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Short communication

Antimicrobial activity of chitosan *N*-betainates

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Abstract

The effect of degree of substitution on the antimicrobial activity of water-soluble quaternary chitosan *N*-betainates was determined against *Escherichia coli* and *Staphylococcus aureus*. Chitosan *N*-betainates showed low antimicrobial activity in neutral conditions and the glucosamine *N*-betainate prepared as a reference compound showed low activity also in acidic conditions. However, the antimicrobial activity increased with decreasing degrees of substitution in acidic conditions (pH 5.5), which suggests that for efficient antimicrobial action, the positive charge should be situated in the amino group of chitosan, rather than in the quaternary substituent.

Keywords: Chitosan; Synthesis; Quaternary; Water-soluble; Antimicrobial

1. Introduction

Antimicrobial properties of chitosan and chitosan derivatives have been widely explored (Lim & Hudson, 2003; Rabea, Badawy, Stevens, Smagge, & Steurbaut 2003; Shahidi, Arachchi, & Jeon, 1999). Several mechanisms have been proposed to explain the antibacterial action of chitosan, but its exact mechanism of action is not known. Chitosan is a positively charged molecule and the target of its antimicrobial action is the negatively charged cell wall of bacteria, where it binds and disrupts the normal functions of the membrane, e.g. by promoting the leakage of intracellular components and also by inhibiting the transport of nutrients into the cells (Chen & Chou, 2005; Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Sudarshan, Hoover, & Knorr, 1992; Tsai & Su, 1999).

The antimicrobial activity of chitosan increases with decreasing pH (Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002; Roller & Covill, 1999; Tsai & Su, 1999; Wang, 1992; Yang, Chou, & Li, 2005). This is due to the fact that the amino groups of chitosan become ionized at pH below 6 and carry a positive charge. Unmodified chitosan is not antimicrobially active at pH 7, since it does not dissolve and also since it does not contain any positive charge on the amino groups (Chung, Kuo, & Chen, 2005; Liu, Guan, Yang, Li, & Yao, 2001; Yang et al., 2005). The antimicrobial activity of chitosan also increases with increasing degree of deacetylation, due to the increasing number of ionizable amino groups (Liu et al., 2001).

Chitosan derivatives with a quaternary ammonium moiety are interesting, since a permanent positive charge is gained on the polysaccharide backbone and, as discussed above, the antimicrobial action of chitosan has been linked to this positive charge. The amino group in chitosan has been quaternized by first reductively alkylating it with aldehydes to form imines, followed by reduction to obtain *N*-alkyl derivatives. These alkyl derivatives have then been quaternized with methyl iodide (Jia, Shen, & Xu, 2001; Kim & Choi, 2002; Kim, Choi, Chun, & Choi, 1997). However, structurally uniform polymer structures cannot be prepared with this strategy. The conditions needed to achieve a high degree of quaternization will also inevitably lead to total alkylation of the hydroxyl groups in chitosan. Since the degree of *N*-alkylation in the first step by reductive alkylation is not complete, the free amino groups can also be mono-, di- and trimethylated after the quaternization step. Thus, this procedure will actually produce a diverse

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heteropolymer with a wide variety of different monomers and thus structure-activity relationships cannot be reliably determined. These derivatives have, however, showed MIC values as low as 0.5 μg/mL in neutral conditions and the MIC values were even lowered by addition of acetic acid (Jia et al., 2001). The overall trend was that the activity of the derivatives increased with increasing length of the alkyl chain (Jia et al., 2001; Kim & Choi, 2002; Kim et al., 1997).

Another approach to prepare quaternary chitosan derivatives is to attach a substituent with a quaternary ammonium moiety. Daly and Manuszak-Guerrini reported MIC values as low as 10–20 μg/mL against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* for *N*-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride at pH 7.2. (Daly & Manuszak-Guerrini, 2001).

Also a physical complex of chitosan acetate and C-12 C-18 alkyl amido prophyl dimethylamino betaine (AAPDB) has exhibited higher antimicrobial action compared to the activity of sole chitosan acetate or AAPDB (Liu, Du, Yang, & Zhu, 2004).

