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Purification of plum pox (sharka) virus with the use of Triton X-100

H. J. VAN OOSTEN

Laboratory of Virology, State Agricultural University, Wageningen

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Abstract

Plum pox virus was purified by adding up to 5% non-ionic detergent Triton X-100 to extracts clarified by low-speed centrifugation. After stirring for 1/2 h, the suspensions were subjected to 2 cycles of differential centrifugation followed by sucrose density-gradient centrifugation. Purity of the product was confirmed by electron microscopy and equilibrium density-gradient centrifugation in CsCl. The virus sedimented in the analytical ultracentrifuge as a single peak with a sedimentation coefficient of about 170 S at infinite dilution. Virus so purified showed an absorption spectrum with a minimum at 247 nm and a maximum at 263 nm. The modal length of the virus particles in purified preparations was 764 nm. Antiserum prepared had a specific titer of 4096.

Introduction

Plum pox virus (PPV) (*/*;*/*; $^{E}/_{E}$; $^{S}/_{Ap}$) has been described as the causal agent of a disease of plums. Fruits of infected trees show a clear discoloration of the fruit surface and in some cases deep irregular grooves and pits (Christoff, 1958). The latter symptom, however, is thought to be non-specific (van Oosten, 1971). Based on the main length of its filamentous particles, Kegler *et al.* (1964) and Kassanis and Šutić (1965) described the virus as a member of the potato virus Y group.

Partial purification of PPV has been done in various ways by Dounine and Minoiu (1968), György and Németh (1968), Schade (1969), Ranković and Jordović (1970) and Babović and Šutić (1971). In preliminary own purification experiments several methods involving clarification with organic solvents and adsorbents, and precipitation by polyethylene glycol were compared. The use of diethylether and carbon-tetrachloride (Wetter, 1960) proved to be the most gentle one. In the present paper this method is compared with a procedure based on the use of the non-ionic detergent Triton X-100.

Materials and methods

Virus and host plants. The isolation and identification of the PPV-isolate used were reported earlier (van Oosten, 1970). The isolate was maintained in *Nicotiana clevelandii* by mechanical transmission and occasionally by aphid transmission. *N. clevelandii* plants were inoculated at the 5–8 leaf stage. Systemically infected leaves were

harvested 16–20 days after inoculation, when the PPV-concentration was highest (Schade, 1969; van Oosten and van Bakel, 1970). Infectivity was tested on *Chenopodium foetidum* using an incomplete block design. Properties of the virus in vitro such as thermal inactivation point, longevity *in vitro* and dilution end-point were determined according to Bos *et al.* (1960). The entire purification procedure was done at 0–2 °C.

Homogenization and determination of extracting buffer. Systemically infected leaves were cut in small pieces and mixed well. Small samples of equal weight were first mixed in a proportion of 1:1 (w/v) with either borate-, citrate-or phosphate buffer at different pH values and molarities and then ground in a mortar. Later on, other ratios of the amount of leaves to buffer were tested. The polyphenoloxidase inhibitor sodium diethyl dithiocarbaminate (Na-Dieca) (Hampton and Fulton, 1961) was added to the homogenization buffer and studied for its effect on infectivity. Because Na-Dieca is unstable (Bode, 1954), it was added to the homogenization buffer just before use.

For the homogenization of large quantities of leaves the use of the Waring blendor was compared with the use of mortar and pestle. Samples were taken for infectivity tests after pressing the extracts through one layer of cheesecloth.

Clarification procedures. Extracts were pressed through one layer of cheesecloth and shaken with 1/4 volume diethylether/carbontetrachloride (1:1) (ether/CCl_4) and subjected to low speed centrifugation (6000 *g*, 15 min) (Wetter, 1960). Triton X-100 (Sigma Chem. Comp., Missouri, USA) was added dropwise up to the desired concentration (v/v) either during homogenization or after clarification of the extracts by ether/CCl_4 treatment or after low speed centrifugation (6000 *g*, 15 min). Extracts were stirred for at least 1/2 h and then subjected to high speed centrifugation.

