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ELECTROPHORETIC INVESTIGATION OF SOME GRAPEVINE FANLEAF VIRUS ISOLATES

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Introduction

Virus infected grapevines of the island of Vis display very different symptoms, such as fanleaf, yellow mosaic, vein banding, cane deformities etc. Virus isolates obtained from such plants were tested serologically by immunodiffusion in agar gel with antisera to twelve isometric viruses. A positive reaction was obtained only with grapevine fanleaf virus (GFV) antiserum. The precipitation bands coalesced pointing out serological identity. However, these serologically indistinguishable GFV differed a great deal in the host range and symptoms in herbaceous hosts (Šarić and Vrdoljak 1973a).

The behaviour of five GFV isolates in immunoelectrophoresis and the study of their protein by polyacrylamide gel electrophoresis are presented in this paper.*

Material and Methods

Material

Viruses used. Five GFV isolates were examined. Two of them, denoted PP and VV, were isolated from cv. Plavac showing typical fanleaf and white mosaic symptoms. The isolate Y originated from cv. Trbljan and displayed fanleaf and cane deformities, and the isolate MM derived

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from cv. Okatac with chlorosis symptoms. The fifth isolate OO was taken from cv. Babić with vein banding, yellow mosaic and leaf deformities.

The isolate from cv. Babić was of particular interest because it provoked symptoms unusual for GFV on different herbaceous plants (Šarić and Vrdoljak 1973 b). Therefore we shall note here the host range of this isolate and the symptoms which appear on a number of plants (Tab. 1).

Table 1. Host range and plant symptoms of the virus isolated from grapevine cv. Babić
L = local symptoms, S = systemic symptoms

Plant names	Symptoms
Amaranthaceae	
Gomphrena globosa L.	L, necrotic lesions; S, mottle, deformity
Chenopodiaceae	
Chenopodium amaranticolor Coste and Reyn.	L, chlorotic sports; S, vein clearing, mottle and deformity
C. capitatum (L.) Aschers.	Same symptoms as in *C. amaranticolor*
C. murale L.	Same symptoms as in *C. amaranticolor*
C. quinoa Willd.	Same symptoms as in *C. amaranticolor*
Cucurbitaceae	
Cucumis sativus L.	L, chlorotic and ring sports; S. mottle
Fabaceae	
Phaseolus vulgaris L. cv. Bountiful	L. chlorotic and necrotic ring spots; S, chlorotic and necrotic lesions (winter)
Vigna sinensis (Torner) Savi cv. Midget	L, chlorotic spots; S, occasional vein clearing, chlorotic sports, blotching
Scrophulariaceae	
Antirrhinum majus L.	L, chlorotic and white necrotic rings
Solanaceae	
Datura stramonium L.	L, chlorotic and white necrotic rings
Nicotiana clevelandii Gray	L, chlorotic rings and spots; S, ring spots and line pattern
N. tabacum L. cvs. Samsun, White Burley and Xanthi-nc	Same symptoms as in *N. clevelandii*
Petunia hybrida Vilm.	L. chlorotic spots and rings; S, mottle

We used a German GFV isolate which served as a type strain in this investigation. Cherry leaf roll virus and tobacco mosaic virus were obtained from the Department of Botany (Zagreb).

Preparation of inocula. Cuttings from labelled stocks were taken in December and January and forced in a greenhouse. The inoculum was prepared from young leaves ground with the addition of a 2.5% nicotine solution. For the transmission to herbaceous hosts the sap of infected leaves was diluted (1 : 10) with 0.02 M phosphate buffer, pH 7.2 In the case of OO isolate 0.2% of 2-mercaptoethanol was used as additive to the buffer.

Antisera employed. As mentioned in the Introduction, many antisera were used for the identification of starting isolates. For this purpose antisera against the following viruses were used: arabis mossaic, artichoke mottle crinckle, cherry leaf roll, GFV, Hungarian chrome mosaic, raspberry ringspot, sowbane mosaic, strawberry latent ringspot, tobacco necrosis, tobacco ringspot, tobacco streak, tomato black ring and tomato ringspot.

Protein markers. Molecular weights of GFV protein subunits were estimated by comparing their relative mobility with that of protein markers (Weber and Osborne 1969). For this estimation protein markers of the following origin and molecular weight were used: bovine serum albumin (Serva, 68,000), ovalbumin (Schwartz/Mann, 43,000), carbonic anhydrase (Serva, 29,000), chymotripsinogen (Serva, 25,000), gamma-globulin L and H chain (Serva, 23,500 and 50,000) and ribonuclease (Sigma, 13,700).

