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Purification of pea enation mosaic virus and the infectivity of its components

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Abstract

A slightly modified procedure for purifying pea enation mosaic virus (PEMV) is described. Essential features are the adding of Mg^{++} ions to the extraction buffer and the use of polyethylene glycol for concentrating the virus; Mg^{++} ions seem to protect virus infectivity. Both components of PEMV were found to be infectious. The infectivity of each component in its band on a sucrose gradient was directly proportional to its optical density. The infectivity was not enhanced when both components were simultaneously inoculated onto *Chenopodium album*. It is concluded that each component carries a complete genome of the virus.

Introduction

Izadpanah and Shepherd (1966) purified pea enation mosaic virus (PEMV; R/1: */29:S/S:S/Ap), and studied some of its biological and immunological properties. Purified preparations of PEMV consist of two centrifugal components with sedimentation coefficients of 86 and 105 S, referred to as top and bottom component, respectively. These authors observed that both components were infectious and claimed that the top component was even more infectious than the bottom component. This was recently confirmed by Gonsalves and Shepherd (1972). Gibbs et al. (1966) reported also that the top component was infectious. In contrast Bozarth and Chow (1966) claimed that the infectivity was restricted to the bottom component only. Recently, Hull and Lane (1972) obtained evidence that mixtures of both components had a higher infectivity than the components separately. However, Gonsalves and Shepherd (1972) found no enhancement of the infectivity in mixtures.

We now describe the results of a study on the separation of both components and their relative infectivities. In addition a modified purification method is given as the methods developed by other authors did not give satisfactory results under our conditions.

Materials and methods

Virus. The isolate E 154 of PEMV used was obtained from L. Bos (Institute of Phytopathological Research, Wageningen). It was maintained in peas (*Pisum sativum*

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'Kelvedon Wonder', and later in 'Koroza'). A sample of the first grown batch of infected plants was stored at −18 °C, to preserve the original isolate.

Infected plant material. Plants were grown in a greenhouse at a temperature of 20–23 °C and inoculated about 8 days after sowing. As the virus content appeared to be considerably lower in the symptomless parts of the plants, only those parts showing symptoms were harvested 11–14 days after inoculation and used in the experiments. Although additional light was given lower yields of virus were obtained during winter.

Infectivity assays. Local-lesion assays were made on 2 or 3 partially and 3 or 2 fully expanded leaves of *Chenopodium album* plants (Hagedorn et al., 1964). Because of the variability in response of the local lesion host to PEMV a latin square (5 × 5) or (6 × 6) design was used for the analysis of the assays. The assays were often made in duplicate. Local lesions appeared within 5 days and were counted 7 days after inoculation.

Purification. The following procedure was adopted as a suitable and rapid method to purify the virus. Harvested plant material was homogenized in chloroform and 0.15 M sodium acetate, pH 6.1, containing 0.015 M magnesium chloride, using 1 ml and 2 ml of these liquids per g tissue. The homogenate was filtered through cheesecloth and centrifuged at 10,000 rpm for 15 min. The supernatant was adjusted to pH 5.3 with 1 N acetic acid and allowed to stand for 60 min. The flocculated material was removed by means of centrifugation at 10,000 rpm for 10 min. After readjusting the pH to 6.0 with 1 N NaOH the supernatant was centrifuged at 30,000 rpm for 3 h. The pellets were resuspended in 0.1 M sodium acetate, pH 6.1, containing 0.01 M magnesium chloride (acetate buffer). Differential centrifugation was repeated once or twice. In resuspended material containing the virus and some F1 protein was layered on a sucrose gradient and centrifuged at 22,500 rpm for 3 h.

In later experiments the virus was concentrated by precipitation with polyethylene glycol (PEG). After readjusting the pH, 6% (w/w) PEG was added and the extract was allowed to stand for one hour before being centrifuged at 10,000 rpm for 10 min. The pellets were resuspended in the acetate buffer and after a further lowspeed centrifugation the supernatant was centrifuged at 40,000 rpm for 2 h. The pellets were resuspended in acetate buffer and given low speed centrifugation. Depending on the amount of plant material used the virus suspension was now centrifuged either on a gradient consisting of 20–50% sucrose in acetate buffer prepared in tubes, or on a gradient consisting of 15–30% sucrose in acetate buffer prepared in a zonal rotor. These were run at 22,500 rpm for 3 h and 33,000 rpm for 4 h, respectively.

