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Bactericidal and fungicidal activities of different molecular weight chitosan samples

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ABSTRACT

The antimicrobial activity of different molecular weight chitosan samples (with approximately the same degree of deacetylation 83 ± 2 %) against some common of plant pathogenic bacteria and fungi was investigated. Average molecular weight of chitosan samples was determined by measurements of intrinsic viscosity and was found to be 3.60×10^5 Da for low molecular weight (LMW), 6.11×10^5 Da for medium molecular weight (MMW) and 9.53×10^5 Da for high molecular weight (HMW) chitosan samples. The antibacterial assessment of the chitosan samples were performed with agar dilution method against cultures of six plant pathogenic bacteria *Agrobacterium tumefaciens, Corynebacterium fascians, Erwinia amylovolora, Erwinia carotovora, Pseudomonas solanacearum* and *Sarcina lutea*. The results are expressed in term of Minimum Inhibitory Concentration (MIC). The results indicated that MMW and HMW were more potent in bactericidal activity than LMW chitosan and a HMW chitosan exhibited a good antibacterial potency especially against *C. fascians* with MIC 500 $mg.L^{-1}$. The fungicidal assessment was assessed using a mycelial radial growth inhibition technique against six plant pathogenic fungi *Alternaria alternata*, *Botrytis fabae*, *Fusarium oxysporum*, *Penecillium digitatum*, *Pythium debrianum* and *Rhizoctonia solani* and the results are expressed as Minimum Effective Concentration of 50% of mycelial growth (EC_{50}). The data also demonstrated that the fungicidal activity was increased with the increasing of the molecular weight and a HMW chitosan was the most potent one against all the tested fungi especially *P. digitatum* with EC_{50} of 510 $mg.L^{-1}$.

Keywords: Bactericidal activity; fungicidal activity; chitosan; viscosity; molecular weight.

INTRODUCTION

Chitosan is a linear polysaccharide composed of randomly distributed *β*-(1-4)-linked D-glucosamine unit and *β*-(1-4)-linked *N*-acetyl-D-glucosamine unit. Chitosan is produced commercially by deacetylation of chitin which is the structural element in the exoskeleton of crustaceans (No and Meyers, 1997).

Chitosan is insoluble in aqueous media at neutral and basic conditions, but is soluble in aqueous diluted acids. However, the application of this polysaccharide is limited by its high molecular weight resulting even at low chitosan concentration (Rathke and Hodson, 1994).

As its unique poly-cationic nature, chitosan has been used as active material such as antifungal activity and the minimum inhibitory concentrations (MICs) reported for tested fungi ranged from 18 to 10000 mg. L^{-1} and are influenced by a multitude of factors such as the pH of the growth medium, the degree of polymerization of chitosan and the presence or absence of interfering substances such as lipids and proteins (Sudarshan *et al*., 1992; Chen, 1998; Tsai and Su, 1999; Roller and Covill, 1999 and 2000; Rhoades and Roller, 2000; Knowles and Roller, 2001; Liu *et al.*, 2001; Muzzarelli *et al*., 2001; Rabea *et al*., 2003 and Tikhonov *et al*., 2005). Besides, the inhibitory effect of chitosan was also demonstrated with soil-borne phytopathogenic fungi (Stossel and Leuba, 1984) which was higher at pH 6.0 than at pH 7.5 when most of the amino groups are in the free base form.

In addition, numerous studies on bactericidal activity of chitosan have been carried out (Jia *et al.,* 2001; No *et al.,* 2002; Rabea *et al*., 2003; Badawy *et al*., 2004; Liu *et al.,* 2004; Qi *et al*., 2004 and Tikhonov *et al*., 2005) and some controversial evidences for a correlation between bactericidal activity and chitosan molecular weight have been found.

Increase in chitosan molecular weight led to a decrease in chitosan activity against *E. coli* in some studies (Zheng and Zhu, 2003 and Gerasimenko *et al*., 2004), while in the others an increased activity for a high molecular weight (HMW) chitosan in comparison with LMW chitosan have been found (Kyung *et al*., 2003). In contrast to the above mentioned results, no differences in HMW and LMW chitosan activities were found

against *E. coli* (Jeon *et al*., 2001 and Zhishen *et al*., 2001) and *Bacillus subtilis* (Jeon *et al*., 2001 and Gerasimenko *et al*., 2004).

In the present study, the antimicrobial activity of different molecular weight chitosan samples was investigated against six plant pathogenic of bacteria *Agrobacterium tumefaciens*, *Corynebacterium fascians, Erwinia amylovolora, Erwinia carotovora*, *Pseudomonas solanacearum* and *Sarcina lutea* as well as six pathogenic fungi *Alternaria alternata*, *Botrytis fabae*, *Fusarium oxysporum*, *Penecillium digitatum*, *Pythium debrianum* and *Rhizoctonia solani*.

