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Preparation of water-soluble *O*-(succinyl) chitosan derivatives and their antimicrobial activity against some plant pathogens

Entsar I. Rabea
Department of Pest Control and Environmental Protection; Faculty of Agriculture, Damanhour, Alexandria University, Egypt

ABSTRACT

In the present study, several water soluble *O*-(succinyl) chitosan derivatives were synthesized to study their enhancement of the antimicrobial activity of chitosan molecule. The chemical structures of the compounds were characterized by ^{1}H-NMR spectroscopy and their degree of substitution ranged from 0.18 to 0.64. The antimicrobial activity was investigated against bacteria of crown gall *Agrobacterium tumefaciens* and soft mold *Erwinia carotovora* and fungi of early blight *Alternaria alternata*, grey mold *Botrytis cinerea* and root rot *Fusarium oxysporum*. *O*-(succinyl) chitosans showed much more enhancement the antibacterial activity than chitosan. A derivative with a DS of 0.18 was the most active with MIC = 600 and 525 mg.L^{-1} against *A. tumefaciens* and *E. carotovora*, respectively. The effects of these derivatives were also assessed on polygalacturonase and pectin-lyase activities in the tested bacteria. *O*-(succinyl) chitosan with a DS of 0.64 caused a high inhibition of the above enzymes activities at 250, 500 and 1000 mg.L^{-1}. The study showed that *O*-(succinyl) chitosans were much more potent as antifungal activity than chitosan and a compound of 0.64 DS was the highest in its mycelial growth inhibition with EC_{50} of 1380, 1945 and 1505 mg.L^{-1} against *A. alternata*, *B. cinerea* and *F. oxysporum*, respectively. The study showed that the spore germination of *B. cinerea* and *F. oxysporum* was significantly affected with the above chitosan derivatives at 50, 125 and 250 mg.L^{-1} and the inhibition activity was increased with an increase in the DS.

Keywords: *O*-(succinyl) chitosan; ^{1}H-NMR spectroscopy; antibacterial activity; antifungal activity; fungal spore germination; polygalacturonase; pectin-lyase.

INTRODUCTION

Chitosan is a linear polysaccharide obtained by deacetylation of a naturally occurring chitin. It is consisting of *β*-(1,4)-2-acetamido-2- deoxy-D-glucose and *β*-(1,4)-2-amino-2-deoxy-D-glucose units. Applications of chitosan in agriculture and environmental protection include its use as a biocontrol agent for plant diseases (Rabea *et al.,* 2003). Chitosan has antimicrobial activity against a variety of bacteria and fungi coming from its polycationic nature (Helander *et al.,* 2001 and Rabea *et al.,* 2003). However, this activity is limited to acidic conditions due to its poor solubility at a pH higher than 6.5 (Liu *et al.,* 2001). Therefore, a special emphasis has been focused on the preparation of chitosan derivatives soluble in water over a wide pH range such as *N*-(sulfonated) and *N*-(sulfobenzoyl) chitosan (Chen *et al.,* 1998), *N*-(alkylated) chitosan (Ma *et al.,* 2008), *N*-,*O*-(carboxymethyl) chitosan (Chen and Park, 2003) *N*-(carboxymethyl) chitosan (Skorik and Gomes, 2003) and quaternary ammonium salt of chitosan (Badawy, 2010). Synthesis of *O*-(succinyl) chitosan by introduction of succinyl groups to the -OH group on the glucosamine units show many advantage such as water solubility, low toxicity to mammals and biodegradability (Zhang *et al.*, 2003).

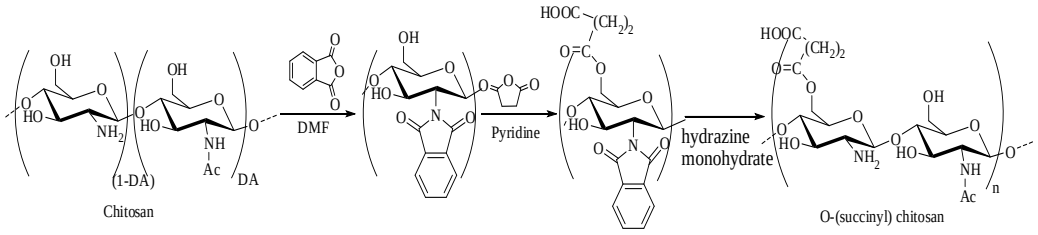
In the current research, water-soluble *O*-(succinyl) chitosans were synthesized according to Zhang *et al.,* (2003) with different degrees of substitution (DS). The antimicrobial activities of these derivatives were evaluated against bacteria of crown gall *Agrobacterium tumefaciens* and soft mold *Erwinia carotovora* and fungi of early blight *Alternaria alternata*, grey mold *Botrytis cinerea* and root rot *Fusarium oxysporum*. Moreover, the activity of these compounds on polygalacturonase and pectin-lyase in bacterial suspension of *A. tumefaciens* and *E. carotovora* was determined.