The top and bottom component were separated by several cycles of centrifugation (22,500 rpm for 3 h) on linear gradients of 20–50% sucrose in acetate buffer. Gradients were fractionated by the method described by van Kammen (1967). After each separation the components were dialysed against acetate buffer, concentrated by centrifugation at 40,000 rpm for 2 or 3 h, resuspended in acetate buffer and layered on gradients for a new cycle of fractionation. To separate the components using CsCl, the virus was layered onto 4.5 ml of a solution of 7.2 g CsCl added to 10 ml buffer, in a 5 ml Lusteroid tube and centrifuged at 35,000 rpm for 24 h.

Serology. Virus obtained from gradients and concentrated as described above was fixed in 2% formaldehyde for two h and then used for injecting rabbits. Nine times with intervals of one week, 2 ml of virus suspension (1 mg/ml) was intravenously injected. This was followed by an intramuscular injection of 3 ml virus (1.5 mg/ml) emulsified with an equal volume of Freund's complete adjuvant. The rabbits were bled 2 weeks after the last injection.

Ouchterlony agar-gel diffusion was performed in 0.8% agar gels in acetate buffer.

Immunoelectrophoresis was performed using a micromethod in which the agar layer was prepared as in the gel diffusion tests. Electrophoresis was carried out at 10 mA and 60 volts per slide (6 volts/cm) for 1 h at 4 °C. A 1.5–2 mm slot then was cut parallel to the direction of current flow and filled with antiserum. The slides were incubated overnight at room temperature and subsequently fixed and stained.

Base composition. The base composition of nucieic acids was estimated by a chromatographic procedure similar to that described by Knight (1963). The RNA (unextracted) was hydrolyzed in 1 N HCl by heating in a closed ampoule for 1 h at 100 °C.

Sedimentation coefficients. Sedimentation coefficients were determined in a Spinco model E analytical ultracentrifuge and calculated by the graphic method of Markham (1960).

Results

Evaluation of the purification procedure. The procedure yielded preparations of high purity. Antiserum against these virus preparations did not react with sap from healthy pea plants indicating that no appreciable amounts of antigens from healthy plants were present in these preparations. This observation was confirmed by Farro (1969) using our antisera.

Extracts obtained at different stages during the purification were assayed for infectivity. No significant loss of infectivity occurred at any stage in the purification (Table 1). The infectivity of crude sap was lower than that of the extracts after the chloroform treatment. Modifications of the pH did also not alter the infectivity. The final virus suspension, diluted to bring it to the original volume of the extract, was only slightly less infectious than the other extracts assayed.

Table 1. Infectivity of extracts obtained at different stages of the purification of PEMV from peas.

Extracts	Number of lesions[1]
1. Crude juice from infected tissue	143
2. Extract after chloroform emulsification	236
3. Extract after pH modification	235
4. Final purified virus preparation[2]	185

[1] Average of 24 leaves of *Chenopodium album.*
[2] Diluted to the volume of crude sap.

Tabel 1. De infectiositeit van de extracten die werden verkregen op verschillende tijdstippen in de zuivering van PEMV uit erwten.

Table 2. Infectivity of the material sedimenting at pH 5.3 and collected by low speed centrifugation, extracted in different media.

Media	Number of lesions[1]	
	Exp. 1[2]	Exp. 2[3]
1. 2% NaCl	24	–
2. 4.5% sucrose	70	–
3. 4.5% glucose	67	–
4. 2% NaCl + 4.5% sucrose	11	–
5. 2% NaCl + 4.5% glucose	6	–
6. 0.1 M acetate buffer	94	61
7. 0.1 M Tris pH 6.0	–[4]	41
8. 4% glycine	–	6

[1] On *Chenopodium album* leaves.
[2] Mean number of local lesions obtained on 18 leaves.
[3] Mean number of local lesions obtained on 12 leaves.
[4] Not tested.

Tabel 2. De infectiositeit van extracten die met verschillende media werden verkregen uit de neerslagen, die bij pH 5.3 ontstonden in het extract na de behandeling met chloroform.