MATERIALS AND METHODS

1. Materials: Chitosan of low, medium and high molecular weight with 85, 81 and 82 % degree of deacetylation, respectively (made from coarse ground crab) were purchased from Sigma-Aldrich Co. (Bornem, Belgium). Ubbelohde viscometer (capillary section size 0.7 mm) was used to determine the intrinsic viscosity.

Six bacteria species, *Agrobacterium tumefaciens*, *Corynebacterium fascians, Erwinia amylovolora, Erwinia carotovora*, *Pseudomonas solanacearum* and *Sarcina lutea*, were used in this study. Microorganisms were provided by Microbiology Laboratory, Department of Plant Pathology, Alexandria University. The bacteria species were maintained on Nutrient Agar medium (NA: peptone 10 g, meat extract 5 g, sodium chloride 2.5 g and agar 10 g in 1000 ml distilled water at pH 6.5 - 6.6).

The six fungi species used, *Alternaria alternata*, *Botrytis fabae*, *Fusarium oxysporum*, *Penecillium digitatum*, *Pythium debrianum* and *Rhizoctonia solani*, were obtained from the Fungicide Bioassay Laboratory, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University. The fungi were maintained during the course of the experiments on Czapek-Dox Agar medium (CDA: sucrose 30 g, sodium nitrate 2 g, potassium monohydrogen phosphate 1 g, potassium chloride 0.5 g, magnesium sulphate 0.5 g, ferrous sulphate 0.01 g and 15 g agar in 1000 ml of distilled water) at 25 ºC. All materials were used without further purification.

2. Measurement of Viscosity and Molecular Weight determination: Dried chitosans were accurately weighed and dissolved in 0.2 mol / L CH_3COONa / 0.5 mol / L CH_3COOH solution. Nine concentrations (0.04 - 0.20 g / 100 ml) of chitosan solution were prepared and the solution was passed through a filter of 0.45 mm to remove insoluble materials. Relative viscosity of chitosan solutions was performed using an Ubbelohde viscometer (capillary section size 0.7 mm) at 25 °C. The capillary was filled with 25 ml of sample and equilibrated in a water bath to maintain respective temperature. The sample was passed through the capillary once before the running time was measured. Each sample was measured 3 times. The running times of the solution and solvent were used to calculate the relative viscosity, specific viscosity, and reduced viscosity as follows:

Relative viscosity (η_{rel}) = t_{ch} / t_{sol}
Specific viscosity (η_{sp}) = (η_{rel}) -1
Reduced viscosity (η_{red}) = η_{sp}/c

where t_{ch} is the running time of the chitosan solution, t_{sol} is the running time of the solvent, and c is the chitosan concentration in g / dl.

Intrinsic viscosity, defined as $[\eta] = C(\eta_{red})_{c=0}$, was obtained by extrapolating the reduced viscosity versus concentration data to zero concentration, the intercept on the ordinate is the intrinsic viscosity (Launay *et al*., 1986; Rinaudo and Domard, 1989 and Chen and Tsaih, 1998).

The molecular weight was calculated based on the Mark Houwaink equation as follows:

$$[\eta] = KM^a$$

Where [η] is the intrinsic viscosity, K and a are viscometric parameters depending on the solvent. For chitosan in 0.5 M CH_3COOH/0.2 M CH_3COONa the K and a constant are found to be $3.5x10^{-4}$ and 0.76 (Wang *et al*., 1991 and Terbojevich *et al*., 1996).

3. Bactericidal Test: Agar dilution method was used, as recommended by European Society of Clinical Microbiology and Infectious Diseases (ESCMID, 2000), for determination of minimum inhibitory concentration (MIC) of the three samples of chitosan. Appropriate volumes of the stock solutions were added to molten nutrient agar to obtain a range of concentrations from 200 to 2400 mg. L^{-1} before pouring to Petri dishes.

After solidifications, 6µl of bacterial cultures grown in a nutrient broth for 12 hours (approximately 10^8 CFU / ml) was spotted (three spots per each plate) using 2 µl standard loop on the surface of agar. The inoculum spots were allowed to dry before inverting the plates for incubation at 35 ºC for 24h. Each sample was tested in triplicate. The control was nutrient agar with a maximum volume of the solvent (acetic acid) which added to the treatments. The MIC was determined as lowest concentration of the chitosan samples showing no visible bacterial growth in the agar plates.

