ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2015, Vol. 41, No. 1, pp. 57–62. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © S.N. Kulikov, V.E. Tikhonov, E.A. Bezrodnykh, S.A. Lopatin, V.P. Varlamov, 2015, published in Bioorganicheskaya Khimiya, 2015, Vol. 41, No. 1, pp. 67–73.

Comparative Evaluation of Antimicrobial Activity of Oligochitosans against *Klebsiella pneumoniae*

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Received May 6, 2014; in final form, June 17, 2014

Abstract—The Antibacterial activity of chitosan of different molecular weights was studied against gram-negative *Klebsiella pneumoniae* at different pH values. It was found that the dependence of the inhibitory activity of chitosan on its molecular weight was undergoes inversion when increasing the pH of the medium above 7.0. In acidic media, chitosan of the higher molecular weight had the higher antibacterial activity, while in weàk alkaline media, oligomeric forms of chitosan displayed only the inhibition effect. Our results showed that the antibacterial activity of chitosan against *Klebsiella pneumoniae* was closely associated with its polycationic nature, and depended on the degree of protonation of the chitosan amino groups, which, in turn was the function of the degree of polymerization and the pH values of the medium. The results allow one to explain, in part, the contradictory literature data concerning the relationship between the antibacterial activity and molecular weight of chitosan.

Keywords: chitosan, oligochitosan antibacterial activity, Klebsiella pneumoniae

DOI: 10.1134/S1068162015010100

INTRODUCTION

Chitosan is a copolymer of glucosamine and acetylglucosamine. It is the product of deacetylation of chitin, which is the component of exoskeletons of crustaceans and insects, squid gladius, fungal cell walls, and some algae. Chitosan is the only positively charged polymer (polycation) of natural origin, which is produced in large quantities with a high degree of chemical purity, and, what is important, with reasonable cost [1]. The world amount of chitin is estimated to be 10^{10} tons, which, together with the fact of its permanent biosynthesis makes it an inexhaustible source for chitosan.

In the past two decades, the interest in the biocidal properties of chitosan has greatly increased, which along with its nontoxic, biocompatible, hypoallergic, and biodegradable properties allow its use for biomedical purposes as an alternative and/or auxiliary substance in antimicrobial therapy, especially against a number of bacterial strains resistant to classical antibiotics [2].

There are many publications in the literature concerning the study of the biocidal properties of chitosan against gram-positive bacteria, such as staphylococci [3–7] and bacilli [8, 9] and gram-negative bacteria, such as *E. coli* [3, 6–11], pseudomonades [2, 10], and salmonellae [9, 10]. Much more limited is the information on the antimicrobial activity of chitosan against *Klebsiella pneumonia*, among which we can mention the work on the cell ultrastructure under the action of the chitosan polymer [4]. There are almost no data about the influence of chitosans of different molecular weight on this type of bacteria.

At the same time, klebsiella is one of the main groups of bacteria causing infectious diseases in humans, which are characterized by a severe course and are often fatal [12]. Among the major intestinal infections, klebsielosis reaches 20%, amounting in some years in some areas to half of all cases of this type of disease. This disease is of particular importance in newborns and young children accounting for two-thirds of all acute intestinal infections [13]. A widespread of multidrug resistant strains of enterobacteriaceae becomes the increasingly serious problem of antibiotic therapy accompanied with dysbacteriosis [14].

Therefore, an urgent problem is the investigation of biocidal properties of the chitosan polymer against *K. pneumonia*, which leads to more than 85% infec-

Abbreviations: MES, morpholino ethansulfonic acid; ACES, *N*-(2-acetamido)-2-aminoethansulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminomethansulfonic acid; MIC, minimal inhibitory concentration.

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Characteristics of chitosan samples

Sample number	M_w, kDa	Polydispersity index	Mean degree of polymerization	Degree of deacetylation, mol %	pK_a
1	0.7	1.41	4	95	7.1 ± 0.05
2	1.5	1.39	8	93	6.7 ± 0.05
3	2.0	1.40	12	97	6.6 ± 0.05
4	2.2	1.34	13	97	6.6 ± 0.05
5	3.5	1.71	20	95	6.5 ± 0.05
6	4.2	1.38	24	97	6.5 ± 0.05
7	5.5	2.28	32	78	6.5 ± 0.05
8	8.3	1.50	49	99	6.4 ± 0.05
9	9.6	1.44	56	97	6.4 ± 0.05
10	12.7	1.39	74	95	6.4 ± 0.05
11	15.0	1.61	87	94	6.4 ± 0.05
12	19.9	1.66	116	98	6.4 ± 0.05
13	600000*	–	3400	85	6.4 ± 0.05

* Average viscous molecular weight (M_U).

tious diseases caused by all other members of this genus [13]. Moreover, the relationship between the antibacterial effect and the molecular weight of chitosan remains to be answered.