The quantity of virus extracted from the pellets obtained by modifying the pH was less than 5% of the virus in the original extracts. The acetate buffer used throughout the purification procedure was more effective in extracting virus from these pellets than other solvents tested (Table 2).

In one experiment the effect of Mg^{++} ions on the infectivity of PEMV was determined. Equal amounts of virus purified as described were suspended in 0.1 M sodium acetate buffer, pH 6.1, containing 0.01 M magnesium chloride, and in 0.1 M sodium acetate, pH 6.1, respectively. The suspensions were stored at 2°C; at various time intervals samples were taken and tested for infectivity. The results show that Mg^{++} ions in the buffer had a protective effect on the infectivity (Table 3). The experiment was terminated after 19 weeks when the virus preparations were exhausted.

The virus preparations obtained by the modified procedure and that of Izadpanah and Shepherd (1966) were compared in one experiment. Equal numbers of infected

Table 3. The effect of magnesium ions on the infectivity of PEMV suspended in 0.1 M sodium acetate buffer, pH 6.1, and stored at 2° C for different periods (weeks).

Virus suspension	Number of lesions[1]							
	1	2	4	7	11	14	17	19
with 0.01 M Mg^{++}	183	185	173	160	155	140	57	63
without Mg^{++}	68	67	70	60	40	37	11	2

[1] On 6 *Chenopodium album* leaves.

Tabel 3. De invloed van Mg^{++}-ionen op de infectiositeit van het erwte- enatiemozaïekvirus (PEMV) dat in 0.1 M natrium acetaat buffer, pH 6,1 gedurende verschillende perioden (in weken) bij 2°C werd bewaard.

Fig. 1. Sedimentation diagram of a virus preparation purified according to methods described by the present authors (a) and by Izadpanah and Shephard (1966) (b). Sedimentation is from right to left. Speed 31,410 rev/min (Spinco model E). Bar angel 60°. Photograph taken 9 min after reaching speed.

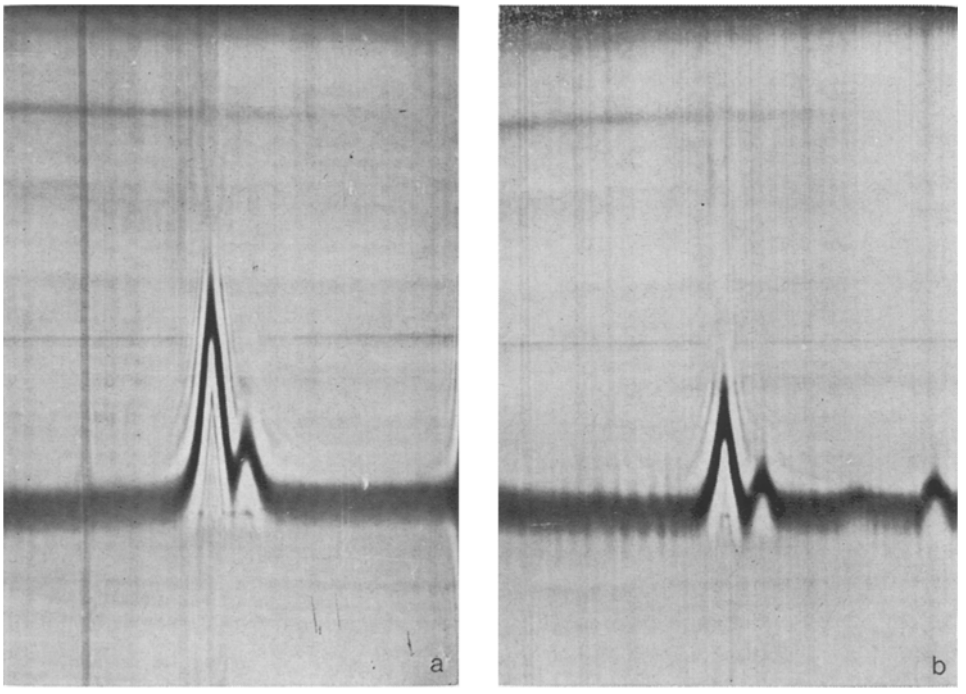

Fig. 1. Sedimentatiediagram van een viruspreparaat gezuiverd volgens de methode (a) beschreven in dit artikel en door Izadpanah and Shepherd (b) (1966). De sedimentatie verloopt van rechts naar links. Snelheid 31.410 (Spinco model E). Spleethoek 60°. De foto werd gemaakt 9 minuten nadat de centrifuge de afgestelde snelheid had bereikt.

