Preparation and Anti-TMV Activity of Guanidinylated Chitosan Hydrochloride

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ABSTRACT: In this study, guanidinylated chitosan hydrochloride (GCH) was synthesized and its structure was characterized by UV–vis and FTIR. The degree of substitution of guanidinylated chitosan was confirmed by elemental analysis. *In vitro* antiviral activity of guanidinium derivative on local infection and systemic infection of tobacco mosaic virus (TMV) inoculated were evaluated by semileaf method using different modes of GCH application and antiserum assay. Meanwhile, the morphological characteristic of virus treated by GCH was performed by transmission electron microscope. The results showed that GCH had better antiviral activity than chitosan. The average inhibitory rate of GCH on local infection was 84%, which was much higher than that of chitosan hydrochloride. It was shown that the guanidinylated chitosan was an efficient passivator, and its antiviral effect decreased after mechanical inoculation. The guanidinylated chitosan increased the resistance of plant against TMV and decreased the infection of the virus. The electron microscope photograph exhibited that GCH not only directly altered the configuration of TMV but also congregated and reduced the virus. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 112: 3522–3528, 2009

Key words: guanidinylated chitosan hydrochloride; TMV; antiviral activity

INTRODUCTION

Chitosan is a natural nontoxic, biocompatible, and biodegradable polymer, which is prepared by N-deacetylation of chitin. Chitosan consists of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units. Chitosan affects the plant itself inducing resistance to the plant with a phytopathogen and chitosan induces a wide spectrum of defensive reactions in the plant,[1–3] which limit a systemic spread of the viruses and viroids over the plant and lead to the development of the systemic acquired resistance.[4] Chitosan applied by spraying or inoculating leaves protected various plant species against local and systemic infection caused by alfalfa mosaic virus (ALMV), tobacco necrosis virus (TNV), tobacco mosaic virus (TMV), peanut stunt virus (PSV), cucumber mosaic virus (CMC), and potato virus X (PVX).[3,6] Chitosan possesses an antiviral activity by its ability to induce resistance toward viral diseases in plants, to inhibit viral infections in animal cells, and to prevent the multiplication of bacteriophages in infected cultures of microorganisms.[6,7] The efficiency of chitosan in the inhibition of viral infection depends upon the host–virus combination, chitosan concentration, and mode of its application.

Guanidinium salts have attracted increasing interest in recent years. Guanidines have long been the focus of considerable attention as a ubiquitous moiety incorporated into many drugs with numerous therapeutic applications and biological activities such as antidiabetic, antimicrobial, and antiviral drugs.[8] Guanidine-containing sugars and sugar-like molecules have a wide range of biologically important uses such as inhibition of inappropriate mitogenic signaling,[9,10] therapy for bacterial infections, treatment of noninsulin-dependent diabetes, and inhibition of enzymes including thrombin, glycosidases, and nitric oxide synthases.[11–18] In most of these cases, the guanidino-sugars have been developed to mimic carbohydrate and peptidic molecules. We synthesized the guanidinylated chitosan sulfite (GCS) with better antimicrobial activity. Because of the possible antiviral activity of guanidinylated chitosan and as there is no previous report about the same, it seemed worth to further study.

This research therefore converted GCS into guanidinylated chitosan hydrochloride (GCH) through a convenient chemical modification to improve the

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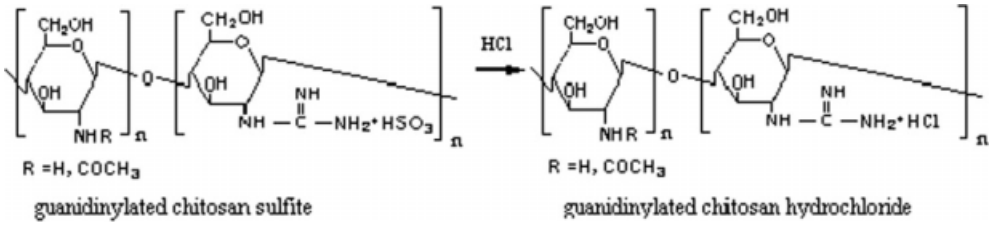

Scheme 1 Synthesis of guanidinylated chitosan hydrochloride.

dissolvability and decrease of toxicity of GCS. Guanidinylated chitosan was comprehensively characterized. *In vitro* antiviral activity of guanidinium derivative on local infection and systemic infection of TMV inoculated were evaluated by semileaf method using different modes of GCH application and antiserum assay. The morphological characteristic of virus treated by GCH was performed by transmission electron microscope.