We have earlier reported the synthetic procedure for the preparation of water-soluble quaternary chitosan *N*-betainates (Holappa et al., 2004). With the reported procedure chitosan *N*-betainates can be prepared having various degrees of substitution and structurally uniform molecular structures can be obtained, thus structure-activity relationships and the effect of degree of quaternization can be exactly determined. In the present study, our aim was to study the effect of the degree of substitution on the antimicrobial activity of these water-soluble chitosan *N*-betainates. We used different molecular weight chitosans as starting materials for the synthesis, to obtain products with various molecular weights. We also prepared and tested glucosamine *N*-betainate monomer as a reference compound.

The antimicrobial activity of these water-soluble chitosan *N*-betainates was measured against *E. coli* and *S. aureus* at pH 7.2 and also at acidic pH 5.5. Minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) were determined according to the NCCLS (now named CLSI) standard (NCCLS, 2003). Growth rates were measured in broths at two different pH values to determine whether the bacteria divide at approximately the same rate at these two pH levels tested.

2. Experimental section

2.1. Materials

ChitoClear™ chitosan donated by Primex Ltd (Reykjavik, Iceland) was used as a starting material for the synthesis. Three different starting materials were used; chitosan a with M_w 201 kDa, M_n 89.8 kDa and degree of deacetylation (dd) of 85%, chitosan b with M_w 380 kDa, M_n 172 kDa and dd 82%, and chitosan c with M_w 519 kDa, M_n 265 kDa and dd 82%. D-glucosamine hydrochloride was purchased from Fluka (Steinheim, Germany). All other reagents were used as received and solvents were purified by common procedures.

2.2. Characterization

^{1}H NMR spectra were recorded on a Bruker AVANCE DRX 500, operating at 500.13 MHz. Chitosan *N*-betainates were measured at 343 K in D_2O using 3-(trimethylsilyl)propionate-d_4 as an internal standard and glucosamine *N*-betainate was measured at 300 K in DMSO-d_6 using tetramethylsilane as an internal stardard. The molecular weights of chitosan *N*-betainates were determined by Viscotek's GPC system, including pump, autosampler, refractive index (RI), right angle light scattering 90° (RALS), low angle light scattering 7° (LALS) and viscometer detectors. A narrow poly(ethylene oxide) standard was used for calibrating the instrument and triple detection (RI, LS and viscometer) were used for the calculations. Two ViscoGEL GMPWXL (300×7.5 mm) columns and guard column were used at an oven temperature of 35 °C. A mobile phase of acetic acid (0.3 M)/sodium acetate (0.2 M) (pH 4.5) at a flow of 0.8 ml/min was used. The refractive index increment (d*n*/d*c*) for each sample was determined by using five different injection volumes. The obtained dn/dc value was then used in the calculations.

2.3. Synthesis

Chitosan *N*-betainates were prepared by a five-step synthetic route as reported earlier, by protection and deprotection of the amino- and primary hydroxyl groups (Holappa et al., 2004). ^{1}H NMR (343 K, D_2O): δ 2.06 (CH_3, *N*-acetyl), 2.77 (H-2, when amino group unsubstituted), 3.34 (H-9, i.e. N^+–CH_3), 3.4–4.0 (H-6), 3.5–3.7 (H-5), 3.55–3.8 (H-4), 3.6–3.85 (H-3), 3.65–3.9 (H-2, substituted), 4.2 (H-8, i.e. CO–CH_2–N^+), 4.4–4.8 ppm (H-1). Degrees of substitution were determined from the ^{1}H NMR spectra by comparing the signal integrals from CH_3-protons attached to the quaternary nitrogen (at 3.34 ppm) to the hemiacetal proton signal (at 4.4–4.8 ppm). Glucosamine *N*-betainate was synthesized via 2-chloroacetyl glucosamine by the method of Giraud, Rapp, Maurizis, and Madelmont (2000).

