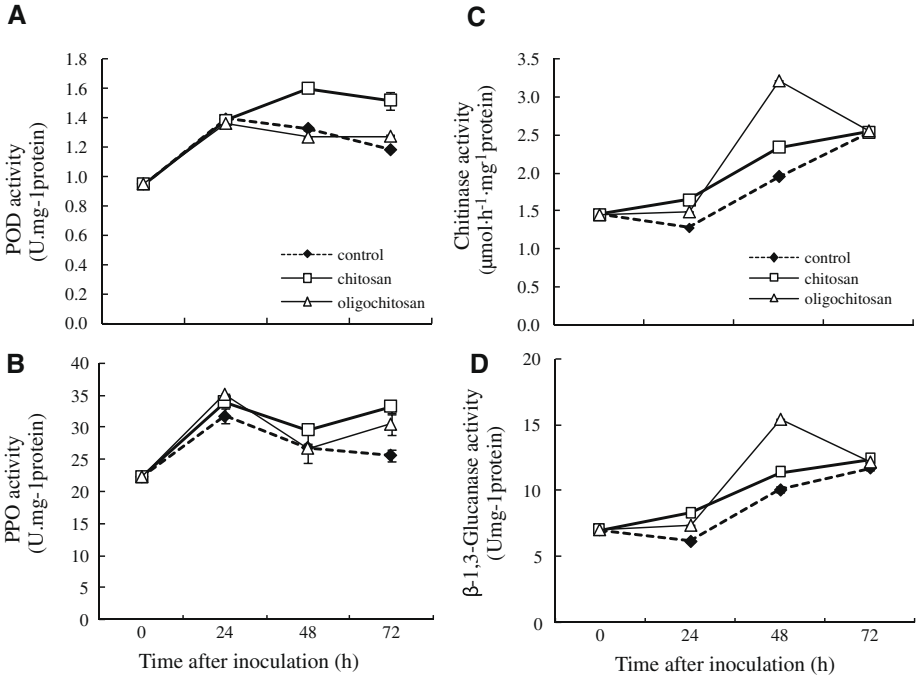

Fig. 4. Effects of chitosan or oligochitosan on activities of POD (A), PPO (B), CHI (C) and β-1,3-glucanase (D) of pear fruit.

P. piricola, on inductive physiological and biochemical change of host and on control of related postharvest diseases. Chitosan and oligochitosan had stronger inhibitory effect on mycelia growth than spore germination, germ tube elongation of both *A. kikuchiana* and *P. piricola* by the comparison of IC_{50}. Moreover, *P. piricola* was more sensitive to chitosan and oligochitosan than *A. kikuchiana*. Oligochitosan treatment had more obvious effect on enhancing activities of CHI and β-1,3-glucanase in pear fruit as compared to chitosan, but chitosan treatment significantly increased POD activity. Chitosan and oligochitosan treatments were effective in controlling postharvest diseases caused by *A. kikuchiana* and *P. piricola* in pear fruit stored at 25 °C, but chitosan had better control effect than oligochitosan. These results suggest that chitosan and oligochitosan could be promising natural fungicides to partially substitute the utilization of synthetic fungicides for fruit, but the mechanism on difference of chitosan and oligochitosan as fungicides, as elicitor and in particular decay control still need further investigation.

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