EFFECT OF pH, IONS, BOVINE SERUM ALBUMIN AND HETEROLOGOUS ANTISERA ON THE STABILITY OF IMMUNOSORBED FLEXUOUS POTATO VIRUSES

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Summary. – Electron microscopic studies on the stability of immunosorbed (trapped) virions of potato viruses X, S and Y° (PVX, PVS and PVY°) revealed disintegration and dislodging of PVY° virions upon incubation with (1) antisera to PVX, PVS, or both diluted in saline, (2) 0.86% NaCl (saline) or 0.1 mol/l $CaCl_2$ but not with 0.1 mol/l $CaSO_4$ or 0.1 mol/l $MgSO_4$. PVX virions, on the other hand, showed partial dislodging upon incubation with an antiserum to PVS diluted in saline, but complete disintegration and dislodging with saline. 0.1 mol/l $CaCl_2$ caused partial dislodging while $MgCl_2$, $CaSO_4$ or $MgSO_4$ (all 0.1 mol/l) had no apparent adverse effect. PVS virions were not affected by saline, $CaCl_2$, $MgCl_2$, $CaSO_4$ or $MgSO_4$ (all 0.1 mol/l) and were only partially dislodged by antisera to PVX or PVY°. Disintegration and/or dislodging of the PVX and PVY° virions was prevented when (1) they were fixed with glutaraldehyde prior to incubation or (2) the virus extract contained bovine serum albumin (BSA) or (3) heterologous antisera were diluted in 0.1 mol/l phosphate buffer (PB) before use except the PVS antiserum which still caused disintegration and dislodging of PVY° virions. Prior fixation of virions prevented their disruption and dislodging by saline only in the case of PVY° but not PVX. On the other hand, BSA reverted the adverse effect of saline but not that of the PVS antiserum on PVY° virions. The results presented here suggest (1) a disruptive effect of Cl^- on PVX and PVY° virions particularly when it was associated with Na^+ and (2) an interaction between the immunosorbed virions of PVX or PVY° and the antiserum to PVS.

Key words: PVX; PVS; PVY°; immunosorbed virions; stability

Introduction

Virus particles are nucleocapsids organized by the interactions between capsid protein subunits and/or between capsid protein subunits and genomic nucleic acid. These interactions based on hydrophobic effects and/or electrostatic attractions are influenced by pH, ions and temperature (Durham, 1978; Durham and Hendry, 1977; Kaper, 1975).

Immunosorption (trapping) is an important step in the diagnosis of viruses by immunosorbent electron microscopy (IEM). Adsorption of globular proteins on a solid phase has been reported to cause their conformational changes or denaturation (Altschuh *et al.*, 1985; Soderquist and Walton, 1980). Vulnerability of immunosorbed virions of PVY° to different pH of the antiserum-diluting buffer and to different titers of the homologous antiserum during decoration was reported previously (Garg and Khurana, 1993). It was also reported that immunosorbed virions of PVY° were highly unstable when incubated with an antiserum to PVS (Garg and Khurana, 1994). However, two other important and ubiquitous potato viruses, PVX and PVS had shown a better stability in those studies.

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Abbreviations: BSA = bovine serum albumin; CPRI = Central Potato Research Institute; IEM = immunosorbent electron microscopy; PB = 0.1 mol/l Na_2HPO_4 pH 7.2; PVX, PVS, PVY° = potato viruses X, S, Y°; saline = 0.86% NaCl; SPB = 0.1 mol/l Sőrensen phosphate buffer

Present studies were aimed at (1) determining the effect of different ions, BSA, heterologous antisera and pH of the antiserum-diluting buffer on the immunosorbed virions of these three important potato viruses and (2) search of a way to overcome the observed vulnerability of PVX or PVY°. Our results suggest that disruption/disorganisation of immunosorbed virions of PVX or PVY° may be the outcome of alterations in the hydrophobic interactions between the capsid protein subunits upon their adsorption on solid phase therby making them more vulnerable to the chaotropic effect of Cl^-. Our results also suggest an interaction between an antiserum to PVS and the immunosorbed virions of PVX or PVY°.