4. Fungicidal Assay: The antifungal activity of chitosan samples was tested using the radial growth technique method (El Ghaouth *et al*., 1992). Appropriate volumes of the stock solutions of the samples in 1 % aqueous acetic acid were added to molten nutrient agar (Czapek-Dox Agar; CDA) to obtain concentrations of 250, 500, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 mg. L^{-1} immediately before pouring into the Petri dishes (9.0 cm in diameter) and the pH was adjusted to 5.5 - 6.0 with 1M NaOH (Stossel and Leuba, 1984 and Badawy *et al*., 2004). Each concentration was tested in triplicate. Parallel control was maintained with 1 % aqueous acetic acid mixed with CDA. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 8-day-old cultures on CDA plates, were transferred aseptically to the centre of Petri dishes. The plates were incubated in the dark at 26 ± 2 °C (El Ghaouth *et al*., 1992). Colony growth diameter was measured after the fungal growth in the control had completely covered the Petri dishes. Inhibition Percentage of mycelial growth was calculated (Pandy *et al*., 1982) as follows:

$$\text{Mycelial growth inhibition (\%)} = [(DC-DT)/DC] \times 100$$

where DC and DT are average diameters of fungal colony of control and treatment, respectively. Inhibiting concentration of 50 % of mycelial growth (EC_{50}) and corresponding 95 % CL was estimated by probit analysis (Finney, 1971).

RESULTS AND DISCUSSION

1. Characterization of chitosan samples: The resulting values from Table 1 permit to qualify three different chitosan samples (with approximately the same degree of deacetylation 83 ± 2 %) as low molecular weight (3.60×10^5 Da), medium molecular weight (6.11×10^5 Da) and high molecular weight (9.53×10^5 Da), when their viscosity average molecular weights are

compared. The curves relating reduced viscosities and chitosan concentrations for the purified polymers (Fig. 1) show that all experimental points are very well aligned along straight lines (r>0.96). The viscosity measurement performed with the purified chitosan samples allowed the determinations of their intrinsic viscosities and viscosity average molecular weights.

The intrinsic viscosity [η] is commonly used to evaluate the average molecular weight of macromolecules, as polymers and polysaccharides and use of purified polymer samples in the determinations of [η] and molecular weight (MW) is called for obtaining a reliable relationship (Launay *et al*., 1986; Rinaudo, and Domard, 1989; Wang *et al*., 1991; Terbojevich *et al*., 1996 and Chen and Tsaih, 1998).

Table (1). Characterization of low, medium and high molecular weight chitosan samples.

Chitosan sample	DDA (%)	[η]	MW (Da)
LMW	85	5.850	3.60×10^5
MMW	81	8.745	6.11×10^5
HMW	82	12.255	9.53×10^5

LMW = Low molecular weight; MMW = Medium molecular weight; HMW = High molecular weight; DDA = Degree of deacetylation; MW = Molecular weight and [η] = Intrinsic viscosity.

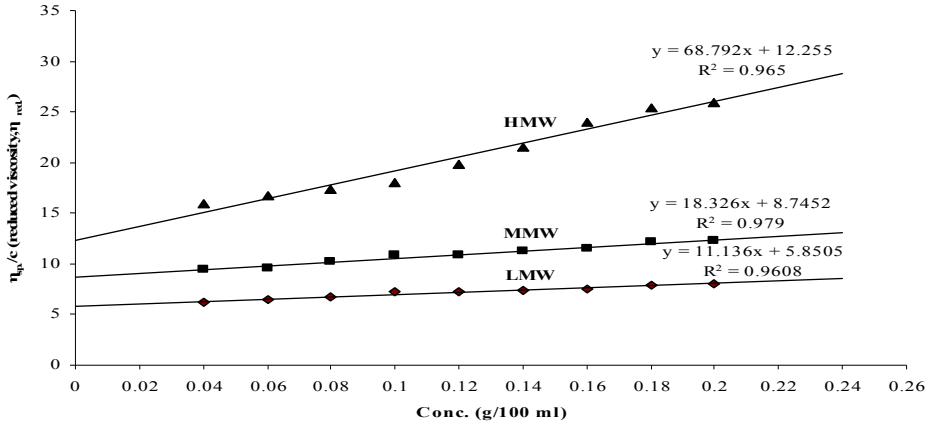

Fig. (1): Curves of reduced viscosity (η_{red}) against concentrations for low molecular weight (LMW), medium molecular weight (MMW) and high molecular weight (HMW) chitosan samples.

