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Protein Composition of Tomato Spotted Wilt Virus

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Analysis of the protein composition of tomato spotted wilt virus (TSWV), purified by an improved procedure, by polycrylamide gel electrophoresis, revealed three major structural proteins (of MW 84,000, 50,000, and 29,000d) and a minor one of MW 220,000d. The three major proteins constitute about 98% of the total viral protein and all three were shown to be glycoproteins. One of the major proteins (MW 29,000d) and the minor protein were shown to be associated with subviral particles isolated by treatment of virus with the nonionic detergent Nonidet P-40. Only traces of the other two proteins were detected in the subviral particles.

Synthesis of virus-induced proteins in TSWV-infected tobacco leaves was studied by labeling infected and healthy tissue with [3H] and [14C]valine, respectively. The labeled tissues were then fractionated into crude subcellular fractions and protein patterns of healthy and infected tissues were compared by coelectrophoresis on polyacrylamide gels. Only one virus-specific protein (of MW 49,000d) was detected in the virus-enriched fractions; this corresponded with the viral structural protein of MW 50,000d.

INTRODUCTION

Tomato spotted wilt virus (TSWV) has been partially purified by several workers (Black et al., 1963; Martin, 1964; Best, 1966, van Kammen et al., 1966; Tsakiridis and Gooding, 1972) but little is known about its detailed structure and composition (Best, 1968). Virus particles, about 75–85 nm in diameter, appear spherical in thin sections of infected plant cells (Milne, 1970), and pleomorphic when examined in negatively-stained preparations (van Kammen et al., 1966; Milne, 1970). The particles have outer envelopes with surface projections but no details of the internal structure have been observed. Milne (1967) noted the similarity of TSWV particles to those of influenza virus and Best (1968) suggested that TSWV is essentially a “pleomorphic myxovirus.” However, in a recent attempt to classify plant viruses, Harrison et al. (1971) proposed a monotypic group for TSWV.

In this paper, we describe the protein composition of TSWV purified by a method modified from that of Black et al. (1963). An investigation into the synthesis of virus-induced proteins in tobacco leaves infected with TSWV is also reported.

MATERIALS AND METHODS

Virus and Plants

An isolate of TSWV from Dahlia, collected at the Waite Institute, was used in all experiments. Virus was maintained on Datura stramonium L. in a glasshouse at 22–30 C. Infectivity was assayed on Nicotiana tabacum L. cv. White Burley (Mohamed and Randles, 1972). For the studies on protein synthesis in virus-infected plants, White Burley plants grown in the glasshouse were inoculated and transferred to a growth cabinet maintained at 28 ± 1 C with a daylength of 14 hr and a light intensity of 23,000 lx. Protein synthesis was studied in the first symptomically infected leaf (Mohamed and Randles, 1972).
Step no.

1. 30 g systemically infected *Datura* leaves +90 ml of solution A; homogenize for 2 min in Waring Blender at low speed, strain through muslin; wash out blender with 30 ml solution A.

2. Centrifuge at 7000g for 20 min

3. supernatant—discard  
   supernatant—resuspend in 30 ml of 0.01 M Na$_2$SO$_4$; homogenize in Servall Omnimix at 140 V for 5 min; stand on ice for 20-30 min

4. Centrifuge at 9000g X 20 min

5. Supernatant—centrifuge at 30,000 rpm for 30 min in Spinco 30 rotor

6. supernatant—discard  
   supernatant—resuspend thoroughly in 3 ml of 0.01 M Na$_2$SO$_4$

7. supernatant—layer 1 ml on 10-40% sucrose density-gradients containing 0.01 M Na$_2$SO$_4$ in 30-ml tubes; centrifuge at 25,000 rpm for 35 min in Spinco SW25 rotor.

8. Collect virus band (2.5-3 cm below the meniscus)

9. Centrifuge at 30,000 rpm for 45 min in Spinco 30 rotor

\[ \text{supernatant—discard} \quad \text{pellet—final virus preparation} \]

\[ ^a \text{Solution A: 0.1 M phosphate buffer, pH 7.0, 0.01 M Na$_2$SO$_4$.} \]

\[ ^b \text{Or layer 200 μ l on 10-40% sucrose density gradients in 5-ml tubes and centrifuge at 38,000 rpm for 20 min in Spinco SW50 rotor.} \]

**Analysis of Viral Protein by Polyacrylamide Gel Electrophoresis (PAGE)**

Purified virus was prepared as described in Fig. 1 and dissociated into polypeptides by heating for 2 hr at 60 C in protein extraction buffer (0.005 M sodium phosphate, pH 7.2, 1% sodium dodecyl sulphate (SDS), 5 M urea, and 0.005 M 2-mercaptoethanol).