MATERIALS AND METHODS

Chemistry: Chitosan of low molecular weight (3.60×10^5 Da, determined by viscometric method), phthalic anhydride and succinic anhydride were purchased from Sigma-Aldrich Co. (USA). Other reagents and commercially available solvents were used without further purification.

Preparation of phthalimide chitosan: Phthalimide chitosan was firstly synthesized according to Zhang *et al.,* (2003) as shown in Scheme 1. Chitosan (6.0 g) was added into a solution of phthalic anhydride (16.8 g) in 100 ml DMF and heated to 130°C with stirring. After 8 h of reaction, the solution was filtered and the filtrate was poured into ice water. The precipitate was collected, simultaneously washed with ethanol and ethyl ether and dried at 60°C overnight to give phthalimide chitosan (~5 g).

Preparation of O-(succinyl) chitosan compounds: One gram of phthalimide chitosan was dissolved in 20 ml DMF with stirring. Succinic anhydride was added, with mol ratios of 0.1, 0.3, 0.5, 0.8 and 1.0 mol/glucoseamine unit (Scheme 1), into the flask by drop-wise at room temperature. Pyridine was subsequently dropped into the solution mixture and the reaction was maintained at room temperature overnight. The mixture precipitated in an excess of acetone, filtered and washed with acetone. The product was dissolved in DMF then hydrazine monohydrate (20 ml) and water (40 ml) were added. The mixture was heated to 100°C with stirring. After 15 h of reaction, the suspension was filtered; 50 ml of water was added into the filtrate and then dried in vacuum. The residue was dissolved in water, dialyzed against distilled water for 4 days and then the product was oven dried at 60°C overnight.



Scheme 1. Preparation of *O*-(succinyl) chitosan

^{1}H-NMR spectroscopy*:* ^{1}H-NMR measurements were performed on a JEOL A-500 NMR spectrometer (Faculty of Science, Alexandria University, Egypt). 20 mg of sample was dissolved in 0.5 ml of 1% CD_3COOD/D_2O solution and the tube was kept at room temperature for one hour to dissolve the compound.
Spectral data of chitosan: δ 2.09-2.12 (br s, NHAc), 3.21-3.27 (br m, H-2 of GlcN residue), 3.57-4.14 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.62-4.93 (m, H-1 of GlcN and GlcNAc units) (Figure 1A).

