Carbohydrate Polymers 87 (2012) 250–256

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Characterization and antimicrobial activity of water-soluble *N*-(4-carboxybutyroyl) chitosans against some plant pathogenic bacteria and fungi

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ARTICLE INFO

Article history:
Received 28 April 2011
Received in revised form 21 July 2011
Accepted 25 July 2011
Available online 3 August 2011

Keywords:
Chitosan
N-(4-Carboxybutyroyl) chitosan derivatives
^{1}H and ^{13}C NMR spectroscopy
Degree of substitution
Antimicrobial activity

ABSTRACT

Water-soluble *N*-(4-carboxybutyroyl) chitosan derivatives with different degrees of substitution (DS) were synthesized to enhance the antimicrobial activity of chitosan molecule against plant pathogens. Chitosan in a solution of 2% aqueous acetic acid–methanol (1:1, v/v) was reacted with 0.1, 0.3, 0.6 and 1 mol of glutaric anhydride to give *N*-(4-carboxybutyroyl) chitosans at DS of 0.10, 0.25, 0.48 and 0.53, respectively. The chemical structures and DS were characterized by ^{1}H and ^{13}C NMR spectroscopy, which showed that the acylate reaction took place at the *N*-position of chitosan. The synthesized derivatives were more soluble than the native chitosan in water and in dilute aqueous acetic acid and sodium hydroxide solutions. The antimicrobial activity was *in vitro* investigated against the most economic plant pathogenic bacteria of *Agrobacterium tumefaciens* and *Erwinia carotovora* and fungi of *Botrytis cinerea*, *Pythium debaryanum* and *Rhizoctonia solani*. The antimicrobial activity of *N*-(4-carboxybutyroyl) chitosans was strengthened than the un-modified chitosan with the increase of the DS. A compound of DS 0.53 was the most active one with minimum inhibitory concentration (MIC) of 725 and 800 mg/L against *E. carotovora* and *A. tumefaciens*, respectively and also in mycelial growth inhibiation against *B. cinerea* (EC_{50} = 899 mg/L), *P. debaryanum* (EC_{50} = 467 mg/L) and *R. solani* (EC_{50} = 1413 mg/L).

1. Introduction

Chitosan is a copolymer of glucosamine and *N*-acetylglucosamine units linked by 1,4-glucosidic bonds and it is obtained through the alkaline hydrolysis of chitin (No & Meyers, 1997). Owing to its high biodegradability and nontoxicity to mammals, chitosan is widely used as an antimicrobial agent either alone or blended with other natural polymers (El Hadrami, Lorne, Adam, El Hadrami, & Daayf, 2010; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). The antimicrobial activity of chitosan against a variety of bacteria and fungi coming from its polycationic nature is well known (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Muzzarelli et al., 1990; Muzzarelli, Muzzarelli, Tarsi, Miliani, & Cartolari, 2001; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Sudarshan, Hoover, & Knorr, 1992; Wang & Wang, 2011; Xia, Liu, Zhang, & Chen, 2011). However, this activity is limited above pH ~ 6.5, where chitosan start to lose its cationic nature and become poor soluble (Liu, Guan, Yang, Li, & Yao, 2001; Rabea et al., 2003). So the water solubility is an important factor in applications of chitosan as an antimicrobial agent, and many researchers have focused on the preparation of derivatives soluble in water over a wide pH range (Lim & Hudson, 2003). Therefore, special attention was paid to chemical modification of chitosan and several derivatives with high solubility in water have been prepared such as *O*-, *N*-(carboxymethyl) chitosan (Muzzarelli & Tanfani, 1982), quaternary ammonium salts (Badawy, 2010; Jia, Shen, & Xu, 2001; Muzzarelli & Tanfani, 1985), *N*-(sulphate) chitosan (Holme & Perlin, 1997), *O*-(butyryl) chitosan (Grant, Blair, & Mckay, 1988), *N*-(methylenephosphonic) chitosan (Heras, Rodriguez, Ramos, & Agullo, 2001; Ramos et al., 2003) and *N*,*O*-(succinyl) chitosan (Zhang, Ping, Zhang, & Shen, 2003). Moreover, chitosan derivatives containing carboxyl groups such as *N*,*O*-(carboxyalkyl and aryl) chitosans, *N*,*N*-(dicarboxyethyl) chitosan and *N*-(carboxyacyl) chitosans have been reported as water soluble compounds (Hirano & Moriyasu, 1981, 2004; Kurita, Ichikawa, Ishizeki, Fujisaki, & Iwakura, 1982; Shigemasa et al., 1995; Yamaguchi, Arai, & Itoh, 1982).

