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EXPERIMENTAL ARTICLES

Effect of Chitosan Derivatives on the Reproduction of Coliphages T2 and T7

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Abstract—The effect of chitosan derivatives with different degrees of polymerization and deamination, as well as of chitosan 6-*O*-sulfate and chitosan *N*-succinate-6-*O*-sulfate, on the reproduction of coliphages T2 and T7 in *Escherichia coli* and on the growth of this bacterium was studied. Chitosan derivatives decreased the yield of coliphages and exhibited antibacterial activity. The efficiency of inhibition of viral infection and the antibacterial activity of chitosan were found to be dependent on the degree of its polymerization. At the same time, there was no correlation between the degree of chitosan deamination and the extent of inhibition of viral infection. Anionic chitosan derivatives virtually did not possess antiviral or antibacterial activity. It is assumed that chitosan blocks some stages of phage reproduction. The decrease in the phage-producing ability of *E. coli* may also be due to the antibacterial effect of chitosan.

Key words: bacteriophages T2 and T7, chitosan, *Escherichia coli*

It is known that chitosan inhibits the infection of *Escherichia coli* by bacteriophages T2 and T7, of *Bacillus thuringiensis* by bacteriophages 1-97A and 1-97B, and of *Gluconobacter oxydans* by Gs bacteriophages [1–3]. Therefore, chitosan effectively inhibits the reproduction of various virulent phages in both gram-negative and gram-positive bacteria and prevents their phagolysis.

It has been found that chitosan produces multiple effects on the development of viral infection. In particular, it inactivates mature phage particles, making them avirulent. At the same time, chitosan inhibits the growth of *E. coli* and probably affects some stages of phage reproduction [1, 2]. Therefore, the degree of inhibition of viral infection by chitosan likely depends on a number of factors; however, it remains unknown which of them are decisive in preventing the formation of virulent phage progeny.

It should also be noted that relevant experiments were carried out with the use of a high-polymeric chitosan preparation. Meanwhile, due to its polymeric nature, many biological activities of chitosan depend on the degree of polymerization of its fragments, the degree of acetylation of amino groups, the net charge of the molecule, and on its conformation in solutions.

Since specific structural properties of chitosan may differently affect bacterial culture, intact phage particles, and the particular stages of viral infection, various chitosan derivatives might appear to be useful for studying these particular stages.

In this work, chitosan fragments with different degrees of polymerization and a number of its chemical derivatives were used to study the process of infection of *E. coli* by bacteriophages T2 and T7.

MATERIALS AND METHODS

The strain *E. coli* B1, bacteriophages T2 and T7, and experimental conditions were described in detail previously [1].

Krill chitosan fragments with different degrees of polymerization (DP), composed of 250, 19, and 15 glucosamine residues, were prepared by hydrolyzing chitosan with hydrochloric acid. Partially deaminated chitosan derivatives with a degree of deamination of 10, 30, and 50% were prepared by hydrolyzing chitosan with nitrous acid. The average DP of these deaminated fragments was 28, 15, and 4, respectively. Two anionic derivatives, chitosan 6-*O*-sulfate and chitosan *N*-succinate-6-*O*-sulfate, were characterized by DP 30 and 28, respectively. The chitosan derivatives used and the methods of their preparation have been described in detail previously [4].

Solutions of chitosan (1 mg/ml) were prepared by dissolving dry chitosan in 0.05% acetic acid or water. When necessary, the pH of the solutions was adjusted to 5.6–5.8 with 1 M NaOH. Solutions were sterilized by autoclaving at 1 atm for 20 min.

Cold-synchronized *E. coli* cultures were incubated in liquid M9 medium for 1.5 h at 37°C, supplemented with chitosan at concentrations ranging from 0.1 to 100 μg/ml, and incubated for the next 20–30 min. Then bacteriophage was added in a proportion of 0.1 phage particles per one cell, as previously described [1].

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Table 1. Effect of chitosan fragments with different degrees of polymerization on the yield of bacteriophages (% of the control level)

Bacteriophage	Chitosan concentration, μg/ml	Average degree of polymerization of chitosan fragments		
		250	19	15
T2	100	10^{-4}	0.4	82.2
	10	4.5	10.5	80.0
	1	75.1	30.3	83.0
	0.1	90.2	40.4	60.6
T7	100	0.02	1.1	80.8
	10	0.03	33.3	80.0
	1	50.2	37.4	73.1
	0.1	50.9	40.4	60.0

Table 2. Effect of chitosan fragments with different degrees of deamination and polymerization on the yield of bacteriophages (% of the control level)

Bacteriophage	Chitosan concentration, μg/ml	Degree of deamination, %/degree of polymerization of chitosan fragments		
		10/28	30/15	50/4
T2	100	1.1	1.4	6.2
	10	30.2	9.6	92.1
	1	80.8	77.3	91.9
	0.1	70.7	92.3	99.0
T7	100	0.1	0.2	0.2
	10	22.2	0.3	1.5
	1	95.0	25.5	75.1
	0.1	95.0	100.0	70.6

The number of virulent phage particles in the experimental and control samples was determined by the method of agar layers [5]. The effect of chitosan on the reproduction of bacteriophages was expressed as the ratio of phage titer in the sample with chitosan to that in the control sample containing no chitosan. All the experiments were performed in several replicates. The tables show the average values of the phage titer ratios expressed as a percentage of the control. Standard deviations are not shown.

The number of viable *E. coli* cells was determined by plating cultures onto agar LB medium [1].