plants from the same batches were subjected to both procedures, compared in the analytical ultracentrifuge, and bioassayed on *C. album*. A higher yield of virus was obtained by the modified procedure (Fig. 1). A third peak consistently present in the preparations obtained by Izadpanah and Shepherd's method, is due to the presence of some plant material (presumably F1 protein). The presence of F1 protein (20S) in a very small amount has only infrequently been seen in our preparations. Furthermore some material sedimenting at 55S was present in the preparations obtained by Izadpanah and Shepherd's method. This component appeared to be sensitive to chloroform. The specific infectivity of preparations made by the modified procedure was higher than that of virus prepared according to Izadpanah and Shepherd. This may be due to the presence of Mg^{++} ions in our buffer. Three other isolates of PEMV could also be purified by the modified procedure with similar results.

Properties of purified virus. In the analytical ultracentrifuge purified virus produced two boundaries with sedimentation coefficients of 91 S (top component) and 107 S (bottom component). The amount of top component was invariably smaller than of the bottom

component. The ratio of top to bottom component did not vary much between preparations, the amount of top component always been less than that of bottom component.

Base ratio analysis gave values of A 24.1; G 26.6; C 24.5 and U 24.8 moles nucleotides per 100 mol. These values resemble those reported by Shepherd et al. (1968).

Electron microscopy showed that PEMV has particles with a diameter of approximately 26 nm. The particles are hexagonal or pentagonal in profile. The latter can be found more frequently in top zone fractions than in bottom zone fractions.

Separation of the centrifugal components and their infectivity. Efforts were made to separate the components on sucrose and CsCl gradients and determine their relative infectivities. The infectivity of top and bottom component was determined after three consecutive cycles of density gradient centrifugation. Thereupon each component showed a single boundary in the analytical ultracentrifuge. The U.V. absorbance profiles of the band formed by the separated top and bottom components indicated that the bottom component was not always entirely free from top component material. Bioassays showed that both components were infectious (Table 4). In these experiments both components exhibited about the same specific infectivity. In most experiments the top component had a higher infectivity than the bottom component. This corresponds with the results of Izadpanah and Shepherd (1966) and Gonsalves and Shepherd (1972).

In several experiments the distribution of infectivity in the bands was determined. The U.V. absorbance in the bands of top and bottom component separated by 3 cycles of density gradient centrifugation is illustrated in Fig. 2. Each band was sampled in a number of fractions which were assayed on *C. album* plants. This showed that the distribution of the infectivity in each band is proportional to the optical density. Even minor contamination of the bottom component with top component material did not enhance infectivity of the first bottom component fractions. The results show that both components are infectious, and that no enhancement of the infectivity of the bottom component was found in the presence of some top component material.

In an experiment to determine whether addition of top component to bottom component would cause a substantial increase of the infectivity, the components were

Table 4. Infectivity of top and bottom component of PEMV, separated by three cycles of density gradient centrifugation and adjusted to the same optical density.

Dilution	Number of lesions[1]	
	top	bottom
undiluted	116	109
1:4	79	77
1:16	32	31
1:64	17	15

[1] Average number of local lesions on 54 leaves of *C. album* in three experiments.

Tabel 4. De infectiositeit van de top- en bodemcomponent van PEMV nadat ze door 3 cycli van dichtheidscentrifugeringen waren gescheiden en op gelijke concentratie gebracht.

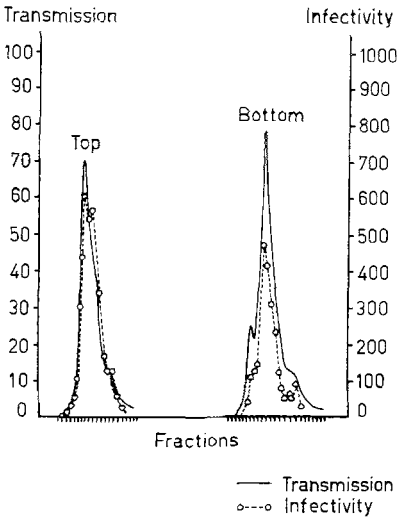

Fig. 2. The distribution of infectivity and optical density in the bands formed on a sucrose gradient by the separated top and bottom components.

