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Purification and some properties of pea leafroll virus

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Abstract

Pea leafroll virus (PeLRV) was purified from infected pea plants using polyethylene glycol precipitation of crude extracts followed by clarification with chloroform-butanol, differential centrifugation and density-gradient centrifugation. Higher yields of virus were obtained from roots than from shoots. The A260/A280 was 1.83. Particles were isometric with a diameter of 27 nm in preparations stained with uranyl acetate.

An antiserum with a homologous titre of 1/512 in gel-diffusion tests was prepared. PeLRV was precipitated by antisera to soybean dwarf virus and beet western yellows virus but did not react with antiserum to a New Zealand strain of barley yellow dwarf virus.

Polyacrylamide gel electrophoresis of disrupted virions revealed a single RNA component with a relative molecular mass of about 2.4×10^6 and a single polypeptide with a relative molecular mass of $30\text{-}35 \times 10^3$.

Additional keywords: Beet western yellows virus, soybean dwarf virus, immune density-gradient centrifugation.

Introduction

Pea leafroll virus (PeLRV) was originally described from Germany by Quantz and Völk (1954) and has been subsequently reported from several European countries (De Fluiter and Hubbeling, 1955; Roland, 1955; Tinsley, 1959; Meier et al., 1959), from Iran (Kaiser, 1972), from New Zealand (Smith, 1966), and from the USA (Thottappilly et al., 1977). The last named authors have reviewed the synonymy of PeLRV and have suggested possible relationships with other legume viruses such as subterranean clover stunt, subterranean clover red-leaf, milk-vetch dwarf and soybean dwarf (SDV).

PeLRV belongs to the luteovirus group (Fenner, 1976), most members of which characteristically produce yellowing symptoms in hosts, are confined to the phloem tissue and are present in low concentration. Virus particles are isometric, 25-30 nm in diameter and transmitted by aphids in a persistent manner. Of the typical luteoviruses only barley yellow dwarf virus (BYDV) (Rochow et al., 1971), beet western yellows virus (BWYV) (Gold and Duffus, 1967) and SDV (Kojima and Tamada, 1976) have been purified and their antigenic properties investigated. It seems likely that potato leafroll virus is atypical of the group and should be considered distinct (Duffus, 1977).

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The present study describes the purification, electron microscopy and serology of PeLRV and also the determination of the molecular masses of its nucleic acid and coat protein.

Materials and methods

Virus propagation. The virus isolate used in this study was obtained in the Netherlands by aphid transfer from naturally infected lucerne showing distinct vein yellowing symptoms as described by Van der Want and Bos (1959). In host range and symptomatology this isolate did not differ markedly from isolates obtained from infected peas and broad beans. Virus transmission was in a persistent manner using *Acyrthosiphon pisum* (Harris). The virus was propagated in *Pisum sativum* cvs Onyx and Eurofin, inoculated two weeks after sowing by transferring 10-15 viruliferous aphids to each plant. These aphids had been propagated on the batch of plants inoculated two weeks previously. Plants were grown at 15-20°C with a 16 h photoperiod (daylight plus fluorescent light). Infected plants were harvested two weeks after inoculation. Roots were separated from potting soil or sand by washing. The weekly yield from 800 plants was approximately 1.2 kg of shoots and 0.6 kg of roots.

Purification. Virus was purified separately from roots and shoots. Initial extraction and the resuspension of the first virus pellets was in 0.1 M phosphate buffer, pH 7.4, containing 0.1% 2-mercaptoethanol and 10 mM EDTA. Subsequent virus pellets were resuspended in 50 mM phosphate buffer, pH 6.5, containing 1 mM EDTA. Resuspension of virus pellets and storage of virus suspensions and buffers was in a cold room at 3°C.

Each batch of 0.3-0.5 kg of infected tissue was first homogenised with 4 times its weight/volume of buffer in a four litre Waring blender for 3 min. The homogenate was divided into 400 ml portions and further disintegrated at 45 000 rev/min for 2 min, using a Virtis homogeniser with the container immersed in an ice bath.

