Structure and Antimicrobial Activity Relationship of Quaternary *N*-Alkyl Chitosan Derivatives Against Some Plant Pathogens

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ABSTRACT: In the present work, quaternary chitosans as water-soluble compounds were prepared based on three-step process. Schiff bases were firstly synthesized by the reaction between the amino groups of chitosan with aliphatic aldehydes followed by a reduction with sodium borohydride ($NaBH_4$) to form *N*-(alkyl) chitosans. *N,N,N*-(dimethyl alkyl) chitosans were then obtained by a reaction of chitosan containing *N*-butyl, *N*-pentyl, *N*-hexyl, *N*-heptyl, and *N*-octyl substituents with methyl iodide. The compounds were characterized using IR and NMR spectroscopy. Subsequent experiments were conducted to test their antimicrobial activities against the most economic plant pathogenic bacteria of crown gall disease *Agrobacterium tumefaciens,* soft mold disease *Erwinia carotovora,* fungi of grey mold *Botrytis cinerea,* root rot disease *Fusarium oxysporum,* and damping off disease *Pythium debaryanum*. Quaternary chitosans enhanced the antibacterial activity and *N,N,N*-(dimethyl pentyl) chitosan was the most active one with Minimum Inhibitory Concentration (MIC) of 750 and 1225 mg/L against *A. tumefaciens* and *E. carotovora,* respectively. All quaternized chitosans gave stronger antifungal activities than chitosan where *N,N,N*-(dimethyl pentyl) chitosan and *N,N,N*-(dimethyl octyl) chitosan were significantly the highest in mycelial growth inhibiation against *B. cinerea* (EC_{50} = 908 and 383 mg/L, respectively), *F. oxysporum* (EC_{50} = 871 and 812 mg/L, respectively), and *P. debaryanum* (EC_{50} = 624 and 440 mg/L, respectively). In addition, spore germination of *B. cinerea* and *F. oxysporum* was significantly affected with the compounds at the tested concentrations and the inhibition activity was increased with an increase in the chain length of the alkyl substituent.
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Key words: quaternary *N*-alkyl chitosans; IR and NMR spectroscopy; antibacterial activity; antifungal activity; fungal spore germination

INTRODUCTION

Chemical modifications of polysaccharides are increasingly studied as they have the potential of providing new applications for such abundant polymers. With regard to its unique properties such as biocompatibility, biodegradability, and no toxicity to mammals, it is widely used in fields like biotechnology, pharmaceutics, cosmetics, and agriculture.[1–4] In particular, the antimicrobial activities of a biopolymer chitosan and its derivatives have aroused considerable recent interest.[4,5] Chitosan is a copolymer of glucosamine and *N*-acetyglucosamine units linked by 1,4-glucosidic bonds and is obtained by *N*-deacetylation of chitin.[6] Unfortunately, in spite of the chitosan advantages, it is only soluble in acidic aqueous solutions with pH values lower than 6.5. However, its solubility is limited at a pH higher than 6.5 where chitosan start to lose its cationic nature. Moreover, the chemical modification by introducing of aromatic or alkyl substituents into chitosan polymer chain results in decreased solubility. This problem is probably the major limiting factor for chitosan utilization, i.e., its application in biology, because many enzyme assays are performed at neutral pH. If water-soluble chitosan would be easily accessible, it is expected that the biological and physiological potential of chitosan would increase dramatically.

One of the strategies to increase both the solubility and positive charge density of chitosan macromolecules is based on the introduction of quaternary ammonium groups in chitosan. This modification has got the commonly accepted term "quaternization of chitosan". Thus, water-soluble chitosan derivatives soluble to both acidic and basic physiologic circumstances may be good candidates for the polycationic biocides.[7] Many efforts to synthesize quaternized chitosan derivatives have been reported. For example, Muzzarelli and Tanfani,[1] reported the formation of *N*-dimethyl chitosan and the preparation of *N*-

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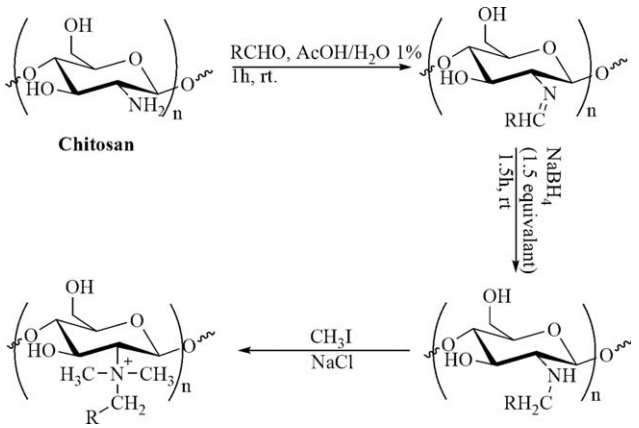
trimethyl chitosan iodide with formaldehyde and sodium borohydride ($NaBH_4$). Trimethyl chitosan ammonium iodide was also obtained by reaction of a low acetyl content chitosan with methyl iodide and sodium hydroxide under controlled conditions.[8,9] *N*-alkyl chitosan derivatives were reacted with methyl iodide to produce water soluble quaternary ammonium salts of *N,N,N*-trimethyl, *N*-*N*-propyl-*N,N*-dimethyl, and *N*-furfuryl-*N,N* dimethyl chitosan.[10,11] Their antibacterial activities increased with an increase in the chain length of the alkyl substitute. Recently, a new, smooth and one-step method of preparation of quaternizated chitosans has been carried out by means of reaction with betaine in the presence of the coupling reagent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline in aqueous media at pH 5.5±0.5.[12] This reaction results in preparation of *N*/(trimethylammonio)acetyl/chitosan chloride and its amphiphilic derivatives. Moreover, many applications of water-soluble chitosan derivatives including antibacterial activity,[11] anticoagulant activity,[13] antioxidant activity,[14] and gene delivery vehicles[15] have been reported.