Concentration procedures. Virus was sedimented by centrifugation at 78,480 *g* for 90 min in the Spinco R 30 rotor. Pellets were resuspended in 1/5–1/10 volume 0.01 *M* citrate pH 6.7, containing 0.0005 *M* EDTA (ethylene diaminetetraacetic acid) or 1 *M* urea and 0.1 % mercaptoethanol (Damirdagh and Shepherd, 1970). Insoluble material was removed by low speed centrifugation at 1000 *g* for 10 min. The clarified virus suspension was carefully layered on 8 ml 20 % sucrose in a SW 25.1 tube and centrifuged for 2 h at 51,500 *g* in the Spinco SW 25.1 rotor. The pellets were resuspended in the same buffer and the suspension was clarified by low speed centrifugation (1000 *g*, 10 min).

Density-gradient centrifugation for further purification. Suspensions were centrifuged on 10–50 % (w/v) linear sucrose gradients for 3 h at 51,000 *g* in the Spinco SW 25.1 rotor. The virus suspension obtained was dialyzed against 0.01 *M* citrate pH 6.7 and concentrated by high speed centrifugation (78,480 *g*, 90 min). Pellets were resuspended in 0.01 *M* citrate pH 6.7 and examined for purity.

Analytical ultracentrifugation. Centrifugal analyses were done in a Spinco Model E centrifuge at 20 °C using Schlieren optics. Sedimentation coefficients were determined by the graphical method of Markham (1960).

Equilibrium density-gradient centrifugation. The concentrated virus suspension (0.5 ml) was layered on 3.8 ml 34% (w/w) CsCl and centrifuged for 24–30 h at 86,180 *g* in the Spinco SW 39 L rotor. Afterwards the bottom of the tubes was punctured and fractions of 10 droplets each were collected. Even numbered fractions were made up to 1 ml with 0.01 *M* citrate pH 6.7 and measured spectrophotometrically for absorbance at 260 nm. Odd-numbered fractions were used for the determination of densities by means of the refractometer.

Electron microscopy. Samples were sprayed on to 400 mesh grids and either stained with 2% PTA pH 6.5 or shadow-cast with palladium. Polystyrene particles with a diameter of 357 nm were often added as an internal standard for rough determination of particle length in shadow-cast preparations. Preparations were examined with a Siemens Elmiskop I.

Serology. About 15 mg of virus of 10 different purification experiments was injected intramuscularly into a rabbit over a period of 7 weeks. The virus suspensions were mixed 1:1 with Freund's incomplete adjuvant. The rabbit was bled one week after the last injection. Sera were collected and then stored at −20 °C. Serological studies were made by microprecipitin tests under paraffin oil. Systemically infected leaves of *N. clevelandii* were homogenized in 0.01 *M* citrate pH 6.7 at a ratio of 1:4 (w/v). The extract was first clarified by low speed centrifugation (6000 *g*, 15 min). Droplets were incubated at 37°C for 2 h and subsequently for 12 h at 0–2°C, then examined for precipitation using a microscope.

Spectrophotometry. Virus concentrations and ultraviolet absorption spectra were determined with a Zeiss spectrophotometer. Corrections for light scattering were made using the graphical method of Treiber and Schauenstein (1949). Absorbance between 230–600 nm was plotted on log/log graph paper. Values for absorbance between 320–600 nm gave a straight line. Light scattering was determined by extrapolation of this line between 320–230 nm. The values obtained were subtracted from the measured absorbance. The RNA-content of PPV was estimated according to Paul (1959).

Results

Properties of the virus in vitro. The dilution end-point of PPV in sap of systemically infected leaves of *N. clevelandii* harvested 16–20 days after inoculation, was about 10^5. The thermal inactivation point was 54–56 °C. Sap from infected leaves lost all its infectivity within 48 h at 20 °C and within 5–7 days at 0–2°C.

Homogenization and determination of extracting buffer. The infectivity of PPV is greatly influenced by the method of homogenization. Extracts obtained after the use of a Waring blendor were notably less infective than those obtained by use of mortar and pestle. Therefore in all experiments leaves were triturated in a mortar. The buffers used for homogenization are another important factor. Best results were obtained at low molarities as shown for phosphate buffer in Table 1. Several other buffers were compared at 0.01 *M*, but only citrate buffer was about equal to phosphate buffer (Table 2). However, if the amount of buffer per gram of leaves was raised, highest

Table 1. Effects of different molarities of phosphate buffer pH 8.0 and distilled water on infectivity of plum pox virus during homogenization at a ratio of 1:1 (w/v).