Since luteoviruses are present in low concentration, three to five batches of material were processed during each purification and the handling of such large quantities was facilitated by concentrating the crude extract prior to clarification (Maat and De Bokx, 1978). The crude extract was first centrifuged at 10 400 *g* for 10 min and then ammonium sulphate (380 kg per m^3 of extract) or a mixture of polyethylene glycol 6000 (PEG) and NaCl (8% and 3% w/v, respectively) was added to the supernatant. The extract was stirred for 1 h and then centrifuged at 16300 *g* for 15 min. The precipitate was resuspended in pH 7.4 buffer, stirred for 1 h and briefly (5-10 sec) emulsified with one-quarter volume of a mixture (1:1) of chloroform and n-butanol. The emulsion was stored for 1 h, after which the phases were separated by centrifuging (10 400 *g*, 10 min) and the aqueous phase containing the virus was recovered and stored overnight. Virus was purified from this phase by two cycles of differential centrifugation (7900 *g*, 10 min; 77 700 *g*, 3 1/2 h and 4140 *g*, 10 min; 122 250 *g*, 2 1/2 h). Virus pellets from each batch of material were resuspended overnight in 0.5 ml phosphate buffer. Each 0.5 ml of partially purified virus was layered onto a 10-40% linear sucrose gradient prepared in 50 mM phosphate buffer, pH 6.5, and centrifuged at 76 000 *g* for 3 1/2 h using a Beckman SW27 rotor. Virus zones were isolated using an Isco density-gradient fractionator and an Isco UA-5

absorbance monitor. The scale absorbance range was 0.5 units at 254 nm wavelength with a 10 mm light path.

Virus concentrations were estimated by comparing the size of peaks in UV scans of density-gradient columns or from UV-absorption spectra. The virus zones from gradients were diluted with buffer and centrifuged at 122 250 *g* for 3 h. The pellets were resuspended in phosphate buffer.

In some cases the virus was further purified by isopycnic density-gradient centrifuging in Beckman SW41 rotor tubes filled with 2.75 ml of caesium sulphate solution (535 kg per m^3 of water), 0.40 ml of water, 0.30 ml of virus suspension and 8.75 ml of paraffin oil. They were centrifuged for 17-18 h at 30 000 rev/min and 5°C. Virus bands were detected by light scattering and removed by puncturing the side of the tube, using a 26 gauge needle attached to a hypodermic syringe.

Infectivity assay. The infectivity of purified virus preparations was tested by allowing aphids to feed for 30-40 h through stretched parafilm M (Marathon products) membranes on drops of virus suspension in 25% sucrose. Aphids were then transferred to test plants (*P. sativum* cv. Eurofin) in groups of five aphids per plant.

Electron microscopy. Purified virus, either directly from sucrose gradients or in buffer, was placed on an electron-microscope grid, and washed with 30 drops of distilled water, 5 drops of 2% aqueous uranyl acetate, and then examined in a Philips EM 300 electron microscope. TMV in crude sap was used as an internal standard (300 nm).

Serology. An attempt to produce antiserum in a rabbit by a series of intravenous injections of small amounts of virus directly from Cs_2SO_4 gradients was unsuccessful. After a period of 2 1/2 months the same rabbit was given two intravenous and one intramuscular injections, each at an interval of one week. In each case the virus was obtained from 0.5 kg of root tissue. The virus for intravenous injections was taken directly from the sucrose gradients whereas for the intramuscular injection, the virus from the sucrose gradient was diluted, centrifuged at 122 250 *g* for 3 h, resuspended in 1 ml phosphate buffer and emulsified with an equal volume of Freund's incomplete adjuvant. The rabbit was bled at varying intervals from two weeks after the third injection and given booster doses as required. Sera were mixed with an equal volume of glycerol and stored at −20°C.