Therefore, we wish to report in this article, the preparation of quaternary alkyl chitosans and their antimicrobial activities of these chitosan derivatives against the bacteria of crown gall *Agrobacterium tumefaciens,* soft mold disease *Erwinia carotovora* , the fungi of grey mold *Botrytis cinerea*, root rot disease *Fusarium oxysporum,* and damping off disease *Pythium debaryanum*.

MATERIALS AND METHODS

Materials and tested microorganisms

Low molecular weight (3.60×10^5 g/mol) of acid-soluble chitosan (made from coarse ground crab with 90% degree of deacetylation), butraldehyde, valeraldehyde, hexaldehyde, heptaldehyde, octaldehyde, methyl iodide, deuterium oxide, deuterated acetic acid, and $NaBH_4$ were purchased from Sigma-Aldrich Co. (USA) Potato Dextrose Agar (PDA), Nutrient Broth (NB), and Nutrient Agar (NA) media were purchased from Oxoid (Basingstoke, Hampshire, UK). All materials were used without further purification. Bacteria of crown gall disease *A. tumefaciens* (Family: Rhizobiaceae; Class: Alpha Proteobacteria), soft mold disease *E. carotovora* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria), fungi of grey mold *B. cinerea* (Family: moniliaceae; Class: Deuteromycetes), root rot disease *F. oxysporum* (Family: Tubercullariaceae; Class: Deuteromycetes), and damping off disease *P. debaryanum* (Family: Pythiaceae; Class: Oomycetes) were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt.



Scheme 1 Synthetic pathway for preparation of *N,N,N*-(dimethyl alkyl) chitosan derivatives.

Synthesis of quaternary *N*-alkyl chitosan derivatives

The *N*-(alkyl) chitosan derivatives were obtained first by a reductive amination following the procedure described by Borch et al.,[16] as shown in Scheme 1. It is a versatile and specific method for creating a covalent bond between a substrate and the amine function of the chitosan. In our work, it involves the reaction between the amine function of the chitosan and an aldehyde function as follows: 18 mmole (3.0 g calculated as glucosamine unit) chitosan was dissolved in 1% (v/v) aqueous acetic acid. One equivalent (calculated as glucosamine unit) of an aromatic aldehyde (butraldehyde, valeraldehyde, hexaldehyde, heptaldehyde, or octaldehyde) was dissolved in methanol and added dropwise to the chitosan solution at room temperature. After 1 h of stirring at room temperature, the pH of the solution was adjusted to 4.5 by addition of 1*M* NaOH solution. To this solution, 10% (w/v) $NaBH_4$ (1.5 equivalents to the aldehyde) was added, and the solution was stirred for 1.5 h. The precipitate of the *N*-(alkyl) chitosan derivative was obtained by adjusting the pH to 10. The precipitate was washed with distilled water to neutralize and the unreacted aldehyde was Soxhlet-extracted with 1 : 1 (v/v) ethanol/diethyl ether for 2 days. The solid residue was then oven-dried overnight at 60°C giving the *N*-(alkyl) chitosan derivative.

For the synthesis of quaternary *N*-(alkyl) chitosan derivatives, the procedure described by Saxena et al.[17] was used with some modification as follows: 2.5% (w/v) *N*-(alkyl) chitosan derivative was dispersed in 42% methanol/water and the mixture was kept under constant stirring for 1 h at 30°C. Then 5% NaCl (w/v) and 5% (v/v) methyl iodide (CH_3I) was added at intervals of 4 h in 2 : 1 : 1 proportion under constant stirring and the reaction mixture was refluxed at 70°C for 12 h. Quaternized chitosan

derivative (*N,N,N*-(dimethyl alkyl) chitosan) was then separated by centrifugation at 4000 rpm and the precipitate was washed several times with acetone and then dried under vacuum at 50°C (Scheme 1). The water-solubility of quaternized chitosan derivates was evaluated as follows: 0.1 g of dried chitosan compound was dispersed in 2 mL of distilled water and stirred at room temperature until the sample was completely dissolved.

^{1}H-NMR and IR spectroscopy

^{1}H-NMR measurements were performed on a JEOL A-500 NMR spectrometer (Faculty of Science, Alexandria University, Alexandria, Egypt) under a static magnetic field of 500 MHz at 25°C. For those measurements, 20 mg of sample was introduced into 5 mm Φ NMR tube, to which 0.5 mL of 0.5% (v/v) CD_3COOD/D_2O solution was added, and finally the tube was kept at room temperature to dissolve the polymer. Fourier transform infrared (FT-IR) spectra were obtained with a Shimadzu 470 Infrared spectrometer.

^{1}H-NMR and IR spectral data

Spectral data for chitosan

δ 2.09–2.12 (br s, $NHCOCH_3$), 3.15–3.30 (br m, H-2 of GlcN residue), 3.57–4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), and 4.88–5.00 (m, H-1 of GlcN and GlcNAc units). IR (KBr): ν 3427 (br, OH/NH), 2855–2920 (m, C—H), 1656 (>C=O stretching, amide I) and 1570 cm^{-1} (NH_2 stretching, amide II), 944–1150 cm^{-1} assigned to the saccharide structure.

Spectral data for *N,N,N*-(trimethyl) chitosan (compound 1)

δ 2.20–2.22 (br s, $NHCOCH_3$), 3.12–3.29 (br m, H-2 of GlcN residue), 3.30–3.40 (s, tertiary *N*-CH_3), 3.63–4.01 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.68–4.71 (m, H-1 of GlcN unit), and 4.85–4.94 (m, H-1 of GlcNAc unit). IR (KBr): ν 3398 (br, OH/NH), 2905 (m, C—H), 1656 (>C=O stretching, amide I) and 1588 cm^{-1} (NH_2 stretching, amide II), 1475 cm^{-1} (tertiary *N*—CH_3) cm^{-1} and 890–1150 cm^{-1} assigned to the saccharide structure.