Protein subunits of tobacco mosaic virus (17,800), sowbane mosaic virus (31,000) and cherry leaf roll virus (54,000) were used too. All these viruses were increased in host plants and purified before use. Tobacco mosaic virus was purified by the method of Gooding and Hebert (1967), sowbane mosaic virus by the method of Kado (1967) and cherry leaf roll virus by the modified method of Wetter (1960).

Methods

Purification. Leaves of *Chenopodium quinoa* frozen immediately after harvesting or fresh White Burley tobacco leaves were used for purification. Clarification was performed by chloroform — butanol method (Steere 1956; Harrison and Nixon 1960) or by diethylether — carbon tetrachloride method (Wetter 1960). In order to preserve the virus during clarification, 0.2% of 2-mercaptoethanol in 0.02 M phosphate buffer, pH 7.0, was added. The virus was precipitated from the solution containing 0.05—0.1 M NaCl and 10% PEG-6000 in the course of 30 min at room temperature and pelleted by centrifugation at 14,000 g for 20 min. The pellets were resuspended in 0.01 M phosphate buffer of pH 7.0 — 0.01 M EDTA and submitted to 2 to 3 cycles of differential centrifugation (10 min at 10,000 g and 2 hrs at 90,000 g). Partially purified virus preparations were further treated by centrifugation in sucrose density gradient (10—40% of sucrose, ribonuclease free, Schwartz/Mann) in a Beckmann SW 25.1 rotor for 3 hours. The purity of the separated components was checked by electron microscope and spectrophotometer.

Electrophoresis. Agar-gel immunoelectrophoresis was performed in a modified "Iskra" apparatus for paper electrophoresis. Microscopic slides were covered with 1% ager-gel according to Feinstein (1968). A potential of 6.5—10 V/cm with current strength of 10—30 mA for 90 min was used. The buffer for electrophoresis was 0.04 M sodium-veronal, pH 8.2. Gels were prepared either with sodium-veronal, pH 8.2, or phosphate buffer, pH 7.2, of ionic strength from 0.025 to 0.25. Electrophoresis was carried out at room temperature on parallel slides. After electrophoresis GFV antiserum was transferred to one series of slides in the central channel. Another series was left without antiserum, but was immediately fixed with 1% acetic acid in 50% ethanol. The slides were then dried and stained with amidoblack 10 B (Spire 1967).

Sodium dodecyl sulphate — polycrylamide (SDS—PAA) gel electrophoresis was performed either in 10% gel columns in 0.1 M phosphate

buffer, pH 7.2 (a Shandon Sc. Co. Ltd. London apparatus) or in 7.5% gel slab in 0.02 M phosphate buffer, pH 7.2 (an 8-cell slab type apparatus). The buffer contained 1% sodium dodecyl sulphate (SDS) and 0.1% 2-mercaptoethanol. Aproximately 1 mg/ml of viruses or protein marker dissolved in 0.01 M phosphate buffer, pH 7.2 (containing 1% SDS and 1% 2-mercaptoethanol) was heated in boiling water for 1 min (Jones and Mayo 1972). Five to ten μl of protein markers and 20—50 μl of virus subunits suspension were placed on each column or slab slot. The current strength was 8 mA/gel tube or 120 mA per slab. All proteins migrate as anions (Maurer 1971). Electrophoresis was stopped when bromphenol tracking dye reached the opposite end of the gel. The migration of the dye was marked in Indian ink. Gels were stained with amidoblack 10 B (Hill and Snepherd 1972) or in Coomasie Brilliant Blue (Koenig et al 1970).

Fig. 1. Immunoelectrophoretic (a) and electrophoretic (b) patterns of the PP-GFV isolate in sodium-veronal buffer pH 8.2, ionic strength 0.025 (current 15 mA).

Fig. 2. Immunoelectrophoretic (a) and electrophoretic (b) patterns of the PP-GFV isolate in phosphate buffer pH 7.2, ionic strength 0.25 (current 30 mA).

Fig. 3. Immunoelectrophoresis of unfractionated PP isolate and its density gradient components. (Phosphate buffer pH 7.2; ionic strength 0.05; current 22 mA). B — bottom component; M — middle component; T — top component; GFV — unfractionated isolate.