Molarity	pH extract	Lesions/leaf at 10^{-2} dilution of the homogenate[1]
0.5	7.4	4
0.2	7.2	38
0.1	7.1	136
0.05	7.0	208
0.025	6.8	220
0.01	6.6	267
distilled water	6.3	185

[1] Mean number of lesions on 12 leaves in 2 experiments. Dilutions were made in the same buffer as used for homogenization.

Tabel 1. Het effect van enkele fosfaatbuffers met pH 8,0 en een verschillende molariteit en van gedestilleerd water op de infectiositeit van het (pruime) sharkavirus gedurende het homogeniseren. De blad: buffer verhouding is 1:1 (w/v).

Table 2. Effect of different 0.01 *M* buffer solutions with different pH on infectivity of plum pox virus during homogenization.

Buffer	Ratio w/v	pH extract	Lesions/leaf at 10^{-2} dilution of the homogenate*
Experiment 1			
citrate pH 6.7	1:1	6.3	48
phosphate pH 6.0	1:1	6.2	28
phosphate pH 8.0	1:1	6.6	53
borate pH 8.0	1:1	6.6	18
borate pH 9.3	1:1	7.5	35
K_2HPO_4 pH 9.4	1:1	6.6	23
Experiment 2			
citrate pH 6.9	1:1	6.4	43
	1:4	6.5	148
	1:9	6.7	201
phosphate pH 8.0	1:1	6.6	39
	1:4	7.0	112
	1:9	7.4	177
Experiment 3			
citrate pH 6.7	1:4	6.3	158
citrate pH 6.7 + 0.01 *M* NaDieca	1:4	6.9	331**

* Mean number of lesions on 6 leaves per experiment. Other experiments showed similar results. Dilutions were made in the same buffer as used for homogenization.
**Dilutions were made in 0.01 *M* citrate pH 6.7.

Tabel 2. Het effect van enkele bufferoplossingen van 0,01 M en een verschillende pH op de infectiositeit van het (pruime) sharkavirus gedurende het homogeniseren.

infectivity was always obtained with 0.01 *M* citrate pH 6.7 (Table 2). For practical reasons a leaf -buffer ratio of 1:4 (w/v) was chosen for purification. The addition of 0.01 *M* Na-Dieca to the homogenization buffer further enhanced infectivity of the extract (Table 2).

Clarification and concentration. Clarification by ether/CCl_4 was only partial, since pellets after high speed centrifugation remained greenish. Low-speed centrifugation of resuspended pellets caused great losses in infectivity. When Triton X-100 was added in concentrations of 0.1–25% during homogenization or immediately after, the pellets were also greenish after high-speed centrifugation. But when Triton X-100 was added after clarification of extracts either by low-speed centrifugation or ether /CCl_4 treatment, pellets were no longer greenish. Infectivity tests were made from resuspended pellets and showed that infectivity was highest when Triton X-100 was added up to a concentration of about 5% to extracts clarified by low-speed centrifugation. Infectivity was also increased when extracts clarified with ether /CCl_4 were supplemented with Triton X-100. Suspensions containing Triton X-100 could not be tested for infectivity before high-speed centrifugation since results were contradictory, apparently due to Triton X-100. The pellets obtained with the use of Triton X-100 were still slightly brown, but could be further cleaned with 0.0005 *M* EDTA in the homogenization buffer. Higher EDTA-concentrations reduced infectivity too much. Prior to density-gradient centrifugation the virus was separated from host material by centrifuging through a layer of 2 cm (8 ml) 20% sucrose allowing the virus to sediment while host material such as ribosomes remained in the sucrose.