Spectral data for *N,N,N*-(dimethyl alkyl) chitosan derivatives (compounds 2–6)

δ 0.87–1.02 (br m, CH_3), 1.30–1.50 (br m, $(CH_2)_n$, n = 2, 3, 4, 5 or 6), 1.64–1.78 (br m, CH_2), 2.03–2.04 (br s, $NHCOCH_3$), 3.10–3.29 (br m, H-2 of GlcN residue), 3.32–3.45 (s, CH_3), 3.57–4.06 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.88–5.00 (m, H-1 of GlcN and GlcNAc units). IR (KBr): ν 3388–3400 cm^{-1} (br, OH/NH), 2880–2910 cm^{-1} (m, C—H), 1650–1660 cm^{-1} (>C=O stretching, amide I) and 1580–1590 cm^{-1} (NH_2 stretching, amide II), 1450–1480 cm^{-1} (methyl groups of substituent) and 890–1150 cm^{-1} assigned to the saccharide structure.

Antibacterial assay on *A. tumefaciens* and *E. carotovora*

The antibacterial activity of chitosan and its derivatives was assayed using NA dilution method against *A. tumefaciens* and *E. carotovora*.[18] Preliminary screening tests were performed at concentrations of 200, 500, 1000, 2000, and 3000 mg/L. For determination of a Minimum Inhibitory Concentration (MIC), NA medium was mixed with a solution (0.5%, v/v, aqueous acetic acid) of chitosan and with 0.01% v/v, aqueous acetic acid of quaternary chitosans as a water-soluble to increase its solubility. The pH was adjusted to 5.5–6.0 with 1*M* NaOH and solutions were then poured into autoclaved Petri dishes. One loopful of microorganism suspensions in NB medium (≈6 μl) was spotted on the surface of NA medium (ten spots per plate) then incubated at 37°C in ISCO Incubator for 24 h. Each concentration was tested in triplicate. The MIC was determined as the lowest concentration of the compound required to completely inhibit a bacterial growth after incubation at 37°C for 24 h.

Antifungal assay on mycelial growth of *B. cinerea*, *F. oxysporum*, and *P. debaryanum*

The antifungal activity of chitosan compounds on the mycelial growth of *B. cinerea, F. oxysporum,* and *P. debrianum* was tested using a radial growth technique.[19] Chitosan was dissolved in 0.5% (v/v) aqueous acetic acid whereas quaternary chitosan compounds as a water-soluble were dissolved in 0.01% (v/v) aqueous acetic acid to increase its solubility in water and the pH was adjusted to 5.5–6.0 with 1*M* NaOH. Serious concentrations of chitosan compounds (250, 500, 1000, 1500, 2000, 2500, and 3000 mg/L) were, respectively, added to sterilized PDA medium immediately before pouring into the Petri dishes. Each concentration was tested in triplicate. Parallel controls were maintained with water and aqueous acetic acid (0.5% and 0.01%) mixed with PDA medium. The discs of mycelial culture (0.5 cm diameter) of fungi, taken from 8-day-old cultures on PDA plates, were transferred aseptically to the centre of the Petri dishes. The plates were incubated in the dark at 26°C using an ISCO Incubator. The colony growth diameter was measured when the fungal growth in the control had completely covered the Petri dishes. Inhibition percentage of mycelial growth was calculated as follows:

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

where DC and DT are average diameters of fungal colonies of control and treatment, respectively. Inhibiting concentration of 50% of a mycelial growth (EC_{50}) and its corresponding 95% confidence limits were estimated by a probit analysis.[20]

Activity of chitosan compounds on spore germination of *B. cinerea* and *F. oxysporum* in liquid medium

Preparation of fungal spores

B. cinerea and *F. oxysporum* spores were harvested from 2-weeks-old PDA culture grown under fluorescent lights in 9-cm diameter petri dishes at 26°C. In this study, the spore germination assay was not evaluated on *P. debaryanum* because this fungus forms various types and shapes of oospores and sporangia (bearing conidia). An amount of 5 mL of sterile water was added to a petri plate culture. The spores were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile water to an absorbance of 0.25 at 425 nm as determined by a Unico 1200-Spectrophotometer. This suspension contained about 1.0×10^6conidia/mL.

Spore germination assay

Aliquots of 50 μl of a spore suspension were placed in Eppendorf tubes containing 500 μl of Potato Dextrose Broth (PDB) medium with a chitosan concentration. Preliminary screening tests were performed at concentrations of 250, 500, and 1000 mg/L. These concentrations caused high mortality of *B. cinerea* spores, therefore; lower concentrations of 50 and 125 mg/L were then tested. The tubes were incubated at 25°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 for spore germination. Spore counting was done using a Neubauer haemocytometer and light microscopy at 40x. All experiments were conducted in four replicates. A spore was considered germinated when the length of the germ tube equaled or exceeded the length of the spore.[21]

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The log dose-response curves allowed determination of the EC_{50} values for the fungal bioassay according to the probit analysis.[20] The 95% confidence limits for the range of EC_{50} values were determined by the least-square regression analysis of the relative growth rate (% control) against the logarithm of the compound concentration. The data of fungal mycelial growth inhibition and spore germination were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Student-Newman-Keuls (SNK) test according to Snedecor and Cochran[22] and differences at $P \leq 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Structure and physicochemical characteristics of chitosan derivatives

The quaternization of chitosan and *N*-alkyl chitosan derivatives with methyl iodide was achieved in the present study as shown in Scheme 1. Synthesis of quaternary *N*-alkyl chitosan derivatives was carried out by three steps. *N*-alkyl chitosan has been firstly prepared by introducing an alkyl (butyl, pentyl, hexyl, heptyl, or octyl) group into the amine group of chitosan via Schiff's base (step one) followed by reducing the C=N bond (step two), and finally reacted with methyl iodide to produce *N,N,N*-(dimethyl alkyl) chitosans (step three). The third stage of synthesis is based on a nucleophilic substitution of chitosan amine protons with methyl groups of a methyl iodide. Therefore, the counterion I^- was exchanged in the presence of sodium chloride in a methanol/water medium with Cl^- to obtain *N,N,N*-(dimethyl alkyl) chitosans chloride having higher solubility than the iodide counterpart.