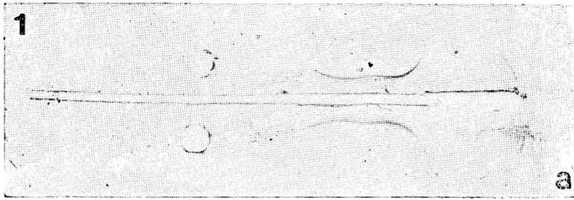
Figures 4—7: SDS-PAA gel electrophoresis of virus proteins of different grapevine isolates. Abbreviations: BSA — bovine serum albumin; O — ovalbumin; Ca — carbonic anhydrase; Ch — chymotripsinogen; R — ribonuclease; γ_1 and γ_2 — gamma-globulin, H and L chains; GFV — grapevine fanleaf virus protein; CLRV — cherry leaf roll virus protein; SMV — sowbane mosaic virus protein; TMV — tobacco mosaic virus protein.

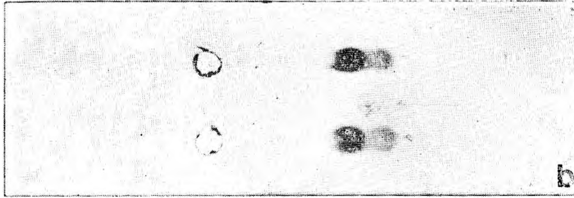
Fig. 4. Migration rate of GFV protein subunit compared with the migration of protein markers (10% gel columns). BSA and Ch in all columns, GFV in column 1 and 6, O in 2 to 6, TMV only in 2, R in 1 to 5.

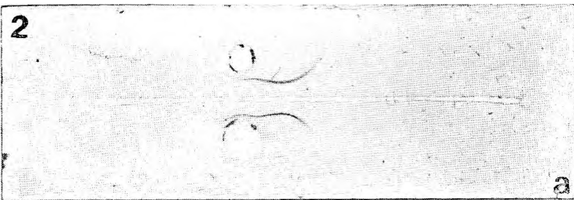
Fig. 5. Migration rates of virus proteins obtained from different grapevine isolates in comparison with CLRV protein subunit and other protein markers (10% gel columns). Columns 1, 2, 3 and 8 with virus subunits and with markers, others with strains MM, Y, OO and VV. Columns 1 and 2 contain CLRV protein subunits (c), column 3 contains GFV strain Y, and column 8 contains GFV strain OO.

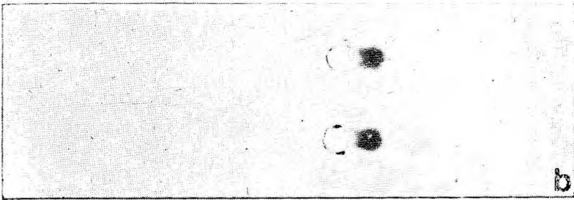
Fig. 6. Migration rate of protein obtained from systemically infected tobacco cv. White Burley (OO isolate) compared with the migration of markers and GFV protein subunit. Columns 4, 5 and 6 with markers; BSA, O, Ch and R are present in 4 to 6; Ca only in 6, and TMV in 4 and 5. In columns 2, 3 and 7 GFV strains without markers. In column 1 additional protein of 41,000 daltons from OO isolate.

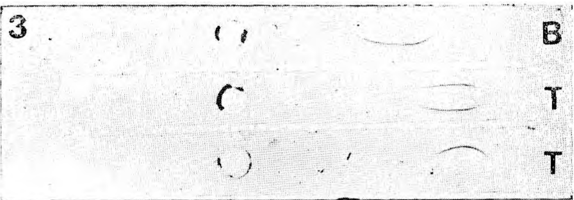
Fig. 7. Electrophoresis of OO and PP isolates of GFV with protein markers in 7.5% gel slab. Migration lines from top to bottom. Line 1: BSA, O, Ch and TMV; Line 2: GFV strain PP, O and Ch; Line 3: GFV strain PP (1) with additional band 2; Line 4: PP, O, Ch and TMV; Line 5: GFV strain OO, O, SMV and Ca; Line 6: γ_1 and γ_2; Line 7: OO and SMV; Line 8: BSA, GFV strain OO, O, SMV and Ca.