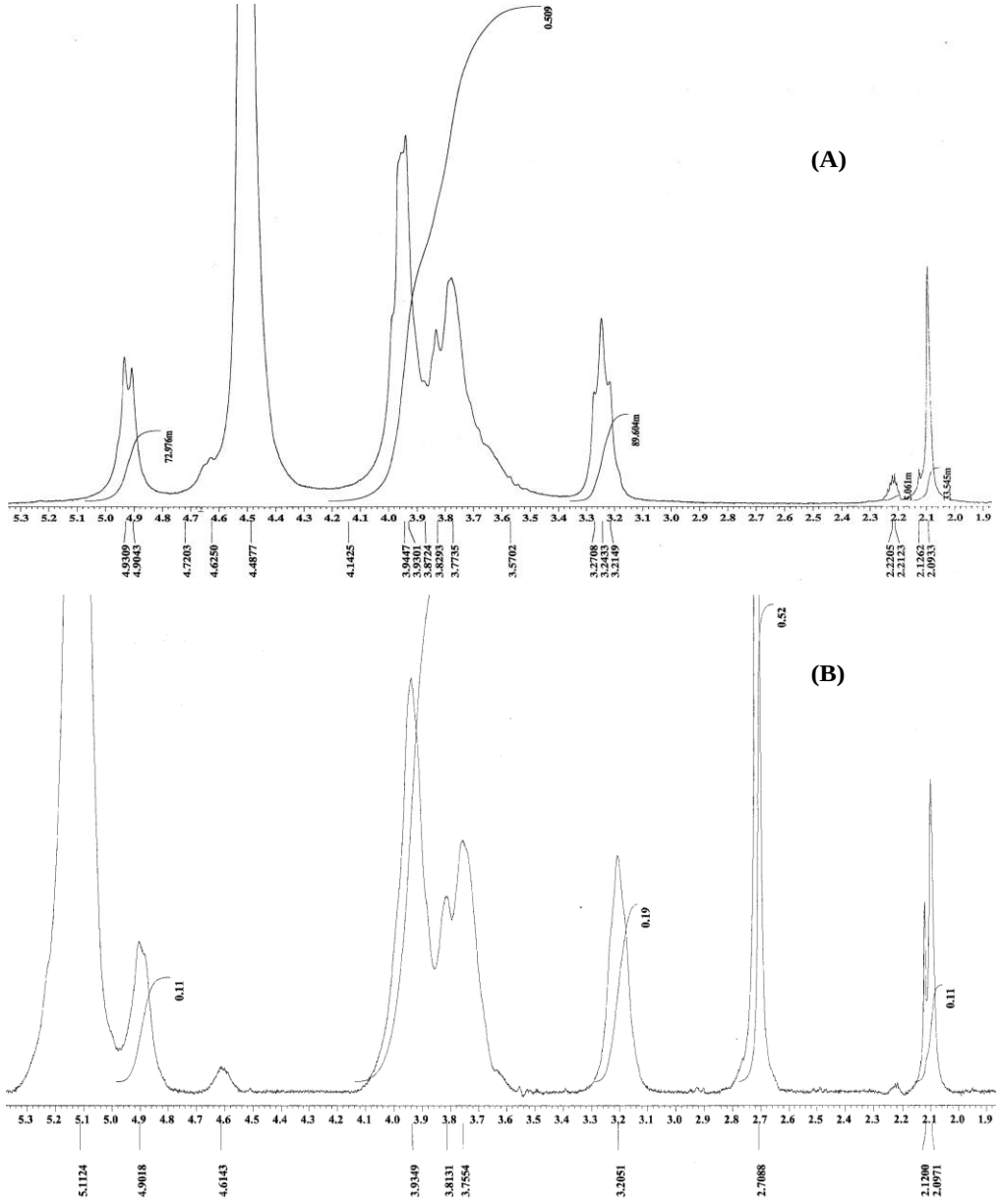


Figure 1. ^{1}H-NMR spectra of chitosan (A), *O*-(succinyl) chitosan (compound 5, B) in 1% CD_3COOD/D_2O at 25 °C.

Spectral data of compound 1 (ratio of 0.1 mol succinic anhydride to one mol phthalimide chitosan): δ 2.0-2.2 (br s, NHAc), 2.7 (br s, $(CH_2)_2$), 3.20 (br s, H-2 of GlcN residue), 3.60-4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.60 (br s, H-1 of GlcNAc residue), 4.90 (br s, H-1 of GlcN residue).

Spectral data for compound 2 (ratio of 0.3 mol succinic anhydride to one mol phthalimide chitosan): δ 2.10-2.12 (br s, NHAc), 2.71 (br, s $(CH_2)_2$), 3.20 (br s, H-2 of GlcN residue), 3.65-4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.62 (br s, H-1 of GlcNAc residue), 4.90 (br s, H-1 of GlcN residue).

Spectral data for compound 3 (ratio of 0.5 mol succinic anhydride to one mol phthalimide chitosan): δ 2.09-2.11 (br s, NHAc), 2.70 (br,s $(CH_2)_2$), 3.20 (br s, H-2 of GlcN residue), 3.60-4.09 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.61 (br s, H-1 of GlcNAc residue), 4.90 (br s, H-1 of GlcN residue).

Spectral data for compound 4 (ratio of 0.8 mol succinic anhydride to one mol phthalimide chitosan):): δ 2.09-2.12 (br s, NHAc), 2.70 (br,s $(CH_2)_2$), 3.20 (br s, H-2 of GlcN residue), 3.65-4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.6 (br s, H-1 of GlcNAc residue), 4.90 (br s, H-1 of GlcN residue).

Spectral data for compound 5 (ratio of one mol succinic anhydride to one mol phthalimide chitosan):): δ 2.09-2.12 (br s, NHAc), 2.70-2.75 (br s, $(CH_2)_2$), 3.20 (br s, H-2 of GlcN residue), 3.70-4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.61 (br s, H-1 of GlcNAc residue), 4.90 (br s, H-1 of GlcN residue) (Figure 1B).

Bioassays: Bacteria of crown gall *Agrobacterium tumefaciens* and soft mold *Erwinia carotovora* and fungi of early blight *Alternaria alternata*, grey mold *Botrytis cinerea,* and root rot *Fusarium oxysporum* were used for antimicrobial assay, and provided by Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. citrus pectin and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Co., Nutrient Agar (NA), Nutrient Broth (NB),

Potato Dextrose Broth (PDB) and Potato Dextrose Agar (PDA) media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK).

Antibacterial assay: The antibacterial activity of chitosan compounds against *A. tumefaciens* and *E. carotovora* was determined using nutrient agar dilution method (EUCAST, 2000). The culture was obtained by growing the bacteria overnight at 37°C in NB. A series of concentrations (200-3000 $mg.L^{-1}$) were prepared in 0.5 and 0.01% (v/v) aqueous acetic acid for chitosan and *O*-(succinyl) chitosans, respectively and mixed with NA medium. The pH was adjusted to 5.5-6.0 with 1M NaOH and solutions were then poured into autoclaved Petri dishes. One loopful of bacterial suspension was spotted on the surface of NA medium (ten spots per plate) then incubated at 37°C for 24h. Each concentration was tested in triplicate. The MIC was defined as the lowest concentration of the tested sample at which the bacterial colonies were not visible with naked eye within 24h.