NMR method is the most effective technique concurrently to determine the structure of chitosan and its derivatives.[9,23] The signal at 0.80–0.90 ppm is attributed to CH_3 group of the alkyl substituent chain, whereas H_2-H_6 protons of the polysaccharide backbone superimpose the CH_3 groups attached to nitrogen atom in quaternary ammonium group. The peak at δ 2.0–2.10 ppm was assigned to the proton of residual CH_3 acetyl. The peak at δ 3.20 ppm was attributed to H-2 of glucosamine residue (Fig. 1). The intense band at 4.8–5.30 ppm is related to OH groups and HDO (solvent). In this region, as observed more clearly from an extended spectrum, some different anomeric protons (H-1 of glucosamine (GlcN) and glucosamine-*N*-acetyl (GlcNAc) units) are appeared at 4.88–5.00 ppm. The chemical structure and the molecular fraction (MF) of GlcN unit (degree of deacetylation, DDA) and MF of *N*-(alkyl) glucosamine unit (degree of substitution, DS) were estimated by ^{1}H NMR spectra in according to the method of Hirai et al.,[24] and Sashiwa and

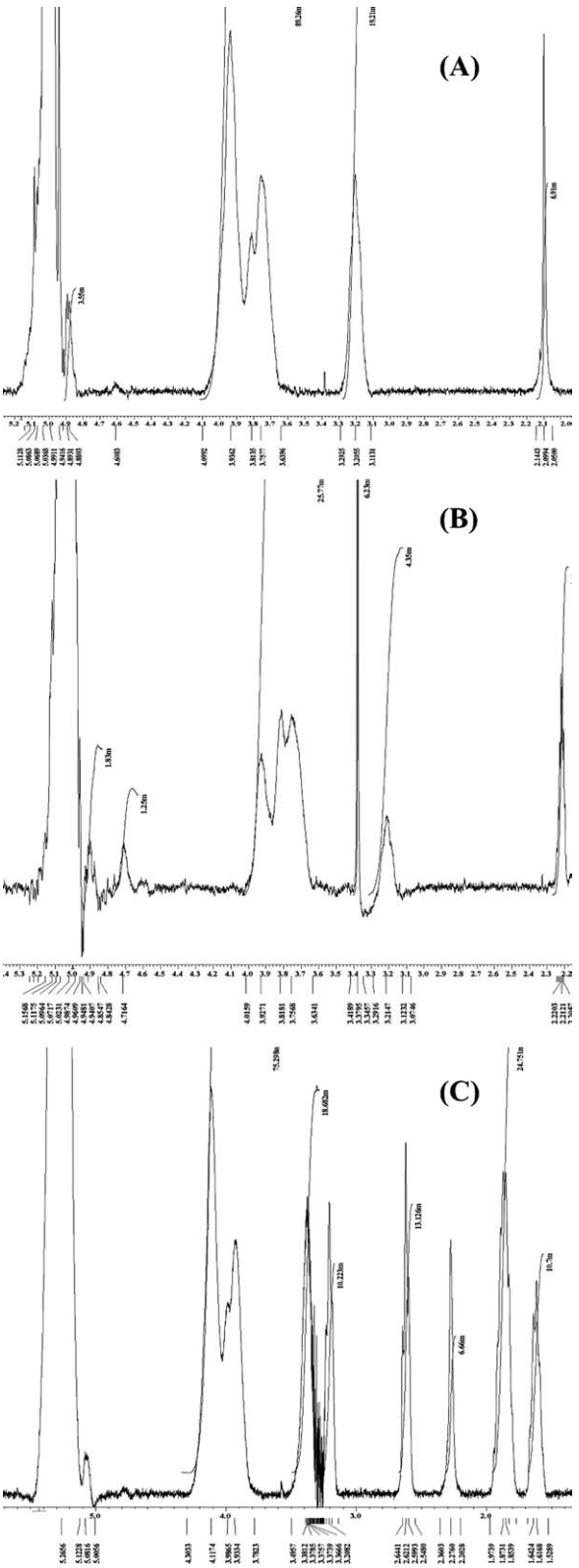


Figure 1 ^{1}H-NMR spectra of chitosan (**A**), *N,N,N*-(trimethyl) chitosan (**B**) and *N,N,N*-(dimethyl hexyl) chitosan (**C**) in 0.5% CD_3COOD/D_2O at 25°C.

Shigemasa,[25] and the data are presented in Table I. DDA of chitosan was calculated to be 0.90 in chitosan from the integral ratio between the proton on the C-2 and the GlcN unit protons and were ranged from 0.64 to 0.75 for *N*-(dimethyl alkyl) chitosans. DS values based on the ratio of the areas of the protons in the alkyl substituent and the protons of the pyranose unit ranged from 0.14 to 0.26. A high DS (0.26) was obtained by a reaction of chitosan with valeraldehyde (pentaldehyde) whereas a DS of 0.14 was obtained by a reaction with butraldehyde.

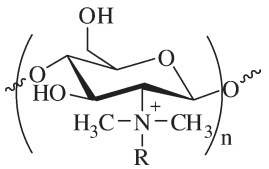
The IR spectral data of chitosan and quaternized chitosan derivatives are shown in Figure 2. The IR spectrum of chitosan powder [Fig. 2(A)] shows characteristic absorption bands at 3427, 2920, and 2855 cm^{-1}, which represent the presence of OH group, CH_2 and CH_3 groups, respectively. The amino group has a characteristic absorption band in the region of 3250- 3500 cm^{-1}, which must have been masked by the absorption band due to the OH group.[26,27] The >C=O stretching (amide I) peak at 1656 cm^{-1} representing the structure of GlcNAc, as well as the NH_2 stretching (amide II) peak at 1579 cm^{-1} representing the GlcN functional group, appeared in the spectrum of chitosan powder. A region of 1375–1414 cm^{-1} representing the structure of methyl groups in GlcNAc units. In addition, the absorption band in the region of 890–1150 cm^{-1} assigned to the saccharide structure.[28] In the spectrum of quaternary chitosan compounds, absorption peak by amino group at 1597 cm^{-1} [Fig. 2(B)] considerably decreased due to the reaction of amino group with aliphatic aldehydes and methyl iodide, and a new peak by the methyl groups appeared at about 1450–1480 cm^{-1}.[11,23] These results demonstrate that the quaternized chitosan derivatives were obtained.