The serological reaction of PeLRV with its homologous antiserum was examined using immune density-gradient centrifugation (Ball and Brakke, 1969) and double-diffusion tests in agar gels. Immune density-gradient centrifugation was used to detect the reaction of PeLRV with various antisera at virus concentrations to low to produce precipitin lines in gel-diffusion tests. Purified virus and antiserum (diluted 1:5) were mixed and incubated at 37° for 30 min. The mixture was layered on top of a linear 20-60% sucrose gradient and centrifuged in a Beckman SW41 rotor at 30 000 rev/min for 1 1/2 h. The gradient columns were then scanned at 254 nm. Ouchterlony double-diffusion tests were done in 1% Oxoid purified agar in 0.85% saline, containing 0.02 % sodium azide. Fifteen ml of melted agar were poured into each 90 mm disposable plastic petri dish and various patterns of 3 mm wells at 2 mm spacings were used.

Fig. 1. UV-absorption patterns of sucrose gradients to compare: the virus yield from roots (A) with that from shoots (B); the $(Nh_4)_2SO_2$ method (C) with the PEG method (D) for purifying from shoots; the $(Nh_4)_2SO_4$ method (E) with the PEG method (F) for purifying from roots. Comparable amounts of tissue were processed for each comparison. Ph = phytoferritin; V = virus; dV = degraded virus.