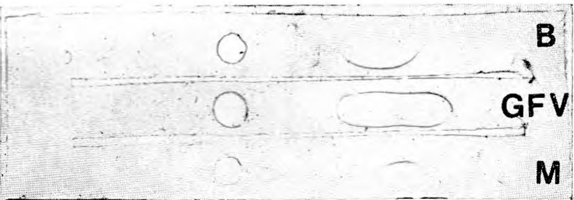




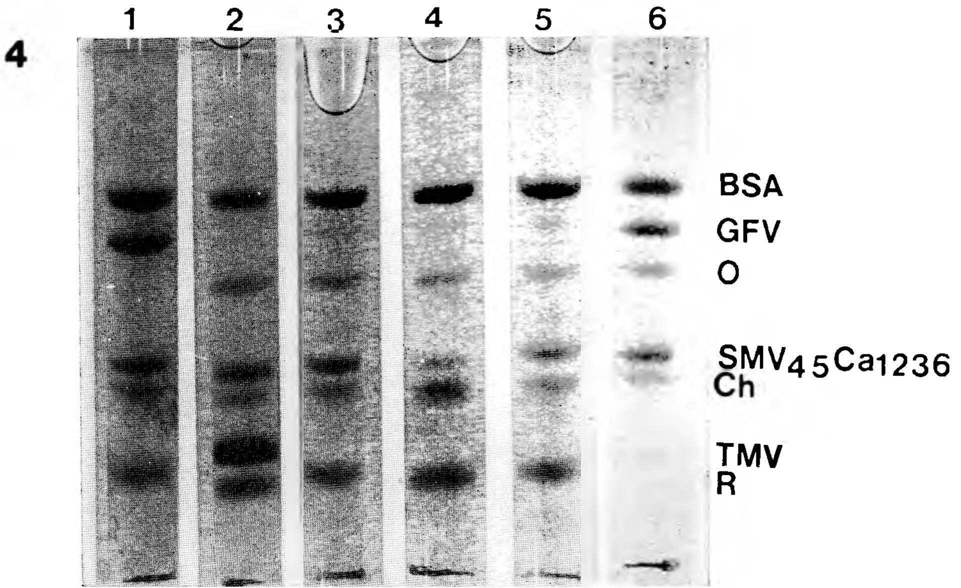


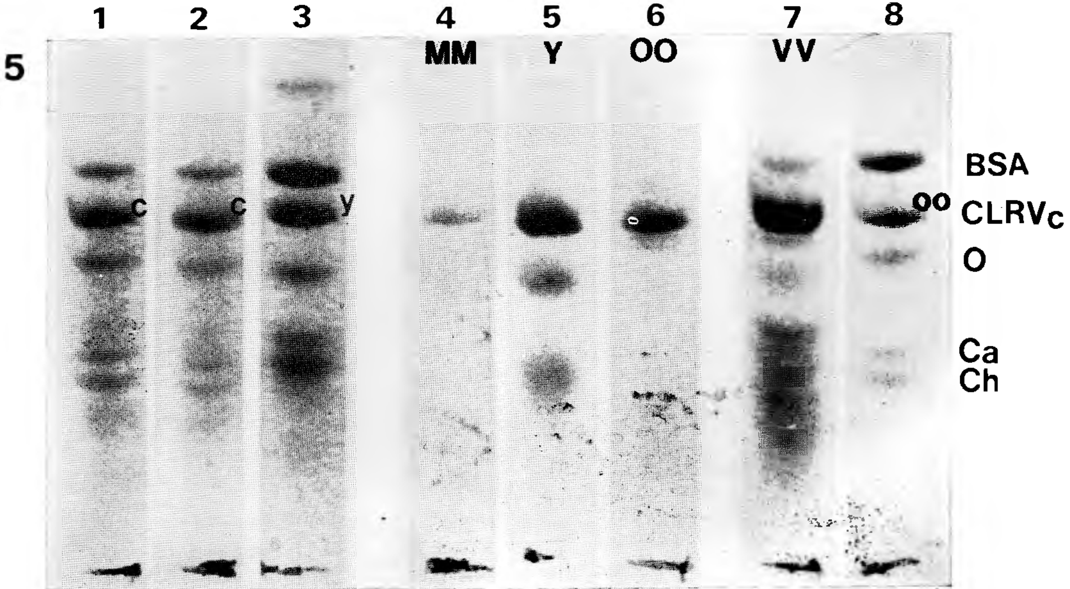






Figs. 1—3.





Figs. 4—5.