EXPERIMENTAL

Materials

Chitosan with molecular weight 210 kDa was supplied by Yuhuan Ocean Biochemistry (Taizhou, China). The deacetylation degree was determined as 91.6% by a pH titration method.[19] Freund's Adjuvant Complete adjuvant was purchased from Sigma Chemical (St. Louis, Missouri). All other chemicals used were of analytical grade.

Preparation of guanidine derivatives

Preparation of guanidinylated chitosan sulfite

GCS was prepared according to the synthetic procedure given in the literature.[20]

Preparation of guanidinylated chitosan hydrochloride

GCS powder was dissolved in distilled water, and the desired amount of hydrochloric acid was added slowly at 80°C with stirring. Then, the reaction was kept for 60 min and cooled to room temperature after reaction. The mixture was poured into ethanol, and the precipitate was filtered off, washed thoroughly with the mixture of water and ethanol, and then dried under vacuum to constant weight to give the product GCH. The replacement reaction of the GCH from GCS was achieved by a convenient procedure as shown in Scheme 1.

Characterization

UV–vis absorption spectra were obtained using dilute aqueous solutions on a Shimadzu 1601 UV–vis spectrophotometer (Shimadzu, Nicolet).

FTIR spectra were recorded in powder form in KBr discs in the range of 4000–400 cm^{-1} on a Nicolet 670 FTIR spectrophotometer.

The degree of substitution was determined by the elemental analysis. The elemental analysis (C, N, H) of samples was performed on a Flash Elemental Analyzer 1112 (ThermoQuest, Milan, Italy).

Plant materials and virus

Plants of tobacco (*Nicotiana glutinos* and *Nicotiana tobacum*) were grown from seeds in a greenhouse and were used at the 4- to 6-leaf stage after 2 months in culture. The plants were kept in a growth chamber at 23°C ± 1°C with a photoperiod of 16 h and 70–80% relative humidity for several days before treatments. *N. glutinos* were used as local infected plants and *N. tobacum* were used as systemic infected plants.

TMV was supplied by China Center for General Viruses, Culture Collection of the Committee on Type Culture Collection of Chinese Academy of Sciences, Wuhan Institute of Virology, CCA. Seeds tested were from this institute's preservation.

TMV was multiplied in *N. tobacum*. Leaves were infected homogeneously by TMV to propagate virus, and the leaves were collected and mashed by DS high-speed masher at 10 days after inoculation. Twice the volume of 0.01 mol/L PBS (pH 7.2) with 0.1% mercaptoethanol was added to the extract. The 8% *n*-butanol was added after it was filtrated through four-ply pledgets. Then, the mixture was centrifuged for 30 min at 10,000 rpm. Polyethylene glycol (PEG; 4%) and 3% NaCl were added to the supernatant. The mixture was mixed to dissolve completely and stored overnight at 4°C. Afterward, it was centrifuged at 10,000 rpm for 15 min. The sediment was dissolved with a small quantity of 0.01 mol/L PBS (pH 7.2). The purified suspension was used for mechanical inoculation. The concentration of TMV was calculated as 6.0×10^{-3} mg/mL by UV spectrophotometer at 260 nm. The TMV solution was preserved at 4°C, and the working solutions were obtained by diluting the stock solution before use.

Evaluation of antiviral activity

Protective effect of GCH against TMV local infection on *N. glutinosa*

GCH and chitosan hydrochloride (CH) solution was spread on the surface of left half-leaf of *N. glutinosa*. The right half-leaf was spread with water as control. All treatments were in quintuplicate. After 12-h application, the leaves were inoculated mechanically with 10 μL TMV solution (concentrate of 1.5×10^{-4} mg/mL). Then, the leaves were washed with tap water. After 3-day incubation at 26°C in a LRH-250-G Light Bioculturer (Guangzhou, China), local lesions on the half-leaves were investigated. The protection efficacies of GCH and CH were calculated from the ratio of the number of local lesion produced on left half-leaves to that on the control right half-leaves.