Density-gradient centrifugation. One single virus band was seen about 3 cm below the meniscus. Above this band there was no opalescence when virus had previously been pelleted through a 20% sucrose layer. Below the virus band a more or less opalescent region or a second band was present due to virus aggregates. This could be partially prevented using buffer with 1 *M* urea and 0.1% mercaptoethanol. Sucrose density-gradient centrifugation clearly demonstrated the superiority of the purification procedure based on Triton X-100 over that employing ether/CCl_4 (Fig. 1). The suggested purification procedure for PPV is presented in Fig. 2.

Spectrophotometry. The ultraviolet spectrum of virus obtained from sucrose gradients measured directly after dialysis showed a minimum at 247 ± 2 nm and a maximum at

Fig. 1. Comparison of the purification procedures based on clarification by ether/tetra and Triton X-100. Scanning patterns of linear sucrose density gradients are given.

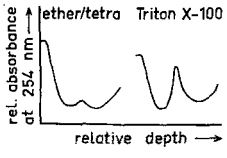

Fig. 1. Een vergelijking van de zuiveringsprocedures gebaseerd op behandeling met ether/tetrachloorkoolstof en Triton X-100 aan de hand van de verdeling van de ultraviolet absorptie in de suikergradiënten.

Fig. 2. Suggested procedure for the purification of plum pox virus.

Homogenize leaves in 0.01 *M* citrate pH 6.7,
containing 0.015 *M* NaDieca and 0.0005 *M* EDTA (w/v 1/4)
press suspension through cheesecloth.

EXTRACT — discard debris

centrifuge at 6000 *g*, 15 min

SUPERNATANT — discard pellet

add 5 % (v/v) Triton X-100
stir for ½ h
centrifuge at 78,480 *g*, 90 min

PELLET — discard supernatant

resuspend in 1/5–1/10 volume
0.01 *M* citrate pH 6.7, containing
1 *M* ureum and 0.1 % mercaptoethanol
let stand for 2 h
centrifuge at 1000 *g*, 10 min

SUPERNATANT — discard pellet

centrifuge suspension over 8 ml 20 %
sucrose in SW 25.1 rotor for 2 h
at 51,500 *g*

PELLET — discard supernatant

resuspend in 1–2 ml 0.01 *M* citrate pH 6.7
containing 1 *M* ureum and 0.1 % mercaptoethanol
let stand for at least 2 h
centrifuge at 1000 *g*, 10 min

SUPERNATANT — discard pellet

density gradient centrifugation in
10–50 % sucrose for 3 h at 51,500 *g*
remove virus band and dialyse overnight
against 0.01 *M* citrate pH 6.7

concentrate virus by centrifugation
at 78,480 *g*, 90 min

Fig. 2. De voorgestelde procedure voor de zuivering van het (pruime) sharkavirus.

263 ± 2 nm (Fig. 3.2). The MAX:MIN ratio varied from 1.06–1.16 and the $E_{260}^{0.1\%}$ ratio from 0.80–0.85 (indicating a RNA-content of 5.5–6.0 %), both after correction for light scattering. The variation might be due to a small amount of impurities. However, when dialyzed virus suspensions were further concentrated by centrifugation they did not show spectra as given above, apparently due to aggregation (Fig. 3.1). Typical virus spectra could be obtained again after corrections for light scattering. Based on corrected values of the absorbance at 260 nm and an $E_{260}^{0.1\%}$ value of 2.4 (Purcifull, 1966) virus yields were estimated on 20–45 mg per kg of leaves during winter.

Fig. 3. Ultraviolet absorption spectra of plum pox virus, obtained from sucrose density gradients (3.2) and after further concentration by high speed centrifugation (3.1), without corrections for light scattering.

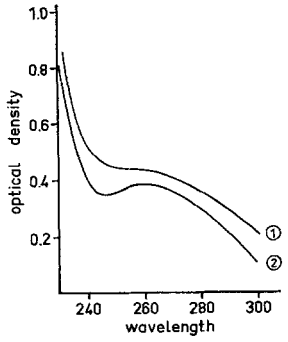

Fig. 3. Ultraviolet absorptiespectra van het (pruime) sharkavirus na centrifugeren in een suikergradiënt (3.2) en na verder concentreren door centrifugeren bij hoog toerental (3.1). De spectra zijn niet gecorrigeerd voor lichtverstrooiing.