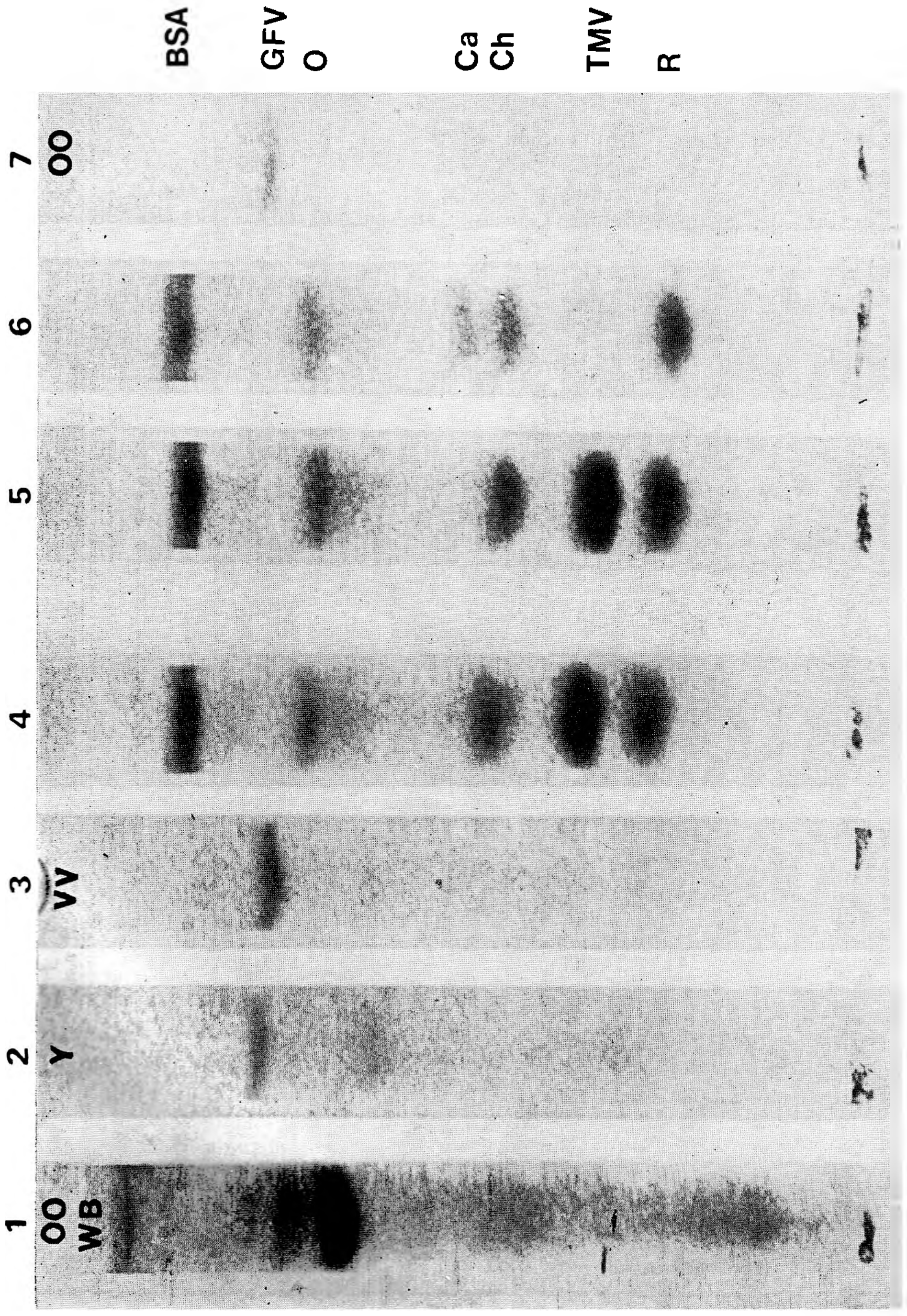


Fig. 6

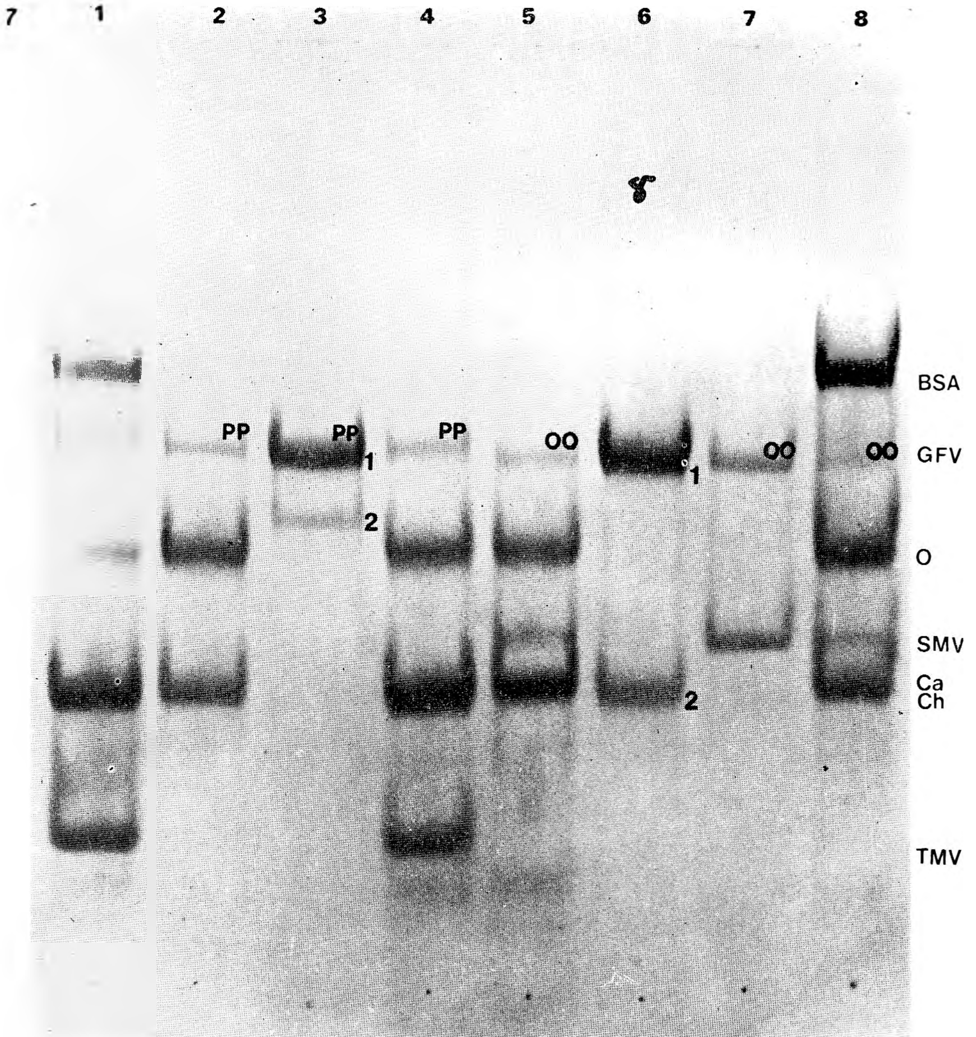


Fig. 7.

Results

Agar-gel immunoelectrophoresis

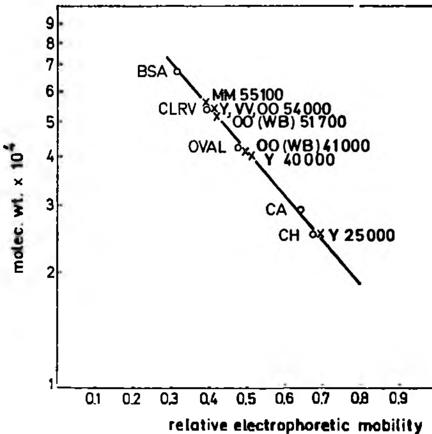
In buffers of 0.025—0.05 ionic strength, partially purified grapevine fanleaf virus usually formed two, sometimes three arcs of precipitation stretching towards the cathode. The electrophoretic patterns of the control microscopic slides (without antiserum) showed well defined, separated spots corresponding to those precipitation lines (Fig. 1). The acid precipitated spots of virus protein showed the quantity of virus particles too. In the stronger buffers, the majority of virus particles remained near the origin well and the degree of separation was less than in the buffers of low ionic strength (Fig. 2).

GFV isolates named MM and VV and Berck's GFV isolate moved somewhat slower towards the cathode.