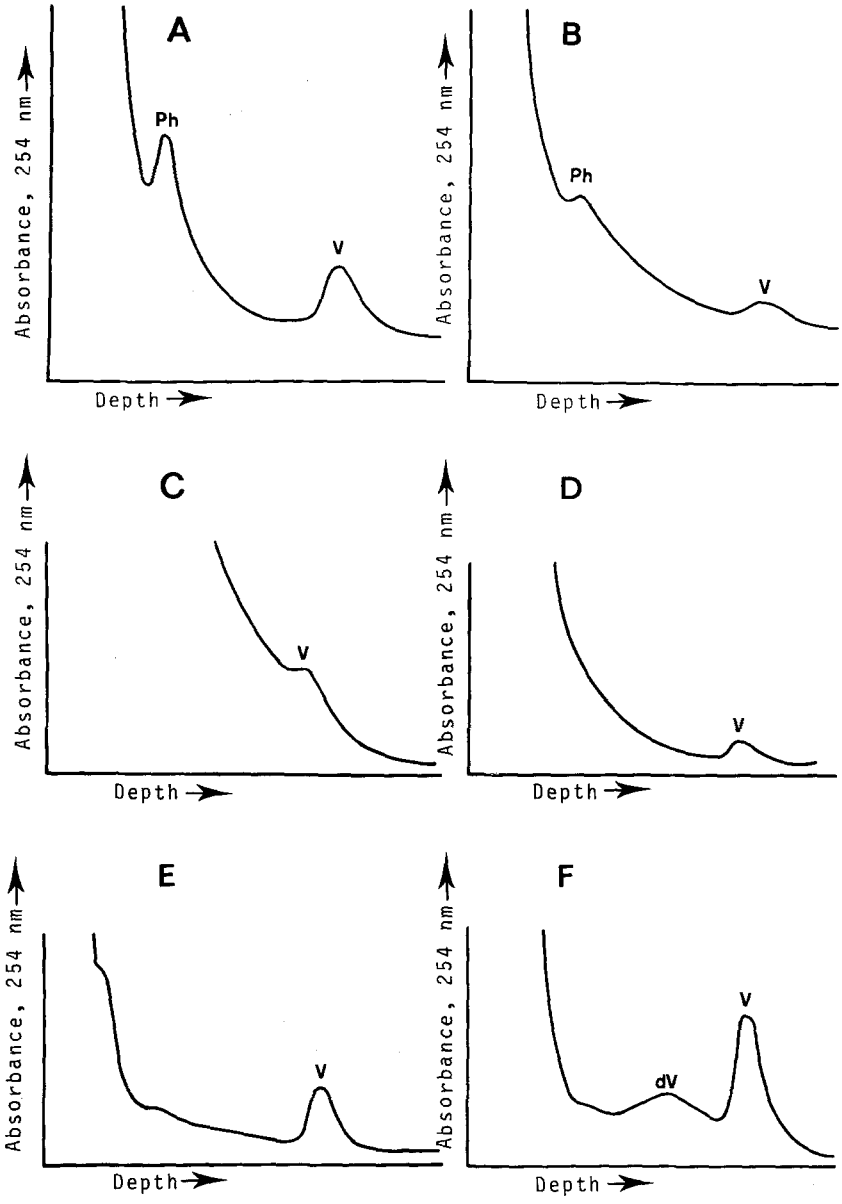

Fig. 1. UV-absorptiepatronen van suikergradiënten om de virusopbrengst te vergelijken van: zuiveringen uitgaande van wortels (A) en scheuten (B); de $(NH_4)_2SO_4$-methode (C) en de PEG-methode (D) voor zuivering uit scheuten; de $(NH_4)_2SO_4$-methode (E) en de PEG-methode (F) voor zuivering uit wortels. Voor elke vergelijking werden vergelijkbare hoeveelheden weefsel verwerkt. Ph = fytoferritine; V = virus; dV = afgebroken virus.

Fig. 2. UV-absorption pattern and density gradient of isopycnic centrifugation of PeLRV in Cs_2SO_4.

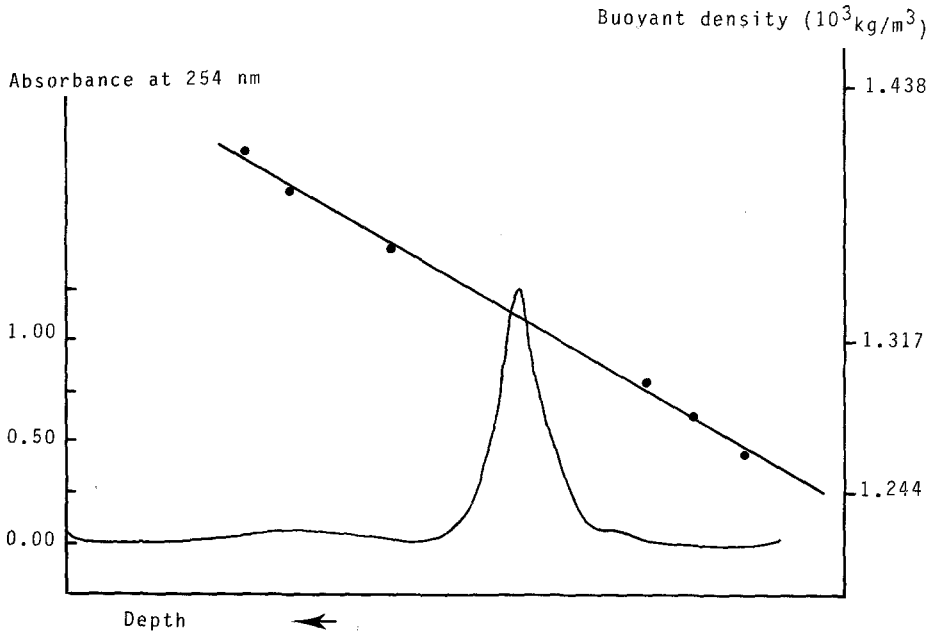

Fig. 2. UV-absorptiepatroon en dichtheidsgradiënt van evenwichtscentrifugering van PeLRV in Cs_2SO_4.

Relative molecular masses of nucleic acid and coat protein. Samples were prepared for nucleic acid and protein relative molecular-mass determinations by resuspending virus pellets, following high-speed centrifugation, in 0.1 M phosphate buffer, pH 7.4, containing 1% sodium dodecyl sulphate (SDS) and 1% 2-mercaptoethanol. The suspensions were incubated at 37°C for 3 h and stored frozen at −20°C until required.