Materials and Methods

Viruses. Pure cultures of PVX (*Potexvirus* genus), PVY° (*Potyviridae* family, *Potyvirus* genus) and PVS (*Carlavirus* genus) maintained in *Nicotiana glutinosa*, *N. tabacum* cv. Havana and potato cv. Craig's Defiance, respectively, at Central Potato Research Institute (CPRI), Shimla were used. Polyclonal antisera to PVX, PVY° and PVS were prepared at CPRI (Khurana *et al.*, 1990).

Virus extracts. Crude extracts of PVX or PVS were prepared by grinding 0.5 g of infected leaf tissue with 4 ml of PB pH of which was adjusted to 7.2 by 0.1 mol/l EDTA (disodium salt). The slurry was filtred and centrifuged at 10,000 x g for 10 mins and 0.5 ml of the supernatant was diluted further with PB (1:50 for PVX and 1:5 for PVS). Crude extract of PVY° was prepared in a different way. Infected leaf tissue (1.0 g) was ground in 5 ml of PB and the obtained slurry was stirred with equal volume of mixture of n-butanol and chloroform (1:1, v/v) followed by centrifugation at 10,000 x g for 10 mins. Polyethylene glycol 6000 and saline were added to the supernatant to final concentrations of 6% and 0.125 mol/l, respectively. The mixture was stirred, allowed to stand at 4°C for 2 hrs and centrifuged at 10,000 x g for 10 mins. The pellet was left to resuspend overnight in 1 ml of PB. This extract was further diluted 1:10 with PB before use. Virus extracts were also prepared in PB supplemented with saline.

Antisera. Each antiserum was diluted either with saline or 0.1 mol/l Sőrensen phosphate buffer (SPB) pH 6, 7.2 or 8 to obtain final microprecipitin titers of 1:1 or 1:2, depending on whether they were used individually or in dual combination.

Preparation of grids. Trapping of virus particles was carried out according to the method described earlier (Garg and Khurana, 1993). Grids with trapped virions were divided in two lots: one was used without fixation while the other was used after fixation with 3% glutaraldehyde prepared in SPB pH 7.2 for 2 hrs at room temperature.

Treatment of virions. Trapped virions, fixed and unfixed, were incubated in Petri dishes at 37°C for 1 hr with an antiserum (simple or mixed) diluted in saline or PB. However, incubation with saline, $CaCl_2$, $CaSO_4$, $MgCl_2$ or $MgSO_4$ (all 0.1 mol/l) involved only the trapped unfixed virions.

IEM. In each experiment, virus particles were counted at a displayed magnification of 21,000 x on a fluorescent screen 5 x 4 cm. Ten counts from each grid hole were made (Cohen *et al.*, 1982) and their means were calculated. Fifteen such means were determined from 15 squares of 3 grids per treatment. The grand means did not differ significantly from individual ones.

Results

Effect of heterologous antisera buffered to different pH on the stability of immunosorbed virions of PVS, PVX and PVY° is presented in Table 1. There was dislodging in 19–44% of unfixed PVX virions by heterologous antisera and PB; the dislodging was weaker with the heterologous antisera than with the buffer. Trapped PVX virions showed extensive

Table 1. Effect of heterologous antisera buffered to different pH on the immunosorbed virions of PVS, PVX and PVY°

Virus	No. of virions left after incubation with antiserum to[a]															No incubation (control)
	PVS (pH)				PVX (pH)				PVY° (pH)				PB[b] (pH)			
	6.0	7.2	8.0	Sln	6.0	7.2	8.0	Sln	6.0	7.2	8.0	Sln	6.0	7.2	8.0	
PVS (unfixed)	14	13	13	8	13	12	13	12	13	12	13	12	10	12	13	14
PVS (fixed)[c]	14	13	13	8	13	13	12	12	12	13	13	13	12	12	13	–
PVX (unfixed)	18[d]	20	26	15	28	29	30	27	29	28	29	30	20	18	20	32
PVX (fixed)[c]	30	29	29	25	30	29	31	29	29	29	30	29	28	30	30	–
PVY° (unfixed)	10	7	0	0	8	9	8	2.5	6	9	8	*	8	9	7	10
PVY° (fixed)[c]	10	9	10	10	10	10	10	9	10	9	10	10	9	9	9	–

[a]Antiserum diluted in SPB.
[b]SPB.
[c]Fixed with 3% glutaraldehyde for 2 hrs at room temperature prior to incubation.
[d]Extensive fragmentation, the count concerns the fragments.
Sln = antiserum diluted in saline pH 7.0.
*Virions showed disorganisation and were difficult to distinguish.