Polygalacturonase and pectin-lyase activities assay*:* Strain of bacteria was used for enzyme production on liquid medium containing citrus pectin as a sole carbon source. Its composition was as follows: 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.20% K_2HPO_4 and 0.02% $MgSO_4.7H_2O$ (the pH was adjusted to 6.5 before autoclaving). 50 ml of the medium containing different concentrations of *O*-(succinyl) chitosans (250, 500 and 1000 $mg.L^{-1}$) was shaking and inoculated with 0.5 ml of bacterial suspension of 24 h old culture. At the end of incubation period (6 days) at 30°C, the biomass was separated by centrifugation at 6000 rpm for 10 min and polygalacturonase (PG) and pectin-lyase (PL) activities were determined colorimetrically according to Miller, (1959). The reaction mixture containing 0.2 ml enzyme and 0.8 ml (1.0%) of citrus pectin in 0.2M acetate buffer (pH 4.5) was incubated for 1 h at 30°C. After that, 2.5 ml of a previous mixture mixed with 2.5 ml of 1N HCl and 5 ml of 0.04M TBA. The tested tube was then placed into a water bath for 30 min, cooled and the absorbance was read at 515 nm for PG activity and at 550 nm for PL activity (Aboaba, 2009).

Antifungal assay*:* The mycelia radial growth inhibition technique was used to determine the antifungal activity of chitosan derivatives against *A. alternata*, *B. cinerea* and *F. oxysporum* as described by El-Ghaouth *et al.*, (1992). Solutions

of compounds were prepared in 0.5% and 0.01% (v/v) aqueous acetic acid for chitosan and *O*-(succinyl) chitosans, respectively. A series of concentrations (100-4000 $mg.L^{-1}$) contained in PDA medium were seeded in sterile culture plates and infected with 6-mm agar plugs taken from the margin of a 7 days old culture. Three replicates were used for each fungus per concentration tested. The plates were incubated in the dark at 26±2°C.

Spore germination of B. cinerea and F. oxysporum in liquid medium*: B. cinerea* and *F. oxysporum* spores were harvested from 2-weeks-old PDA culture. Aliquots of 50 µl of a spore suspension (1.0×10^6 $conidia.mL^{-1}$) were placed in eppendorf tubes containing 500 µl of PDB medium with a compound concentration. Tests were performed at concentrations of 50, 125 and 250 $mg.L^{-1}$. The tubes were incubated at 26°C during 16 h. The samples were placed on both chambers of a hemocytometer by carefully touching the edges of cover slip with the pipette tip and allowed capillary action to fill the counting chambers and observed under the microscope at 40x. The numbers of germinated and non-germinated conidia were recorded and inhibition of spore germination (%) was calculated. All experiments were conducted in three replicates (Griffin, 1994).

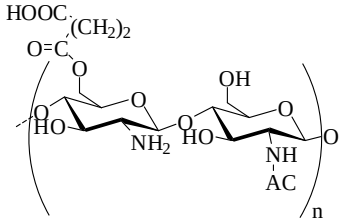
Statistical analysis: Statistical analysis was performed using SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The effective concentration that cause a 50% reduction of mycelial growth (EC_{50}) and corresponding 95% confidence limits were estimated by probit analysis (Finney, 1971). The data of spore germination and enzymes activity were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Student-Newman-Keuls (SNK) test according to Snedecor and Cochran, (1989) and differences at $P \leq 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Characterization of *O*-(succinyl) chitosans: *O*-(succinyl) chitosans derivatives were obtained with different DS values ranged from 0.18 to 0.64 (Table 1). Due to succinyl group introduced into chitosan *O*- terminal of the glucosamine units, the water solubility has been greatly enhanced as shown in Table 1. Compounds 4 and 5 were highly soluble in water and the solubility

was increased with increasing the DS. Compound 1 formed a gel but compounds 2 and 3 formed viscous solutions. The *O*-succinylation was further confirmed by ^{1}H-NMR with the peaks at 2.70 ppm, which corresponds to the methylene protons of the succinyl group. ^{1}H-NMR, analysis was employed for further estimation of the degree of acetylation (DA), degree of deacetylation (DDA), degree of substitution (DS), formula weight (FW) and yield (%) of chitosan and its derivatives according to Badawy *et al.*, (2004). FW for chitosan, compound 1, 2, 3, 4 and 5 are 167, 209, 292, 308, 329 and 342, respectively depending on the DA, DDA and DS. The results indicated that the FW was increased with an increase in the mol ratio between succnic anhydride and chitosan molecule (Table 1). The results also showed that *O*-(succinyl) chitosan derivatives were isolated with 60-80% yields.