2.4. Antimicrobial tests

Organisms. The organisms used were *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. The strains were kept frozen in tryptose–glycerol freezing media (Difco Laboratories, Detroit, MI) at −80 °C, cultured and then subcultured once on 5% horse bloodagar (Oxioid, Hamshire, UK) prior to the testing.

Culture medium. Mueller–Hinton broth (Oxoid) at pH 7.2 with divalent cations Ca^{++} and Mg^{++} was adjusted by the manufacturer. The Mueller–Hinton broth was also adjusted with HCl (5 N) to pH 5.5 for measurements at the lower pH. The pH was measured with a PHM220 Lab pH meter MederLab (Radiometer, Copenhagen, Denmark). For plating, bacterial count and minimal lethal concentration measurements 5% horse bloodagar was used.

Microdilution. The chitosan derivatives were weighed and dissolved in Mueller Hinton broth at pH 7.2 and 5.5 to obtain a concentration of 8192 μg/ml. Then 50 μl of this solution was

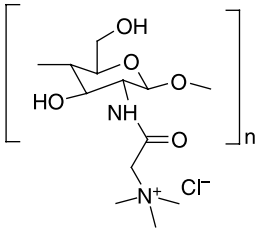

Fig. 1. The chemical structure of chitosan *N*-betainate chloride.

added to the first two wells on a microtiter plate and twofold dilutions were done in 50 μl Mueller Hinton broth from well two.

The final range (after addition of the inoculum) was from 4096 to 2 μg/ml, giving the possibility of reporting ≥8192 μg/ml as the highest result.

Inoculum. The inoculum was made by suspending colonies directly into Mueller Hinton broth. It was adjusted to the turbidity of MacFarland standard 0.5, or approximately $1–2\times10^8$ CFU/ml. The suspension was then diluted 1:100 ($1–2\times10^6$) to aim to achieve 5×10^5 × CFU/ml in the test, as the diluted suspension is further diluted 1:2 when 50 μl of the diluted suspension was added to 50 μl chitosan derivatives creating a twofold dilution in the microtiter wells.

Incubation. The microtiter plates were incubated at 35 °C in a sealed plastic bag in ambient air for 16–20 h.

MIC determination. MIC was determined according to the standard, as the lowest concentration that completely inhibited growth of the organism in the microtiter wells.

MLC determination. MLC was determined after reading the results for the MIC. Blood agar was plated with two times 10 μl of all the dilutions that showed no growth and incubated at 35 °C in ambient air for 16–20 h. MLC was determined as the lowest concentration that achieved a 99.9% decrease of viable bacteria.

Quality control. Growth control was 50 μl of Mueller Hinton broth without chitosan derivatives, but inoculated as the tests. Sterility control was 100 μl of Mueller Hinton broth. Performance control was ceftriaxone 32 μg/ml diluted twofold as the other substrates in the test. Viable cell count was done by making tenfold dilutions of the inoculum. Two times 10 μl of the dilutions were plated on bloodagar, incubated in same conditions as the tests and colonies counted. The results of the counts were then used to calculate the 99.9% killing of the bacteria, which is the criterion for MLC. The criterion for acceptance of results for measurements of bacterial activity was that all controls should be within acceptable limits.

Growth rate at different pH. The growth rate of both organisms was measured at both pH values used in the test. 1 ml Mueller Hinton broth was inoculated to achieve the same concentration of bacteria as in the test. The viable cell count was performed at 2 h intervals for 8 h. Growth curves were plotted.

3. Results and discussion

3.1. Synthesis and characterization

The degrees of substitution (d*s*) of chitosan *N*-betainate chlorides (Fig. 1) ranged from 0.05 to 0.9 (Table 1). Only ^{1}H NMR was measured from the chitosan *N*-betainates, since we have earlier described the detailed NMR characterization of the materials, which confirmed that we could obtain structurally uniform chitosan derivative structures with the synthetic procedure used (Holappa et al., 2004). As described before, the protection-deprotection strategy of chitosan used here degraded the chitosan polymer (Holappa et al., 2004, 2005; Kurita, Akao, Yang, & Shimojoh, 2003; Kurita, Kojima, Nishiyama, Shimojoh, & Nishimura, 2000; Kurita, Shimada, Nishiyama, Shimojoh, & Nishimura, 1998). However, we noticed that when starting materials with different molecular weight were used, end products with relatively small molecular weight differences were obtained (Table 1).