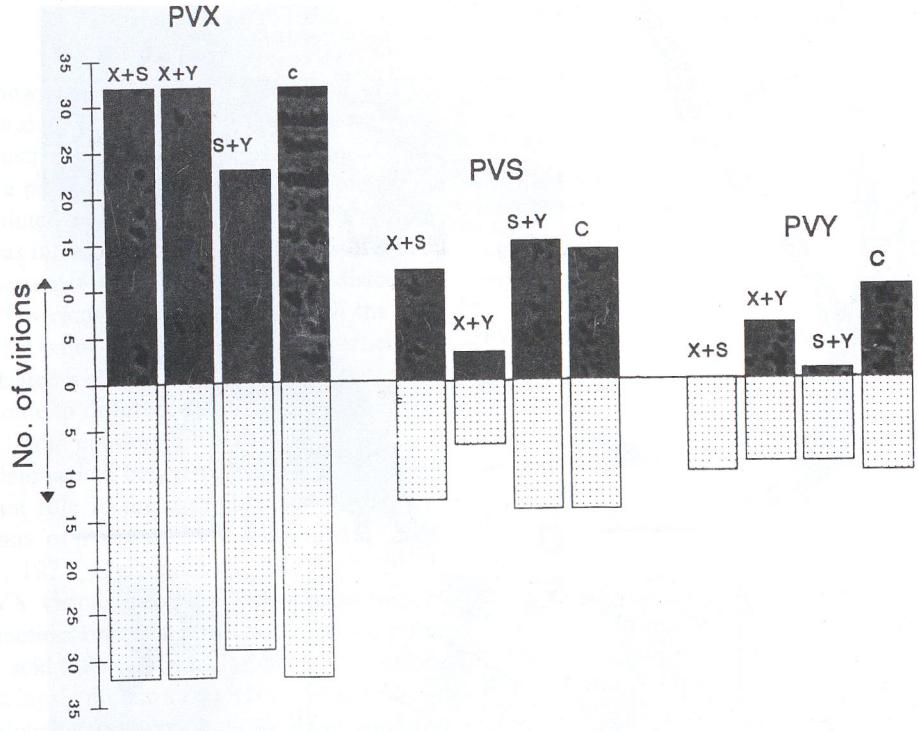

Fig. 1

Effect of antiserum mixtures on stability of immunosorbed virions of PVX, PVS and PVY

Unfixed virions (upper side of the ordinate) and fixed virions (lower side of the ordinate). X, S, Y = antisera to PVX, PVS and PVY° diluted in saline, respectively. C = control.

fragmentation upon incubation with the PVS antiserum at pH 6. Unfixed PVY° virions were completely dislodged by the PVS antiserum at pH 8 only or when the PVS antiserum was diluted in saline. Dislodging of PVY° virions with the PVX antiserum was quite weaker. Prior fixation of the immunosorbed virions with glutaraldehyde markedly prevented their dislodging.

Effect of combinations of antisera as compared to single antiserum on the trapped virions was quite different (Fig. 1). The mixtures of PVS and PVX or PVX and PVY° antisera had no adverse effect on immunosorbed PVX virions; the mixture of PVS and PVY° antisera caused only a slight (28%) dislodging of PVX virions. Immunosorbed PVS virions showed a marked (78%) dislodging only upon incubation with the mixture of PVX and PVY° antisera. Trapped unfixed PVY° virions were markedly sensitive to all combinations of antisera except that of PVX plus PVY° which caused a weak dislodging (Fig. 1, Fig. 2D). Prior fixation with glutaraldehyde imparted maximum stability to trapped virions of PVX and PVY° (Fig. 1).

