Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives

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Received 22 March 2005; received in revised form 26 October 2005; accepted 27 October 2005
Available online 15 December 2005

Abstract

Low molecular weight chitosan (4.6 kDa) and N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives of different degrees of substitution were tested for their antimicrobial activity against Escherichia coli, Pseudomonas aureofaciens, Enterobacter agglomerans, Bacillus subtilis, Candida krusei and Fusarium oxysporum f. sp. radicis lycopersici. The results indicated that the chitosans show high activities against all bacteria, yeast and filamentous fungus. In fact, they suppressed fungal colony growth and inhibited fungal spore germination at 0.01% (w/v) concentration. A higher activity of chitosan and its derivatives was obtained with a substitution degree of 3 mol% for E. coli and B. subtilis, while a substitution degree of 9 mol% was usually preferable for increased activity against P. aureofaciens, E. agglomerans and C. krusei.

Keywords: Chitosan; Chitosan derivatives; Antibacterial activity; Antifungal activity

1. Introduction

Chitosan, β-(1 → 4)-2-acetamido-2-deoxy-d-glucan, is the most abundant among the natural aminopolysaccharides. Chitosan, the poly-(β-1 → 4)-2-amino-2-deoxy-d-glucopyranose, is a collective name for a group of partially and fully deacetylated chitin. This polysaccharide was found to be non-toxic, biocompatible and biodegradable (Lui, Dunn, Grandmaison, & Goosen, 1997). Chitosan has found several of applications being employed either alone or in blends with other natural polymers (starch, gelatin, alginites) in the food and pharmaceutical industries mainly due to its high biodegradability and antimicrobial properties (Arvanitoyannis, 1999; Arvanitoyannis, Nakayama, & Aiba, 1998; Hague et al., 2005; Kim, Chen, Wang, & Rajapakse, 2005; Yamada, Akiba, Shibuya, Kashiwada, Matsuda, & Hirata, 2005). Microbiological activity of chitosan has been detected for many bacteria, filamentous fungi and yeasts (Hirano & Nagao, 1989; Kendra & Hadwiser, 1984; Uchida, Izumi, & Ohtakara, 1989; Ueno, Yamaguchi, Sakairi, Nishi, & Tokura, 1997).

These studies show that the biological activity of chitosan depends significantly on its physico-chemical properties such as molecular weight and molecular fraction of glucosamine-units in the chitosan polymer chain (usually referred as the degree of chitosan N-deacetylation). These factors influence chitosan solubility and interaction with the cell walls of target microorganisms. Chitosan is insoluble in aqueous media at neutral and basic conditions, but is soluble in aqueous diluted acids. However, in some fields the application of this polysaccharide is limited by its high molecular weight resulting in low solubility in aqueous diluted acids and high viscosity of the solution even at low chitosan concentration. Medical chitosan applications require, as a starting material, a low molecular weight (LMW) chitosan with a high solubility and low viscosity in water at physiologically acceptable pH values.

Numerous studies on bactericidal activity of chitosan have been carried out (Badawy et al., 2004; Muzzarelli et al., 1990; Muzzarelli et al., 2001; Rhoades & Roller, 2000) and reviewed (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Chirkov, 2002), and some controversial evidences for
a correlation between bactericidal activity and chitosan molecular weight have been found. Most investigators used the uncertain term ‘low molecular weight chitosan’ for a partially depolymerized chitosan not indicating exactly its molecular weight. Only a few data on bactericidal activity of low molecular weight chitosan could be compared depending on bacteria tested, conditions of biological test and chitosan molecular weight, but even in this case the results did not correspond to each other. Thus, 9.3 kDa chitosan restricted growth of *Escherichia coli* while 2.2 kDa chitosan promoted growth of this bacterium (Tokura, Ueno, Miyazaki, & Nishi, 1997). Increase in chitosan molecular weight led to a decrease in chitosan activity against *E. coli* in some studies (Gerasimenko, Avdienko, Bannikova, Zueva, & Varlamov, 2004; Zheng & Zhu, 2003), while in the others an increased activity for a high molecular weight (HMW) chitosan in comparison with LMW chitosan have been found (Kyung, Thomas, Chan, & Park, 2003). In contrast to the above mentioned results, no differences in HMW chitosan and LMW chitosan activities were found against *E. coli* (Jeon, Park, & Kim, 2001; Zhishen, Dongfeng, & Weiliang, 2001) and *Bacillus subtilis* (Gerasimenko et al., 2004; Jeon et al., 2001). At present, it has been commonly recognized that the biological activity of chitosan depends on its molecular weight, deacetylation degree, chitosan derivatization, degree of substitution, length and position of a substituent in glucosamine units of chitosan, pH of chitosan solution and, of course, the target organism. These variations suggested to lead to two different mechanisms of chitosan and target microorganism interaction: the first—adsorption of chitosan to cell walls leading to cell walls covering, membrane disruption and cell leakage—is mainly connected with HMW chitosan; the second—penetration of chitosan into living cells leading to inhibition of various enzymes and interference with synthesis of mRNA and proteins (Rabea et al., 2003; Chirkov, 2002; Zheng & Zhu, 2003). Experimental conditions of chitosan bioassays can also bias the results of such tests. Moreover, we believe that a suitable method of LMW chitosan preparation for experiments should be adopted. In fact, the method chosen (e.g. acidic or enzymatic hydrolysis, oxidative deamionization by nitrite) will determine the chemical structure of the reducing end of the chitosan polysaccharide chain and may, in turn, affect the biological activity of LMW chitosans.