There are many experimental works concerning antibacterial and antifungal activities of chitosan [2–11, 15–18]. However, the relationship between the chemical structure of chitosan and its biological effect on the cells of microorganisms remains unclear. The establishment of this relationship is complicated by the fact that almost all available chitosan preparations are a heterogeneous set of the molecules differing in molecular weight, deacetylation degree, disposition of remaining acetylated units along the polymer chain, viscosity, and the average value of pK_a.

The biocidal activity of chitosan has been experimentally established to be determined by its polycationic nature [11, 19]. The antimicrobial activity of chitosan increases with increasing its deacetylation degree and pH of the medium when the free amino groups are positively charged [11, 20]. However, information about the influence of the molecular weight of chitosan on its antimicrobial effect still remains controversial [21]. The mentioned enhancement of the antibacterial effect of chitosan with increasing its molecular weight may be associated with a stronger binding of the polymer to the cell surface and, thereby, a higher agglutination of the cells due to the increase of the number of the amino groups [6, 11]. On the other hand, the enhancement of the antibacterial effect of chitosan with decreasing its molecular weight can be due to the fact that low molecular-weight chitosan and its oligomers are able penetrate through the bacterial cell wall more efficiently, interact with nucleic acids and other cytoplasmic substances, and violate their functioning, which leads to cell death [8, 15, 22].

There are no data on the comparative study of the antibacterial properties of chitosan according to both the polymerization degree and the level of acidity of the medium. This analysis is necessary because the chitosan molecules can have different pK_a values depending on its molecular weight, and the difference in the protonation degree may contribute to the biological activity of chitosan.

The goal of this work was to study the antibacterial activity of a number of chitosan samples of different molecular weights at different pH against *K. pneumoniae*.

RESULTS AND DISCUSSION

We used the chitosan samples characterized by both the molecular weight distribution (MWD) and deacetylation degree (table). The samples of low molecular chitosan were prepared by acidic hydrolysis and had a rather narrow MWD (polydispersion), which allowed for the comparative study of the antibacterial properties of 12 samples with the molecular weight from 0.7 to 19.9 kDa. To diminish the influence of the acetyl residues, we used only the samples with the high deacetylation degree (excluding sample 7). Thus, it was possible to evaluate the antibacterial activity of chitosan only depending on the molecular weight at controlled pH.

We showed that the experimental samples had the different pK_a values, which depended on the polymerization degree of chitosan. The oligomeric forms of

chitosan with the minimal molecular weight had higher pK_a values than the higher molecular weight samples (table). The sample with the minimal polymerization degree corresponding to the mixture of approximately tetramer-pentamer had the maximal pK_a value (7.1). This sample was the closest for this value to the monomer of chitosan, i.e., glucosamine ($pK_a = 7.9$). The increase in the polymerization degree led to the predictable decrease in the pK_a value of chitosan, and the sample with the polymerization degree of 49 had $pK_a = 6.40$, which did not change further for the higher molecular-weight samples including the initial high molecular-weight chitosan.

Taking into account that the pK_a value influences not only the protonation degree of the amino groups of chitosan but also its solubility, we studied precipitation of chitosan after alkalization of the solution.

It was found that the highest solubility was typical for the oligomeric forms of chitosan with the polymerization degree from 4 to 13 and the high pK_a values from 6.6 to 7.1 (Fig. 1). These samples had good solubility in the entire range of pH values used in the experiment. Another group of highly soluble samples contained oligochitosans with the polymerization degree from 20 to 32 and the pK_a value of 6.5. Unlike the samples of the first group, these samples formed a noticeable amount of precipitate in mildly alkaline conditions, however, not immediately, but after a few hours. The decrease in the deacetylation degree to 78% (sample 7) did not significantly change both the solubility and pK_a value of chitosan. The last group contained all samples with the polymerization degree of about 50 and more, including the initial high molecular-weight chitosan. They had the lowest pK_a value ($pK_a = 6.4$). These samples formed a significant amount of precipitate during a short period of time, even at a pH close to neutral. In this case, an approximately equal amount of the precipitated chitosan was formed at pH 7.75 and 8.00 (and after 24 h at pH 7.50) indicating almost complete precipitation of the polymer and the absence of a noticeable amount of chitosan in the soluble form. Thus, all samples of chitosan were highly soluble in the acidic medium and differed in this parameter in neutral, and especially alkaline, media.