RESULTS

It can be seen from Table 1 that chitosan derivatives prevent the development of viral infection by decreasing the yield of virulent phage. It is obvious that there exists a direct relationship between the DP of chitosan fragments and the extent of inhibition of phage reproduction, which is most pronounced at high chitosan concentrations (100 and 10 μg/ml) but is still noticeable even at a chitosan concentration of 0.1 μg/ml. The character of decrease in the extent of inhibition with a decrease in the chitosan concentration was dependent on the DP of the fragments. For instance, the effect of the high-polymeric chitosan fragment with DP 250 drastically fell with a decrease in its concentration, whereas the effect of the short fragment with DP 19 was less dependent of its concentration, and the effect of the fragment with DP 15 was very low, whatever its concentration. It should be noted that such a tendency was characteristic of both the phages investigated, despite their distinct differences.

In another series of experiments, the effect of deaminated chitosan derivatives on the reproduction of coliphages was studied. In contrast to treatment with hydrochloric acid, which led to only the fragmentation of chitosan, the treatment of chitosan with nitrous acid not only led to its depolymerization, but also was accompanied by a chemical modification of chitosan: deamination at the polymeric chain breaking sites due to the conversion of the arising terminal glucosamine residue to 2,5-anhydromannose [6, 7]. As a result, deaminated chitosan fragments had a low DP. The data on the antiviral activity of deaminated chitosan fragments are presented in Table 2.

It can be seen that the high concentration (100 μg/ml) of deaminated chitosan fragments inhibited phage infection, decreasing the phage yield by three orders of magnitude. No noticeable correlation was revealed between the degree of deamination and the extent of inhibition of phage reproduction. It is interesting that the chitosan fragment with a 30% degree of deamination and DP 15 taken at high concentrations (10 and 100 μg/ml) was much more effective than the nondeaminated fragment with the same DP (Table 1). Infection induced by phage T7 was inhibited by deaminated chitosan fragments at concentrations of 10 and 100 μg/ml stronger than the infection induced by phage T2.

It has been previously shown that high-polymeric chitosan preparations inhibit the growth of *E. coli* in mineral M9 medium [1], i.e., that they possess antibacterial activity. Since the state of a bacterial culture is of paramount importance for its susceptibility to viral infection, we attempted to elucidate whether the chitosan derivatives used in this study affect the growth of *E. coli*. As can be seen from the results of these experiments presented in Table 3, chitosan derivatives did affect *E. coli* growth, exhibiting a direct correlation between their DP and antibacterial activity. The degree of deamination also influenced antibacterial activity: the chitosan derivative with a 10% degree of deamination did not influence the growth of the culture, whereas the derivative with a 30% degree of deamination caused a transient reduction of culture growth, and the derivative with 50% degree of deamination completely inhibited growth. The control culture (without chitosan)

Table 3. Effect of chitosan derivatives on the growth of *E. coli* in M9 medium

Number of viable cells ($\times 10^8$)	Degree of					
	polymerization			deamination, %		
	250	19	15	10	30	50
At the moment of addition of chitosan	3.0	4.4	4.4	1.3	2.0	1.4
20 min afterwards	0.02	0.1	4.3	–	0.3	–
90 min afterwards	0.1	–	–	4.7	3.5	0.7

Note: Chitosan fragments were added at a concentration of 100 μg/ml; "–" stands for "not determined."

exhibited linear growth (data not shown), which is typical of mineral media.

Even at a high concentration of 100 μg/ml, anionic chitosan derivatives did not affect *E. coli* growth and decreased the yield of bacteriophages by no more than 30% (data not presented).

DISCUSSION

On the basis of our previous experimental data [1–3], it could be suggested that chitosan may prevent the reproduction of phages in several ways: (i) by blocking certain stages of reproduction, beginning from the stage of phage adsorption and ending in the stage of lysis of the infected cell; (ii) by neutralizing the virulence of original or newly formed phage particles; and (iii) by affecting the susceptibility of the bacterial culture to viral infection due to the antibacterial activity of chitosan.

The results presented in this paper show that the extent of inhibition of viral infection by chitosan, as well as its antibacterial activity, directly depend on the DP. Such correlation suggests that the decrease in the number of viable cells in the presence of chitosan is directly related to the decrease in the yield of the virulent phage.

The antibacterial activity of chitosan is well known [8]. In particular, chitosan and its derivatives inhibit the growth of many gram-negative microorganisms [9, 10]. It has also been shown that chitosan forms stable complexes with the lipid A of *E. coli* and *Ersinia* [11]. The antibacterial effect of chitosan is analogous to that of other polycations, which disrupts the external layer of the outer membrane and induce the death of bacterial cells [12, 13].

Therefore, the detrimental effect of chitosan on a bacterial culture may be one of the causes of inhibition of viral infection. The following explanation of the observed correlation between the antibacterial and antiviral activities of chitosan and its DP seems most plausible. It is known that phages can be specifically adsorbed on the cell wall of both viable and dead susceptible cells, but their reproduction is possible only in viable cells. Therefore, the decrease in the number of viable cells and the increase in the number of dead cells with adsorbed phage particles incapable of reproduction will result in a decrease in the phage progeny yield.

The results of experiments with deaminated chitosan derivatives can be summarized as follows. First, no correlation was found between the degree of chitosan deamination and the extent of inhibition of viral infection, which suggests that the number of amino groups does not play an important role in the inhibition of infection. Second, the high concentrations of deaminated chitosan suppressed phage T7 more strongly than phage T2. Third, the antibacterial activity of deaminated chitosan derivatives increased with the increase in the degree of deamination and with the decrease in the DP. These data indicate that the effect of deaminated chitosan derivatives on phage infection differs from that of chemically unmodified chitosan.

Thus, the extent of inhibition of viral infection by chitosan depends on the degree of its polymerization. The antibacterial activity of chitosan can be considered as one of the causes of its antiviral activity. The role of inactivation of phage particles in the inhibition of viral infection is the subject of the accompanying paper.

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