The Use of Protein A, from *Staphylococcus aureus*, in Immune Electron Microscopy for Detecting Plant Virus Particles

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**SUMMARY**

An immune electron microscopic technique for detecting plant viruses is described which involves pre-coating electron microscope grids with protein A (a wall protein of *Staphylococcus aureus*) before coating them with specific antiserum. The method trapped 339 and 51 times more sugarcane mosaic virus and tobacco mosaic virus particles, respectively, than untreated grids. It appears particularly suitable for virus particles occurring in plant extracts in small numbers.

Electron microscopy is commonly used for detecting plant virus particles, but one of its major disadvantages is that when plant virus particles are present in small numbers (10⁹/ml or less) in plant extracts they are difficult to detect directly by this technique. In the past, various methods of immune electron microscopy have been developed to overcome this and other problems (Milne & Luisoni, 1977) but by far the most efficient and sensitive of these techniques is that developed by Derrick (1973). The use of this technique resulted in 40 times more tobacco mosaic virus (TMV) particles and 20 times more potato virus Y (PVY) particles being attached to the antiserum-treated grids than to the untreated grids. Milne & Luisoni (1977) shortened and simplified the Derrick technique while still retaining the same sensitivity; they reduced the total incubation period from 90 min to 20 min. If their technique of subsequently decorating virus particles with specific antibody is combined with the modified Derrick method the total incubation time amounts to 35 min. Using protein A, a wall protein from *Staphylococcus aureus*, we have developed a modification of the previously published methods which further increases the efficiency of this technique and is particularly useful for detecting virus particles present in plant extracts in low concentrations.

A common strain of TMV and the Johnson grass strain of sugarcane mosaic virus (SCMV; a member of the PVY group) were propagated in the glasshouse in Turkish tobacco and sweetcorn cv. Iochief, respectively. Systemically infected leaves from plants infected for about 8 weeks (tobacco) or 4 weeks (sweetcorn) were used as the virus source. Virus extracts were prepared by grinding the leaf tissue in a pestle and mortar in 50 ml/g (TMV) or 5 ml/g (SCMV) of 0.1 M-phosphate buffer, pH 7.0. Protein A (Pharmacia) was tested at three concentrations (0.1, 0.5 and 1.0 mg/ml) in initial experiments. The lowest concentration was found most satisfactory and hence was used in all subsequent experiments; the higher concentration of protein A increased the amount of contaminants attached to the grid and did not increase the number of particles proportionately. Antisera to the two viruses were produced in rabbits. Purified TMV (2 mg/o.5 ml H₂O/injection) emulsified in an equal volume of Freund's incomplete adjuvant was injected intramuscularly into the hind legs. A total of seven injections was given over a period of 13 weeks and the blood was collected from the marginal ear vein 3 weeks after the last injection. The SCMV antiserum was prepared by a series of seven intravenous injections of the virus (1 mg/ml...
Table 1. Relative number of tobacco mosaic virus (TMV) and sugarcane mosaic virus (SCMV) particles trapped after different treatments of the grids

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Range</th>
<th>Mean*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCMV</td>
<td>Untreated</td>
<td>0–13</td>
<td>4·2</td>
</tr>
<tr>
<td></td>
<td>Antiserum</td>
<td>11–32</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Protein A + antiserum</td>
<td>1152–1845</td>
<td>1418</td>
</tr>
<tr>
<td>TMV</td>
<td>Untreated</td>
<td>198–733</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>Antiserum</td>
<td>918–3126</td>
<td>2275</td>
</tr>
<tr>
<td></td>
<td>Protein A + antiserum</td>
<td>13860–18105</td>
<td>16039</td>
</tr>
</tbody>
</table>

* Mean was calculated from 12 micrographs taken randomly from three squares on each of four grids per treatment.

H₂O/injection) over a period of 8 weeks. A final intravenous injection with 2 mg virus was given 1 week later and the blood was taken 9 days after the last injection. The TMV antiserum had a titre of 1:2048 in agar-diffusion tests and the SCMV one of 1:512 in slide precipitin tests. Dilutions of the antisera were prepared in the phosphate buffer. Freshly prepared Formvar–carbon coated 400 mesh electron microscope specimen grids were used after glow discharge treatment (Milne & Luisoni, 1975).