Nucleic acid determinations were done in 2.6% polyacrylamide gels, at room temperature using the method of Loening (1967). The ribonucleic acids of cowpea chlorotic mottle virus (CCMV) and cowpea mosaic virus (CPMV) were used as markers. The relative molecular masses of these RNAs under non-denaturing conditions are as follows: CCMV, 1.15×10^6, 1.00×10^6, 0.85×10^6, 0.32×10^6 (Bancroft, 1971); CPMV, 2.55×10^6, 1.5×10^6 (Geelen, 1974). Gels were prerun at $2^1/_2$ mA per gel for $^1/_2$ h and run at $2^1/_2$ mA per gel for 4 h.

Protein molecular mass determinations were done on 10% polyacrylamide gels at room temperature using the method of Weber and Osborn (1969). BDH molecular mass marker mixture, product number 44223 2U (relative molecular masses 14.3-71.5×10^3), was used as a standard. Gels were run for $4^1/_2$ h at 7 mA per gel.

Results

Virus purification. The highest yield of virus was obtained from roots (Fig. 1A, B). Purification from this source was easier than from shoots since fewer plant constituents co-precipitated with the virus. Concentration of extracts with PEG resulted in

higher yields of virus and also gave cleaner preparations from shoots than were obtained using $(NH_4)_2SO_4$ (Fig. 1C, D).

From roots, however, clean preparations were obtained using $(NH_4)_2SO_4$ and although the yield obtained using PEG was higher, an additional peak containing damaged virus particles was sometimes detected on sucrose gradients (Fig. 1E, F).

Assuming an $E^{0.1\%}_{260nm,\ 10mm} = 5$ (Kojima and Tamada, 1976), yields of up to 668 µg per kg of root tissue were obtained. In most preparations a considerable amount of phytoferritin was obtained (Fig. 1A), but isopycnic centrifugation in Cs_2SO_4 resulted in highly purified monodisperse preparations (Fig. 2). After 14 days symptoms typical of infection with PeLRV were observed in peas inoculated with purified virus acquired by aphids feeding through membranes.

The UV scan of purified preparations was typical of that of a virus with a high nucleic-acid content (Fig. 3).

The A260/A280 was 1.83. The buoyant density of PeLRV in Cs_2SO_4 was about $1.32 \times 10^3 kg/m^3$.

Electron microscopy. The virions of PeLRV are isometric with a diameter of 27 nm in preparations negatively stained with uranyl acetate (Fig. 4).

Serology. The results of the immune density-gradient centrifugations of PeLRV with

Fig. 3. Ultraviolet-absorption spectrum of purified PeLRV.

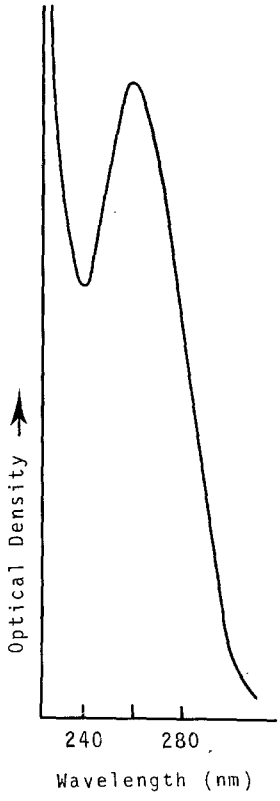

Fig. 3. UV-absorptiespectrum van gezuiverd PeLRV.

Fig. 4. Partially purified preparation of PeLRV stained with 2% aqueous uranyl acetate. Bar represents 100 nm.