Therefore, we wish to report in this paper the preparation of water-soluble *N*-(4-carboxybutyroyl) chitosan derivatives to enhance the antimicrobial activity of chitosan molecule against the most economic plant pathogenic bacteria of crown gall disease *Agrobacterium tumefaciens* and soft mold disease

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doi:10.1016/j.carbpol.2011.07.054

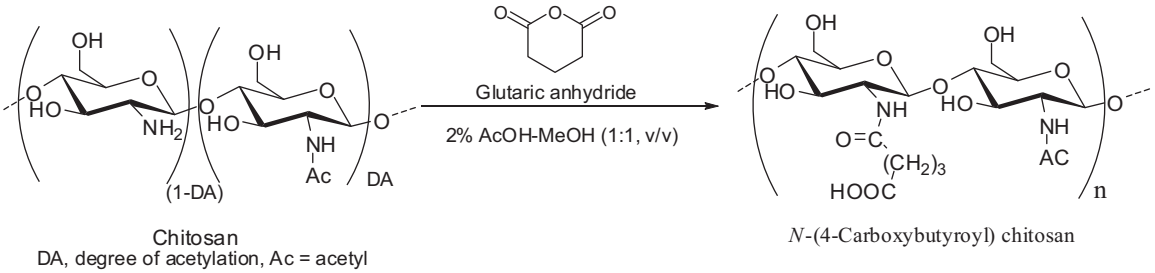


Fig. 1. Synthetic scheme of *N*-(4-carboxybutyroyl) chitosan derivatives using different mol ratios of glutaric anhydride.

Erwinia carotovora and fungi of grey mold *Botrytis cinerea*, damping off *Pythium debaryanum* and *Rhizoctonia solani*.

2. Materials and methods

2.1. Materials and tested microorganisms

Chitosan (viscosity average molecular weight 3.60×10^5 g/mol, and 89% degree of deacetylation), glutaric anhydride, deuterium oxide and deuterated acetic acid were purchased from Sigma–Aldrich Co. (USA). Potato dextrose agar (PDA), nutrient broth (NB) and nutrient agar (NA) media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). All materials were used without further purification. Bacteria of crown gall disease *A. tumefaciens* (Family: Rhizobiaceae; Class: Alpha Proteobacteria) and soft mold disease *E. carotovora* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria) and fungi of grey mold *B. cinerea* (Family: moniliaceae; Class: Deuteromycetes), damping off *P. debaryanum* (Family: Pythiaceae; Class: Oomycetes) and *R. solani* (Family: Ceratobasidiaceae; Class: Agaricomycetes) were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt.

2.2. Synthesis of N-(4-carboxybutyroyl) chitosans

N-(4-Carboxybutyroyl) chitosan derivatives were synthesized according to the method of Hirano and Moriyasu (1981) as follows: a portion (1.7 g) of chitosan was dissolved in 50 mL of 2% aqueous acetic acid (1%, v/v), and the solution was diluted with methanol (50 mL). To this solution, glutaric anhydride was added (0.1, 0.3, 0.6 and 1.0 mol/glucose amine (GlcN) unit) (Fig. 1). The mixture was stirred at ca. 50 °C for about 5 min, and kept at room temperature overnight. The mixture which became to be in gel or solidified form was washed with acetone to give pale yellow solid of *N*-(4-carboxybutyroyl) chitosans.

2.3. NMR spectroscopy

^{1}H and ^{13}C 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 chitosan sample was introduced into 5 mm Φ NMR tube, to which 0.5 mL of 1% CD_3COOD/D_2O solution was added, and finally the tube was kept at room temperature to dissolve the polymer.