The method for processing the specimens was as follows: (1) 5 μl of 0·1 mg/ml protein A were placed on each grid, left for 10 min then drained; (2) 5 μl of antiserum (TMV 1:20, SCMV undiluted) were placed on each, left for 10 min, washed with 20 drops of buffer and drained; (3) 5 μl of diluted crude sap were added to each grid, incubated for 10 min, washed with 30 drops of buffer and then drained; (4) to decorate the virus particles, 5 μl of antiserum (TMV 1:100, SCMV 1:5) were added, incubated for 10 min, washed with 30 drops of the buffer and 30 drops of distilled water; (5) the grids were stained using 5 drops of 2% aqueous uranyl acetate, pH 4·5, drained and air-dried.

The grids were not allowed to dry except after staining. Control grids without protein A and others without antibody were also prepared. All the grids were processed at the same time in the laboratory at about 20 °C. Three randomly selected squares on each grid were photographed at a magnification of about 4000 in a Hitachi HS8 electron microscope.

The mean number of particles on grids treated with both protein A and antiserum was a 339-fold and 51-fold increase for SCMV and TMV, respectively, compared to the untreated grids (Table 1). This contrasts with a fivefold and sevenfold increase for SCMV and TMV, respectively, when the grids were treated with antiserum alone. The increase in the number of particles obtained using a mixture of protein A and antiserum was 68-fold for SCMV and 7-fold for TMV over the grids treated with antiserum only (Fig. 1).

It is well established that protein A binds to the Fc portion of IgG molecules (Forsgren & Sjöquist, 1966); two IgG molecules bind to one molecule of protein A (Sjöquist et al. 1972). Presumably, when a grid is coated with protein A, more IgG molecules are bound to the grid than when only antiserum is added and these in turn bind more virus particles.

To demonstrate that protein A helps in increasing the number of particles only if the grids are subsequently coated with virus-specific antiserum, we applied SCMV to grids that had been treated with protein A and antiserum to TMV, and vice versa. In each case we observed that the number of particles attached was about the same as that to untreated grids.

The difference between the increase in binding of the two viruses could be due to a purely physical phenomenon. It is well known that TMV particles are present in higher numbers in plant extracts than viruses such as SCMV. In our experiments we obtained an average of about 16000 TMV particles per micrographic area at 1:50 sap dilution whereas in the use
of SCMV, at 1:5 sap dilution, we could trap only about 1500 particles using both protein A and antiserum. Thus, in the case of TMV, if there are already large numbers of particles bound to the grid, the binding of more particles could be hindered. If this is the case our technique is especially useful for viruses that are present in plants in low numbers.

The conditions we used were based on those found by Milne & Lusoni (1977) to be the
most appropriate for this technique but differ considerably from those of Derrick (1973), Derrick & Bransky (1976) and Bransky & Derrick (1979) particularly in the antibody–virus reaction time. These workers used 30 min to 24 h reaction times for different viruses whereas we allowed only 10 min in order to make this technique as quick and simple as possible. Milne & Luisoni (1977) pointed out that even their 15 min incubation of virus with antibody was almost certainly too short to give maximum combination. This reduced reaction time may be the most important factor, besides our shortened antiserum adsorption time and different antiserum concentrations, accounting for our grids treated with antiserum alone not being as successful as those of other workers.

The conditions in our experiments may not be optimal. There are many variables including those mentioned above which should be tested. Nevertheless, our full method proved superior to all previously published methods and takes only fractionally longer – a 40 min total incubation period compared to the 35 min of Milne & Luisoni (1977). The results from our method are reproducible not only with TMV and SCMV described here but also with other virus–antibody combinations (unpublished data). Even if the conditions are optimized we would still expect an increase in the number of particles bound to protein A and antiserum-treated grids compared to those treated with antiserum alone. We have found (as did Milne & Luisoni, 1977) that decorating the virus with IgG molecules enhances the contrast and therefore the detectability of the virus particles when stained. Obviously the biggest advantage is that it confirms the identity of the virus being tested.

Although we used plant viruses to test our method there is no reason to believe that animal and insect viruses would behave differently and this technique should have wide use.

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REFERENCES


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