Table 1: Chemical structure and properties of *O*-(succinyl) chitosans



Compound	SA/1 mol GlcN	DDA	DA	DS	FW	Yield (%)	Solubility in	
							water	0.5% (v/v) aqueous acetic acid
Chitosan	-	0.89	0.11	-	167	-	-	+++
1	0.1	0.88	0.12	0.18	209	80	+	+++
2	0.3	0.87	0.13	0.46	292	61	++	+++
3	0.5	0.87	0.13	0.53	308	63	++	+++
4	0.8	0.86	0.14	0.62	329	61	+++	+++
5	1.0	0.89	0.11	0.64	342	60	+++	+++

SA = succinic anhydride, GlcN = glucosamine, DDA = degree of deacetylation, DA = degree of acetylation, DS = degree of substitution and FW= Formula weight. - Insoluble, + low soluble (form a gel), ++ moderate soluble (viscous solution), +++ high soluble (clear solution).

Antibacterial activity of chitosan and *O*-(succinyl) chitosans: The data of the *in vitro* antibacterial activity of chitosan and *O*-(succinyl) chitosan with

different DS values against *A. tumefaciens* and *E. carotovora* are presented in Table 2. The results indicate that *O*-(succinyl) chitosans possessing strong antibacterial activity than chitosan (MIC of chitosan was higher than 2400 $mg.L^{-1}$). Compounds markedly inhibited growth of the two bacteria with MIC values ranging between 525 and 1250 $mg.L^{-1}$. The inhibitory effects differed with regard to the DS and the type of the bacteria. *O*-(succinyl) chitosan of 0.18 DS (compound 1) displayed the highest inhibation activity against *A. tumefaciens* and *E. carotovora* with MIC of 600 and 525 $mg.L^{-1}$, respectively. An increase in the DS led to a decrease in the activity where compound 5 (DS = 0.64) possessed the lowest activity (MIC = 1250 and 1125 $mg.L^{-1}$ to *A. tumefaciens* and *E. carotovora*, respectively). Considering the susceptibility of the microorganisms, it was noticed that *E. carotovora* was more susceptible to these compounds than *A. tumefaciens* which may be attributed to their different cell walls (Xie *et al.,* 2002). This result was also confirmed by our previous study (Rabea *et al.,* 2009) which indicated that *N-(o*-ethylbenzyl) chitosan showed a high antibacterial effect against *E. carotovora* and *A. tumefaciens* (MIC = 500 and 1200 $mg.L^{-1}$, respectively).

Table 2: Antibacterial activity of chitosan and *O*-(succinyl) chitosans against *A. tumefaciens* and *E. carotovora*

Compound	MIC $mg.L^{-1}$	
	A. tumefaciens	*E. carotovora*
Chitosan	>2400	>2400
1	600	525
2	850	725
3	1025	775
4	1100	1025
5	1250	1125

MIC: Minimum inhibitory concentration

Chitosan showed a broad-spectrum antimicrobial activity against both gram-positive and gram-negative bacteria (Chung and Chen, 2007). From the results obtained, these derivatives showed higher antibacterial activity than chitosan. These results are in agreement with Rabea *et al.,* (2009) and Badawy, (2010). They reported that chemical modifications of chitosan molecule dramatically enhanced the antibacterial action against *E. carotovora* and *A. tumefaciens*.

Effect of chitosan and *O*-(succinyl) chitosans on polygalacturonase and pectin-lyase activities: Polygalacturonase (PG) and pectin-lyase (PL) activities were detected in bacterial suspension of *A. tumefaciens* and *E. carotovora* (Table 3). The result revealed that the activity of PG and PL was decreased with increasing in the concentration. In *A. tumefaciens*, compound 5 at all concentrations significantly inhibited both of PG and PL enzyme activities. While there is no significant difference between the others compounds.

Table 3: Effect of chitosan and *O*-(succinyl) chitosans on the activity of polygalacturonase and pectin-lyase in *A. tumefaciens* and *E. carotovora*