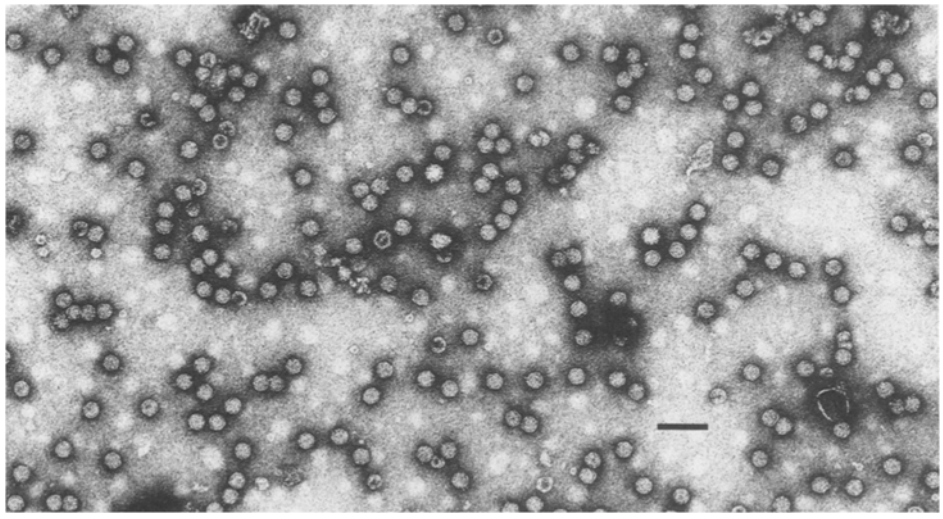

Fig. 4. Gedeeltelijk gezuiverd PeLRV preparaat na kleuring met 2% uranylacetaat in water. De vergrotingsstreep geeft 100 nm weer.

Fig. 5. UV-absorption patterns of immune density-gradients of PeLRV incubated with the antisera indicated. HP = antiserum to healthy pea.

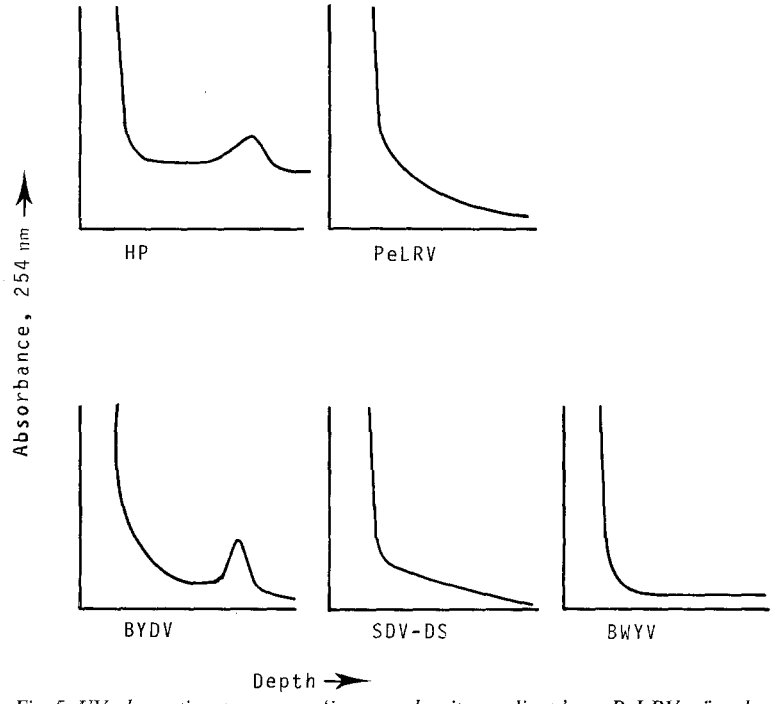

Fig. 5. UV-absorptiepatronen van 'immune density-gradients' van PeLRV geïncubeerd met de aangegeven antisera. HP = antiserum tegen gezond erwtemateriaal.

antisera to some other luteoviruses are shown in Fig. 5. When virus and antiserum reacted, the resulting virus/antibody complex sedimented much more rapidly than unreacted virus and the virus peak disappeared. PeLRV reacted with its homologous antiserum, with antiserum to BWYV (from Dr J. Duffus, Salinas, California) and with antiserum to SDV, dwarfing strain (DS) (from Dr T. Tamada, Hokkaido, Japan). No reaction occurred between PeLRV and antisera to healthy pea (from Ing. D.Z. Maat, IPO) or to BYDV (New Zealand strain; antiserum prepared by Dr M.F. Clark).