Inhibitory effect of GCH against TMV local infection on *N. glutinosa*

The leaves of *N. glutinosa* were inoculated mechanically with 20 μL TMV solution (1.5×10^{-4} mg/mL). Then, the leaves were washed with tap water. The left half-leaves were treated with 10 μL, 2 and 1 mg/mL GCH after different inoculation time. Control half-leaves on the right were treated by water instead of sample. All treatments were repeated in quintuplicate. After 3-day incubation at 26°C in LRH-250-G Light Bioculturer (Guangzhou, China), local lesions on the half-leaves were investigated, and the inhibition efficacy of GCH for different time was counted. The experiment was repeated in triplicate.

Effect of GCH on passivation against TMV *in vitro*

GCH (2 mg/mL) was mixed with equal volume of TMV suspension (1.5×10^{-4} mg/mL) and treated with different time. Then, 10 μL mixture was spread to the left half-leaves, and the control half-leaves on the right were spread by TMV solution of same concentration without GCH. Then, the leaves were washed with tap water. All treatments were performed in quintuplicate. The leaves were washed with water after inoculation. After 3-day incubation, local lesions on the half-leaves were investigated, and the passivation efficacy of GCH for different time was counted.

Circular piece[21]

To evaluate the effect of GCH on systemic viral infection, the relative quantity of the virus in the inoculated plants was determined by circular piece test as follows: TMV was inoculated mechanically on *N. tobacum*. At 6 h after inoculation, circular pieces about 12-mm in diameter were cut. Pieces were immerged in 2 mg/mL GCH and CH solution for 48 h, respectively. The other pieces were immerged in distilled water as control. The pieces were triturated with desired amount of 0.01 mol/L PBS (pH 7.2) and centrifuged for 15 min at 4000 rpm. The quantities of virus in the supernatant of sample and control were determined by semileaf method. The local lesions on leaves were investigated at the 3rd day after inoculation.

Antiserum assay[22]

TMV antiserum assay. TMV antigen was prepared as follows: 150 g of leaves, heavily infected by TMV, were minced to get TMV extract, and then filtrated, centrifuged, and precipitated twice by PEG. The deposit was dissolved with 0.1 mol/L PBS. The liquid containing virus after differential centrifugation must be purified further in 10–40% sucrose by rundle density centrifugation. Rundle density sucrose solution was added by differential density so that the smaller density was above and made a continuing density ladder in 4°C overnight. Then, 1.6 mL of the refine extract was added to the top of rundle density sucrose liquid, centrifuged for 1.5 h at 25,000 rpm on a Coulter Optima™ XL-100K Ultra-ultra centrifuge (Beckman, USA). After super-speed centrifugation, the precipitate at middle superstratum was aspirated by injector and desugared in a super-speed centrifuge at 40,000 rpm for 1.5 h. This precipitate was dissolved with 1 mL 0.01 mol/L PBS (pH 7.2) buffer to obtain a concentrated and refined extract of TMV, which was assayed by ultraviolet spectrophotometer to calculate the concentration of virus protein. The dilute virus was incubated to rabbit at the concentration of virus protein of 400 μg/mL, which was used as the virus solution for TMV antigen preparation.

At first immunity injection, 500 μL Freund's Adjuvant Complete adjuvant was given to the foodpad, lymph node of tickle, and subcutaneous position of healthy male rabbit. After 1 week, the second immunity injection was performed with 750 μL TMV antigen completely mixed with equal volume of Freund's Adjuvant Complete adjuvant; Freund's Adjuvant Incomplete adjuvant were used every 2 weeks at the third and fourth immunity injections. At the 7th and 10th day after the immunity injection, ear blood was collected for assay. Antiserum efficacy was examined by agar diffusivity. The average efficacy was 1 : 128. Blood from the carotid vessel was collected for TMV antiserum assay and preserved in a refrigerator at 4°C.