From highly concentrated virus preparations three centrifugal components were obtained by density gradient centrifugation. Each component gave one arc of precipitation in immunoelectrophoresis (Fig. 3). The top component moved most rapidly, the middle was just slightly slower (about 2 mm difference in migration) and the bottom was the slowest (about 8—10 mm difference in migration from the top component).

Sodium dodecyl sulphate — polyacrylamid (SDS-PAA) gel electrophoresis.

SDS-PAA gel electrophoresis of virus protein of different grapevine isolates showed interesting results as illustrated in Fig. 4—7. Estimations of the molecular weights of protein subunits of the virus isolates investigated are presented in diagrams in Fig. 8 and 9.



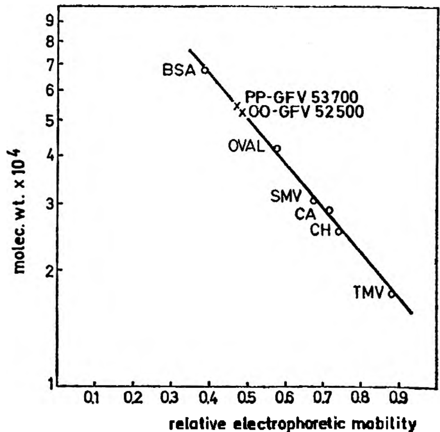


Fig. 8. Determination of molecular weights of proteins, found in various grapevine isolates, in 10% SDS-PAA gel columns.

