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Antiviral activity of chondroitin sulfate against infection by tobacco mosaic virus

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In the course of searching for antiviral substances against the tobacco mosaic virus (TMV), it was found that chondroitin sulfate (Chs) A-type and C-type had a high inhibitory activity against TMV infection. The addition of Chs to the inoculum solution greatly reduced the number of local lesions formed on Xanthi NN tobacco leaves. The degree of inhibition increased with Chs concentration and was higher in C-type than A-type, which suggests that the strength of inhibition relates to the stiffness of the polymer chain of Chs. For Chs C-type inhibition was independent of molecular weight where the molecular weight was between 30 000 and 70 000, and decreased with molecular weight below 30 000. The electron micrograph showed that the TMV suspension itself was almost monodisperse and that the addition of Chs caused TMV to form large raftlike aggregates. The TMV solution became turbid after the addition of a large amount of Chs. The antiviral activity of Chs on the infectivity of TMV may be caused by blocking the decapsulation process of TMV protein on the cell membrane surface. © 1997 Elsevier Science Ltd

INTRODUCTION

Tobacco mosaic virus (TMV) is a rod-shaped virus composed of single-stranded RNA encapsulated in a coat protein capsid. In the TMV and tobacco mesophyll protoplasts, an electron microscopic study (Otuki *et al.*, 1972) suggested that one end of the TMV rod was adsorbed on the cytoplasmic membrane, that few TMV lay on the cell surface and that TMV penetrated into the protoplasts by a pinocytotic process. These phenomena may correspond to the initial phase in the multiplication of TMV. For the infection of TMV to take place in leaves, it is necessary that TMV directly penetrates the cell and makes contact with materials in the plasma membrane or with intracellular organelles.

The TMV coat protein is present in a large number of association states, A-protein (4-7S), double disk (20S) and helical rods (> > 20S). The helical rod aggregates can be formed by hydrophobic interaction (Butler, 1984, Sano *et al.*, 1996). The extremity of the cylindrical rod has, therefore, the hydrophobic sites exposed and these regions can penetrate extensively into the hydrophobic membrane lipid layer. Model experiments with the phosphatidylcholine lipid monolayer have shown that the exposed hydrophobic region of the cylindrical rod could penetrate into the lipid membrane. In addition the A-protein formed after decapsulation from the extremity of the cylindrical rod had interacted with the hydrophobic region of the lipid molecule (Sano, 1989).

The substances inhibiting the process of viral infection are classified according to their antiviral actions, as (i) inhibitors of infection and (ii) inhibitors of virus multiplication. The inhibitors of infection are the substances that prevent infection from occurring when inoculated into leaves simultaneously with the virus. The inhibitors of virus multiplication are the substances that retard the rate of multiplication of the infecting virus. Cationic and anionic polyelectrolytes of both natural and synthetic origin, such as heparin and hyaluronic acid (Cohen, 1942), polylysine and polyvinylamine (Burger and Stahmann, 1951), polyglutamate, polyacrylate and polypectate polymers (Stahmann and Gothoskar, 1958), polyacrylic acid (Gianinazzi and Kassanis, 1974), polycarboxylate (Stein and Loebenstein, 1972) and alginate (Sano, 1992), have been shown to inhibit TMV.

It is well known that sulfated polysaccharides are

potent and selective inhibitors of human immunodeficiency virus, e.g. as dextran sulfate (Baba *et al.*, 1988a; Ito *et al.*, 1987; Mitsuya *et al.*, 1988; Ueno and Kuno, 1987), heparin (Baba *et al.*, 1988a), pentosan polysulfate (Baba *et al.*, 1988b), sulphoevernan (Weiler *et al.*, 1990), sulfated bacterial glycosaminoglycan (Baba *et al.*, 1990a), lentinan sulfate (Yoshida *et al.*, 1988), mannan sulfate (Ito *et al.*, 1989), dextrin sulfate (Ito *et al.*, 1991), sulfated cyclodextrin (Schols *et al.*, 1991), and synthetic sulfated polymers such as sulfated polyvinyl alcohol and sulfated copolymers of acrylic acid with vinyl alcohol (Baba *et al.*, 1990b). Their mechanism of action has been attributed to inhibition of virus adsorption on to the cell membrane.