The solubility of the samples depends on both the molecular weight of chitosan and its pK_a. The latter parameter characterizes the ability of chitosan to bind protons to its free amino groups, which results in the formation of a polycation with the corresponding properties. The difference in the pK_a values for the samples of different molecular weight indicates the variation of the amount of the charged amino groups in these polymers at a certain pH value. For example, exactly half of the amino groups of chitosan with the polymerization degree from 20 to 32 are positively charged at pH 6.5. In these conditions, the oligomeric forms of chitosan contain the higher proportion of the protonated amino groups as compared with the uncharged ones. The fraction of the charged aminosugar residues will increase with decreasing the polymerization degree of the sample. On the other hand, the higher molecular weight samples with the increased polymerization degree (>50) will contain the slightly lower proportion of the protonated amino groups because of the minimal pK_a value (6.4) for these samples.

Since it is the positive charge of the polymeric molecule which determines the antibacterial activity of chitosan, we decided to investigate the antibacterial activity of the chitosan samples differing in molecular weight (and the pK_a value) in the media with a wide pH range.

We used a series of chitosan samples with molecular weights continuously covering the entire range of transfer from oligomers to the typical low molecular-weight forms. We used a buffer system based on three organic acids, which maintained a specified level of acidity in a wide pH range comprising the pK_a values of all chitosan samples under investigation.

The control experiments without chitosan showed the growth of the *K. pneumoniae* culture at all pH values. Consequently, it was the studied compound (chitosan) but not the medium acidity, which inhibited the growth of the culture.

The inhibitory effect of the polymer on the *K. pneumoniae* growth was shown to depend on both the molecular weight of chitosan and the pH in the solution (Fig. 2). The inhibitory effect of each sample increased with decreasing pH, i.e., the necessary level of the inhibition of the microorganism growth required the decreased concentration of chitosan. In this case, the interesting feature of the individual, mostly low molecular-weight, samples was observed. They exhibited the maximal inhibitory effect in the region of pH 6 but not at the maximal acidity of the medium. The similar effect was noticed for other polycations against both gram-negative and gram-positive bacteria [25].

The antibacterial action of chitosan is due to its molecular weight; however, two points may be noted in this relationship. First, the significant difference in the antibacterial properties are typical for the oligomeric forms of chitosan and those with the lowest molecular weight, while the samples with the polymerization degree of 50 and more exhibit an almost equal antibacterial effect. Second, the dependence of the antibacterial effect of chitosan samples on their molecular weights clearly falls into two ranges of the pH values, i.e., in the acidic media with pH below 7 and alkaline media with pH above 7. In the acidic media, the antibacterial activity of chitosan increases with increasing its molecular weight. In this case, the inhibition of the bacterial growth requires the lower concentration (by more than an order of magnitude) of the samples with the high polymerization degree as compared with the oligomeric samples. The antibacterial activity of chi-