Compound	Conc. ($mg.L^{-1}$)	Relative activity (%) ± SE			
		A. tumefaciens		*E. carotovora*	
		PG	PL	PG	PL
Control	0	100.00^{a} ±0.25	100.00^{a}±0.27	100.00^{a}± 0.50	100.00^{a} ±1.08
Chitosan	250	90.29^{c} ± 0.63	92.06^{b}±0.54	99.57^{a}± 0.25	100.00^{a} ±1.35
	500	82.90^{fg} ± 1.01	89.10^{bcde}±1.02	90.87^{b}± 1.00	94.86^{bc} ±0.81
	1000	80.29^{g} ± 0.38	86.14^{def}±0.56	86.52^{c}± 0.50	90.19^{def} ±0.47
1	250	97.83^{a} ± 1.26	91.59^{bc}±0.27	91.30^{b}± 1.26	95.79^{b} ±1.35
	500	95.07^{b} ±1 .24	90.03^{bcd}±1.49	85.22^{cd}± 0.75	91.43^{de} ±0.31
	1000	83.04^{fg} ± 0.66	86.45^{cdef}±0.81	84.35^{cde}± 1.00	89.72^{def} ±0.54
2	250	93.48^{b} ± 0.50	89.88^{bcd}±0.95	89.86^{b}± 1.05	92.37^{cd} ±0.87
	500	86.38^{de} ± 0.38	87.54^{bcdef}±2.29	83.04^{cde}± 0.50	88.79^{defg}±0.54
	1000	81.30^{g} ± 0.50	79.91^{gh}±0.54	82.75^{de}± 0.88	87.85^{efg} ±0.81
3	250	93.48^{b} ± 1.26	89.41^{bcde}±2.04	89.57^{b}± 1.33	91.90^{cd} ±1.09
	500	86.52^{de} ± 0.25	87.07^{bcdef}±1.53	83.33^{cde}± 0.81	88.63^{defg}±0.41
	1000	80.87^{g} ± 0.50	79.44^{gh}±0.54	82.75^{de}± 0.72	86.60^{fg} ±1.36
4	250	90.00^{c} ± 0.75	87.85^{bcde}±0.81	83.04^{cde}± 0.25	86.45^{fg} ±0.81
	500	86.96^{de} ± 0.25	84.27^{ef}±2.45	76.52^{f}± 1.51	80.37^{h} ±0.71
	1000	80.72^{g} ± 0.63	76.17^{hi}±0.27	72.46^{g}± 0.38	72.27^{i} ±0.68
5	250	89.13^{cd}± 0.75	82.71^{fg}±0.27	80.87^{e}± 0.50	85.05^{g} ±1.18
	500	84.78^{ef} ± 1.26	79.75^{gh} ±0.16	76.96^{f}± 0.50	79.91^{h} ± 0.27
	1000	70.43^{h} ± 0.50	73.99^{i}±0.16	69.86^{h}± 0.38	71.96^{i} ± 0.97

Data are average of three replicates ± SE. Values within a column bearing the same letter are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls (SNK) test.

The PG and PL activities of *E. carotovora* showed that the enzymes activity decreased with an increase in the DS and the inhibition of the activity was a concentration dependant. Compounds 4 and 5 possessed significant inhibition

of PG and PL at all concentrations. Compounds 1, 2 and 3 slightly reduced the activity.

Studies of bacterial enzyme activities provide fundamental information relevant to microbial physiology and to a more complete understanding of cell metabolism. PG and PL enzymes are necessary for the hydrolysis of pectin (Gadre *et al.,* 2003). These enzymes have been isolated and studied in a wide variety of bacterial and fungal plant pathogens and has been reported for diverse types of diseases such as soft rot, dry rot, wilts, blights and leaf spots (Buzi *et al.,* 2003). Wegener, (2002) reported that pectate-lyase enzymes were major virulence factors of *E. carotovora* and they degrade plant cell wall pectin into unsaturated oligogalacturonates known to elicit plant defence responses.

Antifungal activity of chitosan and *O*-(succinyl) chitosans: The antifungal activity of chitosan derivatives against *A. alternata, B. cinerea* and *F. oxysporum* is presented in Table 4. All *O*-(succinyl) chitosan compounds were more active than chitosan. It can be notice that the antifungal activity was increased dramatically with an increase in DS value. Compound 5 (DS = 0.64) exerted significantly prominent antifungal activity against *A. alternata, B. cinerea* and *F. oxysporum* with EC_{50} of 1380, 1945 and 1505 $mg.L^{-1}$, respectively. However, compound 1 (DS = 0.18) was the lowest active one with EC_{50} of 2033, 2968 and 2473 $mg.L^{-1}$ against *A. alternata, B. cinerea* and *F. oxysporum*, respectively. In regard to the susceptibility of the three tested fungi, it can be noticed that *A. alternata* and *F. oxysporum* are more susceptible than *B. cinerea* to *O*-(succinyl) chitosans.

Numerous studies on the antifungal activity of chitosan and its derivatives against plant pathogens have been investigated (Reddy *et al.,* 2000; Badawy *et al.,* 2004; Rabea *et al.,* 2006, 2009 and Badawy and Rabea, 2009). Recently, many chitosan derivatives that is soluble in water indicated high antifungal activity (Guo *et al.,* 2007 and Badawy, 2010). They found that all quaternized chitosan derivatives (water-soluble chitosans) gave stronger antifungal activities against *B. cinerea, F. oxysporum, Pythium debaryanum* and *Colletotrichum lagenarium* than the unmodified chitosan.