The mechanism for the inhibitory activity of polysaccharides on TMV is not yet clear. When a mixed solution of TMV and alginate was centrifuged, most infectivity was found in the precipitate (Sano, 1992). This indicates that the aggregation of TMV formed in the presence of alginate greatly affects the infectivity of TMV. Sodium chondroitin sulfate (Chs) is a type of mucopolysaccharide. Like alginate it is an unbranched linear polyelectrolyte. In the course of searching for antiviral substances to TMV, we examined the antiviral activity of Chs (C-type and A-type) because their behavior in solution is relatively well characterized (Sano, 1985, 1986).

MATERIALS AND METHODS

Materials

TMV, Japanese common strain OM, was separated from systemically infected leaves of *Nicotiana tabacum* L. var. Bright Yellow and purified by polyethylene glycol precipitation and differential centrifugation. Chs was of the C-type or A-type. The pH of Chs in distilled water was 6.28. Chs C-type was the same as that used in previous studies (Sano, 1985, 1986) and analytical data for this sample have been previously reported (Sano, 1985). The Chs C-type samples of different molecular weight and Chs A-type were purchased from Seikagaku Co. Ltd and were further purified by Sephadex G-200 gel chromatography. Each Chs sample showed a single sharp peak on gel chromatography and therefore the degree of polydispersity of each sample is small. All other reagents were of the purest grade available.

Solution of TMV and Chs dissolved in double distilled water was dialyzed against the distilled water in a cold room at 4°C. Fresh solutions were prepared for each experiment and were clarified by centrifugation at 13 000 rpm for 35 min before use. Concentration of TMV was determined spectrophotometrically. The weight of Chs was corrected for water content by drying a small quantity of Chs in a vacuum over P_2O_5 at 60°C for three days.

Viscosity

Viscosity measurements were carried out with an Ostwald type viscometer at 25°C. The viscometer was rinsed with concentrated nitric acid, and then washed exhaustively with pure water, and dried with acetone. The intrinsic viscosity of the fractionated Chs-C was measured by changing the concentration of Chs-C in 0.15 M phosphate buffer (pH 7.2) in the presence of 0.2 M sodium chloride at 25°C. The Huggins plots gave straight lines within experimental error. By using the Mark Houwink relationship reported by Mathews (1956) for Chs-C,

$$[\eta] = 3.1 \times 10^{-2} M^{0.74}$$

where the intrinsic viscosity ($[\eta]$) is in ml/g, the molecular weights of the Chs-C fractions were calculated.

Analytical ultracentrifugation

Sedimentation studies were performed in a MSE Centriscan 75 (Scientific Instrument Co. Ltd) ultracentrifuge with photoelectric scanning system. The TMV concentration in 0.1 M tris-HCl buffer (pH 7.0) was about 2 mg/ml. Only a single peak was observed. Plots of the logarithm of the radial distance against time were linear and a sedimentation coefficient of 194S was measured for the TMV particle. This value corresponds to the monodisperse state (Sano, 1987). The Chs also showed only one single and symmetric peak in sedimentation velocity runs.

Electron microscopy

A drop of TMV alone and the mixed solution of TMV and Chs was placed on a carbon-coated grid and negatively stained with a few drops of 3% of uranyl formate. After excess fluid had been absorbed on filter paper, the specimen was observed with an electron microscope type H-500 (Hitachi, Co. Ltd).

Inhibition tests

The inhibitory effect of Chs on the infectivity of TMV was assayed on *Nicotiana tabacum* var. Xanthi NN by inoculating opposite halves of a leaf (half leaves method) with TMV (0.05 μg TMV/ml in 0.1 M sodium phosphate buffer, pH 7.0) alone and with TMV and Chs mixtures. Xanthi plants were grown in a glasshouse and selected for uniformity in each experiment. After inoculating and then washing with water, half leaves were put on wet paper in flat boxes and kept at 23°C.