The solubility of these compounds in water is an interesting parameter in this study. As Table I showed, chitosan could not dissolve in water but it was completely soluble in aqueous acetic acid (0.5%, v/v). Moreover, quaternary chitosan compounds were also soluble in aqueous acetic acid. *N,N,N*-(trimethyl) chitosan (1) was highly soluble in water and *N,N,N*-(dimethyl alkyl) chitosans were also water-soluble but the solubility was decreased with the introduction of an alkyl chain, which is hydrophobic group. *N,N,N*-(dimethyl heptyl) chitosan (5) and *N,N,N*-(dimethyl octyl) chitosan (6) formed a gel. Experiment results indicated that chitosan became a water-soluble polyelectrolyte with a high charge density after a quaternization process.

Antibacterial activity of chitosan derivatives

The data of the *in vitro* antibacterial activity of chitosan and *N,N,N*-(dimethyl alkyl) chitosan compounds against *A. tumefaciens* and *E. carotovora* are presented in Table II. The results indicated that the quaternized chitosan compounds showed higher inhibition than chitosan (MIC was higher than 3000 mg/L).

TABLE I
Chemical Structures and Characterization Data of Chitosan and Quaternary *N*-alkyl Chitosan Derivatives



Compound	*R*	DDA[a]	DS[b]	Solubility in water	Solubility in 0.5 % (v/v) aqueous acetic acid
Chitosan	–	0.90	–	–	+++
1	CH_3	–	–	+++	+++
2	$CH_3(CH_2)_3$	0.75	0.14	++	+++
3	$CH_3(CH_2)_4$	0.64	0.26	++	+++
4	$CH_3(CH_2)_5$	0.64	0.24	++	+++
5	$CH_3(CH_2)_6$	0.70	0.18	+	+++
6	$CH_3(CH_2)_7$	0.73	0.18	+	+++

[a] Degree of deacetylation (DDA) was calculated from the integral ratio between the proton on C−2 and the glucose unit protons.
[b] Degree of substitution (DS) was calculated from the ratio of the areas of the protons in alkyl substituent and the protons of the pyranose unit. − Insoluble, + low soluble (form a gel), ++ moderate soluble (viscous solution), +++ high soluble (clear solution).

This would suggest that an increase in the solubility of chitosan molecule and a substitution with alkyl groups led to an increase in the antibacterial activity against the tested bacteria. The quaternized chitosan compounds showed antibacterial activities against not only *A. tumefaciens* but also *E. carotovora*, which were used in the test, although differences existed among them. *N,N,N*-(dimethyl pentyl) chitosan (3) had much a better antibacterial activity among the derivatives, whose MIC values were 750 and 1225 mg/L for *A. tumefaciens* and *E. carotovora*, respectively. It was also noticed that the introduction of alkyl groups especially pentyl (3) and hexyl (4) with quaternized group to the chitosan molecule greatly enhanced the antibacterial activity. In contrast, the activity was slightly reduced with an increase in the alkyl chain (higher than C5), where *N,N,N*-(dimethyl octyl) chitosan (6) was the lowest active one (MIC = 2300 and 2580 mg/L for *A. tumefaciens* and *E. carotovora*, respectively)

When we consider the susceptibility of the microorganisms, another point deserves attention; the compounds had more effective inhibition on *A. tumefaciens* than *E. carotovora*. Moreover, the activity of chitosan against these bacteria still low (MIC > 3000 mg/L) compared with that obtained in the literatures with other bacteria such as *Staphylococcus aureus* and *Escherichia coli*.[10,11,29] The fact may be attributed to the cell wall of *A. tumefaciens* and *E. carotovora*, which are typical Gram-negative bacteria. The cell wall of which is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein, and phospholipids. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules. Sajomsang et al.,[30] synthesized quaternized *N*-(4-*N,N*-dimethylaminobenzyl) chitosan and quaternized *N*-(4-pyridylmethyl) chitosan and the antibacterial activity was determined using *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) bacteria. They found that the MIC of these derivatives ranged from 32 to 128 μg/mL. They also reported that the presence of the *N,N*-dimethylaminobenzyl and *N*-pyridylmethyl substituents on chitosan backbone after methylation did not enhance the antibacterial activity against *S. aureus*. However, *N*-(4-*N,N*-dimethylaminobenzyl) chitosan with degree of quaternization of 17% and 16–30% at the aromatic substituent and the primary amino group of chitosan, respectively, exhibited a slightly increased antibacterial activity against *E. coli*.

In the present study, the antibacterial mechanisms of these derivatives suggested to being on one hand, the positive charge of the group at C-2 resulted in a polycationic structure which can be expected to interact with the predominantly anionic components (lipopolysaccharides, proteins) of the microorganisms surface as previously investigated by Helander et al.[31] and Sudarshan et al.[32] Helander et al.[33] added that the interaction resulted in great alteration of the structure of outer membrane, which caused release of major proportion of proteinaceous material from the cells when the quaternized group was introduced onto the molecular chain, the positive charge was strengthened.