2. Antibacterial activity: Minimum inhibitory concentrations (MICs) values of the three chitosan samples LMW, MMW and HMW was evaluated against six plant pathogenic bacteria using agar dilution assay are shown in Table 2. The result indicates the HMW chitosan exhibits a good antibacterial potency with MIC values ranged between 500 and 600 mg. L^{-1} against all the tested bacterial species and slightly differed with the bacteria tested and MW of chitosan used. In contrary, we observed no noticeable antibacterial activity difference when a LMW chitosan was tested against the tested bacteria at concentrations up to 2400 mg. L^{-1}.

When we consider the susceptibility of the microorganisms, another point deserves attention. In general, *E. carotovora, P. solanacearum* are similar and more resistant than the others to all chitosan samples. These results indicate that the activity of chitosan is related to its MW and the bactericidal activity increases with an increasing of the MW.

Table (2). Minimum inhibitory concentrations (MICs, mg. L^{-1}) of different chitosan samples.

Bacteria	MIC (mg.L^{-1}) of chitosan samples		
	LMW	MMW	HMW
Agrobacterium tumefaciens	>2400	850	525
Corynebacterium fascians	2400	850	500
Erwinia amylovolora	2400	850	525
Erwinia carotovora	>2400	850	600
Pseudomonas solanacearum	>2400	850	600
Sarcina lutea	>2400	850	525

LMW = Low molecular weight; MMW = Medium molecular weight and HMW = High molecular weight**.**

According to the literature (Uchida *et al*., 1989; Jeon and Kim, 2000; Zhishen *et al*., 2001; No *et al*., 2002 and Avadi *et al*., 2004), the main factors affecting the antibacterial activity of chitosan are MW, pH and concentration. There are some reports that chitosan is more effective in inhibiting growth of bacteria than chitosan oligomers (Uchida *et al*., 1989 and No *et al*., 2002) and the MW of chitooligosaccharides is critical for microorganism inhibition and required higher than 10,000 Da (Jeon and Kim, 2000). The MIC of chitosan ranged from 5 to 100 mg.L^{-1} depending on the species of bacteria and MWs of chitosan samples (No *et al*., 2002)

and was varied depending upon the pH of chitosan solution (Liu *et al*., 2001).

In the present study revealed that LMW chitosan (MW = 3.60×10^5 Da) possessed weak or no antibacterial activity against the tested bacteria at tested concentrations up to 2400 mg. L^{-1}. This observation is in agreement with those reported by No *et al*., (2002). According to the result of No *et al*., (2002) the inhibitory effects of different MW chitosan samples against four gram-negative (*Escherichia coli*, *P. fluorescens*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*) and seven gram-positive bacteria (*B. cereus*, *Bacillus megaterium*, *Listeria monocytogenes*, , *Lactobacillus plantarum*, *L. brevis*, *L. bulgaricus* and *Staphylococcus aureus*) was differed with regard to the MW of chitosan and the type of bacterium. With gram-negative bacteria, chitosan of 7.46×10^5 Da appeared most effective against *E. coli* and *P. fluorescens*, compared with chitosan of 4.70×10^5 Da against *S. typhimurium* and *V. parahaemolyticus*. Chitosan of MW = 11.06×10^5 and 2.24×10^5 Da possessed weak or no antibacterial activity compared with chitosan of MW = 2.8×10^4 Da against *S. typhimurium*. Chen (1998) added that chitosan with MW ranging from 10,000 to 100,000 Da would be helpful in restraining the growth of bacteria. Tokura *et al*., (1994) reported that chitosan with an average-MW of 9300 Da to be effective in restraining *E. coli* while that with a MW of 2200 Da accelerated growth.

Generally, the exact mechanism of the antibacterial action of chitosan is still unknown, although different mechanisms have been proposed. Interactions between positively charged chitosan and negatively charged bacterial cell membranes lead to altered cell permeability, which prevents the transport of essential solutes into the cell (Choi *et al*., 2001; Hu *et al*., 2003 and Rabea *et al*., 2003) and results in leakage of proteinous and other intracellular components, thus killing the bacteria cells (Jung *et al*., 1999).

3. Antifungal effect of chitosan samples: Table 3 represents the fungicidal activity of the three chitosan samples against six plant pathogenic fungi (*A. alternata*, *B. fabae*, *P. digitatum*, *F. oxysporum*, *P. debrianum* and *R. solani*) in term of EC_{50} (50 % reduction in a radial hyphal growth) with the corresponding 95 % confidence limits.

