Stability of Southern Bean Mosaic and Carnation Mottle Viruses during the Preservation by Freeze-drying

Fumiyoshi FUKUMOTO* and Hiroshi TOCHIHARA**

Abstract

The infectivity of purified southern bean mosaic virus (SBMV) in 10 mM sodium phosphate buffer, pH 7.0, decreased to 11% after freeze-drying. Such partially swollen virions were found to be markedly susceptible to RNase and sodium dodecyl sulfate (SDS). However, in the preparations supplemented with 0.5% lysine prior to freeze-drying the conformational changes of the virions were suppressed and the effect of RNase and SDS was well tolerated. On the other hand, there were little differences in infectivity of extracted RNA from virions between the preparations with or without lysine. It was suggested that the alterations of the conformation of the SBMV virions caused mainly the decrease of the infectivity of the virus preparations. During storage at 65°C, the preparations of freeze-dried viruses lost their infectivity within one day and all the virions became markedly swollen. RNA extracted from such virions was degraded completely and did not display any infectivity. On the other hand, the virions of the preparations supplemented with 0.5% lysine retained their conformation and a high level of infectivity was maintained in both the preparations of virions and RNA extracted from the virions compared with the preparations without any additives. In the freeze-dried preparations of SBMV-RNA, lysine showed a protective effect during storage. Freeze-dried preparations of carnation mottle virus during storage at 65°C showed a response similar to that of SBMV.

(Received September 7, 1989)

Key words: southern bean mosaic virus, carnation mottle virus, swelling, infectivity, preservation.

INTRODUCTION

Southern bean mosaic virus (SBMV) and carnation mottle virus (CarMV) are small isometric viruses, and consist of an RNA genome of molecular weight (MW) $1.4 \times 10^6$ and $1.3 \times 10^6$ and coat protein of MW 29,000 and 38,000, respectively7,10,19. SBMV is stabilized by both protein-protein interactions (divalent ion-dependent and pH dependent) and protein-RNA interactions12. The properties of CarMV virions are similar to those of SBMV except that RNA-protein interactions are important in maintaining the virion conformation in SBMV12.

We reported that the infectivity of SBMV and CarMV decreased by freeze-drying5,6, and that the virions of both viruses showed conformational changes in the profiles of sucrose density gradient centrifugation. However, the changes of the conformation of both viruses associated with freeze-drying could be significantly prevented by the addition of lysine and the infectivity was maintained at a high level. SBMV virions frozen in water17 or treated with EDTA became...
swollen\textsuperscript{11,12}, and their infectivity decreased markedly. Since it appeared that the cause of decrease of the infectivity of freeze-dried virions was closely associated with the alterations in the conformation of the virions, the relationship between the changes in the viral conformation, the susceptibility of SBMV and CarMV to RNase or sodium dodecyl sulfate (SDS) and the infectivity of both virus preparations was studied. Moreover, the properties of RNA and coat protein extracted from frozen or freeze-dried virions were also examined along with the properties of the freeze-dried viruses preserved at 65°C.

**MATERIALS AND METHODS**

**Virus source and purification.** The preparations of purified SBMV and CarMV employed in previous reports\textsuperscript{5,6}, were mostly suspended in 10 mM sodium phosphate buffer (NaPB) pH 7.0, instead of KPB because SDS precipitated due to reaction with potassium when SBMV-RNA was extracted from the virions.

**Freezing and freeze-drying.** The freeze-dried viruses were prepared and stored at 65°C for assessing promptly the protective effect of additives during the preservation as described previously\textsuperscript{6}.

**Treatment of viral preparations with RNase and sodium dodecyl sulfate.** To determine the effect of RNase on both the frozen or freeze-dried viruses, the preparations of SBMV and CarMV (1 mg/ml) were exposed to RNase A at the concentration of 10 μg/ml at 4°C for 6 hr and then diluted in ice-cold NaPB, to assay the viral infectivity. The sensitivity of both viruses to SDS was analyzed by calculating the remaining virions of SDS-treated preparation to that of the corresponding preparation without SDS in 10-40% sucrose density gradient sedimentation profiles after exposure to SDS at 22-24°C for 20 min.