Results of the effect of ions on the immunosorbed virions are depicted in Table 2. Cl^- in association with Na^+ caused the maximum destabilization of unfixed immunosorbed virions of PVX and PVY° leading to their disintegration (Fig. 2B); prior fixation stabilized the trapped virions of PVY° but not those of PVX (Table 2). Even the heterologous antisera diluted in saline had a severe disintegrating and/or dislodging effect on immunosorbed virions of PVY° or PVX (Table 1, Fig. 2C). The effect of 0.1 mol/l $CaCl_2$ was less severe on PVX virions as compared to PVY° virions which were completely dislodged while the former showed only fragmentation (Table 2). There was a great stabilizing effect of 0.1 mol/l $Mg Cl_2$ on the virions of PVX but not on those of PVY° which showed extensive fragmentation (Table 2; Fig. 2F). There was no effect of any of the salts tested on PVS, and of $CaSO_4$ and $MgSO_4$ on PVX or PVY°. Table 3 shows effect of BSA on the stability of immunosorbed virions. A lesser number of virions were trapped when the virus extract contained BSA and the reduction corresponded to the increase in the concentration of BSA. PVX virions trapped from virus extract containing only 0.25% BSA showed good stability against saline. The presence of BSA in the PVY° extract, on the other hand, enhanced the stability of PVY° virions in saline but not in the PVS antiserum.

Trapping of virions from extracts prepared in PB containing saline did not show any reduction in the number of trapped virions of PVS or PVY° while there was a 40% reduction with PVX virions (data not shown).