Agar gel immunodiffusion. Leaves of *N. tobacum* were inoculated mechanically with TMV. At 72 h after

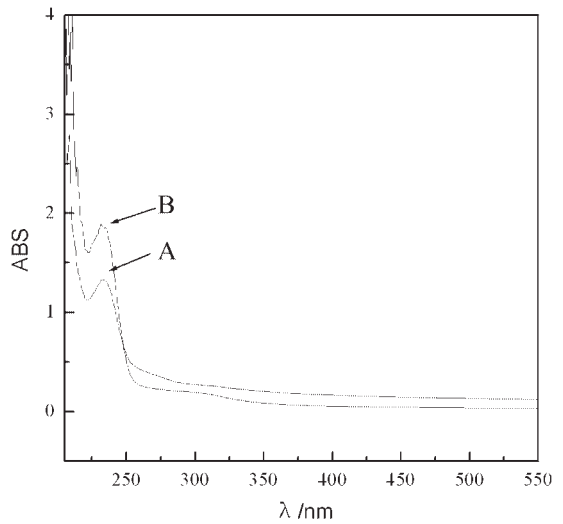

Figure 1 UV–vis spectra of (A) guanidinylated chitosan sulfite (GCS) and (B) guanidinylated chitosan hydrochloride (GCH).

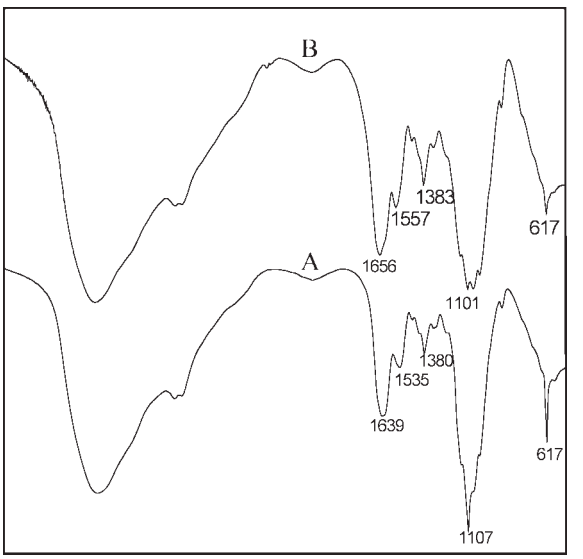

Figure 2 FTIR spectra of (A) guanidinylated chitosan sulfite (GCS) and (B) guanidinylated chitosan hydrochloride (GCH).

inoculation, five circular pieces about 12-mm diameter were immerged in 2 mg/mL GCH solution for 72 h and the other five circular pieces were treated in water. Then, the pieces were collected and added in 500 μL and 0.1 mol/L PBS (pH 7.2), minced and centrifuged, respectively. The extracts were prepared for agar gel immunodiffusion measurement.

Agar plate (1.5 g agar, 100 mL PBS) with 3-mm thickness was stroked seven holes. The hole in the center was half-diluted antibody. Six holes around were grouped into three groups as antigen. The left two holes were extracts treated by the sample. The right two holes were extracts treated by water as positive control, and the top two holes was extract of health leaves as negative control. By the antigen–antibody reaction for 12 h at room temperature with moisturizing environment, the precipitation line was observed.

Electron microscope and morphological measurements

TMV suspension (20 μL; 6×10^{-4} mg/mL) and GCH solution (20 μL; 2 mg/mL) were mixed fully for 4 h. Morphological measurements of the virus were performed by Hitachi-H-7000FA Electron Microscope.

RESULTS AND DISCUSSIONS

Characterization of guanidinylated chitosan hydrochloride

UV–vis absorption spectra of GCS and GCH dilute aqueous solution are shown in Figure 1. In the UV spectra of GCS and GCH, the same peaks at 233 nm suggested the existence of the guanidine groups.[23] It indicated that the GCS was replaced into hydrochloride without a change in the guanidine groups.