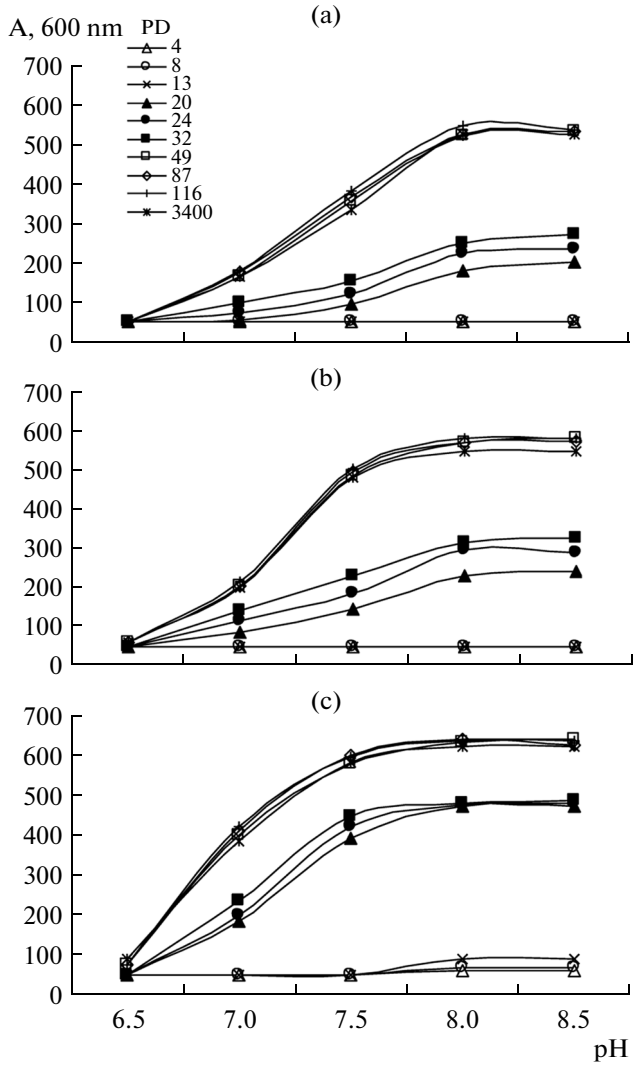

Fig. 1. Dependence of formation of chitosan precipitates on their polymerization degree and pH value in the medium after incubation for 0.5 h (a), 3 h (b), and 24 h (c). Polymerization degrees (PD) are given in the figure legend.

tosan also increases in the range of alkaline pH with increasing molecular weight. However, this effect is observed only for oligomers with the polymerization degree below 32 (5.5 kDa), while the higher molecular-weight samples lose completely their antibacterial properties (Fig. 2).

The minimal inhibitory concentrations (MIC) for oligomers at high pH are significantly lower as compared with those in the acidic media. Nevertheless, the observed inversion effect of the dependence of the antibacterial activity of chitosan on its molecular weight can clarify to some extent the accumulated contradictions in the literature in the explanation of this dependence. We described previously the similar effect for *Staphylococcus aureus* [26], which leads to the conclusion about a certain universality in the relationship between the physico-chemical properties of

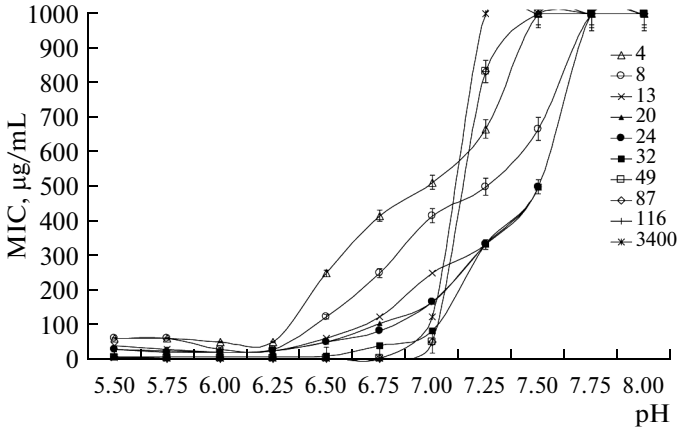

Fig. 2. Dependence of MIC values of chitosan samples with different polymerization degree on the medium pH with respect to *K. pneumonia*. Polymerization degrees are given in the figure legend.

chitosan and its inhibitory activity against both gram-negative and gram-positive bacteria.

The inversion of the dependence of the antibacterial activity of chitosan on its molecular weight indicates the coincidence of the MIC values for any two samples differing in the polymerization degree in the pH range from 7.00 to 7.50. In other words, at a certain pH these samples exhibit the same antibacterial effect despite the difference in their protonation degree as follows from the pK_a data. In this case, it seems likely that the contribution of the polymerization degree along with the protonation degree of chitosan to its inhibitory effect may be the same in the sum value, which will result in the coincidence of the MIC values. This also means that the main factor for the antibacterial effect under acidic conditions is the polymerization degree of chitosan, while the influence of the protonation degree predominates under alkaline conditions.

It should be noted that the initial sample of chitosan with the average-viscosity molecular weight of 600 kDa was close to low molecular-weight samples (10–20 kDa) in its antibacterial action but, nevertheless, was inferior to them in the activity under weakly acidic conditions against *K. pneumoniae* (Fig. 2). This is probably due to a poor solubility of this sample with the higher polymerization degree as compared with low molecular-weight chitosan samples, which can be caused by a substantial amount of crystalline regions.