In the present study, biocidal activities of chemically prepared LMW chitosan of narrow molecular mass distribution and some of its synthetic lipo-derivatives were tested against important species of Gram-positive and Gram-negative bacteria, the yeast *Candida kruisei* and *Fusarium oxysporum* f. sp. *radicis lycopersici*, a common fungal pathogen of plants distributed worldwide. The results were compared with bactericidal activity of enzymatically prepared LMW chitosan.

2. Materials and methods

Chitosan was produced from Kamchatka-peninsula-shelf crab shell chitin with a degree of deacetylation (DD) of 82% and viscosity–average molecular weight of 300 kDa (BioChit, Moscow, Russia).

2.1. Sample preparation

A series of N-/2(3)-(dodec-2-enyl)succinoyl/chitosans (DDC-chitosans) were prepared via reaction of HMW chitosan with 2-(dodecen-1-yl)succinic anhydride (SIGMA) at the conditions described by Hirano, Ohe, & Ono (1976). LMW chitosan and a series of N-/2(3)-(dodec-2-enyl)succinoyl/derivatives (DDC–LMW chitosans) were prepared by acidic hydrolysis of the chitosan and DDC-chitosans following procedure described by Domard & Carter (1989).

DDC content (n) in DDC–LMW chitosans (Fig. 1) was determined by titration of carboxyl-groups after conversion of DDC–LMW chitosan to the corresponding DDC–LMW Chitins following the procedure described by Tikhonov, Radigina, & Yamskov (1996).

Amino-group contents (p) in LMW chitosan and DDC–LMW chitosans (Fig. 1) was determined by elemental microanalysis of chloride ions.

Acetyl-group content (m) in LMW chitosan and DDC–LMW chitosans (Fig. 1) was calculated as a difference:

![Fig. 1. Chemical structure of the DDC–LMW chitosans (2-isomer is only shown in fragment m).](image-url)
m = 1 − (n+p) since no difference in DD was found for the starting chitosan and the prepared LMW chitosan.

2.2. Surface tension activity

The surface tension at the air–water interface of aqueous solutions of chitosans was measured by Wilhelmy’s plate method using tensiometer K100 Mk1 (Kruss, Germany). All the measurements were made for a sufficiently long time (usually longer than 10,000 s) in order to follow the effect of ageing on the surface tension of the adsorption layers.

2.3. Cultures for antimicrobial tests

Bacteria and yeast were obtained from the Russian collection of industrial microorganisms (VKPM) and the American type culture collection (ATCC):

- E. coli ATCC 5945, Pseudomonas aureofaciens VKPM B-7542, Enterobacter agglomerans VKPM B-7541, (all Gram-negative), B. subtilis VKPM B-7540 (Gram-positive), and C. kruisei VKPM Y-2594. F. oxysporum f. sp. radicis lycopersici (isolate 127) was from the culture collection of the Laboratory for Plant Pathology, Department of Marine Sciences and Applied Biology, University of Alicante, Spain.