Structural changes of chitosan derivatives were confirmed by FTIR spectra (Fig. 2). In the spectra of GCS, the strong peaks at 1639, 1535, and 1380 cm^{-1} suggested that guanidinylation reaction had been successful.[24] In the spectra of CGH, these peaks shifted to higher frequency 1656, 1557, 1383 cm^{-1}. The new stronger peak at 1107 cm^{-1} shifted to a lower frequency, and the peak at 1107 cm^{-1} and 617 cm^{-1} was sharply weakened, suggesting that the

TABLE I
Elemental Analysis and Substitution Degree of Chitosan and GCS and GCH

	Analysis found (Calc.) %				
Sample	C	N	H	C/N	DS[a]
Chitosan	37.0 (36.7)	6.70 (6.96)	7.55 (7.53)	5.57	
GCS	31.8 (31.1)	9.19 (9.00)	6.31 (6.31)	3.46	0.30
GCH	25.6 (25.6)	7.26 (7.22)	6.08 (5.52)	3.53	0.28

[a] Substitution degree calculated according to the C/N wt % from the elemental analysis.

conversion of sulfite into hydrochloride and the replacement reaction have been successful.

Experimental evidence from the UV and FTIR indicated that the sulfite of guanidinylated chitosan was replaced by the hydrochloride. The degrees of substitution of GCS and GCH were calculated from the elemental analysis data. As can be seen from Table I, the C/N wt % of GCH is close to that of GCS and DS of GCH was 0.28.

Analyses by UV, IR, and elemental analysis indicated the success of the replacement reaction.

Antivirual activity

Protective effect of GCH against TMV local infection on *N. glutinosa*

Pretreatment with CGH solution on the half-leaf before inoculation with TMV and subsequent protective effect of GCH against TMV are shown in Table II. Average local lesions on the half-leaves that were treated by the sample were much less than that of the control half-leaves. The protective effect of GCH was 84%, which was much higher than that of CH (36%). The higher protection efficacy showed that CGH could induce the resistance of the plant.

There have been several reports about the antiviral activity of chitosan and its oligosaccharide. Chitosan is a deacetylation product of chitin when it is degraded into amino polysaccharides, which can produce a defense response, which is indirect in nature. It can induce the defense reaction of plant growth and resistance against disease. As an effective elicitor of the plant defense mechanism, chitosan can quickly stimulate a plant defense response and activate the defense system. A general type of resistance against a broad range of pathogens is known as nonhost resistance. This inherent plant resistance response is a complicated interaction that involves the recognition of multiple elicitors. Such elicitors may be components of the pathogen such as chitosan, a deacetylated derivative of fungal cell wall chitin, or pathogen exudates. These elicitors may also be derived from the host and include various plant polysaccharides from the digested cell wall matrix.[25] In pea, such recognition signals the transcriptional induction of at least 20 defense genes.[26–29] Different explanations of these observations are possible. Chitosan could block the replication of viral RNA, inhibit the synthesis of a virus-specific proteins, or damage the protoplasts in some manner, for example, because of an increase in the cell membrane permeability.[30] Hadwiiger et al.[31] showed that chitosan, when applied to plant cells, localizes in the nucleus and can activate specific genes in plants. One can speculate that the action of chitosan on virus replication is indirect and is due to the induction of general defensive reactions in plants. The polycationic compound chitosan released from Fsph has a high affinity for DNA and has been shown to enter the plant nucleus[32] and induce pisatin synthesis.[33] Moreover, chitosan is also capable of causing DNA strand breakage *in vitro*.[34]

TABLE II
Protective Effect of 2 mg/mL GCH and CH Solutions Against TMV

Sample	Pretreatment time (h)	Average local lesion[a]		Protective effect
		Sample	Control	
GCH	12	18	108	83%
CH	12	71	111	36%

[a] The protection efficacy of local lesions for at least 10 half-leaves.

TABLE III
Inhibitory Effect of GCH for TMV with Different Times

Concentration (mg/mL)	Interval between virus inoculation and GCH treatment (h)	Average inhibitory rate[a] (%)
2	1	76
2	3	69
2	6	68
2	9	66
1	1	64
1	3	52
1	6	50

[a] The average inhibitory rate of local lesions for at least 10 half-leaves.