2.4. ^{1}H and ^{13}C NMR spectral data

2.4.1. Spectral data for chitosan

^{1}H NMR (25 °C): δ 2.09–2.12 (br s, NHCO$\underline{C}H_3$), 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), 4.88–5.00 (m, H-1 of GlcN and GlcNAc units). ^{13}C NMR (25 °C): δ 22.08 (NH(CO)$\underline{C}H_3$), 56.59 (C-2), 61.02–61.28 (C-6), 70.59–70.76 (C-3), 75.15–75.44 (C-5), 77.78 (C-4), 98.24 (C-1), and 174.79 ($\underline{C}$(O)CH_3).

2.4.2. Spectral data for N-(4-carboxybutyroyl) chitosan derivatives (compounds **1**–**4**)

^{1}H NMR (25 °C): δ 1.90–1.98 (m, CH_2 (β)), 2.10 (br s, NHAc), 2.34–2.55 (m, CH_2 (α and γ)), 3.21 (br s, H-2 of GlcN residue), 3.50–4.19 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.62 (br s, H-1 of GlcNAc residue), 4.91 (br s, H-1 of GlcN residue). ^{13}C NMR (25 °C): δ 20.91 (CH_2 (β)), 22.75 (NH(CO)$\underline{C}H_3$), 30.50 (CH_2 (α)), 35.37 (CH_2 (γ)), 56.34 (C-6), 60.97 (C-2), 70.50 (C-3), 75.25 (C-5), 75.76 (C-4), 97.80 (C-1), 175.13 ($\underline{C}$(O)CH_3), 176.79 ($\underline{C}$OOR), and 178.08 ($\underline{C}$OOH).

2.5. The solubility test

The solubility of chitosan and *N*-(4-carboxybutyroyl) chitosan derivatives was examined in water, aqueous acetic acid (0.1, 0.5 and 1%, v/v) and 1% NaOH (w/v). A sample was soaked in each solvent at the concentration of 10 mg/mL and the solubility was checked after standing for 24 h at room temperature (Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998).

2.6. The antibacterial assay

The antibacterial activity of chitosan and its derivatives was assayed using NA dilution method (EUCAST, 2000) against *A. tumefaciens* and *E. carotovora*. For determination of minimum inhibitory concentration (MIC), the chitosan and its derivatives were dissolved in 0.5% aqueous acetic acid and 0.01%, respectively and were added to NA medium. The pH was adjusted to 5.5–6.0 with 1 M NaOH and the solutions were then poured into autoclaved Petri dishes. Parallel controls were maintained with water and aqueous acetic acid mixed with NA medium. One loopful of microorganism suspensions in NB medium (≈6 μL, obtained by shaking at 37 °C till late exponential phase, $O.D._{550} = 0.9$) was spotted on the surface of NA medium (10 spots per plate) then incubated at 37 °C 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.

2.7. The antifungal assay

The antifungal activity of chitosan compounds on the mycelial growth of *B. cinerea*, *P. debaryanum* and *R. solani* was tested using a radial growth technique (El-Ghaouth, Arul, Grenier, & Asselin, 1992). The compounds were dissolved as described in the antibacterial assay and serial concentrations of 250, 500, 1000, 1500, 2000, 2500 and 3000 mg/L were, respectively, added to 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 in 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. 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:

$$\text{Mycelial growth inhibition}\,(\%) = \frac{DC - DT}{DC} \times 100$$

where DC and DT are average diameters of fungal colonies of control and treatment, respectively. Effective concentration that caused 50% inhibition of a mycelial growth (EC_{50}) and its corresponding 95% confidence limits were estimated by probit analysis (Finney, 1971).

2.8. 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 (Finney, 1971). 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.