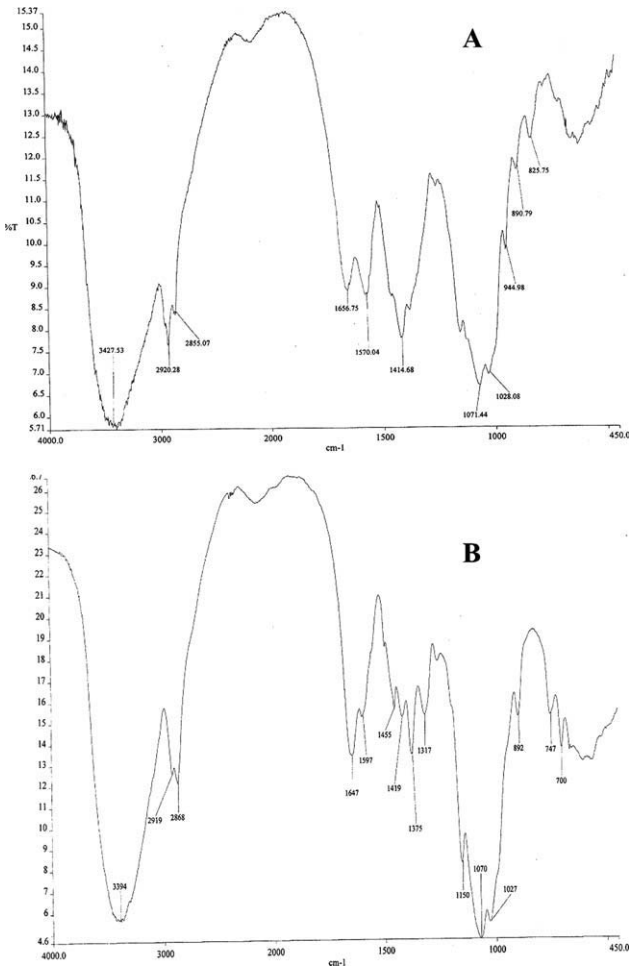


Figure 2 The IR spectra of chitosan and *N,N,N*-(dimethyl hexyl) chitosan (compound 4).

Antifungal activity of chitosan derivatives on mycelial growth of *B. cinerea, F. oxysporum,* and *P. debaryanum*

The *in vitro* antifungal activity of chitosan and its quaternized derivatives against the plant pathogenic fungi of grey mold *B. cinerea*, root rot disease *F. oxysporum,* and damping off disease *P. debaryanum* are presented in Figure 3 and Table III. As can be seen in Figure 3, chitosan slightly inhibited the growth of the tested fungi and the inhibition percentages were 49.26, 40.74, and 51.85 at 2000 mg/L for *B. cinerea, F. oxysporum,* and *P. debaryanum*, respectively compared to quaternized chitosans which showed a high inhibition (> 65%) of fungal growth at the same concentration (2000 mg/L) except of compound 5. In addition, fungal growth inhibition was increased significantly with an increase in the concentration. On the basis of the effective concentration that caused 50% inhibition of mycelial growth (EC_{50} with its 95% confidence limits), the results are shown in Table III to compare statistically between the compounds. As can be seen in the result, all the quaternized chitosan derivatives gave stronger antifungal activities fungi than the unmodified chitosan (EC_{50} higher than 3000 mg/L). The potential reason is higher charge densities of such derivatives. The result indicated that *N,N,N*-(dimethyl pentyl) chitosan (3) and *N,N,N*-(dimethyl octyl) chitosan (6) were significantly the highest in activity against *B. cinerea* (EC_{50} = 908 and 383 mg/L, respectively), *F. oxysporum* (EC_{50} = 871 and 812 mg/L, respectively), and *P. debaryanum* (EC_{50} = 624 and 440 mg/mL, respectively). In contrast, *N,N,N*-(dimethyl heptyl) chitosan (5) was the lowest active one with EC_{50} = 2161,

TABLE II
Antibacterial Activity of Chitosan and Quaternary *N*-alkyl Chitosan Derivatives Against *A. tumefaciens* and *E. carotovora*

Compound	MIC (mg/L)	
	A. tumefaciens	*E. carotovora*
Chitosan	>3,000	>3,000
1	2,425	2,680
2	1,750	2,380
3	750	1,225
4	850	1,380
5	2,175	2,460
6	2,300	2,580

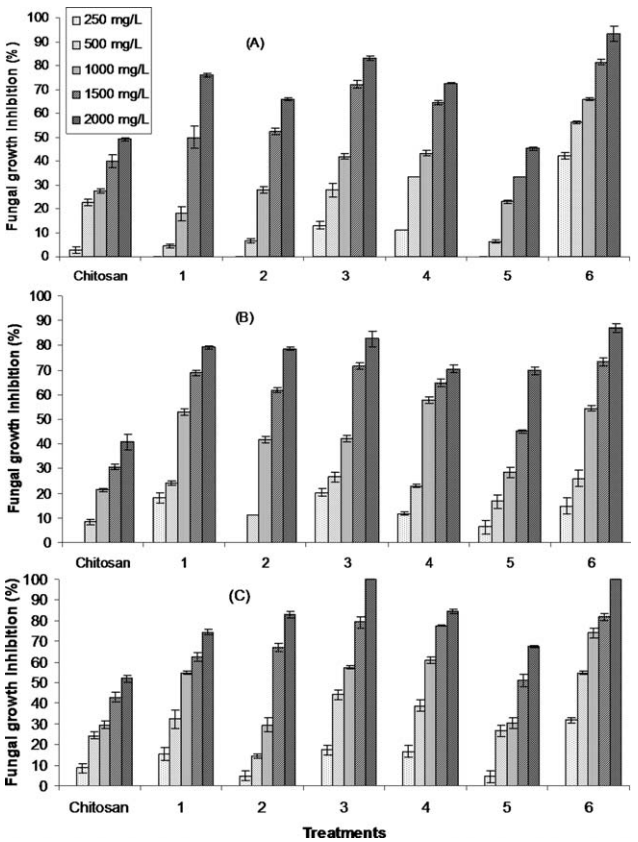


Figure 3 Inhibition of mycelial growth of *B. cinerea* (A), *F. oxysporum* (B), and *P. debaryanum* (C) by chitosan compounds at 250, 500, 1000, 1500 and 2000 mg/L. Data are averageof three replicates ±SE.