Alternatively, in some experiments, preparations were heated at 100 C for 2 min. The extracts were then dialysed for 16 hr against dialysis buffer (0.005 M phosphate buffer, pH 7.2, 0.1% SDS, 0.5 M urea, and 0.005 M 2-mercaptoethanol). Sucrose was added to the samples to a final concentration of 10% before electrophoresis. Protein markers, used in determining molecular weights, were treated in the same way.

Cylindrical gels (8 cm long) containing
either 5 or 10 \% acrylamide were prepared in glass tubes 9 cm x 0.6 cm, as described by Dunker and Ruekert (1969). The electrophoresis buffer contained 0.1 \text{M} sodium phosphate, pH 7.2, 0.1 \% SDS, and 0.5 \text{M} urea. Fifty to 100 \mu l of the samples containing 10–50 pg of protein were layered on top of the gels and electrophoresis was carried out. Bromophenol blue was added to all gels as a marker.

For protein staining, gels were immersed in a solution of 50 \% methanol–7 \% acetic acid containing 0.2 \% Coomassie Blue (Maizel, 1971) for 2–4 hr at 40 C. Gels were destained in 7 \% acetic acid at 40 C over a period of several days. For staining of glycoproteins, gels were oxidized in 1 \% periodic acid in 3\% acetic acid at 10 C for 1 hr, rinsed in water for 1 hr and stained with Schiff’s reagent for 45 min (Clarke, 1964). Gels were destained in water, and stored in 1 \% sodium metabisulphite.

**Analysis of Virus Induced Proteins in Infected Tissues**

Leaf tissue from healthy and infected tobacco plants was sliced into strips 1 mm X 5 mm and infiltrated with 0.01 \text{M} phosphate buffer, pH 7.0, containing 60 \mu g/ml actinomycin D (AMD), 300 \mu g/ml cephaloridine, and 10 \mu g/ml rimocidin (Jensen et al., 1971). Infiltration was carried out by applying vacuum with an air pump, three times for 30 sec each time. The tissues were incubated in the dark at 25 C for 4 hr and then 20 \mu Ci of [3H]valine was added to the infected tissue and 3 \mu Ci of [14C]valine to the healthy tissue. The infiltration was repeated and the tissues were incubated at 25 C under a light intensity of 2500 lx.

After incubation, the tissues were homogenized in 10 vol (w/v) of “grinding buffer” (Zaitlin and Hariharasubramanian, 1972) and centrifuged at 1,000g for 10 min to sediment intact nuclei and chloroplasts. The pellet was resuspended in “grinding buffer” and Triton x-100 was added to 1 \% to solubilize chloroplasts. The solution was recentrifuged at 1,000g for 10 min and the resultant pellet and supernatant were classed as crude “nuclear” and “chloroplast” fractions, respectively. The supernatant from the first centrifugation was centrifuged at 20,000g for 10 min to sediment a crude “mitochondrial” pellet while the supernatant was classed as the “cytoplasmic” fraction. The “nuclear” and “mitochondrial” pellets were resuspended in protein extraction buffer while SDS and urea were added to 1 \% and 5 \text{M}, respectively, to the “chloroplast” and “cytoplasmic” supernatants.

Proteins were extracted as described above and the protein patterns of healthy and infected tissues were compared by coelectrophoresis in 10 \% polyacrylamide gels. Samples (30–60 \mu l) were loaded on the same gel and after electrophoresis the gels were frozen, sliced, and the radioactivities measured (Mohamed and Randles, 1972).

**Sources of Chemicals**

Markers proteins (\% -globulin, catalase, pepsin, myoglobin, lysozyme, and cytochrome C) were from the Sigma Chemical Co; radioactive isotopes ([3H] and [14C]valine) from the Radiochemical Centre, Amersham, England; Nonidet P-40 from the Shell Company; and AMD was a gift from Merck, Sharp and Dohme.

**RESULTS**

**Purity of Virus Preparation**

Final preparations of virus, purified as described in Fig. 1, were pale green although a high yield of virus was obtained (Fig. 2) and electron microscopic examination revealed only a little host membranous material (Fig. 3). Subsequent analysis of the protein composition of TSWV purified by this method indicates that there is very little contamination with host protein. A further advantage of this method is that it can be carried out in about 5 hr, an important consideration because of the instability of TSWV.