2.4. Bacteria and yeast cultivation

E. coli, P. aureofaciens, E. agglomerans were grown on slanted agar under aerobic conditions as described in Bergey’s manual of Systematic Bacteriology (Sneath, 1986). C. kruisei was cultivated on Sabouraud agar slants. B. subtilis was grown in flasks containing LB Broth (SIGMA). The flasks were inoculated shaking (100 rpm) for 24 h at 37°C. The cultured cells (bacteria and yeast) were suspended into 0.1 M sodium acetate buffer (pH 6.5) at a concentration of 109 cells/ml.

2.5. Bactericidal test

A 0.1 ml suspension of the microorganism to be tested was added to 0.9 ml LMW chitosan or DDC–LMW chitosan solution (0.1 or 1% w/v) in 0.1 M sodium acetate buffer (pH 6.5) under sterile conditions. The mixture was incubated shaking for 1 h at 37°C, and the number of viable cells was calculated. Bactericidal activity was expressed as percentage of cell death.

2.6. Fungistatic activity test

Fungistatic activity of LMW chitosan and DDC–LMW chitosans was determined by a radial hyphal growth bioassay. A sample of chitosan to be tested was dissolved in 0.25 M HCl, and the pH was adjusted to 5.0–5.5 with 1 M NaOH. Potato dextrose agar (PDA) media including 0.1, 0.5 or 1.0 mg/ml of a given chitosan solution was sterilized at 121°C for 20 min and then poured onto sterile petri dishes (9-cm diameter). The plates were inoculated with 6-mm-diameter plugs taken from the margins of 3–5-days old colonies of F. oxysporum on PDA. Three replicates for each sample concentration were used. Control plates (PDA only) were also inoculated with the fungus. All plates were incubated in the dark at 24°C. The radial colony growth was measured daily for 2 weeks.

2.7. Germination of conidia of F. oxysporum

LMW chitosan or DDC–LMW chitosan were dissolved in 0.25 M HCl and the pH was adjusted to 5.6 with 1 M NaOH. The chitosan solutions were filter-sterilized (Acrodisc, Gelman, 0.22 μm pore size). These experiments were performed using multi-well microscope slides each containing 10 wells (ICN Biomedicals, Aurora, OH USA). Each well was filled with 25 μl of the chitosan solutions (0, 0.01, 0.001, 0.0001 mg/ml) containing 106 conidia/ml. After 24 h, the numbers of germinated and non-germinated conidia were recorded and the percentage germination was calculated (Laflamme, Benhamou, Bussieres, & Dessureault, 1999).

3. Results and discussion

3.1. LMW chitosan and DDC–LMW chitosans preparation

Reactions of HMW chitosan with 2-dodecenediamine anhydride were carried out in a 1% aqueous acetic acid/methanol (1:2 v/v) solution (pH 4.5) at 500°C led to N-substituted chitosans bearing 2 (3)-(dodec-2-enyl)succinoyl-groups in correspondence with the numerous studies on reaction of chitosan with anhydrides (Hirano et al., 1976). After acidic hydrolysis of chitosan and DDC-chitosans, hydrochlorides of LMW chitosan and DDC–LMW chitosans were separated by size exclusion chromatography (Domard et al., 1989). Chemical structures of DDC–LMW chitosans are shown in Fig. 1. Their fractional compositions determined by chemical analysis of C/N ratio, chlorine content and titration of the substances obtained are presented in Table 1. Carboxylic acid anhydride following Hirano’s method of chitosan acetylation (Hirano et al., 1976). The presence of a band at 1715 cm−1 in IR spectra of a DDC–LMW chitosan

<table>
<thead>
<tr>
<th>Sample</th>
<th>m (mol%)</th>
<th>n (mol%)</th>
<th>p (mol%)</th>
<th>Mw (kDa) andPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW chitosan</td>
<td>0</td>
<td>20</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>DDC(13%)–LMW</td>
<td>3</td>
<td>20</td>
<td>77</td>
<td>4.6/1.6</td>
</tr>
<tr>
<td>chitosan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDC(9%)–LMW</td>
<td>9</td>
<td>20</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>chitosan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDC(16%)–LMW</td>
<td>16</td>
<td>20</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

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Table 1

DCC-chitosans fractional composition, molecular weight (Mw) and polydispersity index (PI)
hydrochloride confirmed the presence of carboxyl-groups in DDC substituted chitosan hydrochloride. Gel permission chromatography revealed that LMW chitosan and DDC–LMW chitosan had average molecular weights of 4.6 kDa and a narrow molecular mass distribution with polydispersity index of 1.6 (Table 1).