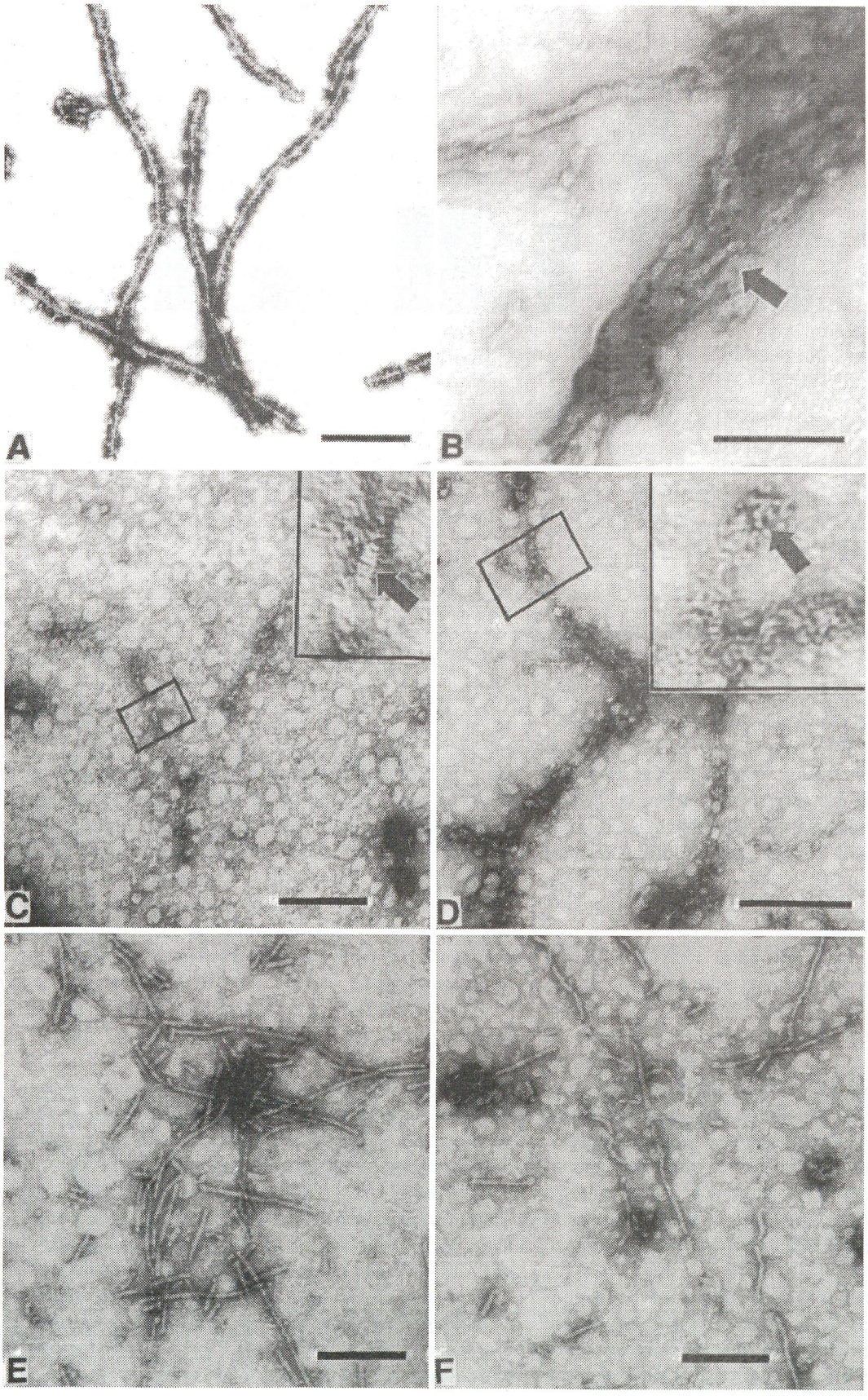

Fig. 2
Immunosorbent electron microscopy of PVY^o and PVX
Parts A–F show trapped virions after incubation under following conditions.
A: PVY^o, PVY^o antiserum (microprecipitin titer of 1:1), pH 7.2.
B: PVY^o, saline. Virus-like particles undergo disintegration.
C: PVY^o, PVX antiserum diluted in saline. Insert: a disintegrated virion (arrow).
D: PVY^o, mixture of PVS and PVY^o antisera. Insert: fragment of a disintegrated virion (arrow).
E: PVX, PVS antiserum (microprecipitin titer of 1:1), pH 6. Extensive fragmentation of virions.
F: PVY^o, 0.1 mol/l $MgCl_2$. Fragmentation of virions.
Bar = 200 nm (A, C–F) or 100 nm (B).

Discussion

Our results show differences in the behaviour of PVX and PVY° virions and these differences appear to be virus-specific as demonstrated by a complete dislodging of PVY° virions but only a partial disloding of PVX virions by the PVS antiserum diluted in saline. In the case of PVY° virions, the dislodging was influenced by the pH of the buffer used to dilute the antiserum; it was 100% at pH 8. The dislodging of PVX or PVY° virions upon incubation with the PVS antiserum diluted in saline was due largely to Cl' particularly when it was associated with Na^+. However, the adverse effect of Cl' was overcome to different extent in the presence of Ca^{++} or Mg^{++}. SO_4" did not destabilize the virions.

Hydrophobic interactions between capsid protein subunits play an important role in the assembly and stability of assembled virions of PVX or PVY (Goodman, 1977; Goodman *et al.*, 1975; McDonald and Bancroft, 1977). Assembly of PVX virions has been proposed to involve electrostatic interactions between capsid protein subunits and genomic nucleic acid followed by hydrophobic interactions between adjacent capsid protein subunits (Goodman, 1977). Adsorption of globular proteins on solid phase has been reported to cause their conformational changes or even denaturation (Altschuh *et al.*, 1985; Soderquist and Walton, 1980). Virions resemble globular proteins and consequently are expected to behave like the former upon adsorption to solid phase. Thus, it is highly probable that adsorption of PVX or PVY° virions causes changes in their 3-dimensional structure as a result of disturbance in hydrophobic interactions amongst protein subunits. This disturbance seems to make the adsorbed virions more vulnerable to the chaotropic effect of Cl' which might disrupt the virions by dissolving their protein subunits. Highly concentrated NaCl is known to disrupt PVX virions (Goodman *et al.*, 1975). However, PVY° virions are known to be stable in 2 mol/l NaCl and unstable in NaSCN, the SCN' being the most chaotropic anion (Hatefi and Hanstein, 1969). Thus hydrophobic interactions between the capsid protein subunits of PVY appeared to be stronger than those of PVX. Difference in the stability of PVX and PVY° virions observed in the present study may at least partly be due to this fact cited above.

Though association of Ca^{++} with Cl' did not counteract the adverse effect on PVY° virions, it certainly provided some protection to PVX virions. On the other hand, association of Mg^{++} with Cl' though partially counteracted the adverse effect on PVY° virions, completely counteracted the adverse effect on PVX virions. Divalent cations, particularly Ca^{++} and Mg^{++} are known to stabilize the virion structure (Powell, 1975; Proll and Schmidt, 1990). Furthermore, SO_4" is a known antichaotropic anion thereby stabilizing the quaternary protein structure by strengthening hydrophobic interactions (Hatefi and Hanstein, 1969). This was probably the reason of stability of virions in the presence of SO_4". Our results, thus, corroborated these earlier findings on other plant viruses. Though disintegration of PVY° virions upon incubation with the PVS antiserum diluted with saline was complete, it was only partial upon incubation with the PVX antiserum. Again, though 1.0 or 0.5% BSA did not protect PVX virions against saline, 0.25% BSA did protect them. Some serum protein(s) present in the PVX antiserum and absent in the PVS antiserum appeared to provide protection to the PVY° virions. Protection of TMV virions by certain basic proteins and polyamines against the degradative effect of EDTA at alkaline pH has been reported earlier (Brakke and VanPelt, 1969; Taniguchi *et al.*, 1967).