Assessment of purity by:

Analytical ultracentrifugation. The purified virus sedimented as a single peak with a sedimentation coefficient of about 170 S at infinite dilution (Fig. 4). During the first minutes of centrifugation the virus peak was sometimes preceded by a broad shoulder, possibly due to aggregates and impurities.

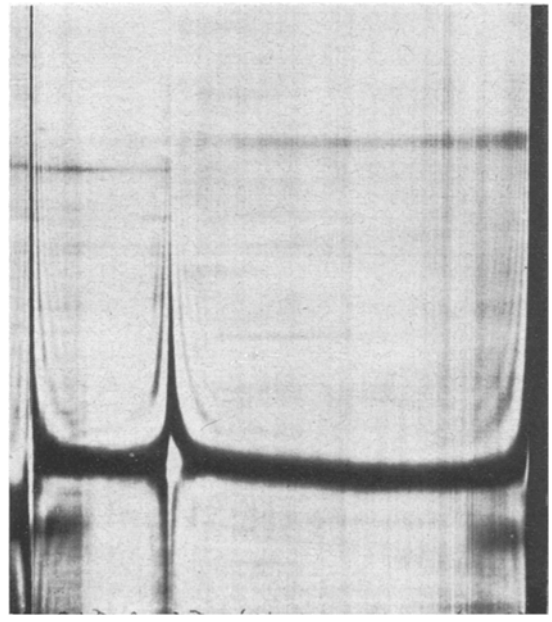

Fig. 4. Schlieren diagram of purified plum pox virus. Sedimentation from left to right. Photograph was taken after 5 min at 31.410 rpm, bar angle 30°. Virus was dissolved in 0.01 *M* citrate pH 6.7. The run was made at 20 °C.

Fig. 4. Schlieren diagram van het gezuiverde (pruime) sharkavirus. De sedimentatie is van links naar rechts. De foto werd genomen na 5 min bij 31.410 rpm. De hoek bedroeg 30°. Het experiment werd uitgevoerd bij 20° C. Het virus was gesuspendeerd in 0.01 M citrate pH 67.

Fig. 5. Electron micrographs of plum pox virus obtained by equilibrium density gradient centrifugation in CsCl. Aggregates of virus and impurities were found in the highest band (top), while pure virus could be obtained from a band at lower position (bottom).