Fig. 9. Determination of molecular weights of protein subunits of PP and OO isolates in 7.5% SDS-PAA gel slab.

All five GFV isolates obtained by direct transmission from grapevine to *Chenopodium quinoa* and increased in it, gave one well defined protein band which migrated similarly to the cherry leaf roll virus (CLRV) protein subunit. The estimated molecular weight was 53,000—55,100. This band appeared regardless of whether partially purified preparation or the density gradient zone was used (Fig. 4) and it corresponded to the molecular weight of GFV protein subunit (Quacquarelli et al. 1975, Dias 1976). Three of these five GFV isolates gave other, less prominent, protein bands. These isolates were Y, VV and PP. In Y isolate two bands were observed corresponding to molecular weights of 40,000 and 25,000 daltons (Fig. 5). VV isolate displayed a number of faster moving bands with smaller molecular weights in the lower part of the gel column (Fig. 5) and PP isolate showed one band of about 45,000 daltons (Fig. 7, PP 1 and 2).

OO isolate was also propagated in tobacco cv. White Burley directly infected from grapevine. When this isolate was purified from the inoculated leaves of tobacco, a protein of about 52,000 daltons was obtained. However, the purified virus preparation from systemically infected tobacco leaves gave another prominent protein band corresponding to the molecular weight of about 41,000 daltons (Fig. 6, first column). This protein band was not obtained when CLRV was purified from tobacco under the same conditions.

Discussion

These investigations were performed in order to search for the presence of another virus or viruses besides the GFV in the material containing various GFV isolates. We used two methods, immunoelectrophoresis and SDS-PAA gel electrophoresis to check the homogeneity of the virus material isolated from the grapes displaying various symptoms of virus diseases.

The presence of two well separated electrophoretic forms of GFV as revealed by immunoelectrophoresis have been noticed since 1973. The migration rates of the virus particles and separation of the components depended on the ionic strength of the buffer (Fig. 1 and 2). The best results were obtained with 0.01 M phosphate buffer (ionic strength 0.024), while in the 0.1 M phosphate buffer the migration and separation were low. Later on it was established that the faster and the slower electrophoretic components corresponded to the top (protein shells) and the bottom (nucleoprotein) components of the density sucrose gradients (Vrdoljak and Šarić 1974). The pattern of acid precipitated virus proteins (Fig. 1b) shows that the quantity of the top component was considerably smaller, which had already been revealed by the density sucrose gradients. As the middle centrifugal component was present in very small quantities, it escaped identification in the first experiments of immunoelectrophoresis. From the highly concentrated virus preparations three eletrophoretic components, which corresponded to the three centrifugal components, were obtained (Fig. 3).

Different results have been reported for the electrophoretic mobility of the viruses belonging to the nepovirus group. Three components of the raspberry ringspot virus migrate homogenously as one particle in immunoelectrophoresis (Murant et al. 1972). Walkey et al. (1973) reported for CLRV that the centrifugal components from the density gradient did not correspond to the electrophoretic components. However, Clark (1976)

reported that the three centrifugal components of arabis mosaic and strawberry latent viruses did correspond to their three electrophoretic components. It seems probable that the electrophoretic heterogeneity of our GFV particles results from the same cause as in Clark's experiments with arabis mosaic virus. Clark thinks that the cause of different electrophoretic mobility is the difference in protein surface charge. The faster moving of the GFV top component may be due to the specific arrangement of the protein molecules, resulting in the change of its electric charge (Kaper 1975).

In tobacco streak virus the electrophoretic heterogeneity of the virus preparations is caused by differences in the particle morphology (Lister et al. 1972) and in cowpea mosaic virus by the composition of the protein coat (Geelen et al. 1972).

GFV isolates MM and VV and Bercks' GFV isolate moved more slowly in immunoelectrophoresis. These slight differences in the moving rates may also be due to the differences in the protein coat charge.

The electrophoretic separation in agar-gel and its comparison with the immunoelectrophoretic patterns of the same sample proved to be an efficient tool to check the purity and the homogeneity of the virus material examined. In the separation of the components, besides the buffer system, the role of agar-gel as an acid polysaccharide was important too.

The molecular weights of the coat protein subunits of five GFV isolates, determined by SDS-PAA gel electrophoresis, are in good agreement with those obtained by Quacquarelli et al. (1975) and Dias (1976) for their GFV isolates. The small differences in the molecular weights obtained for the GFV protein are probably caused by the subunit charge effects which are not eliminated by this method (Tung and Knight 1971).