Table 1
Weight average molecular weights (M_w), number average molecular weights (M_n) and MIC values (μg/ml) and MLC values (μg/ml) of chitosan *N*-betainate chlorides, chitosans and glucosamine *N*-betainate monomer

D*s*	M_w (kDa)	M_n (kDa)	*Staphylococcus aureus* ATCC 29213				*Escherichia coli* ATCC 25922			
			pH 5.5		pH 7.2		pH 5.5		pH 7.2	
			MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
0.05[c]	42.1	27.6	512	512	2048	2048	128	256	2048	2048
0.15[b]	48	24.8	512	1024	2048	2048	256	256	1024	2048
0.4[a]	25	11.8	2048	2048	≥8192	≥8192	256	512	1024	1024
0.6[b]	48.5	20.8	4096	4096	≥8192	≥8192	512	1024	≥8192	≥8192
0.6[c]	137	38.1	4096	4096	4096	≥8192	2048	2048	2048	2048
0.8[a]	45.3	19.5	≥8192	≥8192	≥8192	≥8192	2048	2048	1024	1024
0.8[c]	73.6	52.6	4096	≥8192	≥8192	≥8192	2048	2048	2048	2048
0.9[a]	71.4	27.2	≥8192	≥8192	≥8192	≥8192	2048	4096	512	512
Chitosan a.	201	89.8	64	64	1024	≥8192	256	1024	2048	2048
Chitosan b.	380	172	128	128	2048	4096	128	1024	1024	1024
chitosan c.	519	265	256	256	2048	4096	128	1024	1024	1024
Monomer[d]			≥8192	≥8192	≥8192	≥8192	2048	2048	2048	≥8192

[a,b,c] Starting material used to prepare the derivatives (i.e. chitosan a, b and c).
[d] Glucosamine *N*-betainate.

3.2. Antimicrobial activities

We have encountered difficulties in comparing and interpreting results from other published studies regarding the antimicrobial activity of chitosan and chitosan derivatives. The activities of chitosan and chitosan derivatives vary considerably depending on the different physical and chemical properties of the materials, e.g. their origin, molecular weight, degree of substitution, and solubility. Activity may also depend on the test conditions, for example pH and the bacteria to be tested. Therefore we decided to use an approved and well known standard for measurements of MIC- and MLC-values (NCCLS, 2003). No significant differences were found at different pH values for either bacteria when the growth rates were measured (Fig. 2). This is consistent with earlier reports (Gudmunsson, Erlendsdottir, Gottfredsson, & Gudmundsson, 1991).

Overall, the chitosan *N*-betainates showed low activity at pH 7.2, whereas the glucosamine *N*-betainate was quite inactive at both measured pH values (Table 1). Chitosan *N*-betainates with low d*s* values showed higher antimicrobial activity at pH 5.5 than at pH 7.2. Chitosan *N*-betainates were slightly more active against *S. aureus* than against *E. coli* (Table 1). The lowest MIC at pH 7.2 was 512 μg/ml for derivative with d*s* 0.9 against *S. aureus*.