Turbidity

Turbidimetric measurements of the mixture of TMV and Chs were carried out with a High Sens SM-401 spectrophotometer (Union Gikken) at 350 nm. The turbidity was measured immediately after Chs was added to purified TMV solution. The dead time was about 7 s. The reading of turbidity was automatically recorded at various reaction times.

RESULTS

Inhibition tests

The inhibitory effect of Chs on the infectivity of TMV was assayed by inoculating opposite halves of leaves with TMV alone and with TMV and Chs mixtures. The inhibitory activity of Chs was calculated as the percentage reduction in the number of local regions produced on leaves after 3 days as compared with their controls. To test whether Chs directly inactivates TMV, the suspension of Chs and TMV was incubated at room temperature, and then TMV was pelleted by ultracentrifugation, dissolved in water and inoculated on leaves. Chs did not inactivate TMV, even when incubated for 3 days, suggesting that the Chs does not combine irreversibly with the TMV particle. Circular dichroism spectra of TMV in both cases showed the same pattern. Therefore, the interaction of TMV and Chs was reversible at least on dilution. As is shown in Fig. 1, the infectivity of Chs continuously decreased with increasing concentration of Chs. The degree of inhibition was higher in C-type than A-type. However, Chs did not completely prevent TMV entry into the leaves.

The dependence of infectivity on molecular weight M of Chs C-type showed a constant value between 30 000 and 70 000, and decreased with the decrease of molecular weight below 30 000, as is shown in Fig. 2.

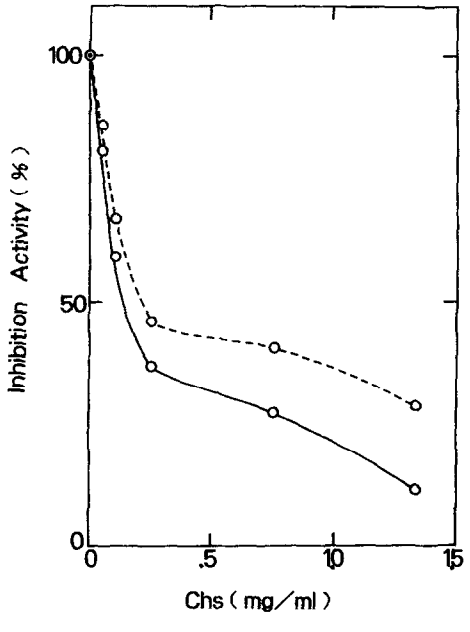

Fig. 1. Infectivity of Chs C-type (real line) and A-type (dotted line).

Electron microscopy

Figure 3 represents a scheme based on typical electron micrographs of TMV in the absence and in the presence of Chs obtained by the negative staining method. In the absence of Chs the electron micrograph of TMV showed that the TMV suspension was almost monodisperse and was free of aggregation. The addition of Chs, however, caused TMV particles to form large raftlike aggregates. The effect of Chs on infectivity may be ascribed to the aggregation formation of TMV particles. The present results are consistent with the view that TMV is precipitated by Chs through mutual incompatibility and spatial exclusion (Sano and Inoue, 1980).

Effect of Chs on TMV aggregation

The addition of Chs to the TMV solution in distilled water leads to gradual formation of turbidity. The kinetics of aggregation of TMV in the presence of Chs were

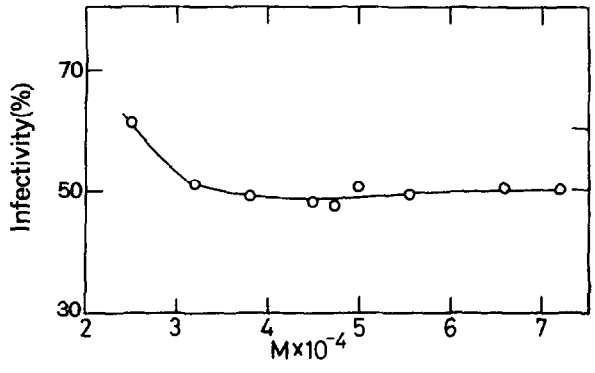

Fig. 2. Infectivity of Chs C-type having different molecular weights.