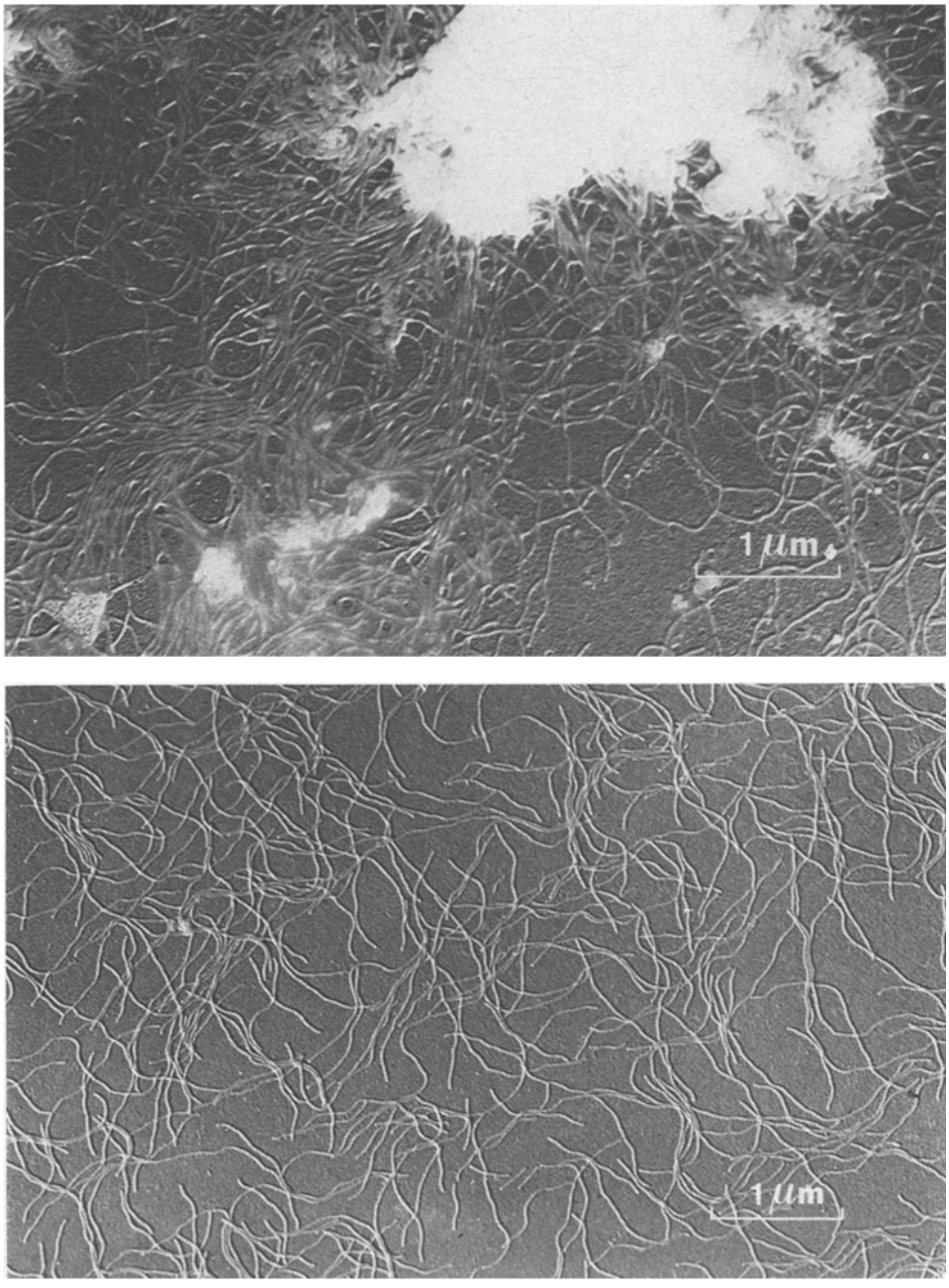

Fig. 5. Electronenmicroscopische foto's van het (pruime) sharkavirus na evenwichtscentrifugering in CsCl. Fig. 5 (boven) toont aggregaten van virus en verontreinigingen uit de hoogst gelegen band, terwijl Fig. 5 (beneden) zuiver virus laat zien, voorkomend in een iets lager gelegen band in dezelfde gradiënt.

Equilibrium density-gradient centrifugation. After reaching equilibrium in CsCl 2 bands were usually seen 0–2 mm apart. The lower band (at a density of approximately 1.31 g/ml) contained pure virus only (Fig. 5, bottom), while the other band, which was very irregular, contained some aggregates of virus with impurities (Fig. 5, top).

Electron microscopy. The presence of some impurities was independently confirmed by electron microscopy of virus suspensions obtained from sucrose gradients. Particles were measured and from a total of 200 particles a modal length of 764 nm was determined.

Serology. The titer of the prepared antiserum was 4096 as determined with clarified sap of leaves of *N. clevelandii*, diluted 1:4 with buffer. With sap of healthy plants it reacted only at dilutions of 1:16 or less.

Preliminary experiments showed that serological testing was unreliable for assaying the various steps of the purification procedure when Triton X-100 was used, because of non-specific reactions.

Discussion

One of the main problems in the purification of PPV was the enormous loss of infectivity during the subsequent steps of purification. It was demonstrated here, that infectivity was already greatly influenced by the methods used for homogenization, i.e. Waring blendor and mortar and pestle. It is not certain whether or not this is due to an effect on the virus itself or on the efficiency of the homogenization. The use of large amounts of buffer during homogenization remarkably increased infectivity; this is thought to be due to a better dispersion of virus and host material. In preliminary experiments infectivity of PPV was greatly reduced by a number of clarification procedures (van Oosten, unpublished). One of the most gentle methods, viz treatment with ether and CCl_4 was inefficient in removing host material, since pellets after high-speed centrifugation remained greenish. The loss in infectivity was mainly ascribed to aggregation of virus and host material in the pellets after high-speed centrifugation and subsequent removal of large aggregates by low-speed centrifugation. Adding Triton X-100 resulted in dissolution of cell membranes and chloroplasts before high speed centrifugation. This apparently led to a release of more virus and a reduction of the losses by aggregation of virus and host material in the high-speed centrifugation pellet.

