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EXPERIMENTAL ARTICLES

Inactivation of Coliphages by Chitosan Derivatives

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Abstract—The effect of chitosan fragments with different degrees of polymerization and the chemical derivatives of chitosan differing in the number of amino groups and total molecule charge on phages T2, T4, and T7 was studied. The interaction of chitosan with bacteriophage particles inactivated them to the extent dependent on the chemical properties of chitosan and its concentration. Phage T2 was found to be most susceptible to inactivation by chitosan. The polycationic nature of chitosan plays an important role in the inactivation of phages. It is assumed that the abnormal rearrangement of the basal plate of phages, the loss of long tail fibers, and, probably, modification of the receptor-recognizing phage proteins may be responsible for the inactivation of coliphages by chitosan.

Key words: bacteriophages T2, T4, and T7, inactivation, chitosan

Chitosan has been shown to affect the viability of bacterial cells and certain stages of phage reproduction, as well as to neutralize the virulence of mature phage particles [1, 2]. The latter effect manifests itself in a decrease in the phage titer after the addition of chitosan to phage suspensions.

The mechanisms underlying the inactivation of bacteriophages by chitosan have not yet been studied. Moreover, the role of inactivation of phage virulence in the inhibition of phage infection by chitosan remains unknown.

In the accompanying paper [3], we showed that the efficiency of inhibition of coliphage-induced infection strongly depends on the chemical properties of chitosan molecules. For understanding the mechanism of action of chitosan, it was necessary to determine the role of its particular structural features.

The aim of this work was to study the effect of chitosan derivatives differing in the degree of polymerization, the number of amino groups, and the net charge of their molecules. Another aim was to search for chitosan derivatives lacking bactericidal activity and the capacity to inactivate mature phage particles but still able to inhibit viral infection. Such derivatives would enable the study of the direct effect of chitosan on the infection process.

MATERIALS AND METHODS

The strain *Escherichia coli* B1, bacteriophages T2 and T7, and relevant experimental methods have been described previously [1–3]. Bacteriophage T4 was kindly provided by A.F. Bobkova (Department of Virology, Moscow State University).

The preparation and main characteristics of the derivatives of krill chitosan with different degrees of polymerization (DP) and deamination, the anionic derivatives chitosan 6-*O*-sulfate (DP 30) and chitosan *N*-succinate-6-*O*-sulfate (DP 28), and water-soluble chitin (DP 1500) have been described in detail previously [3, 4].

To study the effect of chitosan on bacteriophages, 0.9 ml of M9 medium [5] was mixed with 100 μl of chitosan solution of the desired concentration and 50–100 μl of phagolysate and incubated for 20–30 min at 37°C. The phage titer was then determined by the method of agar layers [6]. The extent of inactivation of phage virulence was assessed from the amount of virulent phage remaining in a sample after its incubation with chitosan. The relative amount of virulent phage was determined as the ratio of the phage titers of the experimental and control samples. All experiments were performed in several replicates. The tables show average experimental values (standard deviations not shown).

Structural alterations in the bacteriophage T2 particles incubated with chitosan were analyzed by electron microscopy. Specimens were contrasted with 2% phosphotungsten acid, pH 7.0, and examined at 80 kV in a JEM-100CX electron microscope at a magnification of 50000×.

RESULTS

It can be seen from Table 1 that the virulence of all phages was reduced in the presence of chitosan and that the extent of phage inactivation by chitosan fragments was dependent on their DP. The chitosan fragment with DP 19 showed the highest capacity to inactivate phage T2, almost completely neutralizing its virulence when

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taken at a concentration of 100 μg/ml. Phage T7 was much more resistant to chitosan fragments.

Morphologically, phage T7 strongly differs from phage T2. Therefore, the difference in the extent of inactivation of these two phages by chitosan might be due to their morphological peculiarities. To verify this suggestion, we studied the inactivation of phage T4, which is morphologically close to phage T2. Unexpectedly, the extent of inactivation of phage T4 by chitosan was similar to that of the morphologically dissimilar phage T7. Therefore, the extent of the chitosan-induced inactivation of phage virulence weakly depends on their morphology.