**Extraction and analysis of viral RNA.** The extraction of viral RNA was performed according to the procedure of Sehgal\textsuperscript{16} and the method of phenol extraction. In the former method, the preparations of preserved SBMV and CarMV were mixed with 0.5 volume of dissociative medium (0.2 M NaPB, pH 7.5 containing 20 mM EDTA and 1.5% SDS, and 10 mM NaPB containing 15 mM EDTA and 3% SDS), respectively, and incubated at 20-24°C for 2-3 hr. These preparations were then centrifuged in 10-40% sucrose gradients for 16 hr at 111,700 × g in a Hitachi RPS40T rotor at 4°C. The gradients were scanned as reported previously\textsuperscript{5}. The viral RNA fractions collected were diluted several-fold in NaPB to assay their infectivity.

Another method of extraction of SBMV-RNA consisted of the two phenol method, which was used as described by Harrison\textsuperscript{8}, except for the omission of m-cresol. After addition of 1% SDS, RNA was extracted by mixing the virus samples with one volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline. After shaking and low-speed centrifugation, the aqueous phase was reextracted with one volume of water-saturated phenol. After ethanol precipitation, SBMV-RNA was dissolved in 10 mM NaPB, pH 7.0. The 0.1 ml RNA preparations were freeze-dried at the concentration of 50 μg/ml and stored at 65°C. These preparations preserved were analysed by sucrose density gradient centrifugation for 3.5 hr at 235,000 × g in a Hitachi RPS50-2 rotor at 4°C.

**Analysis of viral coat protein.** Polyacrylamide gel electrophoresis of the viral coat protein was performed according to the method of Laemmli\textsuperscript{14} by using a slab gel in 10% acrylamide containing 0.1% SDS.

**Electron microscopy.** The virions of purified viruses were stained with 1% phosphotungstic acid, pH 6.5 and were examined with a Hitachi H-500 electron microscope.

**Infectivity assay.** Infectivity of the preserved preparations was determined according to the methods routinely used for SBMV and CarMV\textsuperscript{5,6}. 
RESULTS

Alterations of the conformation of frozen or freeze-dried virions

The preparations of purified SBMV at a concentration of 1 mg/ml in 10 mM NaPB, pH 7.0, did not show any significant alterations in the infectivity and in the sucrose density gradient sedimentation profiles when frozen at −20°C for one hr and subsequently thawed, as previously reported in using KPB\textsuperscript{5}. In the case of CarMV the area of the fractions of virions decreased, a fast-sedimenting peak appeared, suggesting that this component may be built up the aggregation of virions. The infectivity also decreased to 57%, which differed from the result of KPB\textsuperscript{9}. On the other hand, the conformation of the SBMV and CarMV virions was altered by freeze-drying, in particular in the preparations where NaPB was used (Fig. 3A) compared with the preparations in which KPB was used (Fig. 1A). The freeze-dried preparations of SBMV supplemented with 0.5% lysine in NaPB were protected from the alterations of the conformation of the virions but were less preserved than those in which KPB was used\textsuperscript{5,6}.

The virus preparations dialyzed to 0.1 M KPB, pH 7.5 for two days, revealed a partial swollen conformation (Fig. 1B). In the case of freezing, the sucrose density gradient sedimentation profiles did not show any changes. However, by freeze-drying all the virions sedimented slowly. Swollen SBMV virions, stabilized mainly by protein-RNA bonds\textsuperscript{13} as in the case of the Cucumoviruses\textsuperscript{13}, were prepared by eliminating the divalent cation with 10 mM EDTA in 0.1 M PB, pH 7.5 and dialyzed to 0.1 M KPB, pH 7.5. In such slow-sedimenting virions, no alterations were observed in the sedimentation profiles by freezing. In the freeze-dried preparations, the fraction of SBMV virions showed slightly broad profiles (Fig. 1C) but was not dissociated into RNA and coat protein such as in the case of the Nepo- and Comoviruses\textsuperscript{4}, which were stabilized by protein-protein interactions.