3.2. Bactericidal and antifungal activities of LMW chitosan and DDC–LMW chitosans

In our study, LMW chitosan and three LMW chitosan derivatives bearing long aliphatic chains and differing in the degree of N-substitution were tested for their activity against Gram-negative, Gram-positive bacteria, yeast and filamentous fungus. Low molecular weight chitosan appeared to better adsorb to and penetrate cell walls of microorganisms (Zheng & Zhu, 2003), so that chemical modification of chitosan (i.e. N-substituted chitosan) by aliphatic chains may affect the antimicrobial properties of DDC–LMW chitosans.

Our results indicate that after a short exposure time (1 h), 1% (w/v) LMW chitosan and its derivatives exhibited high antimicrobial activity against the Gram-negative, Gram-positive bacteria, yeast and filamentous fungus. Low molecular weight chitosan appeared to better adsorb to and penetrate cell walls of microorganisms (Zheng & Zhu, 2003), so that chemical modification of chitosan (i.e. N-substituted chitosan) by aliphatic chains may affect the antimicrobial properties of DDC–LMW chitosans.

Table 2
effect of LMW chitosan and LMW chitosan derivatives on bacteria and yeast (sample concentration: 1% w/v)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Viable cells (CFU/ml±error)/cell death (%)</th>
<th>Control</th>
<th>DDC(9%)-LMW chitosan</th>
<th>DDC(16%)-LMW chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>(4.7±0,1)10^9% (1,6) 98,5%</td>
<td></td>
<td>(1.4±0,1)10^8 75,0%</td>
<td>(1.3±0,1)10^8 76,8%</td>
</tr>
<tr>
<td><strong>P. aerofaciens</strong></td>
<td>(1,7±0,1)10^9% (90,4) 96,0%</td>
<td></td>
<td>(1.3±0,1)10^8 96,0%</td>
<td>(4.5±0,1)10^7 92,0%</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>(3,6±0,1)10^9% (64,6) 99,9%</td>
<td></td>
<td>(4.0±0,1)10^8 96,0%</td>
<td>(2.0±0,1)10^8 98,0%</td>
</tr>
<tr>
<td><strong>E. agglomerans</strong></td>
<td>(3,0±0,1)10^9% (99,91%)</td>
<td></td>
<td>(1.0±0,1)10^8 99,99%</td>
<td>(1.8±0,1)10^8 99,99%</td>
</tr>
<tr>
<td><strong>C. kruisei</strong></td>
<td>(2,1±0,1)10^2 99,9989%</td>
<td></td>
<td>(1.2±0,1)10^2 99,9994%</td>
<td>(2.7±0,1)10^1 99,987%</td>
</tr>
</tbody>
</table>

DDC–LMW chitosans against B. subtilis (up to 99.9% cell death) in comparison with activity against E. coli (60% cell death) and B. subtilis (70% cell death) of enzymatically prepared 5 kDa chitosan (Jeon et al., 2001). Although these differences in antimicrobial activities of chemically and enzymatically prepared low molecular weight chitosans still remain unknown, they may be due to differences in N-acetyl groups distribution along polymer chains of these chitosans (this hypothesis needs to be verified).