Chitosan fragments inactivated phages in a dose-dependent manner, except for the inactivation of phage T2 by the chitosan fragment with DP 15 taken at concentrations of 10 μg/ml and lower (Table 1).

It can be seen from Table 2 that deaminated chitosan derivatives, especially those deaminated by 20 and 30% (DP 19 and 15, respectively), exhibited significantly lower activity than unmodified chitosan fragments. At a concentration of 100 μg/ml, the phage-inactivating activity of deaminated derivatives was several orders of magnitude lower (phage T2) and three to four times lower (phages T4 and T7) than that of unmodified fragments with the same DP. For all the phages investigated, the antiviral activity of deaminated derivatives decreased with the decrease in the number of amino groups, so that chitosan derivatives deaminated by 50% were completely unable to inactivate phages.

Table 3 shows that chitin and anionic chitosan derivatives possessed low antiviral activity.

Electron-microscopic examination of phage T2 particles incubated with the chitosan fragment with DP 19 revealed distinct alterations in their structure. In most particles, long tail fibers were absent, and the basal plate was deformed (Fig. 1a). Some phage particles had contracted tail sheaths (Fig. 1b), although the heads remained electron-dense, indicating a delay in the release of the phage DNA. Incubation with the chitosan derivative deaminated by 50% did not induce noticeable alterations in the structure of phage T2 particles; however, the long tail fibers were completely unwound (Fig. 1c), whereas they are usually folded round the intact phage tail [5].

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	68.1	1.0×10^{-5}	2.0×10^{-2}
	50	–	3.0×10^{-3}	–
	10	92.2	10.1	42.6
	1	100.0	100.0	46.3
	0.1	100.0	100.0	40.8
T4	100	–	21.1	90.4
T7	100	75.4	30.1	24.5
	10	85.0	70.2	60.3
	1	100.0	85.9	79.9
	0.1	100.0	100.0	100.0

Note: "–" stands for "not determined."

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	20/19	30/15	50/4
T2	100	56.1	65.2	77.8	100.0
	10	88.7	91.2	77.3	100.0
	1	89.6	87.4	–	–
T4	100	80.4	80.7	–	100.0
T7	100	71.8	92.6	93.4	100.0
	10	86.5	92.0	98.3	100.0
	1	88.2	100.0	–	–

Note: "–" stands for "not determined."

Table 3. Effect of chitin and anionic chitosan derivatives on the yield of bacteriophages (% of the control level)

Bacteriophage	Concentration of compound added, μg/ml	Chitin	Chitosan sulfate	Chitosan *N*-succinate-6-*O*-sulfate
T2	100	3.0×10^{-1}	88.6	66.2
T7	100	96.8	80.1	92.4

DISCUSSION

The results obtained in this work show that the interaction of chitosan with bacteriophages reduces their virulence to the extent dependent on the structure of the chitosan derivative used, its concentration, and the bacteriophage type. Although phages T2 and T7 were more strongly inactivated by shorter chitosan fragments, a monotonic (inverse) dependence on DP was characteristic only of phage T7. Phages T2 and T4 were inactivated most efficiently by the chitosan fragment with DP 19.

Phage T2 was found to be most readily inactivated by chitosan. The several-orders-of-magnitude decrease in the virulance of this phage is possibly related to alterations in its structure. It is known that the long tail