Thus, the evaluation of the influence of the molecular weight of chitosan and pH of the medium on the antibacterial activity of a number of chitosan samples against gram-negative *K. pneumoniae* showed that the chitosan pK_a value was of great importance. For the first time, we found the inversion effect in the dependence of the inhibitory activity of chitosan on its molecular weight with the change of pH in the medium. This allows one to explain to some extent the

contradiction in the consideration of this fact in the literature and may provide more rational use of chitosan as an antibacterial agent to obtain the maximal effect under certain conditions by using a minimal amount of the sample of the optimal molecular weight. There is also the possibility of enhancing the antibacterial properties of chitosan by its chemical modification, thereby changing its pK_a value.

EXPERIMENTAL

Analysis of chitosan samples. We used the sample of high-molecular-weight crab chitosan with the average molecular weight of 600 kDa and deacetylation degree of 85% (Heppe, Germany). The low molecular-weight chitosan samples were submitted from the collection of the laboratory of physiologically active biopolymers (Institute of Organoelement Compounds of the Russian Academy of Sciences, Russia). The deacetylation degree was evaluated by the PMR method [23]. The average molecular weight and polydispersity index of chitosan were evaluated by high performance gel permeation chromatography as described in [24].

Evaluation of pK_a values and solubility of chitosan samples. The dissociation constant values of conjugated acids were evaluated by titration. The studied sample of chitosan hydrochloride (50 mg) was dissolved in distilled water (50 mL), and after the complete dissolution, hydrochloric acid was added to pH 3.00.The solution was then titered under constant stirring by adding 0.5 M NaOH in portions. The pH value was monitored by a Hanna pH meter. The pK_a values were calculated using the pH values corresponding to the middle of the plateau on the titration curve.

The solubility of chitosan samples was studied in a complex buffer system based on organic acids, i.e., morphilinethan sulfonic acid (MES) (Helicon), *N*-(2-acetamido)-2-aminoethan sulfonic acid (ACES) (Sigma-Aldrich), and 2-[tris(hydroxymethyl)methylamino]-1-ethan sulfonic acid (TES) (Sigma-Aldrich). We used 0.15 M MES-ACES-TES-Na buffer containing the equimolar amounts of all three acids with different pH values from 5.50 to 8.00 with increments of 0.25. The buffer solution was placed in 96-well flat-bottom microplates (180 μL/well), followed by the addition of the chitosan solution (20 μL/well). The mixtures were incubated at room temperature, and the optical absorption was measured in 0.5, 3, and 24 h on a spectrophotometer with the vertical beam for measurement of light absorption at 600 nm.

Preparation of working solutions of low-molecular-weight chitosan samples. Low-molecular-weight chitosan hydrochlorides were dissolved in distilled water to the concentration of 8 mg/mL (the weight of chitosan was calculated without taking into account the counterion) and sterilized by filtration through membranes with a pore diameter of 0.22 μm, followed by heating in a water bath for 5 min. The resultant solutions were stored before use at 4°C.

Bacterial strains and their cultivation. We used the *K. pneumoniae* ATCC 13884 strain, which was stored in a semiliquid meat peptone agar (MPA) at –20°C. The working cultures of bacteria were stored on MPA at 4°C. The 18-h bacterial culture was prepared in 100-mL flask with meat peptone broth (MPB) by adding 5% (in volume) the suspension of the bacterial culture, followed by the incubation of the mixture at 37°C on a shaker at 150 rpm.

Minimal inhibitory concentration (MIC) was evaluated by the modified method described in [5]. The substance was diluted two-fold with MPB containing 0.15 M TES-ACES-MES-Na buffer (0.05 M TES, 0.05 M ACES, 0.05 M MES) at a certain pH from 5.50 to 8.00 with an increment of 0.25 and placed into 96-well round-bottom plates, followed by the addition of the suspension of the bacterium in the same medium to the final concentration of 2.5×10^5 cfu/mL. The MIC values of chitosan samples were evaluated in three independent experiments after the incubation (24 h at 37°C on a shaker at 200 rpm) by the absence of the culture growth in wells containing the minimal concentration of the substance. The wells without chitosan containing the nutrient medium, a buffer with corresponding pH, and inoculated as in the experiment, were used as controls.

ACKNOWLEDGMENTS

The work was funded by the subsidy of the Russian Government to support the Program of Competitive Growth of Kazan Federal University among World's Leading Academic Centers and the Russian Foundation for Basic Research (project no. 12-04-97039 r_povolzhie_a).

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Translated by A. Levina