OPTIMIZATION OF PURIFICATION PROCEDURE FOR POTATO VIRUS Y STRAIN NN

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Summary. – Potato virus Y strain NN (PVY^{NN}) was purified from mechanically infected plants *Nicotiana tabaccum* cv. Samsun by extraction of the plants with various buffers, clarification of the suspensions with chloroform or Triton X-100, high speed centrifugation of the virus through sucrose cushion and resuspension of the sedimented virus in different buffers. The two optimal combinations of different procedures tested were either (1) extraction of the plants with the buffer containing 0.3% ascorbic acid, 0.3% mercaptoethanol, 0.01 mol/l diethyl pyrocarbonate (DEPC) and 5 mmol/l phenylmethylsulphonyl fluoride (PMSF), pH 5.3, clarification with cold chloroform, PEG precipitation, high speed centrifugation through sucrose cushion and resuspension of the sedimented virus in 0.02 mol/l Na-borate buffer pH 7.8, or (2) extraction of the plants with the buffer containing 0.5 mol/l Na-citrate, 1% mercaptoethanol and 5 mmol/l PMSF pH 6.5, clarification with 2% Triton X-100, PEG precipitation, high speed centrifugation through sucrose cushion and resuspension of the sedimented virus in 0.02 mol/l K-phosphate, 0.5 mol/l urea and 0.1% mercaptoethanol, pH 7.5.

Key words: potato virus Y strain NN; purification; infectivity; serological activity

Introduction

PVY^{NN} has been found first in Hungary and Germany (Beczner *et al.*, 1984; Radtke, 1984; Weidemann, 1985). At present, there is an evidence about its occurrence around the whole Europe. The disease is characterized by a superficial tuber necrosis occurring at harvest or some weeks later during storage. PVY isolates inducing necrosis on tubers belong to the PVY^{N} subgroup according to their reactions on *Nicotiana tabaccum* (Beczner *et al.*, 1984). They are not serologically distinct from standard PVY^{N} isolates (Le Romancer and Kerlan, 1991; Le Romancer, 1993, Le Romancer *et al.*, 1994).

In the present paper we focused on optimization of the PVY^{NN} purification. We tried to prepare this virus in sufficient quantity and quality. We compare several purification (clarification) procedures, including various extraction and storage buffers.

Materials and Methods

Virus isolates. We used three isolates of PVY^{NN}, Nicola, Lukava (both obtained from Dr. P. Dědič, Czech Republic) and Lebanon (obtained from Dr. C. Kerlan, France). Each virus isolate was maintained on *N. tabaccum* cv. Samsun as follows: 1 g of infected leaf tissue was grinded with 5 ml of 50 mmol/l phosphate buffer pH 7.0 and the homogenate was rubbed onto the leaves of test plants previously dusted with carborundum powder.

Buffers. We used various extraction and storage buffers, which have been tested in our previous work on purification of various potyviruses (Filigarová, 1975; Čeřovská *et al.*, 1991) and tried to choose their best combination for PVY^{NN}, an extremely unstable virus.

Abbreviations: CFA = complete Freund's adjuvant; DEPC = diethyl pyrocarbonate; ELISA = enzyme-linked immunosorbent assay; PMSF = phenylmethylsulphonyl fluoride; PVY^{NN} = potato virus Y strain NN; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

Extraction buffers:

(I) 0.03% ascorbic acid, 0.3% mercaptoethanol, 0.01 mol/l DEPC and 5 mmol/l PMSF, pH 5.3

(II) O.5 mol/l K-phosphate, 0.5 mol/l Na-sulphate, 1 mol/l urea, and 5 mmol/l PMSF, pH 7.5

(III) 0.5 mol/l Na-citrate, 1% mercaptoethanol and 5 mmol/l PMSF, pH 6.5.

Storage buffers:

(A) 0.1 mol/l Tris-HCl pH 9.0

(B) 0.02 mol/l Na-borate pH 7.8

(C) 0.02 mol/l K-phosphate, 0.5 mol/l urea and 0.1% mercaptoethanol, pH 7.5.

To inhibit the proteases and to stabilize the virus, all the extraction buffers used contained 5 mmol/l PMSF (Čech *et al.*, 1977).

Virus purification. Infected plants were harvested when the symptoms were considered optimal, mostly on the 12th day p.i. The virus was purified from plant tissue stored for one day at -20°C. Fifty g of leaves was homogenized in 1 - 3 volumes of chilled extraction buffer in a blender for 2 mins. The brei was then filtered through the cheese cloth and the filtrate was clarified either by extraction with 10% (v/v) of chloroform and then by centrifugation at 10,000 x g for 10 mins, or by centrifugation and then by extraction with 2% (v/v) Triton X-100.

In the latter case, the supernatant was stirred with Triton X-100 for 15 mins. Then NaCl was added to 2% (w/v) and polyethylene glycol (PEG, MW 6,000) to 7% (w/v), followed by stirring for 1 hr on ice (Dougherty and Hiebert, 1980). The precipitated material was collected by centrifugation at 10,000 x g for 10 mins and resuspended in 20 ml of respective storage buffer. The resuspension consisted of the first extraction for 60 mins on ice bath by gentle stirring and centrifugation at 10,000 x g for 10 mins, and of the second extraction for 30 mins and centrifugation under the same conditions.

Then, a high speed centrifugation through 30% sucrose cushion (2 hrs, 27,000 rpm in Beckman Ti 50.2 rotor) was carried out. Each pellet was resuspended in the same buffer as after PEG precipitation and let soaking overnight. The virus purificates were spectrophotometrically characterized and the virus yields were calculated using the formula $A_{260}(0.1\%) = 2.86$ (Stace-Smith and Tremaine, 1970).