Effect of chitosan and *O*-(succinyl) chitosans on fungal spore germination: The effect of chitosan and *O*-(succinyl) chitosans on spore germination of *B. cinerea* and *F. oxysporum* are shown in Table 5. The results showed that a complete inhibition (100%) of fungal spores of *B. cinerea* was found with a high tested concentration (250 $mg.L^{-1}$) of all compounds except unmodified chitosan. An increase in the DS values led to an increase in the inhibition of spore germination. It can also see that compound 5 was significantly the most potent one (58.05, 67.48 and 100% inhibition at 50, 125 and 250 $mg.L^{-1}$, respectively). However, the lowest inhibition values were observed with chitosan (27.75, 42.75 and 60.83 % at 50, 125 and 250 $mg.L^{-1}$, respectively).

Table 4: Antifungal activity of chitosan and *O*-(succinyl) chitosans against fungi of *A. alternata*, *B. cinerea* and *F. oxysporum*.

Compound	EC_{50} ($mg.L^{-1}$)	95% confidence limits ($mg.L^{-1}$)		Slope ± SE	Intercept of regression line ± S.E	Chi square (χ^2)
		Lower	Upper			
			A. alternata			
Chitosan	> 3000	-	-	-	-	-
1	2033	1604	3085	1.81±0.33	-5.81±0.33	0.59
2	1562	1345	1906	2.55±0.35	-8.14±1.06	0.03
3	1484	1289	1775	2.66±0.35	-8.46±1.06	0.05
4	1464	1268	1758	2.60±0.34	-8.21±1.04	1.12
5	1380	1194	1652	2.50±0.33	-7.84±1.02	0.01
			B. cinerea			
Chitosan	> 3000	-	-	-	-	-
1	2968	2449	3844	1.85±0.22	-6.42±0.73	1.30
2	2123	1820	2541	2.06±0.22	-6.87±0.71	0.71
3	2068	1797	2426	2.29±0.23	-7.59±0.74	1.44
4	2044	1786	2371	2.41±0.23	-7.98±0.75	1.96
5	1945	1688	2278	2.24±0.22	-7.35±0.72	0.90
			F. oxysporum			
Chitosan	> 3000	-	-	-	-	-
1	2473	2155	2909	2.49±0.24	-8.46±0.8	1.44
2	1812	1518	2217	1.70±0.20	-5.54±0.65	1.89
3	1618	1383	1911	1.95±0.21	-6.26±0.67	0.83
4	1587	1308	1950	1.55±0.20	-4.97±0.64	0.64
5	1505	1278	1781	1.87±0.21	-5.96±0.66	0.93

From the statistical analysis, it can be noticed that there is no significant difference between compounds 1, 2 and 3. The spores of *F. oxysporum* (Table 5) were affected significantly with all compounds compared to the control and chitosan. The results revealed that compound 5 (high DS) was significantly the most potent (94.48% at 250 $mg.L^{-1}$) followed by compound 4 at the same concentration. In regard to the susceptibility of fungi, it can also notice that spores of *F. oxysporum* were high sensitive to these compounds compared to *B. cinerea*. These results are in agreement with those of Rabea *et al.,* (2009) and Badawy, (2010). They reported that chitosan derivatives including quaternary and *N*-(benzyl) chitosans had a better inhibition of spore germination of *B. cinerea* and *F. oxysporum* compared to the unmodified chitosan.

Table 5. Effect of chitosan and *O*-(succinyl) chitosans on spore germination of *B. cinerea* and *F. oxysporum*

Compound	Concentration $mg.L^{-1}$	Inhibition of spore germination (%) ± SE	
		B. cinerea	*F. oxysporum*
Control	0	26.50^{h}±1.51	31.00^{j}±1.66
Chitosan	50	27.75^{h}±1.65	25.92^{k}±0.34
	125	42.75^{g}±0.48	33.06^{j}±0.94
	250	60.83^{c}±0.93	45.94^{h}±2.16
1	50	28.75^{h}±0.87	38.85^{i}±0.75
	125	43.50^{fg}±0.87	60.59^{f}±1.11
	250	100.00^{a}±0.00	83.73^{c}±0.73
2	50	29.68^{h}±0.62	38.93^{i}±0.78
	125	46.63^{ef}±1.25	64.13^{e}±1.85
	250	100.00^{a}±0.00	85.00^{c}±0.41
3	50	30.00^{h}±1.47	45.60^{h}±0.95
	125	47.85^{e}±1.15	65.65^{e}±1.73
	250	100.00^{a}±0.00	86.15^{bc}±0.62
4	50	43.43^{fg}±1.26	48.25^{h}±1.03
	125	53.35^{d}±0.90	72.26^{d}±0.94
	250	100.00^{a}±0.00	89.35^{b}±0.79
5	50	58.05^{c}±1.79	52.25^{g}±1.44
	125	67.48^{b}±1.21	73.63^{d}±0.51
	250	100.00^{a}±0.00	94.48^{a}±0.38

Data are average of three replicates ± SE. Values within a column bearing the same letter are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls (SNK) test.