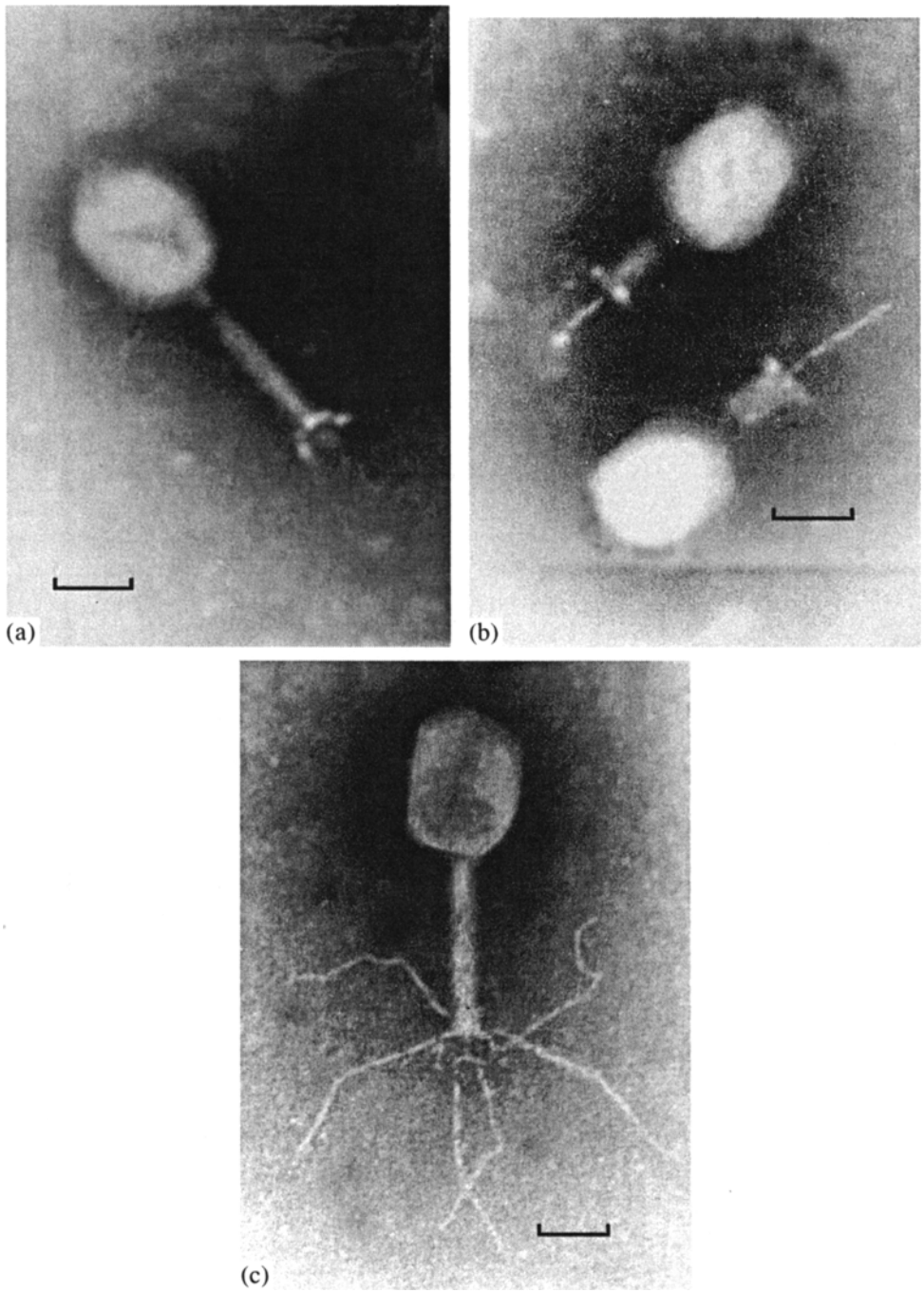

Fig. 1. Bacteriophage T2 particles after incubation with (a, b) chitosan fragment with DP 19 and (c) chitosan deaminated by 50%. Bars represent 50 nm.

fibers of phages are responsible for their binding to bacterial receptors, and that several fibers must simultaneously bind to one bacterial receptor for phage adsorption to be irreversible [7]. Therefore, it would be reasonable to suggest that the loss of the long fibers results in the inability of phages to attach to bacterial cells and, hence, to infect them.