Protection of PVY° virions by BSA against the PVS antiserum in our experiments was none. Trapping of PVY° virions from the mixture of PVY° extract and PVS antiserum was also markedly (by 60%) inhibited. Furthermore, PVY° virions were completely dislodged by the PVS antiserum at

Table 2. Effect of ions on the immunosorbed virions of PVX, PVS and PVY°

Virus	No. of virions left after incubation with						No incubation (control)
	$CaCl_2$[a]	$CaSO_4$[a]	$MgCl_2$[a]	$MgSO_4$[a]	Saline	SPB	
PVS (unfixed)	13	12.5	12.0	12.5	10	13	14
PVS (fixed)	NT	NT	NT	NT	13	13	14
PVX (unfixed)	12[b]	31.5	31.4	32.0	0.5	21	32
PVX (fixed)	NT	NT	NT	NT	0.8	31	32
PVY° (unfixed)	0	9.5	7.4[b]	10.0	0.6	9	10
PVY° (fixed)	NT	NT	NT	NT	10	10	10

[a]0.1 mol/l.
[b]Extensive fragmentation, the count concerns the fragments. pH of $MgCl_2$ and $CaCl_2$ solutions in distilled water was 6.5 while that of $MgSO_4$ and $CaSO_4$ was 7.0.
NT = not tested.

Table 3. Effect of BSA on stability of immunosorbed virions of PVX and PVY°

Percentage of BSA in virus extract	No. of immunosorbed virions of					
	PVX			PVY°		
	after incubation with					
	PVS antiserum[a]	Saline	–	PVS antiserum[a]	Saline	–
1.0	10	0	12	0	4	6
0.5	12	0	12	0	8	8
0.25	12	10	16	0	9	9
0	10	0	20	0	0	10

[a]Diluted in saline.
(–) = no incubation.

pH 8. Thus, there appeared to be an interaction between PVY° virions and the PVS antiserum. A pronounced adverse effect of a PVS antiserum on the trapping of PVY° was also reported in our earlier studies (Garg and Khurana, 1992).

The protection of PVY° virions against saline by all concentrations of BSA tested as compared to that of PVX virions by only 0.25% BSA and the protection of glutaraldehyde-fixed PVY° virions against saline as compared to the weak protection of PVX virions against saline also indicated differences in the organization of virions of these two viruses.

There was the reduction in the number of virions trapped in the presence of BSA which was proportional to the concentration of BSA. This might be due to competition for binding sites on the grid between the virions and the BSA molecules as well as due to steric hindrance. PVS virions differed from both PVX and PVY° in their stability in saline and against the PVX or PVY° antisera. Thus, the forces stabilizing the virion structure of PVS appear to differ from those involved in PVX and PVY°. Disorganization of virions of PVS but not PVX or PVY° upon storage of infected leaf tissue at -20°C for one month, reported in our earlier studies (Khurana *et al.*, 1992), suggest that the forces involved in the assembly of PVS virions may differ from those involved in the assembly of PVX or PVY°

In simultaneous detection of more than one virus by double-antibody sandwich enzyme linked immunosorbent assay (ELISA), researchers have tested use of antiserum mixtures. San Roman *et al.* (1988) have reported use of antiserum mixtures for reliable detection of PVX, PVS, and PVY both in sprout and leaf ELISA. Salazar (1979), in fact, found that when testing mixtures of 8 different antibodies, two of the viruses escaped detection. Our previous studies (Garg and Khurana, 1992, 1993, 1994) as well as the present have suggested a sort of adverse effect of a PVS antiserum on trapping and/or stability of immunosorbed PVX or PVY° virions. Such an adverse interaction can either inhibit or prevent the adsorbtion of a specific virus, particularly when it is present in low concentration, or dislodge the virus during binding of enzyme-antibody conjugates leading to non-detection of the vulnerable virus.

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