3. Results and discussion

3.1. Characterization of N-(4-carboxybutyroyl) chitosans

Chitosan was *N*-carboxyacylated at different degrees of substitution by its treatment with 0.1, 0.3, 0.6 and 1.0 mol of glutaric anhydride per glucose amine (GlcN) unit in a solution of 2% aqueous acetic acid–methanol (1:1, v/v). A hydrogel was produced in the reaction mixture with each of compounds **1**, **2** and **3** however, a white precipitate in that with compound **4**. As the results, *N*-(4-carboxybutyroyl) chitosans with DS = 0.10, 0.25, 0.48 and 0.53 were isolated with 79.28, 83.75, 93.75 and 93.93% yield, respectively. The chemical structure of the compounds, degree of deacetylation (DDA) and degree of substitution (DS) were estimated by ^{1}H NMR spectra according to the method of Hirai, Odani, and Nakajima (1991) and Sashiwa and Shigemasa (1999) and the data are presented in Table 1. The peak at δ 2.0–2.10 ppm was assigned to the proton of residual CH_3 in acetyl group. In chitosan spectrum (Fig. 2A), the peak at δ 3.20 ppm was attributed to H-2 of glucosamine residue. 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) appeared at 4.88–5.00 ppm. The DDA was calculated to be 89% in chitosan (Table 1) from the integral ratio between the proton on the C-2 and the GlcN unit protons. However, DDA of *N*-(4-carboxybutyroyl) chitosans ranged from 0.37 to 0.81. Determination of the DS value was based on the ratio between the areas of the protons in the alkyl substituent and the protons of the pyranose unit. The calculated DS values were 0.10, 0.25, 0.48 and 0.53 for compounds **1**, **2**, **3** and **4**, respectively. This result demonstrates that the increasing of the DS value resulting in the decreasing of the DDA, which confirms that the reaction mainly occurred on the amino group on C-2 of the GlcN (Fig. 2B–E). The formula weights (FW) of chitosan and its derivatives were calculated using the following equation depending on the DA, DDA and DS (Badawy et al., 2004; Sashiwa & Shigemasa, 1999).

$$FW = 161 \times DDA + 203 \times DA + \text{MW of } N\text{-(4-carboxybutyroyl) glucosamine unit} \times DS$$

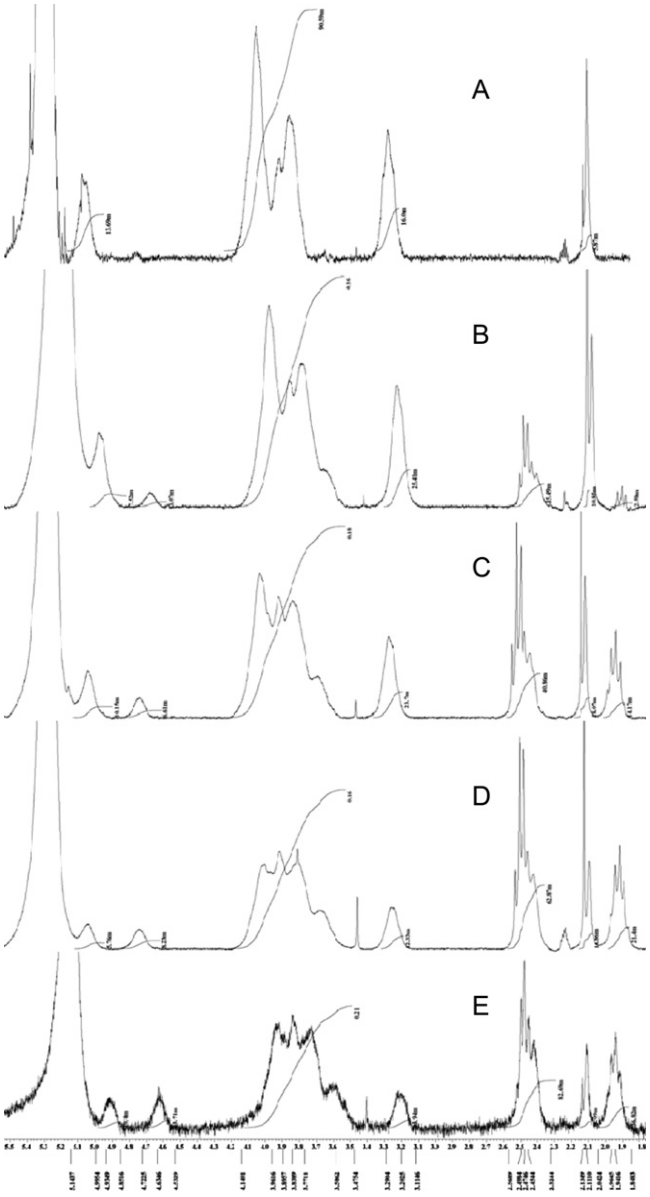
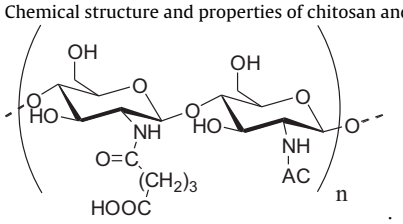