The result indicates that there is no clear activity with LMW chitosan against all the tested fungi and inhibition percentages are lower than 50 % at 3000 mg. L^{-1}. Increase of the MW and viscosity led to dramatically increase

of the activity as shown in the case of MMW and HMW chitosan samples. When we consider the susceptibility of the microorganisms, another point deserves attention; *P. digitatum*, *R. solani* and *B. fabae* are more susceptible in the descending order than the others to MMW and HMW chitosan samples. Moreover MMW and HMW chitosan samples show the higher activity against *P. digitatum* with EC_{50} of 1287 and 510 mg. L^{-1}, respectively than the others. In contrast, all chitosan samples showed no fungicidal activity against *P. debrianum* and *F. oxysporum* when compared to the others.

Table (3). Fungicidal activity of different chitosan samples.

Fungi	EC_{50} (95% CL) (mg.L^{-1}) of chitosan samples		
	LMW	MMW	HMW
Alternaria alternata	> 3000	> 3000	1934 (1262-4947)
Botrytis fabae	> 3000	2858 (2217-4356)	1070 (736-1989)
Fusarium oxysporum	> 3000	> 3000	2905 (1805-8301)
Penicilium digitatum	> 3000	1287 (1025-1765)	510 (337-688)
Pythium debrianum	> 3000	> 3000	> 3000
Rhizoctonia solani	> 3000	2362 (2036-2937)	1270 (1184-1363)

LMW = Low molecular weight; MMW = Medium molecular weight and HMW = High molecular weight.

A report by Benhamou *et al*., (1994) indicated that chitosan derived from crab-shell at concentration of 500 and 1000 mg. L^{-1} was effective in reducing disease incidence caused by *F. oxysporum* f. sp. *radicis-lycopersici*. At the same time El-Ghaouth *et al*., (1994) revealed that chitosan was effective in inhibiting mycelial growth of *P. aphanidermatum* completely at a concentration of 400 mg. L^{-1}. While at a concentration of 100 mg. L^{-1}, it causes a 75 % reduction of the mycelial dry weight. Our result in agreement with El Ghaouth *et al*., (1992) found that chitosan concentration increased (750 - 6000 mg. L^{-1}), the radial growth of *A. alternata*, *B. cinerea*, *Colletrotichum gloeosporioides* and *Rhizopus stolonifer*, were decreased. The same effect was reported on *Sclerotinia sclerotiorum* when chitosan concentrations increased from 1 % to 4 % (Cheah *et al*., 1997). Other studies showed a linear decrease of growth of *R. solani* as the chitosan concentration gradually increased from 0.5 to 6.0 mg ml^{-1} (Wade and Lamondia, 1994). Other studies reported a complete growth inhibition of fungi such as *F. oxysporum*, *R. stolonifer*, *Penicillium*

digitatum and *C. gloeosporioides* at concentrations of 3 % (Bautista-Baños *et al*., 2003 and 2004).

In general, chitosan is already known to interfere with the growth of several phytopathogenic fungi including *B. cinerea, F. oxysporum* and *Pyricularia oryzae* (Allan and Hadwiger, 1979; El Ghaouth *et al*., 1994; Du *et al*., 1997; Oh *et al*.,1998 and Rabea *et al*., 2003), but the mechanism by which it affects several phytopathogenic fungi has not been fully elucidated. Because of its polycationic nature, it is believed that chitosan interferes with negatively charged residues of macromolecules exposed on the fungal cell surface. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Leuba and Stossel, 1986 and Rabea *et al*., 2003). Other mechanisms mentioned in the literature are the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of mRNA and protein synthesis (Hadwiger *et al*., 1986) and the chelation of metals, spore elements and essential nutrients (Cuero *et al*., 1991).

CONCLUSION

Three different molecular weight chitosan samples, low, medium and high were determined by measurements of their intrinsic viscosities and was found to be 3.60×10^5 6.11×10^5 and 9.53×10^5 Da, respectively. The biological activities were evaluated against important economic plant pathogenic bacteria and fungi. The antibacterial assessment was performed against six plant pathogenic bacteria *Agrobacterium tumefaciens, Corynebacterium fascians, Erwinia amylovolora, Erwinia carotovora, Pseudomonas solanacearum* and *Sarcina lutea* and the result showed that medium molecular weight (MMW) and high molecular weight (HMW) chitosans were more potent as bactericides than low molecular weight (LMW) chitosan and a HMW chitosan exhibited a good antibacterial potency especially against *C. fascians*. The fungicidal assessment was also assessed against six plant pathogenic fungi *Alternaria alternata*, *Botrytis fabae*, *Fusarium oxysporum*, *Penecillium digitatum*, *Pythium debrianum* and *Rhizoctonia solani*. The result also demonstrated that the fungicidal activity was increased with the increasing of the molecular weight and a HMW chitosan was the most potent one against all the tested fungi especially *P. digitatum*.

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