Susceptibility of frozen or freeze-dried virions to RNase and SDS

There were some differences in the deformation of the virions between the freeze-dried preparations supplemented with or without lysine. It thus appeared that the changes in the virion conformation were associated with the susceptibility to RNase and SDS.

No decrease in the infectivity was observed in intact and frozen-thawed SBMV exposed to RNase in 10 mM NaPB, pH 7.0. However, the swollen EDTA-treated virions in 0.1 M NaPB, pH 7.5 became susceptible to RNase, and the infectivity of such preparations decreased to 13% of the corresponding RNase-untreated values in the swollen control. The infectivity

![Fig. 1. Sucrose density gradient centrifugation profiles of SBMV virions. Upper profiles: non-freeze-dried virions. Lower profiles: freeze-dried virions. Left panel (A): virions suspended in 10 mM KPB, pH 7.0. Central panel (B): virions dialyzed to 0.1 M KPB, pH 7.5 for two days. Right panel (C): elimination of divalent cation of the virions with 10 mM EDTA in 0.1 M KPB, pH 7.5 and virions dialyzed to 0.1 M KPB, pH 7.5.](image-url)
of the freeze-dried preparations without any additives also decreased to 25% of the corresponding values in the freeze-dried control. In contrast, the preparations supplemented with 0.5% lysine retained 79% of the infectivity compared with the control.

Intact or frozen-thawed CarMV virions were not susceptible to RNase. However, the infectivity of the preparations treated with RNase after freeze-drying decreased to 59% of that of the corresponding freeze-dried control.

In the presence of 0.1% SDS, the SBMV virions did not show alterations in the sucrose density gradient sedimentation profiles but a proportion of the compacted virions decreased slightly when the concentrations of SDS increased to 1–3%. Although the frozen-thawed SBMV preparations were not susceptible to RNase, the virions became slightly more sensitive to SDS at 0.1–3% compared with the intact virions. SBMV virions showed a relaxation of the capsids due to the EDTA treatment and became sensitive to SDS, as reported previously11,12,15). Similar results were obtained in the current studies (Fig. 2). Like the swollen virions treated with EDTA, the freeze-dried virions without additives were completely degraded when exposed to 0.1% SDS. When the preparations were supplemented with 0.5% lysine before freeze-drying the degradation of the viruses was inhibited. In the preparations supplemented with lysine, the proportion of compacted virions reached 72%, 37% and 24% with 0.01%, 0.1% and 1% SDS compared with that of the corresponding preparations without SDS, respectively (Fig. 2).

Although the intact CarMV virions were not affected by the addition of 0.1% SDS, the increase of the SDS concentration caused a dissociation of the virions. However, approximately 20% of the virions remained stable even in 3% SDS. The CarMV virions in the freeze-dried preparations became more SDS-sensitive and almost all the virions were dissociated by 0.1% SDS.

**Viral RNA and coat protein of frozen or freeze-dried virus preparations**

The SBMV virions were completely disintegrated in the dissociative medium. There were little differences among the sedimentation profiles of SBMV-RNA extracted from intact, frozen and freeze-dried virions. The infectivity of RNA extracted from frozen preparation, freeze-dried preparations supplemented with 0.5% lysine and without any additives was 99%, 82% and 77% of the values of the untreated control, respectively, and RNA extracted from the virions applied to freeze-drying affected a little. When the freeze-dried preparations were assayed to observe the conditions of the virions, the infectivity of the freeze-dried preparations supplemented with 0.5% lysine and without any additives decreased to 64% and 11% of the values of the untreated control, respectively (Table 1).