Fig. 2. De verdeling van de infectiositeit en de optische dichtheid in de banden die werden gevormd in een suikergradiënt door de top- en bodemcomponent, nadat deze waren gescheiden.

separated in two cycles on a sucrose gradient in a zonal rotor and subsequently one cycle on a gradient in a tube. Four fractions (T1–T4) of the upper part of the top component band and four fractions (B1–B4) of the lower part of the bottom component band were taken. These fractions and mixtures containing equal amounts of these top and bottom component fractions were assayed. All mixtures approximately exhibited the infectivity that could be expected when the components had only an additive effect on the infectivity (Table 5). Thus it appeared from these experiments that the components did not enhance the infectivity of mixed inocula.

When purified preparations were centrifuged in CsCl density gradient two bands were formed. Electron microscopy revealed virus particles in the lower band. These particles were found to be bottom component as they had a sedimentation coefficient of 110 S. Infectivity and serological activity were also found in association with this band. No exhaustive study was made on the infectivity of this zone, but in the two experiments conducted considerable loss of infectivity was indicated. The upper layers

Table 5. Infectivity of purified top and bottom component fractions of PEMV and of mixtures prepared by mixing equal volumes of these fractions.

Fraction	O.D.	Infectivity[1]	B1 0.024 30	B2 0.020 13	B3 0.016 5	B4 0.013 5
T1	0.014	41	13	19	47	33
T2	0.017	90	46	31	34	51
T3	0.019	50	63	52	67	17
T4	0.022	56	100	54	31	–[2]

[1] Average of six half-leaves per inoculum.
[2] Not tested.

Tabel 5. De infectiositeit van gezuiverde top- en bodemcomponent fracties en van inocula die werden gevormd door deze fracties in gelijke hoeveelheden te mengen.

appeared to be protein. No top component could be isolated from CsCl gradients. A pellet containing RNA was collected from the bottom of these tubes. It was concluded that the top component and possibly also some bottom component material were degraded in CsCl.

Serology. In gel double diffusion tests, antiserum against purified virus gave only one precipitation line indicating that both components are antigenically identical. In tests with purified virus two sharp precipitation lines appeared after immunoelectrophoresis at pH 6.0. The quantity of antigen did not affect the pattern of lines. Their position suggested a slight electrophoretic movement towards the negative pole. The pattern indicates that the components which are antigenically identical, differ in their electrophoretic mobilities.

Two lines are formed in diffusion tests with sap extracted from infected pea plants. The additional line was equidistant from the antiserum and antigen wells which suggests that this antigen has a low molecular weight. This soluble antigen could be eliminated from the virus during the first high speed centrifugation. It could be precipitated from the supernatant by adding 12% PEG. Izadpanah and Shepherd (1966) also reported the occurrence of soluble antigen with a low molecular weight in sap from infected plants, which was antigenically related to, but not identical with, the virus.

Discussion

PEMV has been purified by various procedures (Bozarth and Chow, 1966; Gibbs et al., 1966; Gonsalves and Shepherd, 1972; Izadpanah and Shepherd, 1966, and the present authors). All procedures, except those described by Gibbs et al. (1966), use an extracting buffers at pH 6; Gibbs et al. (1966) preferred a buffer with a pH of 7.5. Izadpanah and Shepherd (1966) found that the infectivity of PEMV in crude sap decreased considerably at pH 7 or above. We noted that PEMV precipitated when purified preparations were dialyzed in buffers at pH 7.5.

Preparations of PEMV obtained by the procedure of Izadpanah and Shepherd (1966) still contained some plant material. When comparing an antiserum from Izadpanah and Shepherd (1966) and from the present authors Farro (1969) concluded that the latter antiserum did not contain antibodies against material of healthy pea plants. The purity of our virus preparation might be due to the addition of Mg^{++} ions to the buffer, but also to the chloroform treatment of crude sap and the removal of the precipitate formed at pH 5.3.

PEMV seems to be unique among the multi-particulate viruses. According to Gonsalves and Shepherd (1972) the complete genome of this virus seems to reside in a single particle. These authors demonstrated that both purified components were infectious and that the infectivity was not enhanced by the addition of one component to the other. We arrived at the same conclusion.