However, at pH 5.5 it was observed that the antimicrobial activity increased with decreasing degree of substitution. This has been previously reported for non-quaternary chitosan derivatives (Liu et al., 2001). This is due to decreasing number of free ionizable amino groups with increasing degree of substitution, leading to a decreased positive charge on the polymer (Liu et al., 2001). However, the low degree of the cationic character is not the explanation for the low activity for chitosan *N*-betainate, since the betaine substituent itself has a positive charge. At pH 5.5, the charge ratio and the amount of positive charge is approximately the same for all chitosan *N*-betainates regardless of the degree of substitution, since the free amino groups are also ionized. Thus, it can be concluded, that in terms of antimicrobial action, the quaternary ammonium moiety of chitosan *N*-betainate is unfavorably placed in relation to the polymer backbone. For efficient antimicrobial activity, the positive charge should be situated in the amino groups of the chitosan backbone. This has been previously shown in numerous studies reporting the antimicrobial activity of chitosan in acidic environment and also when quaternizing the amino groups of chitosan (Jia et al., 2001; Kim & Choi, 2002; Kim et al., 1997). Thus, at least for *E. coli*, the low activity cannot be attributed to the low molecular weight of the derivatives, because the M_w under 100 kDa has been reported to be the optimum M_w in several antimicrobial studies (Liu et al., 2001; Zheng & Zhu, 2003).

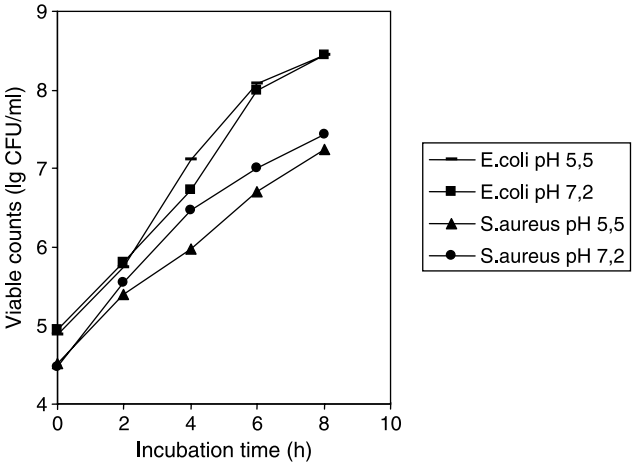

Fig. 2. Growth rates of *Escherichia coli* and *Staphylococcus aureus* at pH 5.5 and 7.2.

Overall, the result that chitosan *N*-betainates were not very active in neutral conditions was quite surprising, since *N*-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride has exhibited very high antimicrobial activities at pH 7.2 (Daly & Manuszak-Guerrini, 2001) and also in alkaline conditions (Qin et al., 2004). This derivative possesses the quaternary moiety, which is attached via three methylene long spacer to the polymeric backbone.

Unmodified chitosans showed an expected antimicrobial activity profile at pH 5.5, i.e. activity against *E. coli* increased with decreasing molecular weight, whereas activity against *S. aureus* increased with increasing molecular weight. However, the activity changes were rather small, especially for *S. aureus*. Molecular weight dependence was not found with chitosan *N*-betainates. This is probably due to the relatively small molecular weight differences of the derivatives. Chitosans were more active than chitosan *N*-betainates against *E. coli*, but for *S. aureus* some chitosan *N*-betainates with low degrees of substitution showed comparable antimicrobial activities to unmodified chitosans.

4. Conclusion

Even though water-soluble quaternary chitosan derivatives can be obtained by *N*-acylation with betaine, these chitosan *N*-betainates are not very active antimicrobials at neutral conditions. Low activity cannot be attributed to the low molecular weights of these derivatives, since the present molecular weight range for derivatives has been shown to be effective against both bacterial species tested, especially against *E. coli*. However, the antimicrobial activity of chitosan *N*-betainates increased with a decreasing degree of substitution in acidic conditions, which suggests that the positive charge has to be situated on the amino group in the chitosan backbone if one wishes to achieve efficient antimicrobial activity. We conclude that just the introduction of a quaternary ammonium moiety into chitosan is not sufficient to obtain antimicrobial action, but the key issue is the optimal positioning of the positive charge in relation to the polymer backbone.

Unmodified chitosans showed an expected molecular weight dependent activity, i.e. the antimicrobial activity against *E. coli* increased with decreasing molecular weight whereas activity against *S. aureus* increased with increasing molecular weight. However, molecular weight dependence

was not obtained for chitosan *N*-betainates. In the future, more derivatives over a wider molecular weight scale need to be evaluated before a reliable determination of the M_w-activity relationships can be constructed.

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