In the OO isolate obtained from the tobacco White Burley (cf. Results), apart from the GFV protein (MW 52,000) another protein with molecular weight of 41,000 was detected too. The protein of this molecular weight does not belong to the proteins present in healthy tobacco leaves (cf. Paul 1974); neither could it be attributed to the presence of a satellite virus since the RNA of the satellite of two nepoviruses, i. e. of tomato black ring virus and tobacco ringspot virus, is packed in the shells of their helper virus (Harrison and Murant 1977). The detection of protein of 41,000 MW in OO isolate may indicate the presence of another virus associated with GFV, but we were unable to separate it.

Moreover, the patterns produced by PP and Y isolated showed more than one protein band, and isolate VV displayed as many as six faster moving bands. It is difficult to conclude only from the results of SDS-PAA gel electrophoresis whether these bands present proteins of another virus or something else.

A similar situation has recently been revealed in a virus isolate originating from grapevine with both 'legno riccio' and 'corky bark' symptoms in Northern Italy (Belli 1980). This isolate showed in SDS-PAA gel electrophoresis, besides the band of 54,000 daltons, another prominent band of MW 40,000 and one of 25,000—26,000daltons.

For tobacco ringspot virus, Chu and Francki reported (1979) that in SDS-PAA gel electrophoresis the original protein subunit consisting of a single polypeptide band of 12,988 daltons appeared in six polymeric components with 85% of protein in the band of 57,000. The SDS-PAA gel pattern obtained from VV-isolate apparently looks like the pattern from tobacco ringspot virus, but the calculated weights showed that the bands of VV protein could not be considered as regular polymers.

Summary

Grapevine fanleaf virus (GFV) isolates originating from the island of Vis were examined by agar-gel immunoelectrophoresis and by sodium dodecyl sulphate polyacrilamide (SDS-PAA) gel electrophoresis. Immunoelectrophoresis of the majority of isolates examined usually displayed two, sometimes three, electrophoretical components moving towards the cathode. Three centrifugal components from the density sucrose gradient had different velocities of migration. The top component was the fastest and the bottom was the slowest. Some strains including Bercks' GFV isolate moved more slowly towards the cathode.

SDS-PAA gel electrophoresis of the grapevine isolates studied, in 7.5 and 10% slab and column gels, revealed one well defined band of grapevine fanleaf virus protein subunit which corresponded to the molecular weight of c. 53,700 daltons. In electrophoresis GFV protein migrated similarly to the cherry leaf roll virus protein. From the OO isolate, which had a wide herbaceous host range, unusual for GFV, the GFV band of about 52,000 and an additional prominent band of 41,000 daltons were obtained. In other isolates, besides the GFV protein subunit band, some less prominent bands were found.

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SADRŽAJ

ISTRAŽIVANJE NEKOLIKO IZOLATA VIRUSA INFEKTIVNE DEGENERACIJE VINOVE LOZE S POMOĆU METODA ELEKTROFOREZE

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Izolati virusa infektivne degeneracije loze (VIDL) s otoka Visa istraživani su s pomoću metoda agar-gel imunoelektroforeze i SDS-poliakril amid gel elektroforeze (SDS-PAA). VIDL se u imunoelektroforezi razdvajao u dvije, katkad tri elektroforetičke komponente koje su putovale prema katodi. Razdvojene tri centrifugalne komponente virusa (gornja, srednja i donja) putovale su različitom brzinom. Gornja komponenta, sastavljena od praznih virusnih proteinskih omotača, kretala se najbrže, a donja komponenta je bila najsporija. Također je zapažena razlika u brzini putovanja prema katodi između različitih izolata VIDL. Metodom SDS-PAA gel elektroforeze u 7,5 i 10%-tnim gelovima dobivena je vrijednost za molekularnu težinu proteinske podjedinice omotača VIDL-a od 53.700 daltona. Ta se vrijednost podudara s molekularnom težinom od 54,000 koju su u svojim istraživanjima dobili Quacquarelli et al. (1976) i Dias (1976). Posebno je istraživan OO izolat koji je imao širok i vrlo neobičan krug domaćina za VIDL. Iz purificiranog materijala OO izolata dobivena je proteinska podjedinica VIDL od 52.000 daltona i protein od 41.000. Taj manji protein mogao bi pripadati drugom virusu koji se prenaša samo zajedno sa VIDL, jer ih nije bilo moguće razdvojiti na test biljkama. Iz drugih izolata dobiveno je također, osim proteinskih podjedinica VIDL-a, i nekoliko drugih polipeptida koji zahtijevaju dalja istraživanja.

Obje metode elektroforeze, opisane u našem radu, pokazale su se korisnima u istraživanju homogenosti virusnog materijala izoliranog iz vinove loze s raznim simptomima zaraze.

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