![Fig. 2. Percentage of remaining virions in frozen, freeze-dried SBMV, or EDTA-treated virions after exposure to sodium dodecyl sulfate. O: untreated virus, X: frozen virus, □: freeze-dried virus supplemented with 0.5% lysine, ●: freeze-dried virus without additives, △: 10 mM EDTA-treated virus.](image-url)
Table 1. Infectivity of SBMV virions and RNA extracted from virions immediately after freezing or freeze-drying

<table>
<thead>
<tr>
<th>Treatment and additives</th>
<th>Infectivity</th>
<th>RNA fractions after SDGC a,b,c,d</th>
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<td></td>
<td>Virion</td>
<td>RNA</td>
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<tr>
<td>Freezing</td>
<td></td>
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<tr>
<td>no additives</td>
<td>99 b)</td>
<td>99 c)</td>
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<tr>
<td>Freeze-drying</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no additives</td>
<td>11</td>
<td>77</td>
</tr>
<tr>
<td>0.5% lysine</td>
<td>64</td>
<td>82</td>
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a) Sucrose density gradient centrifugation.
b) Numbers represent percentage of infectivity of frozen or freeze-dried virus compared with that of untreated control. The values are averages of 4 experiments.
c) Numbers represent percentage of infectivity of RNA extracted from treated virions compared with that of untreated control. The values are averages of 4–6 experiments.
d) Numbers represent percentage of the area of RNA fractions of treated preparations in sucrose density gradient centrifugation (SDGC) compared with that of untreated control. The values are averages of 4–7 experiments.

Table 2. Infectivity of CarMV virions and RNA extracted from virions immediately after freezing or freeze-drying

<table>
<thead>
<tr>
<th>Treatment and additives</th>
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<td>Freezing</td>
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<tr>
<td>no additives</td>
<td>57 b)</td>
<td>101 c)</td>
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<tr>
<td>Freeze-drying</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no additives</td>
<td>21</td>
<td>73</td>
</tr>
<tr>
<td>0.5% lysine</td>
<td>55</td>
<td>86</td>
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Legends in this table: see Table 1. The values are averages of 3–7 experiments.

The relationship between the infectivity of the virions and CarMV-RNA extracted from the freeze-dried virions showed a similar tendency to that observed for SBMV as shown in Table 2.

During acrylamide gel electrophoresis in 10% gel, the coat protein of SBMV and CarMV migrated as a single band corresponding to a MW of about 29,000 and 38,000 respectively, which did not change by freezing or freeze-drying.

Stability of freeze-dried SBMV and CarMV preparations preserved at high temperature

The freeze-dried preparations of purified SBMV lost their infectivity within one day, and the fraction of the SBMV virions gave a broad and slow sedimenting peak in the profiles of sucrose density gradient centrifugation (Fig. 3). All the virions of those preparations were completely penetrated by the stain (phosphotungstic acid), whereas, the preparations supplemented with 0.5% lysine maintained slight infectivity even after 7 days which eventually disappeared after 14 days. The part of the fraction of freeze-dried SBMV after 14 days sedimented more slowly with a shoulder of unaffected virions. More than 50% of the virions of the freeze-dried preparations were not penetrated by the stain after 28 days.

During the storage at 65°C for one day, the peak of the fraction of SBMV-RNA extracted after the disintegration of the freeze-dried virions in the dissociative medium disappeared almost completely in the sucrose density gradient sedimentation profiles and the infectivity was lost. When the preparations were supplemented with 0.5% lysine, however, the infectivity of SBMV-RNA was maintained slightly and the peak of the fraction of SBMV-RNA was considerably
recovered in the sedimentation profile even after 3 days. However, almost all the SBMV-RNA extracted from the preparations was degraded after 7 days and lost its infectivity.

No alterations were detected in the acrylamide gel electrophoresis profiles of coat protein subunits of the preparations of SBMV supplemented with no additives or 0.5% lysine during the storage for one day and 7 days at 65°C, respectively.