**Protein Composition of TSWV**

When protein extracts of TSWV preparations were analysed by PAGE on 5 \% gels (Fig. 4a), 7 proteins were detected. Three of these (nos. 2, 3, and 4) were major proteins and constituted about 98 \% of the total protein of the virus preparation (Table 1).
TSWV treated with Nonidet P-40 sedimented more slowly than intact virus on sucrose density gradients (Fig. 6) and four components of slightly different sedimentation rates were detected. Electron microscopic examination of Nonidet-treated virus showed that the outer layer of projections and the outer envelope had been removed (Fig. 7) releasing the core which was apparently still enclosed within a coat (Fig. 7B). The subviral particle was 30% smaller than the intact virus, with a mean diameter of 60 nm (av for 20 particles) compared with a mean diameter of 85 nm for the intact virus.
Fig. 4. Densitometer traces of the proteins of TSWV analysed by electrophoresis on 5% polyacrylamide gels. (a) Total viral protein stained with Coomassie blue, (b) viral glycoproteins stained with Schiff's reagent, (c) proteins associated with the internal component isolated by treatment of virus with 1% Nonidet P-40 and stained as in (a). The structural proteins (nos. 1-4) are ringed; proteins 5 and 6 are probably aggregates and 7 a host contaminant. The two high MW proteins in (c) of MW 105,000 to 115,000 are probably aggregates.

particle. There is a marked resemblance between the subviral particle and the 55-nm particles reported by Best and Palk (1964) suggesting that these authors were examining particles that had lost their envelope and projections during purification. Infectivity of the virus was considerably reduced on treatment with Nonidet—one lesion was produced by Nonidet-treated virus while an equivalent amount of intact virus produced a total of 143 lesions on four tobacco half-leaves.

Analysis of the proteins from Nonidet-treated virus showed that proteins 1 and 4 were present in amounts comparable with those found in the intact virus (Fig. 4c); only traces of proteins 2 and 3 were present. Proteins 5, 6, and 7 were not detected although two other minor bands, of MW 105,000d and 115,000d were present; these were probably aggregates as they were not detected in preparations of intact virus.

Virus-Induced Proteins in Infected Tissues

Attempts were made to detect viral structural and other virus-induced proteins in infected tissue by using a double-labeling technique to compare protein patterns from healthy and infected tissues. Leaf strips were excised 92 hr after inoculation at the time of appearance of systemic vein-
Fig. 5. Relative mobilities versus the log of molecular weight of marker proteins and the structural proteins of TSWV. The relative mobilities were calculated by assuming the fastest moving component of bromophenol blue marker to have an \( R_f \) of 1.0. The structural proteins are: (1) 220,000d, (2) 84,000d, (3) 50,000d, and (4) 29,000d. The marker proteins are: (a) \( \gamma \)-globulin (160,000d), (b) \( \gamma \)-globulin—dimer of heavy and light chains (80,000d), (c) catalase (60,000d), (d) \( \gamma \)-globulin—heavy chain (55,000d), (e) pepsin (35,000d), (f) \( \gamma \)-globulin—light chain (25,000d), (g) myoglobin (17,600d), (h) lysozyme (14,600d), (i) cytochrome c (12,600d).

The tissues were labeled for 8 hr (96–104 hr after inoculation)—a time at which virus synthesis is rapid (Mohamed and Randles, 1972). The tissues were fractionated into crude subcellular fractions, proteins were extracted and compared by coelectrophoresis on polyacrylamide gels.

The results (Fig. 8) show that no proteins specific to diseased tissues could be detected in the “chloroplast” and “cytoplasmic” fractions while one virus-specific protein was detected in both the “nuclear” and “mitochondrial” fractions. The molecular weight of this virus specific protein was 49,000d corresponding with protein 3 (Table 1) found in purified preparations of TSWV. Infectivity assays of the four subcellular fractions (Fig. 8) showed that virus was associated with the mitochondrial fraction (46 lesions) and the nuclear fraction (10 lesions) only. The lower apparent virus concentration in the "nuclear" fraction was probably due to a reduction in infectivity following treatment with Triton X-100. As the virus-specific protein is associated with the virus-enriched fractions and its molecular weight is similar to that of protein 3, it is probable that this protein is a virus structural protein. The minor peaks in Fig. 8 were not considered to be significant as they were not detected consistently although one of them (at fraction 9) corresponds in molecular weight to structural protein number 2.