CONCLUSION

O-(succinyl) chitosans were synthesized to improve the antibacterial and antifungal activity of chitosan. The antimicrobial activities against bacteria of *A. tumefaciens* and *E. carotovora* and fungi of *A. alternata, B. cinerea* and *F. oxysporum* were investigated. The compounds markedly inhibited the growth of bacteria than chitosan and *O*-(succinyl) chitosan of 0.18 DS was the most effective one with MIC of 600 and 525 $mg.L^{-1}$ against *A. tumefaciens* and *E. carotovora*, respectively. Furthermore, the inhibitory effect of the compounds on PG and PL enzymes was increased with a DS increase. The biological activity of *O*-(succinyl) chitosans against fungi was also increased with increasing of DS where *O*-(succinyl) chitosan of 0.64 DS was the most potent (EC_{50} = 1380, 1945 and 1505 $mg.L^{-1}$) against *A. alternata, B. cinerea* and *F. oxysporum*, respectively. In addition, the effect of the compounds on spore germination showed a wide range of inhibitions against *B. cinerea* and *F. oxysporum*. The present study suggests that water-soluble *O*-(succinyl) chitosans can be used to control some plant pathogens that cause destruction of crops and vegetables.

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تحضير مشتقات سكسينيل كيتوزان القابلة للذوبان فى الماء وتقييم النشاط الإبادى الميكروبى لها ضد بعض الممرضات النباتية

د. إنتصار إبراهيم ربيع
قسم مكافحة الآفات وحماية البيئة-كلية الزراعة (دمنهور)-جامعة الاسكندرية-مصر

تعتبر البكتيريا والفطريات الممرضة للنبات من الكائنات التى تؤثر بطريقة سلبية على عدد كبير من الخضروات والفاكهه التى لها أهمية إقتصادية . لذا تتركز هذة الدراسة على تحضير مشتقات من مركب الكيتوزان الطبيعى والتى لها قابلية للذوبان فى الماء عن طريق إدخال مجموعة السكسينيل بدرجات إستبدال مختلفة بهدف زيادة الكفاءة البيولوجية للكيتوزان ضد بعض الممرضات . تم التأكد من التركيب الكيميائى لهذة المركبات وحساب درجة الإستبدال على جزيئ الكيتوزان بواسطة جهاز الرنين النووى المغناطسى NMR وكانت درجات الإستبدال تتراوح من 0.18 إلى0.64 . بعد ذلك تم التقييم البيولوجى ضد البكتيريا المسببة لمرض التدرن التاجى *Agrobacterium tumefaciens* والبكتيريا المسببة للعفن الطرى لثمار الخضروات والفاكهه *Erwinia carotovora* و ضد كلا من فطر *Alternaria alternata* المسبب للندوة المبكرة وفطر *Botrytis cinerea* المسبب للعفن الرمادى وفطر *Fusarium oxysporum* المسبب لعفن الجذور وسقوط البادرات وأوضحت النتائج أن المركب الذى له درجة إستبدال تساوى 0.18 هو الأعلى تأثير إبادى ضد بكتيريا *A. tumefaciens* و *E. carotovora* حيث كانت قيمة التركيز المسبب لموت 100% من البكتيريا(MIC) هى 600 و 525 ملجم/لتر على التوالى . بالإضافة إلى ذلك فقد تم دراسة تأثير تلك المركبات على إنزيمات polygalacturonase و pectin-lyase وأوضحت النتائج أن *O*-(succinyl) chitosan الذى له درجة إستبدال تساوى 0.64 أعطى أعلى تأثير تثبيطى على نشاط هذة الإنزيمات . أوضحت النتائج أيضا أن المركب الذى له درجة إستبدال تساوى 0.64 هو الذى أعطى أعلى تأثير إبادى فطرى حيث كانت قيم التركيز المسبب لتثبيط 50% من النمو الفطرى (EC_{50}) هى 1380 ، 1945 و1505 ملجم/لتر لكل من *A. alternata*، *B. cinerea* و *F. oxysporum* على التوالى. أيضا تم إختبار كفاءة المركبات المحضرة ضد إنبات الجراثيم لفطريات *B. cinerea* و *F. oxysporum* وكانت معظم المركبات المحضرة لها تأثير معنوى فى تثبيط نمو تلك الجراثيم على كل التركيزات وكان هذا التأثير يزيد بزيادة درجة الإستبدال.