During the storage at 65°C, the infectivity of the freeze-dried preparations of CarMV was similar to that of SBMV as shown in Fig. 4. Moreover, the changes in the conformation of

![Sucrose density gradient centrifugation profiles](image)

**Fig. 3.** Sucrose density gradient centrifugation profiles of SBMV virions and SBMV-RNA extracted from virions after the preservation at 65°C under freeze-dried conditions. Left panel (A, C): no additives, Right panel (B, D): 0.5% lysine. Upper panel (A, B): virions, Lower panel (C, D): RNA extracted from virions. a: immediately after freeze-drying, b: 8 hr after the preservation, c: 1 day, d: 3 days, e: 7 days.

![Infectivity graph](image)

**Fig. 4.** Infectivity of CarMV virions (——) and RNA extracted from virions (-----) after the preservation of purified virus at 65°C under freeze-dried conditions. ○: 0.5% lysine, ◯: no additives.
Fig. 5. Sucrose density gradient sedimentation profiles of SBMV-RNA preserved at 65°C under freeze-dried conditions. A: frozen for 1 day, B: freeze-dried without additives, C: freeze-dried with 3% lysine. a: immediately after freeze-drying, b: 8 hr after the preservation, c: 1 day, d: 3 days.

the virions, the rate of RNA degradation and electrophoresis pattern of the viral coat protein in the CarMV preparations during the storage showed a response similar to that of SBMV except that the virions of CarMV were less penetrated by the stain than those of SBMV as revealed by electron microscopic observation.

**Effect of various additives during the preservation of freeze-dried SBMV-RNA**

In the profile of sucrose density gradient centrifugation, the peak of freeze-dried SBMV-RNA was slightly lower than that of the frozen preparation at -70°C for one day, suggesting that SBMV-RNA was partially degraded by freeze-drying. The preparations of freeze-dried SBMV-RNA underwent a gradual process of degradation during the 3-day storage at 65°C. The addition of 3% lysine gave a protective effect (Fig. 5) whereas the effect of the addition of 0.5% lysine was less conspicuous.

**DISCUSSION**

Swollen EDTA-treated\(^{11,13,15,18}\) or frozen-thawed SBMV virions in deionized water\(^{17}\) became susceptible to the proteolytic enzymes, SDS and RNase due to the alteration of the viral conformation and the infectivity of these preparations decreased markedly. No alterations of the viral infectivity of frozen-thawed SBMV preparations in 10 mM NaPB, pH 7.0 and of the conformation of the virions in the profiles of sucrose density gradient centrifugation were detected. However, the preparations showed a slight susceptibility to SDS but not to RNase, suggesting that the small alterations of the conformation were caused by freezing and thawing.

Freeze-dried SBMV virions, which showed a decrease of the level of infectivity, were susceptible to the effect of SDS and RNase but to a lower extent than the EDTA-treated virions. On the other hand, the preparations of freeze-dried SBMV supplemented with 0.5% lysine maintained a high level of infectivity and were considerably inhibited from the effect of RNase and SDS. From these results, the effect of RNase remained in the process of virus purification is suspected of causing the decrease in infectivity. However, no differences were detected in
both the coat protein and the infectivity of SBMV-RNA extracted from freeze-dried virions in the preparations with or without supplementation of 0.5% lysine. Subsequently, it was suggested that the cause of the decrease of the infectivity was associated with the alterations in the conformation of the SBMV virions, especially the capsids.

Since in the swollen SBMV virions treated with EDTA divalent cation was eliminated, there was a relaxation of the virus capsids and the infectivity decreased to about 10% of the value of the untreated control\(^{(1)}\). Recently, Brisco et al.\(^{(1,2)}\) have reported that encapsidated SBMV-RNA could be translated in a cell free system but it was necessary that the SBMV virions be preswollen with EDTA before they could synthesize proteins in the cell-free extract. Based on this observation, it is unlikely that the infectivity of swollen SBMV virions by EDTA treatment or freeze-drying would have decreased. It is more probable that \(\text{Ca}^{++}\) was associated with the virus penetration into the cells and RNA release from the virions\(^{(3)}\). However, it has not been confirmed hitherto whether the divalent cation was related to the infectivity, since it was not detected in the freeze-dried SBMV virions.