One could oppose that the separated components were still contaminated. Then the contaminating material should not have the same distibution in the bands as the component in question. Our results show that the distribution of infectivity in the top component band as well as that in the bottom component band is proportionally related to the optical density. This indicates that a contamination, if occurring, does

not affect the infectivity of either component. This supports the suggestion put forward by Gonsalves and Shepherd (1972) that both components are infectious. It is further shown by the behaviour of the virus on CsCl gradients, that the bottom component is infectious by its own. This low infectivity does not seem to represent a contamination with top component as this degrades in CsCl. Infectivity of the bottom component was strongly reduced on these gradients as is explained by a deleterious effect of CsCl on the infectivity of bottom component.

The virus showed electrophoretic heterogeneity in immunoelectrophoresis. No experiments were performed to demonstrate whether this was related to the two different centrifugal components on polyacrylamide gels.

Samenvatting

De zuivering van het erwte-enatiemozaïekvirus en de infectiositeit van zijn beide componenten

Een methode, die was beschreven om erwte-enatiemozaïekvirus (PEMV) te zuiveren, werd gewijzigd. De toevoeging van Mg^{++}-ionen bleek gunstig te zijn voor de zuiverheid van het preparaat en voor de infectiositeit van het virus. Het virus kan met polyethyleenglycol worden geconcentreerd zonder dat NaCl behoeft te worden toegevoegd.

Gezuiverde preparaten van PEMV bestaan uit twee componenten met sedimentatiecoëfficienten van 107 en 96 S (Fig. 1). Beide componenten bleken na zuivering infectieus te zijn (Tabel 4). Bij scheiding van de componenten op een suikergradiënt waren de optische dichtheid en de infectiositeit van beide componenten op gelijke wijze over de gevormde zones verdeeld (Fig. 2). Bovendien bleek dat de infectiositeit van beide componenten niet kon worden verhoogd door toevoeging van de een aan de ander (Tabel 5).

Wanneer het virus op een CsCl-gradiënt werd gecentrifugeerd werd alleen de bodemcomponent teruggevonden. Deze bleek infectieus te zijn. De topcomponent wordt waarschijnlijk vernietigd door CsCl.

In de agargeldiffusietoets bleek het virus-antigeen homogeen te zijn. Het virus viel in twee componenten uiteen bij immuno-elektroforese. Mogelijk is dit te verklaren door het bestaan van twee centrifugale componenten.

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References

Bozarth, R. F. & Chow, C. C., 1966. Pea enation mosaic virus: Purification and properties. Contrib. Boyce Thomson Inst. Pl. Res. 23:301–309.

Farro, A., 1968. Étude sérologique du virus de la mosaïque énation du pois. Annls. Phytopath. 1: 181–194.

Gibbs, A. J., Harrison, B. D. & Woods, R. D., 1966. Purification of pea enation mosaic virus. Virology 29: 348–351.

Gonsalves, D. & Shepherd, R. J., 1972. Biological and physical properties of the two nucleoprotein components of pea enation mosaic virus and their associated nucleid acids. Virology 48: 709–723.
Hagedorn, D. J., Layne, R. E. C. & Ruppel, E. C., 1964. Host range of pea enation mosaic virus and use of *Chenopodium album* as a local-lesion host. Phytopathology 54: 843–848.
Hull, R. & Lane, L. C., 1972. The multicomponent nature of pea enation mosaic virus. Ann. Rep. John Innes Hort. Inst. 62: 114–115.
Izadpanah, K. & Shepherd, R. J., 1966. Purification and properties of the pea enation mosaic virus. Virology 28: 463–476.
Kammen, A. van, 1967. Purification and properties of the components of cowpea mosaic virus. Virology 31: 633–642.
Knight, C. A., 1963. Chemistry of viruses. Protoplasmatologia 4 (2). Springer, Vienna.
Markham, R., 1960. A graphical method for the rapid determination of sedimentation coefficients. Biochem. J. 77: 516–519.
Shepherd, R. J., Wakeman, R. J. & Ghabrial, S. A., 1968. Preparation and properties of the protein and nucleic acid components of pea enation mosaic virus. Virology 35: 255–267.

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