TABLE III
In vitro* Antifungal Activity of Chitosan and Quaternary *N*-alkyl Chitosan Derivatives Against *B. cinerea*, *F. oxysporum*, and *P. debaryanum

Compound	EC_{50} (mg/L)	95% confidence limits (mg/L)		Slope ± standard error (SE)	Intercept of regression line ± S.E	Chi square (χ^2)
		Lower	Upper			
			B. cinerea			
Chitosan	> 3000	-	-	-	-	-
1	1467	1358	1596	4.31 ± 0.42	−13.65 ± 1.32	4.99
2	1472	1339	1639	3.36 ± 0.33	−10.66 ± 1.02	0.83
3	908	625	1337	2.27 ± 0.21	−6.74 ± 0.61	8.42
4	1006	872	1172	1.90 ± 0.20	−5.71 ± 0.59	3.32
5	2161	1843	2720	2.51 ± 0.31	−8.37 ± 0.97	1.74
6	383	106	616	1.58 ± 0.19	−4.08 ± 0.55	7.95
			F. oxysporum			
Chitosan	> 3000	-	-	-	-	-
1	875	763	1005	2.02 ± 0.20	−5.94 ± 0.58	4.66
2	1184	1083	1297	3.48 ± 0.30	−10.70 ± 0.92	1.55
3	871	469	1663	1.97 ± 0.19	−5.80 ± 0.57	13.39
4	982	858	1130	2.04 ± 0.20	−6.11 ± 0.60	3.63
5	1485	1068	2677	2.16 ± 0.23	−6.84 ± 0.68	7.03
6	812	720	913	2.40 ± 0.21	−6.99 ± 0.61	4.65
			P. debaryanum			
Chitosan	> 3000	-	-	-	-	-
1	907	779	1059	1.79 ± 0.19	−5.31 ± 0.57	0.63
2	1138	793	1763	3.02 ± 0.25	−9.24 ± 0.77	12.65
3	624	256	1064	2.58 ± 0.21	−7.21 ± 0.62	20.77
4	696	606	791	2.17 ± 0.20	−6.17 ± 0.58	0.41
5	1374	929	2900	2.03 ± 0.22	−6.38 ± 0.65	9.16
6	440	164	688	2.21 ± 0.21	−5.83 ± 0.59	12.91

1485, and 1374 mg/L to *B. cinerea*, *F. oxysporum*, and *P. debaryanum*, respectively. *N,N,N*-(trimethyl) chitosan (1) had a good antifungal activity against *B. cinerea*, *F. oxysporum*, and *P. debaryanum* (EC_{50} = 1467, 875, and 907 mg/L, respectively) compared to *N,N,N*-(dimethyl butyl) chitosan (2) and *N,N,N*-(dimethyl heptyl) chitosan (5).

The conceivable mechanism for the antifungal action of quaternary chitosan is that the ammonium groups interact with the anionic groups on the microbial cell surface, which forms a layer around the cell and prevents nutrients from entering.[34] Bearing this in mind, it can be conclude that the activity of quaternized chitosans should increase by the ammonium ion density on the molecule. The results in the present work are in agreement with the results obtained by Guo et al.,[35] whose evaluated the antifungal properties of Schiff bases of chitosan with benzaldehyde, and salicylaldehyde and their quaternized derivatives against *B. cinerea* and *Colletotrichum lagenarium*. They found that quaternized chitosans had better inhibitory properties than chitosan, Schiff bases of chitosan and *N*-substituted chitosans. Therefore, the present study proposes that the antifungal activity of the quaternized chitosan derivatives could also be caused by the cationic charge that present in these derivatives, which are higher than that in chitosan as shown in Scheme 1.

Effect of chitosan derivatives on spore germination of *B. cinerea* and *F. oxysporum*

The biological activities of chitosan and its derivatives on spores of *B. cinerea* and *F. oxysporum* are presented in Tables IV and V, respectively. As can be seen that the spore germination was affected significantly at the tested concentrations and all the quaternized chitosan derivatives had a better inhibition compared to the control and the unmodified chitosan. Spores of *B. cinerea* were high sensitive to these compounds compared to *F. oxysporum*. Therefore, the test was done at concentrations of 50, 125, and 250 mg/L against *B. cinerea* spores (Table IV), whereas concentrations of 250, 500, and 1000 mg/L were selected to *F. oxysporum* spores (Table V). In *B. cinerea*, *N,N,N*-(dimethyl pentyl) chitosan (3) and *N,N,N*-(dimethyl hexyl) chitosan (4) markedly reduced spore germination with significant effects, which were found among concentrations and 96.54 and 97.08% inhibitions, respectively, were observed in spores treated with a 250 mg/L.

The activity of chitosan and its quaternized derivatives against spore germination of *F. oxysporum* was also increased with an increase in the chain length of alkyl substituent (from butyl to heptyl) and then slightly decreased with octyl group (6) as shown in Table V. *N,N,N*-(dimethyl hexyl) chitosan (4) and

TABLE IV
Effect of Chitosan and Quaternary *N*-alkyl Chitosan Derivatives on Spore Germination of *B. cinerea*

Treatment	Concentration (mg/L)	Spore germination (%) ± SE	Inhibition of spore germination (%) ± SE
Control	0	$73.50^{a} \pm 1.51$	$26.50^{i} \pm 1.51$
Chitosan	50	$65.33^{b} \pm 3.19$	$34.67^{h} \pm 3.19$
	125	$55.40^{cd} \pm 3.00$	$44.60^{fg} \pm 3.00$
	250	$39.17^{e} \pm 1.19$	$60.83^{e} \pm 1.19$
1	50	$64.09^{b} \pm 2.88$	$35.92^{h} \pm 2.88$
	125	$51.90^{cd} \pm 1.87$	$48.10^{fg} \pm 1.87$
	250	$35.75^{ef} \pm 1.70$	$64.25^{de} \pm 1.70$
2	50	$63.78^{b} \pm 3.24$	$36.23^{h} \pm 3.24$
	125	$49.29^{d} \pm 3.41$	$50.71^{f} \pm 3.41$
	250	$14.57^{h} \pm 1.23$	$85.43^{b} \pm 1.23$
3	50	$57.88^{bc} \pm 1.23$	$42.13^{gh} \pm 1.23$
	125	$29.74^{f} \pm 2.27$	$70.26^{d} \pm 2.27$
	250	$3.46^{i} \pm 2.19$	$96.54^{a} \pm 2.19$
4	50	$22.00^{g} \pm 1.78$	$78.00^{c} \pm 1.78$
	125	$8.75^{hi} \pm 1.39$	$91.25^{ab} \pm 1.39$
	250	$2.92^{i} \pm 1.85$	$97.08^{a} \pm 1.85$
5	50	$47.17^{d} \pm 2.35$	$52.83^{f} \pm 2.35$
	125	$34.60^{ef} \pm 1.94$	$65.41^{de} \pm 1.94$
	250	$8.90^{hi} \pm 1.12$	$91.10^{ab} \pm 1.12$
6	50	$48.61^{d} \pm 2.53$	$51.39^{f} \pm 2.53$
	125	$35.06^{ef} \pm 1.50$	$64.94^{de} \pm 1.50$
	250	$9.79^{hi} \pm 1.23$	$90.21^{ab} \pm 1.23$