The properties of freeze-dried CarMV virions showed a similar tendency to that of SBMV. However, the virions of CarMV experienced less conformational damage, the susceptibility of CarMV to RNase and SDS was lower.

The infectivity of both the freeze-dried SBMV virions and SBMV-RNA extracted from the freeze-dried virions, preserved at 65°C, decreased rapidly within one day and the rate of decrease of the infectivity in both preparations was almost similar. In the preparations supplemented with 0.5% lysine, the decrease of the infectivity of both the virions and SBMV-RNA extracted was delayed compared with that of the preparations without additives. Moreover, all the freeze-dried virions without additives became completely swollen during the storage for one day at 65°C whereas in the preparations supplemented with 0.5% lysine the conformational changes of the virions were minimal even after 28 days. However, SBMV-RNA extracted from the virions in the latter preparations was completely degraded even 7 days after the storage and lost its infectivity. It thus seems that the maintenance of the viral conformation plays a role in suppression of the rate of decrease of the infectivity, as the conformation of viral RNA is maintained. On the other hand, the current results also implied that the maintenance of the viral conformation was not always directly associated with the infectivity.

Freeze-dried SBMV-RNA underwent a considerable degradation during the storage for several days at 65°C. The degradation of SBMV-RNA took place regardless of the presence of capsid protein. However, the addition of lysine afforded a protective effect in both preparations of SBMV virions and SBMV-RNA when freeze-dried and stored at 65°C. The concentration of lysine in the coat protein subunits which is high at the protein-RNA interface\(^{(9)}\) might be related to the protective effect of lysine.

The factors responsible for the stabilization of the structure of small icosahedral RNA viruses include (1) protein-protein interactions (Nepo- and Comoviruses), and (2) protein-RNA interactions (Cucumoviruses)\(^{(10)}\). SBMV and CarMV were stabilized by both interactions\(^{(19)}\). Viruses belonging to Nepo- and Comovirus groups are dissociated into RNA and coat protein by freeze-drying\(^{(9)}\), but in the case of the Cucumoviruses the virions became swollen (Fukumoto, unpublished observations). On the other hand, in the swollen SBMV virions, in which the divalent cation was removed by 10 mM EDTA in 0.1 M KPB, pH 7.5 and which were dialyzed to 0.1 M KPB, pH 7.5, RNA-protein interactions were mainly involved as in the case of the Cucumoviruses\(^{(13)}\). The preparations of such viruses never become dissociated by freeze-drying unlike those of the Nepo- and Comoviruses and the virions remain swollen. The behaviour of the swollen virions was similar to that of the Cucumovirus virions. These results suggest clearly that the viruses with RNA-protein interactions are able to tolerate freeze-drying.

We would like to thank Dr. J. Hashimoto, National Institute of Animal Health, for his valuable suggestions.
Literature cited


和文摘要

福本文良・橋原比呂志：インゲンマメ南部モザイクウイルスとカーネーション斑紋ウイルスの凍結乾燥保存中の安定性

純化したインゲンマメ南部モザイクウイルスは、凍結乾燥処理によって病原性が11％に低下した。そのような標品のウイルス粒子は部分的に膨潤し、RNaseとsodium dodecyl sulfateに顕著な感受性を示した。しかし、凍結乾燥前にリジンを添加した標品では、これらの影響が抑制された。一方、無添加とリジン添加の凍結乾燥標品から抽出されたRNAの間に差のないことから、凍結乾燥処理による病原性の低下の原因はウイルス粒子の構造の変化によると考えられた。65Cに保存した凍結乾燥標品は1日で病原性が消失し、ウイルス粒子が膨潤した。そのような粒子内のRNAは完全に崩壊していた。一方、リジンを添加した場合、ウイルス粒子および粒子内のRNAは比較的よく保持され、無添加の標品に比べて病原性も高く維持された。このような傾向はカーネーション斑紋ウイルスでも認められた。