Similar experiments were carried out at other times after infection of the leaf to determine whether this or any other virus-induced proteins could be detected. No virus-induced proteins were detectable until 96 hr after inoculation although virus entered the leaf at 48 hr and was detectable by infectivity assay 72 hr after inoculation (Mohamed and Randles, 1972). Labeling of tissues at a later time (104–116 hr after...
Fig. 7. Electron micrographs of (A) intact TSWV particles and (B) the internal component of TSWV isolated by treating intact virus with Nonidet P-40, stained with 2% uranyl acetate for 1 min. Bar represents 100 nm. t—particle with tail; h—host contaminant; c—viral core enclosed by a coat.

inoculation) revealed only the one virus-specific protein.

DISCUSSION

Analysis of the protein composition of purified preparations of TSWV showed that the virus has three main structural proteins and at least one minor one (Table 1). Protein 4, a glycoprotein, is associated with the detergent-resistant subviral particles (Fig. 4c) and may be a constituent of the coat surrounding the viral core (Fig. 7B). Proteins
The molecular weight determinations of the proteins can be considered only approximations as there are several errors inherent in the PAGE technique (Maizel, 1971), especially when estimating molecular weights of glycoproteins (nos. 2–4) or high molecular weight proteins (no. 1).

The unusual feature of the proteins of TSWV is that all three major proteins are glycoproteins, including protein 4 which is associated with the detergent-resistant subviral particle. These results suggest that TSWV may have three layers surrounding a core of "naked" nucleic acid as suggested by Best (1968). The outermost layer is composed of projections, the middle layer is an envelope soluble in Nonidet, and the innermost layer is a glycoprotein coat surrounding the nucleic acid core. Protein 1, which constitutes only about 1% of the total viral protein, may be associated with either the nucleic acid core or with the coat surrounding the core. Comparison of the protein composition of TSWV with that of other membrane-bound viruses (Table 2) shows that TSWV cannot be grouped with any of them on the basis of protein composition. Hence the classification of TSWV into a monotypic group (Harrison et al., 1971) can be justified.

Virus-induced proteins have been successfully demonstrated in TMV-infected tissue using double-labeling techniques and PAGE (Zaitlin and Hariharasubramanian, 1972; Singer, 1971). However, in TSWV-infected leaf tissue, the synthesis of only one protein (corresponding to structural protein no. 3) was detected. This is surprising as TSWV particles are composed of three major proteins in roughly equal proportions (Table 1). As these experiments were done with two amino acids, leucine and valine, both of which have been shown to be constituents of TSWV protein (Jennings and Best, 1964), it is unlikely that they are incorporated into only one protein and not into the other structural proteins. One possible explanation for these results is that greater quantities of protein 3 are synthesized than are incorporated into the viral particle.
### Table 2

Comparison of Protein Compositions of Membran-bound Viruses with that of TSWV

| Virus (group)                  | No. of proteins | Molecular weights (× 10^3) daltons | Reference                        |
|--------------------------------|-----------------|-----------------------------------|----------------------------------|
| 1. Influenza (myxovirus)       | 7               | 83.5 (74), 60, (55), (51), (30), 26.5 | Compans et al. (1970)            |
| 2. Newcastle disease (paramyxovirus) | 4–6             | (74), 56, (56), 41.               | Mountcastle et al. (1971)        |
| 3. Vesicular stomatitis        | 5               | 190, (69), 50, 40, 29.            | Wagner et al. (1972)             |
| (Rhabdoviruses)                |                 |                                   |                                  |
| 4. Coronavirus OC43 (coronavirus) | 7               | (191), (104), (60), 47, 30, (15)  | Hierholzer et al. 1972          |
| 5. Mouse mammary tumour        | 5               | 90, 70, 52, 33, (23)              | Nowinski et al. (1971)          |
| (Leukovirus)                   |                 |                                   |                                  |
| 6. St. Louis Encephalitis      | 3               | 63, 18, 8.5.                     | Trent and Qureshi (1971)        |
| (Arbovirus group 2)            |                 |                                   |                                  |
| 7. Tomato spotted wilt virus   | 1               | 220, (81), (60), (29).           |                                  |

* ( ) Indicates glycoproteins.

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