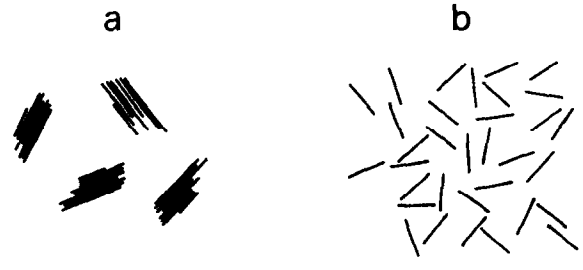

Fig. 3. Schematic representation based on electron micrographs of purified TMV in the presence (a) and the absence (b) of Chs.

Table 1. Maximum turbidity at 350 nm of TMV in the presence of Chs[a]

TMV (mg/ml)	C-type	A-type
0.064	0.154	0.044
0.097	0.237	0.060
0.129	0.315	0.065

[a] Final concentration is 1.3 mg/ml.

determined. In all cases a smooth monotonic rise in turbidity was seen with time. The intensity of turbidity seemed to reach an apparent equilibrium in 3 h. The maximum turbidity after Chs was added to the TMV solution is shown in Table 1. The turbidity of TMV in Chs C-type is much higher than in A-type, which corresponds to the order of inhibitory activity shown in Fig. 1.

DISCUSSION

Chs is known to be an unbranched linear anionic polyelectrolyte. The Chs molecule is fully extended at a very low ionic strength by intramolecular electrostatic repulsion and contracts with increasing ionic strength because of the suppression of the repulsive force by ion-pair formation or by the electrostatic screening effect of the ion atmosphere. Chs takes the form of a semiflexible coil in solution (Tanaka, 1978)

Chs has three isomers, A-, B- and C-types. B-type has an axial carboxyl and an axial sulfonic acid group, A-type has an equatorial carboxyl and an axial sulfonic acid group, and C-type has an equatorial carboxyl and an equatorial sulfonic acid group. These differences in primary structure are considered to influence the behavior of the molecules in solution. It has been shown that the solubility in aqueous ethanol is greater for A-type and C-type than for B-type and that the value of pK_a on the potentiometric titration curve is larger for B-type than for A-type (Mathews, 1956). The affinity of the trivalent cation Co^{3+} to Chs at relatively high ionic strength increases in the order of B-type > A-type > C-type (Mathews, 1956). It may be supposed from these facts that the C-type has the least flexible structure of the three isomers, while the B-type is the most flexible. The strength of inhibitory activity may be related to the stiffness of the Chs chain.

TMV can be precipitated by a wide variety of reagents to form needlelike paracrystalline precipitates. When Chs molecules are added to the TMV solutions, Chs molecules occupy the part of the space previously available to TMV and compress TMV particles into a small volume fraction. As the Chs concentration increases, the fractional volume occupied by TMV becomes smaller until the interparticle distance of TMV molecules is decreased sufficiently for crystallization of TMV to occur (Sano and Inoue, 1980). The strength of the spatial exclusion effect is higher in the case of stiff polymer chains such as the Chs C-type than for flexible polymer chains such as the Chs A-type. The strength of interaction between TMV and Chs is related to the degree of infectivity of Chs A-type and C-type as shown in Figs 1 and 2 and also the degree of turbidity as shown in Table 1. The antiviral activity of Chs on infectivity of TMV may be caused by the large aggregation formation of TMV particles on the cell surface, as is shown in Fig. 3. These large aggregates of TMV prevent the penetration of TMV-RNA into the cell membrane, because they block the decapsulation process of TMV which occurs on the cell membrane in the initial stage of TMV infection (Sano, 1989).

Chs also may affect the interaction between TMV and cell through the strong anionic charge of Chs. Adsorption of TMV to the cell surface is thought to be achieved by interaction between the cationic amino groups of TMV and the anionic phosphate groups of the cell membrane. Polyanions such as Chs interact with both TMV and the cell surface, and suppress adsorption owing to reinforcement of the net negative charge of both cell and TMV.

Mucopolysaccharide such as Chs seems to affect the TMV at its earliest infection stage.

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