Fig. 2. 500 MHz ^{1}H NMR spectra of chitosan (A) and *N*-(4-carboxybutyroyl) chitosan derivatives with DS of 0.10 (B), 0.25 (C), 0.48 (D) and 0.53 (E) in 1% CD_3COOD/D_2O at 25 °C.

where 161 and 203 are the FW of GlcN unit and GlcNAc, respectively. The results indicate that the FW was increased with increasing of the mol ratio between glutaric anhydride and chitosan molecule. FW for chitosan, **1**, **2**, **3** and **4** were 167.35, 175.57, 192.75, 219.70 and 225.20, respectively (Table 1). The average molecular weight of the compounds that depending on the FW was ranged from 3.78 to 4.84×10^5 g/mol. Further evidence for confirmation the chemical structure was obtained from ^{13}C NMR spectroscopy. The carbon peaks due to C=O and CH_3 were found at 174.97 and 22.08 ppm in chitosan, respectively. When we grafted carboxybutyroyl in the amino group of chitosan, new chemical shifts at 176.79 and 178.08 ppm appeared due to the carbonyl group (C=O) and carboxyl group (COOH) in carboxybutyroyl moiety, respectively. Strong and intense peaks at 20.91, 30.50 and 35.37 ppm were obtained due to the presence of methyl carbon units in the carboxybutyroyl moiety [CH_2 (β), CH_2 (α) and CH_2 (γ), respectively]. In addition, assignments of ^{1}H and ^{13}C NMR spectra of *N*-(4-carboxybutyroyl)

Table 1
Chemical structure and properties of chitosan and *N*-(4-carboxybutyroyl) chitosan derivatives



Compound	GA[a]/mol GlcN[b]	DDA[c]	DA[d]	DS[e]	FW[f]	MW[g] (g/mol) × 10^5	Yield[h] (%)
Chitosan	–	0.89	0.11	–	167.35	3.60	–
1	0.1	0.81	0.09	0.10	175.57	3.78[i]	79.28
2	0.3	0.68	0.07	0.25	192.75	4.15[i]	83.75
3	0.6	0.43	0.09	0.48	219.70	4.73[i]	93.75
4	1.0	0.37	0.10	0.53	225.20	4.84[i]	93.93

[a] Glutaric anhydride.
[b] Glucosamine.
[c] Degree of deacetylation.
[d] Degree of acetylation (calculated as: 1 – DDA).
[e] Degree of substitution.
[f] Formula weight.
[g] Molecular weight in g/mol and it was determined for chitosan by measuring its intrinsic viscosity according to the Mark–Houwink–Sakurada equation (Wang, Bo, Li, & Qin, 1991).
[h] Yield was determined by weight recovery in accordance with the change in FW (Badawy et al., 2004; Sashiwa & Shigemasa, 1999).
[i] MW of compounds **1**–**4** was calculated as follows: MW = (FW of the compound × MW of chitosan)/FW of chitosan.

chitosan were also investigated by two-dimensional homonuclear and heteronuclear chemical shift correlation spectroscopy (Fig. 3).

Solubility of chitosan and *N*-(4-carboxybutyroyl) chitosan derivatives (**1**–**4**) was evaluated in water, aqueous acetic acid solutions (0.1, 0.5 and 1%, v/v) and aqueous sodium hydroxide solution (1%, w/v). The data in Table 2 show that the derivatives were more soluble than chitosan in water and diluted aqueous acetic acid solutions. These derivatives also showed solubility in basic media (sodium hydroxide solution). This refers to the presence of carboxyl group in the resulted molecule. Compounds **1**, **2** and **3** were gel formed in water, whereas compound **4** was swelling in water