Inhibitory effect of GCH against TMV local infection on *N. glutinosa*

Inhibitory effect of 2 and 1 mg/mL GCH at 1, 3, 6, and 9 h after inoculation is shown in Table III. The results showed that the inhibitory effect increased with decreasing interval. The shorter the interval between virus inoculation and GCH treatment, the better inhibitory efficacy was shown. Inhibitory efficacy of 2 mg/mL GCH was 76% at 1 h and 66% at 9 h after inoculation. However, 2 mg/mL GCH still inhibited the growth of TMV 9 h after inoculation.

TABLE IV
Passivation Effect of GCH with Different Passivation Time Against TMV

Concentration (mg/mL)	Passivation time (h)	Passivation efficacy[a] (%)
2	1	71
2	3	69
2	6	71
2	9	65

[a] The passivation effect of local lesions for at least 10 half-leaves.

TABLE V
Inhibitory Effect of GCH Against TMV

Concentration (mg/mL)	Interval between virus inoculation and GCH treatment (h)	Average local lesion[a]		Inhibitory effect (%)
		Sample	Control	
2	6	0	113	100
2	48	42	120	65

[a] The average number of local lesions for at least 10 half-leaves.

On the other hand, inhibitory effect of GCH at a concentration of 2 mg/mL was distinctly higher than that of 1 mg/mL after same interval. The inhibitory effect of same interval at concentrations of 2 mg/mL was higher than that for 1 mg/mL. The inhibitory effect of GCH was related to the interval and concentration of GCH.

Effect of GCH on passivation against TMV *in vitro*

Mixed with 2 mg/mL GCH, TMV suspension was passivated for 1, 3, and 6 h. As shown in Table IV, the results indicated average passivation effect of GCH with passivation time of 1, 3, and 6 h that approximated to 70%, which showed little difference. When mixed for 9 h, the average inhibitory effect was 65%, which was lower than that of the shorter passivation time distinctly.

Inhibitory effect of GCH against TMV systemic infection

The inhibitory effect of 2 mg/mL CGH against TMV on *N. tobacum* of systemic infection was investigated by circular pieces test. The relative quantity of the virus after 6- and 48-h incubation in the inoculated plants was determined by the local lesion of semileaf on *N. glutinosa*. As shown in Table V, after 6-h incubation, 2 mg/mL CGH completely inhibited the propagation of TMV, and the inhibitory effect was 100%. However, the inhibitory effect of TMV after 48-h incubation was 65%. It indicated that the inhibitory effect of GCH against TMV-systemic infection decreased with the increase of virus to a certain extent.

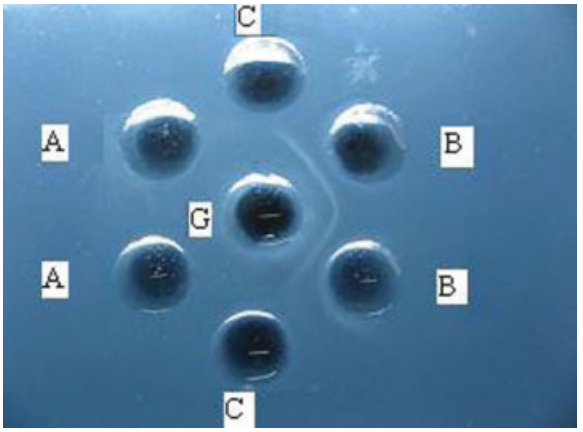

Figure 3 Agar gel immunodiffusion result of GCH (A), positive control (B), negative control (C), and TMV antibody (G). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Agar gel immunodiffusion reaction

The immunodiffusion reaction of antibody–antigen on agar plate is shown in Figure 3. In the center hole was the antibody. C was the control of health leaf, A was the GCH solution treatment, and B was the diluted water treatment. A precipitation line was apparent between B and G for a positive result but unapparent between A and G or C and G for a