Another important consequence of the interaction of chitosan with phage T2 is an aberrant contraction of its tail sheath, which is not accompanied by the release of DNA from the phage head. These results show that chitosan, similarly to other factors, such as elevated temperature or urea, can induce the abnormal rearrangement of the phage tail. Similar abnormal contraction of the tail sheath has also been observed during the rearrangement of the tail appendages in the triple heat-sensitive ts mutant of phage T4, which bears mutations in the genes 5, 6, and 12 that encode proteins of the distal part of the basal plate [8].

It is the basal plate that is responsible for the synchronism of the structural transformations of a phage, so that even small alterations in the proteins of the distal part of the basal plate cause a significant dysfunction of the phage [8, 9]. Therefore, the possibility cannot be excluded that the interaction of chitosan with the proteins of the basal plate of phage T2 destabilizes it and provokes its premature (before contact with the bacterial cell wall) and abnormal rearrangement, which results in the inability of the phage to infect bacterial cells. Abnormal rearrangement may also be responsible for the loss of the long tail fibers which are normally attached to the basal plate.

It should be noted that chitosan fragments with DP 19 induced the rearrangement of only part of the phage particles. In spite of the close relatedness and morphological similarity of phages T2 and T4, the degrees of their inactivation by chitosan were significantly different. Therefore, the involvement of other, less pronounced, consequences of the interaction of chitosan with phage particles resulting in the loss of their virulence cannot be excluded. For instance, chitosan might irreversibly change the conformation of the receptor-recognizing proteins of phage T2 and thus prevent the specific binding of phage particles to the bacterial surface.

Phages T2 and T4 recognize quite different receptors on the bacterial cell surface. Phage T2 recognizes the outer membrane hydrophobic FadL protein, which is a transporter of fatty acids, and porin OmpF, a trimeric protein localized in the outer membrane of *E. coli* [10, 11], whereas phages T4 and T7 recognize a polysaccharide of the outer membrane [7, 12]. It should be emphasized that phages T7 and T4 are similar in recognizing the same receptor and in being weakly susceptible to short chitosan fragments.

The amino groups of chitosan may play an important role in its interaction with phage particles. It was established that deaminated derivatives were much less active than chemically unmodified fragments. Moreover, the antiviral activity of deaminated chitosan derivatives directly correlated with their degree of deamination. Chitin fragments exhibited low antiviral activity; this finding agrees with the fact that more than 80% of amino groups in chitin were acetylated [4]. Therefore, it is highly probable that the decrease in the antiviral activity of deaminated chitosan derivatives is due to a low content of amino groups.

Shielding of the positive charge of chitosan by the negatively charged sulfo groups in chitosan sulfate or the reversal of its charge in chitosan *N*-succinate sulfate led to an almost complete loss of antiviral activity (Table 3). Therefore, one of the causes of phage inactivation may be the ionic interaction of chitosan with phage particles, inducing their rearrangement; this event has been registered by electron microscopy in the case of the interaction of phage T2 and the chitosan fragment with DP 19.

The results presented here and in the accompanying paper indicate that the inhibition of coliphage reproduction and the inactivation of phage virulence by chitosan are rather independent processes determined by different mechanisms. For instance, the reproduction of coliphages was most strongly inhibited by high-polymeric chitosan preparations, whereas their virulence was most strongly suppressed by chitosan fragments with a low DP. Deaminated chitosan derivatives possessed low antiviral activity, which inversely correlated with the degree of deamination. At the same time, no noticeable correlation was found between the extent of inhibition of viral infection by deaminated chitosan derivatives and the degree of their deamination. Furthermore, the extent of inhibition of viral infection was, as a rule, significantly higher than the extent of suppression of phage virulence [3]. These data indicate that the inhibition of phage reproduction by chitosan cannot be explained by only the inactivation of mature phage particles or the bactericidal activity of chitosan [3]. To elucidate the whole mechanisms of inhibition of viral infection by chitosan, its effect on the particular stages of the life cycle of bacteriophages should be studied.

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