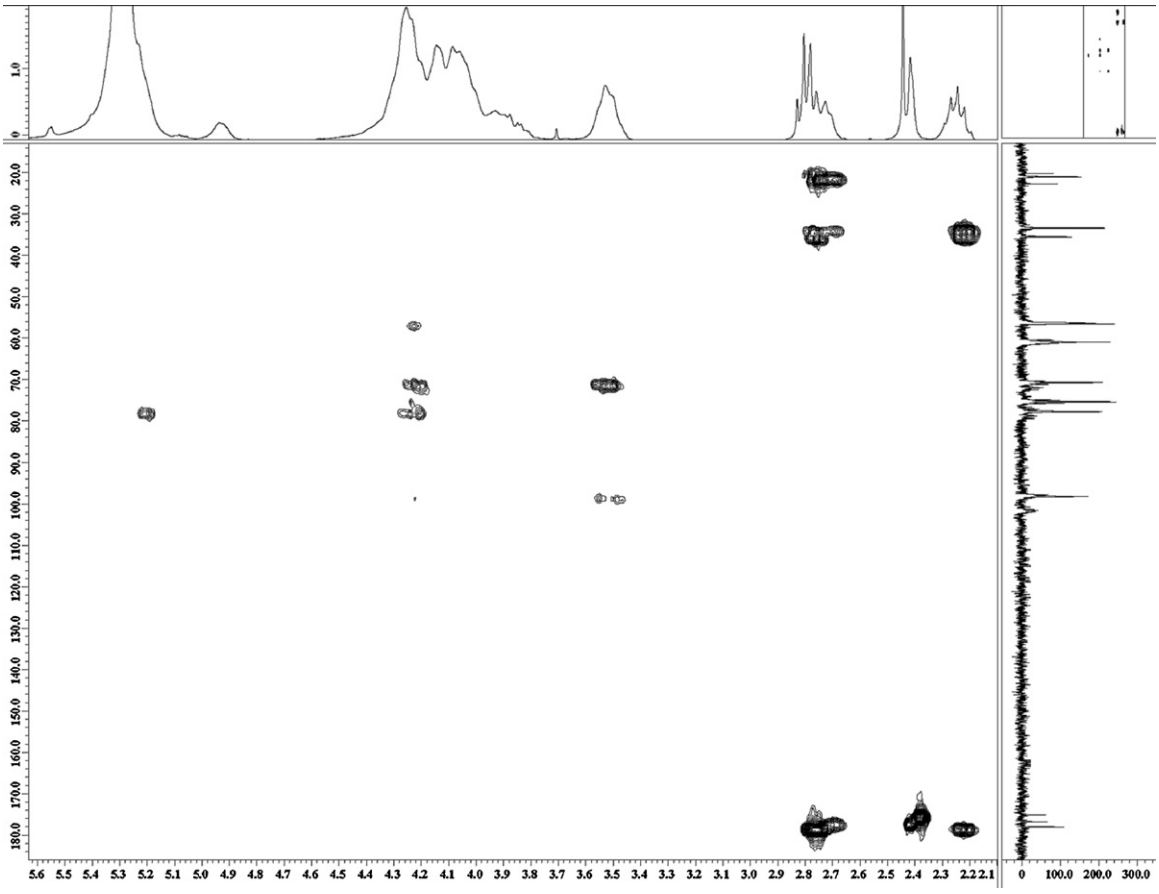


Fig. 3. ^{13}C–^{1}H heteronuclear chemical shift correlation spectrum of *N*-(4-carboxybutyroyl) chitosan (compound **2**) in 1% CD_3COOD/D_2O at 25 °C.

Table 2
Solubility of chitosan and *N*-(4-carboxybutyroyl) chitosan derivatives.

Compound	Solubility[a]				
	H_2O	CH_3COOH (0.1%)	CH_3COOH (0.5%)	CH_3COOH (1%)	NaOH (1%)
Chitosan	i	sw	s	s	i
1	g	g	s	s	sw
2	g	s	s	s	g
3	g	s	s	s	g
4	sw	g	g	g	g

[a] The solubility was evaluated at the concentration of 10 mg/mL in each solvent and was checked after standing for 24 h at room temperature. i, insoluble; sw, swelling; g, gel; and s, soluble.

Table 3
The *in vitro* antibacterial activity of chitosan and *N*-(4-carboxybutyroyl) chitosan derivatives against *A. tumefaciens* and *E. carotovora*.

Compound	MIC[a] (mg/L)	
	A. tumefaciens	*E. carotovora*
Chitosan	>3000	>3000
1	1200	1100
2	1150	1050
3	900	800
4	800	725

[a] Minimum inhibitory concentration.

and formed gel in aqueous acetic acid (0.1, 0.5 and 1%) sodium hydroxide solutions.