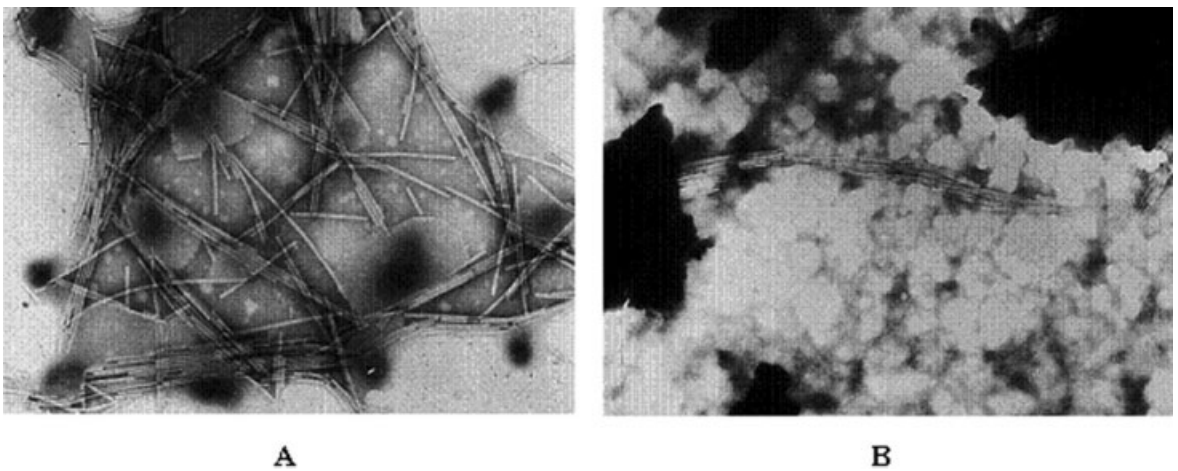

Figure 4 Electron microscope photos of (A) TMV suspension and (B) TMV treated by GCH (magnification 3×10^4 times).

negative result. It indicated that antigen level was decreased after treatment by GCH, and the growth of virus was inhibited to a certain degree.

Morphological measurements

Electron microscope photographs of TMV suspension are shown in Figure 4(A). TMV virus particle is rod-shaped and well dispersed. The virus showed single- or multiple-dispersed particles predominantly of length of 300 nm and 13 nm width. After treatment with GCH, as shown in Figure 4(B), the number of TMV virus particles was notably decreased when compared with TMV particle. Meanwhile, the morphological characteristics were also changed, most TMV particles twisted together and bound into a bundle. Flocculation appeared indicated that GCH could denature the protein shell of TMV, which is the main component of TMV.

Reports on antiviral activity of chitosan derivatives are rarely reported. From the results of the TMV experiment, it was shown that guanidinylated chitosan was an efficient passivator, and the antiviral efficacy before mechanical inoculation was better than after mechanical inoculation. It suggests that guanidinylated chitosan increased the resistance of plant against TMV and decreased the infection of the virus. Apart from the improvement in plant resistance, GCH killed directly and destroyed the virus. From the electron microscope photographs of TMV treated by GCH, the ultrastructure of virus indicated that GCH had a direct effect on TMV. It did not only directly altered the configuration of TMV but also congregated and reduced the virus. The antiviral mechanism of GCH should be studied further to elucidate these mechanisms.

CONCLUSIONS

For the first time, GCH has been successfully synthesized and characterized by UV, FTIR, and elemental analysis. Additionally, the antiviral activity of GCH against TMV was systematically studied. Guanidinylated chitosan showed better antiviral activity than chitosan. All the results imply that GCH will be useful as a potential new antiviral agent.