Data are average of four 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.

TABLE V
Effect of Chitosan and Quaternary *N*-alkyl Chitosan Derivatives on Spore Germination of *F. oxysporum*

Treatment	Concentration (mg/L)	Spore germination (%) ± SE	Inhibition of spore germination (%) ± SE
Control	0	$69.00^{a} \pm 1.66$	$31.00^{i} \pm 1.66$
Chitosan	250	$58.78^{b} \pm 1.43$	$41.23^{h} \pm 1.43$
	500^{cd}	$47.15^{cd} \pm 1.26$	$52.85^{fg} \pm 1.26$
	1000	$41.20^{de} \pm 1.08$	$58.80^{ef} \pm 1.08$
1	250	$50.93^{c} \pm 2.55$	$49.08^{g} \pm 2.55$
	500	$44.15^{cde} \pm 1.18$	$55.85^{efg} \pm 1.18$
	1000	$37.75^{e} \pm 1.03$	$62.25^{e} \pm 1.03$
2	250	$44.25^{cde} \pm 2.78$	$55.75^{efg} \pm 2.78$
	500	$31.90^{f} \pm 1.89$	$68.10^{d} \pm 1.89$
	1000	$17.33^{hi} \pm 1.61$	$82.68^{ab} \pm 1.61$
3	250	$47.75^{cd} \pm 0.85$	$52.25^{fg} \pm 0.85$
	500	$28.30^{fg} \pm 2.60$	$71.70^{cd} \pm 2.60$
	1000	$16.98^{hi} \pm 0.78$	$83.02^{ab} \pm 0.78$
4	250	$45.08^{cde} \pm 2.13$	$54.93^{efg} \pm 2.13$
	500	$20.83^{h} \pm 1.93$	$79.18^{b} \pm 1.93$
	1000	$12.83^{i} \pm 2.28$	$87.18^{a} \pm 2.28$
5	250	$44.05^{cde} \pm 2.06$	$55.95^{efg} \pm 2.06$
	500	$20.75^{h} \pm 1.55$	$79.25^{b} \pm 1.55$
	1000	$11.78^{i} \pm 2.15$	$88.23^{a} \pm 2.15$
6	250	$44.20^{cde} \pm 1.29$	$55.80^{efg} \pm 1.29$
	500	$23.72^{gh} \pm 0.91$	$76.28^{bc} \pm 0.91$
	1000	$18.50^{hi} \pm 1.01$	$81.50^{ab} \pm 1.01$

Data are average of four 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.

N,N,N-(dimethyl heptyl) chitosan (5) were the highest significantly in inhibition of spore germination at 500 mg/L (79.18 and 79.25% inhibition, respectively) and 1000 mg/L (87.18 and 88.23% inhibition, respectively).

El Ghaouth et al.,[19] found that a chitosan at concentrations ranged from 750 to 6000 mg/L was very effective in inhibiting spore germination and germ tube elongation of *B. cinerea* and *Rhizopus stolonifer*. Furthermore, this biopolymer at a concentration greater than 1500 mg/L induced morphological changes in *R. stolonifer*. Hernández-Lauzardo et al.,[36] added that the spore germination of *R. stolonifer* was affected by different molecular weights chitosan (1.74×10^4, 2.38×10^{-4} and 3.07×10^{-4} g/mol). They found that chitosan of 1.74×10^{-4} and 2.38×10^{-4} g/mol markedly reduced spore germination, but there no significant effects were found among the tested concentrations (1.0, 1.5, and 2.0 mg/L). However, they observed that a complete inhibition of spore germination with a chitosan of 3.07×10^{-4} g/mol.

CONCLUSION

The present study describes the antibacterial and antifungal effects of low molecular weight chitosan and its quaternized derivatives against bacteria of *A. tumefaciens* and *E. carotovora* and fungi of *B. cinerea*, *F. oxysporum* and *P. debaryanum* as the most economic plant pathogens. The quaternized chitosans were found to be much effective than chitosan because of their high polycationic charge densities. It was appeared that quaternized chitosans at the applied concentrations exhibited a wide range of antibacterial and antifungal activity *in vitro*, affecting different plant pathogens. *N,N,N*-(dimethyl pentyl) chitosan was the most effective one against *A. tumefaciens* and *E. carotovora* with MIC of 750 and 1225 mg/L, respectively. However *N,N,N*-(dimethyl octyl) chitosan was the highest significantly in mycelial growth inhibiation of *B. cinerea*, *F. oxysporum*, and *P. debaryanum* (EC_{50} = 383, 812 and 440 mg/L, respectively). This research work also describes the complex effect of chitosan compounds on fungal spore germination and exhibited a wide range of inhibition effect against *B. cinerea* and *F. oxysporum*. These results demonstrated that the chemical modification of chitosan led to an enhancement of the biological activity against plant pathogens. The results suggest that quaternized chitosans as water-soluble compounds can be used to control a number of some plant pathogenic bacteria and fungi that cause destruction of crops and vegetables. Thus such compounds have potential applications in agro-industry and as alternatives for disease control instead of some harmful pesticides.

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