Acylation of chitosan was the usual method involves reacting chitosan under homogeneous reaction conditions with either an acid chloride or acid anhydride (Hirano & Moriyasu, 1981, 2004; Kurita, Mori, Nishiyama, & Harata, 2002; Satoh, Vladimirov, Johmen, & Sakairi, 2003). It was shown to proceed smoothly at the free amino groups preferentially and then more slowly at the hydroxyl groups. Acylation with acid chlorides was reported to take place on both *O*- and *N*-positions under a large excess of acid chloride (Badawy et al., 2004; Sashiwa et al., 2002). The major concern in this method is a chemical degredation of chitosan molecule as a result of dissolving it in $MeSO_3H$ that give low molecular weight (Sashiwa et al., 2002). However, we used in the present study a safely acylation method that preserve the molecular weight of chitosan. Furthermore, this acylation method led to increase in the water solubility of the product as a result of carboxyl group formed.

3.2. The antibacterial activity of chitosan derivatives

The data of the *in vitro* antibacterial activity of chitosan and *N*-(4-carboxybutyroyl) chitosan compounds against *A. tumefaciens* and *E. carotovora* are presented in Table 3. The results indicated that the derivatives showed higher inhibition than chitosan. This would suggest that an increase in the solubility of chitosan molecule and a substitution with carboxyacyl group led to an increase in the antibacterial activity. Compounds markedly inhibited growth of both bacteria with MIC values between 725 and 1200 mg/L. The inhibitory effects differed with regard to the DS, MW and the type of the bacteria. Increase in the DS and MW led to an increase in the activity where *N*-(4-carboxybutyroyl) chitosan of 0.53 DS (MW = 4.84×10^5 g/mol) displayed the highest inhibition activity with MIC of 800 and 725 mg/L against *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, Xu, Wang, & Liu, 2002). The fact may be attributed to the cell wall of *A. tumefaciens* and *E. carotovora*, which are typical Gram-negative bacteria. It was generally recognized that the chitosan and its derivatives more pronounced against Gram-positive than against Gram-negative bacteria, and the possible mechanism for antimicrobial activity was that the chitosan on the surface of the cell could form a polymer membrane, which prevented nutrients from entering the cell (Zheng & Zhu, 2003). The cell wall of Gram-negative bacteria 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 (Ratledge & Wilkinson, 1988).

The antibacterial activity of *N*-(4-carboxybutyroyl) chitosan derivatives has not been studied in detail especially against plant pathogens and therefore it is difficult to compare with other studies on their antimicrobial activity. For example, the effect of DS on antibacterial activity has not been reported. In the current study, the antibacterial activity was determined according to the NA dilution method. Thus we can better compare the activity in the current study to the activity found in other studies performed according to this method. Our previous studies on the antibacterial effect of *N*,*N*,*N*-dimethylalkyl chitosans as water soluble derivatives and *N*-(benzyl)chitosan derivatives was done under the same conditions as the present study (Badawy, 2010; Rabea, Badawy, Steurbaut, & Stevens, 2009). In these cases the synthesized compounds significantly increased the antibacterial activity and the effects were higher to *E. carotovora* than *A. tumefaciens* as that obtained in the present study.

3.3. The antifungal activity of chitosan derivatives

The antifungal activity of chitosan and its carboxyacyl derivatives against *B. cinerea*, *P. debaryanum* and *R. solani* is presented in Table 4. All derivatives were more active than chitosan. It can be noticed that the antifungal activity was increased dramatically with an increase in DS and MW values. Compound **4** with a MW of 4.84×10^5 g/mol and DS 0.53 exerted significantly prominent antifungal activity with EC_{50} of 899, 467 and 1413 mg/L against *B. cinerea*, *P. debaryanum* and *R. solani,* respectively. However, compound **1** with a MW of 3.78×10^5 g/mol and DS 0.10 was the lowest active one with EC_{50} of 2170, 814 and 2395 mg/L against *B. cinerea*, *P. debaryanum* and *R. solani*, respectively. In regard to the susceptibility of the three tested fungi, it can be noticed that damping off *R. solani* was more susceptible than *B. cinerea* and *P. debaryanum* to *N*-(4-carboxybutyroyl) chitosans.