References

1. Shibuya, N.; Minami, E. Physiol Mol Plant Pathol 2001, 59, 223.
2. Ren, Y.; West, C. A. Plant Physiol 1992, 99, 1169.
3. Henryk, P.; Sergei, C.; Joseph, A. Plant Sci 1991, 79, 63.
4. Rabea, E. I.; Badawy, M. E. T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W. Biomacromolecules 2003, 4, 1457.
5. Pospieszny, H. Crop Prot 1997, 16, 105.
6. Chircov, S. N.; Illina, A. V.; Surgucheva, N. A.; Letunova, E. V.; Varitsev, Y. A.; Tatarinova, N. Y.; Varlamov, V. P. Russ J Plant Physiol 2001, 48, 774.
7. Chircov, S. N. Appl Biochem Microbiol 2002, 38, 1.
8. Greenhill, J. L.; Lue, P. In Progress in Medicinal Chemistry; Ellis, G. P.; Luscombe, D. K., Eds.; Elsevier Science: New York, 1993; Vol. 30, Chapter 5.
9. Cotner, E. S.; Smith, P. J.J Org Chem 1998, 63, 1737.
10. Hauser, S. L.; Cotner, E. S.; Smith, P. J. Tetrahedron Lett 1999, 40, 2865.
11. Streicher, W.; Loibner, H.; Hildebrandt, J.; Turnowsky, F. Drugs Exp Clin Res 1983, 9, 591.
12. Delaware, D. L.; Sharma, M. S.; Iyengar, B. S.; Remers, W. A. J Antibiot 1986, 39, 251.
13. Reitz, A. B.; Tuman, R. W.; Marchione, C. S.; Jordan, A. D. Jr.; Bowden, C. R.; Maryanoff, B. E. J Med Chem 1989, 32, 2110.
14. Wessel, H. P.; Banner, D.; Gubernator, K.; Hilpert, K.; Müller, K.; Tschopp, T. Angew Chem Int Ed Engl 1997, 36, 751.
15. Chan, A. W.-Y.; Ganem, B. Tetrahedron Lett 1995, 36, 811.
16. Jeong, J.-H.; Murray, B. W.; Takayama, S.; Wong, C.-H. J Am Chem Soc 1996, 118, 4227.
17. Merrer, Y. L.; Gauzy, L.; Gravier-Pelletier, C.; Depezay, J.-C. Bioorg Med Chem 2000, 8, 307.
18. Gravier-Pelletier, C.; Bourissou Merrer, Y. L.; Depezay, J.-C. Synlett 1996, 275.
19. Tetsuaki, T. Biocontrol Sci 2003, 8, 25.
20. Hu, Y.; Du, Y. M.; Yang, J. H.; Kennedy, J. F.; Wang, X. H.; Wang, L. S. Carbohydr Polym 2007, 67, 66.
21. Liu, G. K.; Xie, L. H.; Lin, Q. Y.; Wu, Z. J.; Chen, Q. J. Acta Phytopathol Sinica 2003, 33, 279.
22. Liang, X. S.; Zhang, C. L.; Zhang, Z. F. Plant Virus Serological Technology; Agriculture Press: Beijing, China, 1985; p 109.
23. Chinese Pharmacopoeia 1990, Pharmacopeia Commission of PRC; Xueyuan Huagong Press, People's Medical Publishing House: Beijing, 1990; p 498.
24. Miller, A. E. Synthesis 1986, 9, 777.
25. Dixon, R. A.; Lamb, C. J. Annu Rev Plant Physiol Plant Mol Biol 1990, 41, 339.
26. Wagoner, W.; Loschke, D. C.; Hadwiger, L. A. Physiol Plant Pathol 1982, 20, 99.
27. Hadwiger, L. A.; Wagoner, W. Plant Physiol 1983, 72, 553.
28. Loschke, D. C.; Hadwiger, L. A.; Wagoner, W. Physiol Plant Pathol 1983, 23, 163.
29. Riggleman, R. C.; Fristensky, B. W.; Hadwiger, L. A. Plant Mol Biol 1985, 4, 81.
30. Brodelius, P.; Funk, C.; Haner, A.; Villegas, M. Phytochemistry 1989, 28, 2651.
31. Hadwiger, L. A.; Kendra, D. F.; Fristensky, B. W.; Wagoner, W. Chitin in Nature and Technology; Muzzarelli, R.; Jeuniaux, C.; Gooday, G. W., Eds.; Plenum Press: New York, 1986; p 209.
32. Hadwiger, L. A.; Beckman, J. M.; Adams, M. J. Plant Physiol 1981, 67, 170.
33. Hadwiger, L. A.; Beckman, J. M. Plant Physiol 1980, 66, 205.
34. Kashige, N.; Yamaguchi, T.; Ohtakara, A.; Mitsutomi, M.; Brimacombe, J. S.; Miake, F.; Wantanabe, K. Carbohydr Res 1994, 257, 285.