The infectivity of the virus purificates was assessed by their inoculation onto *N. tabaccum* cv. Samsun using a sterile glass rod dipped in a purificate aliquot. The purity of virus preparations was further examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) (Laemmli, 1970) and enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977), using antibodies prepared according to the following immunization scheme. Approximately 1.3 mg of virus emulsified in 500 µl of complete Freund's adjuvant (CFA) (Calbiochem) was injected into New Zealand white rabbits intramuscularly on day 0, and an additional 1 mg of virus emulsified in 500 µl of incomplete CFA was injected on days 14, 28 and 50 subcutaneously. The antisera had titres of 1/1024 in the Ouchterlony gel diffusion test with sonicated virus.

Results and Discussion

First we examined the optimal conditions for plant tissue extraction (homogenization). In our hands, the best yields were obtained using the extraction buffer I and subsequent chloroform clarification or the extraction buffer III in the case of subsequent Triton X-100 clarification (Table 1 and 2).

Table 1. Influence of various buffers on virus yields and on the activity of virus preparations in purification procedures using chloroform clarification

E	S	Y	R	A_{405}	Inf
I	A	–	–	0	0
	B	14.3	1.35	1.22	+
	C	19.0	1.65	0.68	0
II	A	–	–	0	0
	B	–	–	0	0
	C	6.0	0.9	0.79	+
III	A	–	–	0	0
	B	–	–	0	0
	C	-	-	0	0

E = extraction buffer; S = storage buffer; Y = yield in mg of virus/100 g of starting; material (average from 3 values); $R = A_{260}/A_{280}$; A_{405} = absorbance at 405 nm determined by ELISA after 60 mins of incubation, virus concentration 1 µg/ml; Inf = infectivity; (–) = the maximum of absorbance at 260 nm was not obtained.

The following step was the clarification of the homogenate with chloroform or Triton X-100. The Triton X-100 treatment was more effective than the similar procedure with chloroform. In the latter case, a spectrum characteristic for nucleoproteins was often not obtained. On the other hand, the absorbance values in ELISA were better in the case of the chloroform treatment.

A further critical step was the dissolution of the PEG-precipitated sediment. When it was resuspended in the storage buffer C, highest virus yields (A_{260}) were obtained with most purification protocols, the preparations were active in ELISA, however, in some cases, their UV-spectra were not characteristic for the viral nucleoprotein with a maximum at 260 nm. For these reasons we chose the storage buffer B (0.02 mol/l borate buffer pH 7.8) for dissolution of the virus sediment, resulting in smaller yields of the virus, but we gained a typical viral UV-spectrum, and also the virus activity in ELISA measured immediately or after one month storage was also very satisfactory (Table 1).

There were no significant differences between respective virus purificates in their SDS-PAGE patterns (data not shown). The major band (capsid protein) had M_r about 31 K, and some minor bands of higher (about 70 K) and smaller (14 – 20 K) size were observed too. Upon comparing all the examined procedures for PVY^{NN} purification and summarizing their results, we suggest two purification schemes:

1.(a) Homogenization of the leaves in the extraction buffer I in the ratio of 1:1.5.

(b) Clarification of the homogenate by cold chloroform (10%, v/v) extraction and centrifugation.

Table 2. Influence of various buffers on virus yields and on the activity of virus preparations in purification procedures using Triton X-100 clarification

E	S	Y	R	A_{405}	Inf
I	A	–	–	0	0
	B	10.6	1.61	0.62	+
	C	14.8	1.11	0.70	+
II	A	–	–	0	0
	B	10.0	0.75	0.48	0
	C	–	–	0	0
III	A	2.1	1.5	0	0
	B	4.5	1.38	1.08	0
	C	7.0	1.70	1.43	+

For the legend see Table 1.

(c) Precipitation of the virus with PEG 6,000 (7%) in NaCl (2%, w/v), resuspension of the precipitate in 0.02 mol/l borate buffer pH 7.8 (storage buffer B).

(d) Ultracentrifugation of the virus through 30% sucrose cushion, resuspension of the sediment in the same buffer (storage buffer B) supplemented with 1 mmol/l PMSF. The yield of the virus was 14 mg per 100 g of fresh leaves, the ELISA activity A_{405} was 1.22 after 1 hr of incubation of a substrate with 1 µg/ml virus.

2.(a) Homogenization of the leaves in the extraction buffer III in the ratio of 1:1.5.

(b) Centrifugation of the homogenate and clarification of the supernatant with 2% (w/v) Triton X-100.

(c) PEG precipitation of the virus as under (1), except the resuspension of the precipitate in 0.02 mol/l K-phosphate buffer, 0.5 mol/l urea and 0.1% mercaptoethanol, pH 7.5 (storage buffer C).

(d) Ultracentrifugation and resuspension of the virus in the same buffer (storage buffer C).

The yield of the virus was 7 mg per 100 g of fresh leaves. The ELISA activity A_{405} was 1.43 under the same conditions as those mentioned in the first scheme. We chose this combination of steps considering the fact that the purificates kept their serological activity at least for one month of storage.

Although the purificates prepared according to the scheme I/C (Table 2) had better parameters when measured immediately after purification, they lost completely their serological activity during one month of storage. The infectivity of the purificates prepared by the two recommended procedures was similar.

The virus yields shown in this study are comparable to those achieved by others, e.g. 3 – 4 mg/kg for PVY (Stace-Smith and Tremaine, 1970) and 8 mg/kg for turnip mosaic virus (TuMV) (Hill and Shepherd, 1972; Thompson *et al.*, 1988).

Our results show that it is very useful to optimize the procedure of purification for distinct members of the same genus of the viruses, in our case for the potyvirus group.

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