The present study confirms that un-modified chitosan has low activity the tested against plant pathogens. This finding in agreement with other studies that showed a little decrease of growth of *R. solani* as the chitosan concentration gradually increased from 500 to 6000 mg/L (Wade & Lamondia, 1994). However, a complete growth inhibition was recorded against *F. oxysporum*, *R. stolonifer*, *P. digitatum* and *Colletotrichum gloeosporioides* at concentrations of 30,000 mg/L (Bautista-Baños, Hernández-López, Bosquez-Molina, & Wilson, 2003; Bautista-Baños, Hernández-López, & Bosquez-Molina, 2004). According to this fact, several research groups have started to modify a chitosan molecule to

Table 4
The antifungal activity of chitosan and *N*-(4-carboxybutyroyl) chitosan derivatives against *B. cinerea*, *P. debaryanum* and *R. solani*.

Compound	EC_{50} (mg/L)	95% confidence limits (mg/L)		Slope ± SE	Intercept of regression line ± SE	Chi square (χ^2)
		Lower	Upper			
B. cinerea						
Chitosan	>3000	–	–	–	–	–
1	2170	1727	3057	1.97 ± 0.26	−6.58 ± 0.78	1.31
2	1695	1311	2509	1.43 ± 0.21	−4.61 ± 0.62	0.67
3	1130	920	1475	1.50 ± 0.21	−4.57 ± 0.60	0.58
4	899	749	1105	1.64 ± 0.21	−4.85 ± 0.59	0.30
P. debaryanum						
Chitosan	2180	1761	2822	1.22 ± 0.18	−4.08 ± 0.59	1.91
1	814	438	1800	2.85 ± 0.25	−8.29 ± 0.71	7.33
2	668	214	1994	2.45 ± 0.23	−6.94 ± 0.65	8.82
3	529	178	1019	2.28 ± 0.23	−6.21 ± 0.64	6.25
4	467	138	836	2.18 ± 0.23	−5.82 ± 0.64	5.49
R. solani						
Chitosan	>3000	–	–	–	–	–
1	2395	1904	3407	2.16 ± 0.29	−7.28 ± 0.89	3.04
2	1918	1556	2591	1.99 ± 0.25	−6.52 ± 0.75	2.45
3	1582	1308	2049	1.93 ± 0.24	−6.19 ± 0.70	3.02
4	1413	1169	1820	1.81 ± 0.22	−5.71 ± 0.65	1.79

produce high antimicrobial active compounds. For example, we have prepared in our laboratory some of hydrophobic chitosan derivatives through the reductive amination reaction with aldehydes and hydrophilic derivatives through the quaternization process. We noted that *N*-alkylation or -arylation of chitosan with aliphatic or aromatic aldehydes, respectively, effectively enhanced the antifungal activity of chitosan against *B. cinerea*, *F. oxysporum* and *P. debaryanum* (Badawy et al., 2005; Rabea et al., 2005, 2006, 2009). *N*,*N*,*N*-dimethylpentyl chitosan and *N*,*N*,*N*-dimethyloctyl chitosan as water soluble derivatives were significantly the highest in fungal mycelial growth inhibiation of *B. cinerea*, *F. oxysporum* and *P. debaryanum* (Badawy, 2010).

4. Conclusion

The ntimicrobial activity is one of the most important bioactivities of chitosan and it will be improved in some of the derivatives, which is determined by the groups grafted to chitosan. In the present study, chitosan was *N*-carboxybutyrated with glutaric anhydride under the homogeneous conditions in the presence of methanol. The DS was determined by a proposed ^{1}H NMR spectrometric procedure and ranged from 0.10 to 0.53. The synthesized derivatives were higher soluble than chitosan in water and diluted aqueous acetic acid solutions. These derivatives also showed solubility in aqueous sodium hydroxide solution. The chitosan derivatives at the applied concentrations exhibited a wide range of antibacterial and antifungal activity *in vitro*. The current study verifies this conclusion that it can be observed since the highest DS value is more active than the lowest one. Therefore, we suggest that *N*-carboxybutyroyl chitosan at high DS value can be used for controlling some of plant pathogens that cause destruction of crops and vegetables.

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