In gel-diffusion tests the homologous titre of the PeLRV antiserum was 1/512 (the titre to normal plant protein was 1/4). The titres of antisera of BWYV and SDV-DS against purified PeLRV were 1/8 and 1/128, respectively. The homologous titre of BWYV was 1/625 in infectivity neutralization tests (Duffus and Rochow, 1978); that of SDV-DS was 1/1024 in gel-diffusion tests. No precipitin lines were observed when purified PeLRV was tested against BYDV antiserum.

Relative molecular masses of nucleic acid and coat protein. Polyacrylamide gel electrophoresis of SDS-disrupted virus on 2.6% gels revealed a single nucleic acid component (Fig. 6A). Using RNAs 1, 2 and 3 of CCMV as internal markers, the relative molecular mass of PeLRV RNA was calculated to be 2.4×10^6. PeLRV RNA co-migrated with the RNA of the bottom component of CPMV, when this virus was also used as a marker. Geelen (1974) determined the relative molecular mass of this RNA (CPMV-B) to be 2.55×10^6 under non-denaturing conditions but Reijnders et al. (1974) proved it to be 2.02×10^6 when gels were run under denaturing conditions (60°C and 8 M urea).

Incubation of nucleic acid containing gels with bovine pancreas ribonuclease type 1A (10 g per m^3 of 10 mM phosphate buffer, pH 7) for $3^1/_2$ h at 37°C eliminated the nucleic acid band.

On 10% gels a single polypeptide with a relative molecular mass of $30\text{-}35 \times 10^3$ (Fig. 6B) was detected.

Fig. 6. Polyacrylamide gel electrophoresis of SDS-treated PeLRV. A) RNA component on 2.6% gel. Positions of CPMV RNAs (relative molecular masses: 2.55×10^6 and 1.5×10^6) are indicated. B) Polypeptide component in 10% gel. Position of markers (relative molecular masses: 71.5×10^3, 57.2×10^3, 42.9×10^3, 28.6×10^3 and 14.3×10^3) are indicated.

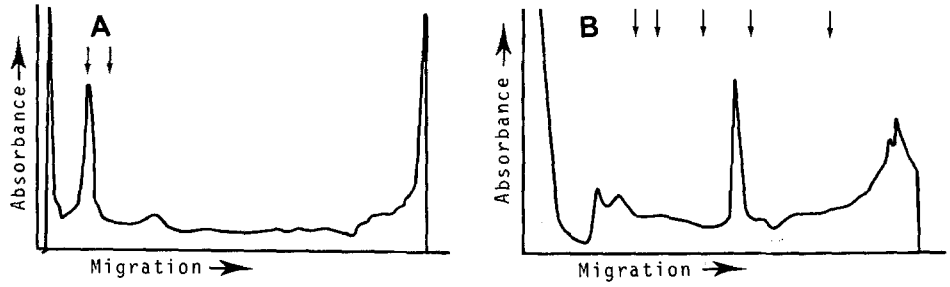

Fig. 6. Polyacrylamide-gelelektroforese van in SDS-buffer gedissocieerd PeLRV. A) RNA-component in 2,6% gel. Posities van de CPMV-RNA's (relative moleculaire massa's: $2{,}55 \times 10^6$ en $1{,}5 \times 10^6$) zijn aangegeven. B) Eiwitcomponent in 10% gel. Posities van de merkers (relatieve moleculaire massa's: $71{,}5 \times 10^3$, $57{,}2 \times 10^3$, $42{,}9 \times 10^3$, $28{,}6 \times 10^3$ en $14{,}3 \times 10^3$) zijn aangegeven.

Discussion

The development of purification techniques for luteoviruses (Rochow and Brakke, 1964; Kojima and Tamada, 1976) has previously relied on infectivity assays of virus by membrane feeding. Using the information obtained by these workers suggesting the stability of luteoviruses it was possible to omit intensive assaying of virus during purification and to base development of the technique on the final yield of virus, as detected by photometric scanning of density-gradient columns. Membrane feeding in this study was used only to prove that the virus preparations were infective.