High antimicrobial activity of LMW chitosan and DDC–LMW chitosans was demonstrated against C. kruisei: the number of viable cells was reduced about 5 log unit for all synthesized chitosans. It should be mentioned that very low activity (about of 1% cell death) was found for enzymatically prepared 5 kDa chitosan at the same concentration (Gerasimenko et al., 2004). Our experiments also show that DDC–LMW chitosans caused C. kruisei cell death depending on the contents of DDC-residues. As shown in Fig. 2, C. kruisei cell death increased from 55.2 to 95.4% when 3 mol% of DDC was introduced. Further increase in DDC contents led to a decrease in activity. Although there was no correlation between activity towards C. kruisei and surface tension activities of DDC–LMW chitosans with different DDC contents (Fig. 3), the presence of a long aliphatic chain should at least facilitate absorption of a substituted LMW chitosan onto cell walls via hydrophobic...
interaction with cell wall proteins and thus increase their activity. Also, the presence of $3\div 9$ mol% DDC may be considered as a preferable DDC-content for cell wall binding and penetration into the cells in spite of that LMW chitosan and DDC(16%)–LMW chitosan demonstrated even higher activity against some species tested in comparison with DDC(3%)–LMW chitosan and DDC(9%)–LMW chitosan. The observed decrease of activity of LMW chitosans with increased DDC contents may be explained by formation of micelle-like structures and interpolyelectrolyte aggregates (Fig. 4) in aqueous solution via hydrophobic interaction between long alkyl chains (Lui, Sun, Zhang, & Yaok, 2003). These alkyl chains hidden inside aggregates (Fig. 4(b)) could not interact with cell wall proteins by forming complexes. Following this hypothesis, we may conclude that the conformation of DDC–LMW chitosans in solution may be a factor, which at least should be taken into account for prediction of antimicrobial activity of low molecular weight chitosan and its hydrophobically modified derivatives. The verification of this hypothesis requires usage of fluorescent labeled LMW chitosan and DDC–LMW chitosans and dynamic light scattering investigations (to be published).

Activity of LMW chitosan and DDC–LMW chitosan containing 3 mol% of DDC residues against *F. oxysporum* f. sp. *radicis lycopersici* was determined by measuring the evolution of colony radius at several concentrations of chitosans tested. Comparative data on fungistatic activities of the substances are shown in Fig. 5(a)–(c). Growth of *F. oxysporum* hyphae was suppressed by LMW chitosan and DDC(3%)–LMW chitosan. Fungicidal activity of DDC(3 mol%)-LMW chitosan was more active against *F. oxysporum* than that of LMW chitosan at all concentrations (Fig. 5(b) and (c)).

The effect of antifungal compounds on fungal spore germination was higher than that on hyphal growth. The effect of LMW chitosan and DDC(3%)–LMW chitosan on germination of conidia of *F. oxysporum* was studied at a concentration range from 0.01 to 0.0001 mg/ml and the results are shown in Table 3. Our data demonstrate that both LMW chitosan and DDC(3%)–LMW chitosan abolished germination of *F. oxysporum* conidia at 0.01 mg/ml (0.001% solution). When conidia were treated with any of the chitosans at 0.01 mg/ml no germination was found upon plating on different growth media (Palma-Guerrero et al., unpublished).
results). This was found for incubation up to 30 days at 240 °C. All the substances tested were fungistatic (i.e. reduced growth rate of developing mycelia), but they also possessed fungicidal activity, since they completely inhibited spore germination at a concentration of 0.01 mg/ml. This inhibitory effect was decreased significantly with concentration.

4. Conclusion

LMW chitosan and its DDC-derivatives showed high activity against bacteria, yeast and fungus tested. The preferment of chitosan derivatization was found for a substitution degree of 3 mol% for E. coli and B. subtilis while substitution degree of 9 mol% was usually preferable for activity against P. aureofaciens, E. agglomerans and C. kruisi. DDC(3%)–LMW chitosan, in particular, inhibited growth of F. oxysporum f. sp. radicis lycopersici.

From comparison of these results and previously published data on bactericidal activity of enzymatically prepared 5 kDa chitosan (Gerasimenko et al., 2004) we could conclude that in spite of lack of knowledge on the antimicrobial action mechanism, the origin of low molecular weight chitosan and its derivatives as well as the method used for low molecular weight chitosan and its derivatives preparation are the important issue especially in biomedical applications.

The results of this study suggest new means to investigate the mode of action of these biocidal substances of natural origin.

Acknowledgements

This work was partly supported by a grant from the Spanish Ministry of Science and Education (AGL 2004-05808).

References


Table 3

Effect of different LMW chitosans on spore germination of F. oxysporum f.sp. radicis lycopersici conidia

<table>
<thead>
<tr>
<th>Type of chitosan</th>
<th>Chitosan concentration (mg/ml)</th>
<th>Germination, % (mean ± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>84.01 ± 2.03</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>99.07 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>99.67 ± 0.33</td>
</tr>
<tr>
<td>DDC(3%)–LMW</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>82.06 ± 4.65</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>98.98 ± 4.94</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>99.25 ± 0.44</td>
</tr>
</tbody>
</table>