Thus far detergents are little used in virology ,but very commonly in enzymology and biology for selective solubilization of membrane systems in plant and animal tissues. Anionic and cationic detergents usually cause also denaturation and precipitation of proteins (Morton, 1955). Non-ionic detergents do not possess strong polar groups and therefore have no strong electrostatic interaction with proteins. Since the report of Glassman (1950) on the lysis of animal tissues by non-ionic detergents like Triton X-100 they became frequently used for solubilization of plant tissues (Bottomley, 1970).

Some reports about the effects of detergents on viruses were summarized by Brakke (1959). He was able to break up barley stripe mosaic virus aggregates by the anionic detergent Igepon T 73. However, infectivity of PPV was greatly reduced by Igepon T

73 (van Oosten, unpublished). During the preparation of this manuscript Nozu and Yamaura (1971) reported the successful use of Triton X-100 in the extraction of tobacco mosaic virus from *Zinnia* and spinach. Preliminary experiments with other elongated viruses such as *Phaseolus* virus 2, potato virus Y, *Sorghum* red stripe virus, tobacco etch virus and beet yellows virus gave high virus yields using the described purification method (van Oosten and Peters, unpublished).

Attention should be drawn to the reduction of the number of lesions in infectivity tests and the non-specific precipitations in serological tests in the presence of Triton X-100. Removal of Triton X-100 was necessary for obtaining reproducible results. Non-specific serological reactions due to detergents have been described earlier (Putnam, 1948).

Data about the *in vitro* properties of PPV correspond closely with those given by Kassanis and Šutić (1965) and Cropley (1968). The modal length of 764 nm was also reported by Kegler *et al.* (1964), while Kassanis and Šutić (1965) and Babović and Šutić (1971) have given a value of 725 nm.

Thus far no other data than those given in this report are known about the ultraviolet absorption spectrum for PPV. However, the values obtained agree with those of other members of the potato virus Y group, like tobacco etch virus (Purcifull, 1966) and maize dwarf mosaic virus (Seghal, 1968; Seghal and Jean, 1970)

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Samenvatting

De zuivering van het (pruime) sharkavirus met behulp van Triton X-100

In een mortier werden systemisch geïnfecteerde bladeren van *Nicotiana clevelandii* gehomogeniseerd in een overmaat buffer. Na centrifugeren bij laag toerental werd aan de bovenstaande vloeistof Triton X-100 toegediend tot een concentratie van 5%. De suspensie werd een half uur geroerd en vervolgens onderworpen aan differentieel centrifugeren, centrifugeren door een 20% suikeroplossing en centrifugeren in een 10–50 % lineaire suikergradient (Fig. 2).

De infectiositeit van het virus bleek zowel afhankelijk van de wijze van homogeniseren als van de samenstelling en de hoeveelheid van de buffer tijdens het homogeniseren (Tabel 1 en 2). Door het gebruik van Triton X-100 werden veel hogere virusopbrengsten verkregen dan met behulp van ether/tetrachloorkoolstof (Fig. 1). Het gezuiverde virus vertoonde een absorptiespectrum met een minimum bij 247 nm en een maximum bij 263 nm (Fig. 3) en sedimenteerde in de analytische ultracentrifuge als een enkele piek met een sedimentatie coëfficiënt van ongeveer 170 S (Fig. 4). De hoge mate van zuiverheid werd tevens aangetoond met behulp van evenwichtscentrifuge-

ring in CsCl en elektronenmicroscopie (Fig. 5). De gemiddelde lengte van de virusdeeltjes in gezuiverde preparaten was 764 nm. Een antiserum kon worden verkregen met een titer van 4096.

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Present adress

Proefstation voor de Fruitteelt, Wilhelminadorp, the Netherlands