Using roots rather than shoots as the main source of virus enabled larger amounts of virus to be more easily obtained. It is possible that by further manipulation of propagative conditions, the virus content of the roots could be increased.

Freezing of tissue, even for short periods, resulted in a slightly reduced virus yield. With a virus limited to phloem tissue it is important that efficient extraction is obtained. Repeated extraction of the fibres remaining after the two homogenisation steps failed to yield more virus. Like other luteoviruses, PeLRV appears to be stable in the presence of chloroform and n-butanol and tolerates a rather lengthy purification procedure. The high A260/A280 of purified PeLRV is similar to the ratios for BYDV (Brakke and Rochow, 1974) and SDV (Kojima and Tamada, 1976) and suggests that these viruses have a high nucleic acid content.

Duffus and Rochow (1978) have stated that the yellowing diseases caused by luteoviruses are emerging as the most important group of virus diseases in plants and it has been shown that BWYV from the USA is serologically related to SDV from Japan and also to European strains of BWYV, turnip yellows virus, beet mild yellowing virus and to the RPV strain of BYDV.

On the basis of host range and symptomatology PeLRV appears very similar to SDV and this is reasonably substantiated by the close serological relationship of PeLRV with SDV-DS. The serological relationship to BWYV is more distant.

BYDV is the only other typical luteovirus for which molecular mass estimates of the nucleic acid are available and like PeLRV it appears to have a single component of single-stranded RNA (Brakke and Rochow, 1974). The relative molecular mass determined for the RNA of BYDV by gel electrophoresis was about 1.85×10^6 which is rather lower than the relative molecular mass of 2.4×10^6 determined for PeLRV. However, it is apparent from the results of Reijnders et al. (1974) that the conditions under which polyacrylamide gel electrophoreses are performed can markedly affect the values obtained. The relative molecular mass reported for the protein of BYDV is 24×10^3 (Scalla and Rochow, 1977) and that reported for SDV is 22×10^3 (Tamada and Kojima, 1977) which are considerably lower than the 30-35 $\times 10^3$ determined for the protein of PeLRV.

The poperties determined for PeLRV are nevertheless consistent with it being a member of the luteovirus group, and within this group it would appear to be closely related to SDV. It is hoped that recently initiated co-operative studies of the host range and serological relationships of the legume-infecting luteoviruses will further elucidate the interrelationships of these viruses.

Samenvatting

Zuivering en enkele eigenschappen van het erwtetopvergelingsvirus

Erwtetopvergelingsvirus (PeLRV) werd gezuiverd uit geïnfecteerde erwteplanten met behulp van polyethyleenglycolprecipitatie uit ruwe extracten, gevolgd door klaren met chloroform en butanol, differentiëel centrifugeren en scheiden op dichtheidsgradiënten. Uit wortels werden hogere virusopbrengsten verkregen dan uit scheuten (Fig. 1). Het virus bereikte een evenwicht in één band bij evenwichtscentrifugering in Cs_2SO_4 (Fig. 2). De zweefdichtheid bedroeg $1{,}32 \times 10^3$ kg/m^3. De A260/A280 was 1,83 (Fig. 3). In met uranylacetaat gekleurde preparaten waren de deeltjes isometrisch met een diameter van 27 nm (Fig. 4).

Er werd een antiserum bereid dat een homologe titer van 1/512 in de gel-diffusietoets had. PeLRV reageerde zowel in de 'immune density-gradient'-toets (Fig. 5) als in de gel-diffusietoets met antisera tegen 'soybean dwarf virus' en slavergelingsvirus ('beet western yellows virus'), maar het reageerde niet met een Nieuwzeelandse stam van het gerstevergelingsvirus.

Polyacrylamide-gelelektroforese van gedissocieerd virus toonde de aanwezigheid aan van één RNA-component met een relatieve moleculaire massa van $2{,}4 \times 10^6$ en één eiwitcomponent met een relatieve moleculaire massa van $30\text{-}35 \times 10^3$ (Fig. 6).

Acknowledgment

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