Techniques

An Indirect Radioimmunoassay of Cauliflower Mosaic Virus

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ABSTRACT

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A radioimmunoassay procedure was developed that allows detection of 10-300 ng of cauliflower mosaic virus (CaMV) per sample. The assay utilized ^{125}I-CaMV, a rabbit antiserum to the virus, and either a second antibody or heat-killed *Staphylococcus aureus*. Triton X-100 and urea at concentrations of 0.2% and 0.2 M, respectively, lowered the amount of radioactive virus precipitated in the absence of, but not in the presence of, specific rabbit antiserum. At pH values of 7.0 and below, no competition could be seen on the addition of nonradioactive virus; therefore, assays were conducted at pH 8.0. Extracts of turnip leaves did not interfere with the assay except at very low virus concentrations. Virus was detected in extracts of leaves of plants infected with CaMV. The addition of Triton X-100 and urea to the leaf extracts was necessary for maximum detection of viral antigen. The levels of virus detected in turnip leaves by this assay exceeded the levels that were obtained on isolation and purification of CaMV from these leaves. The assay allows the detection of less than 1 μg of virus per gram of tissue.

Additional key words: inclusion bodies.

The DNA-containing plant viruses are potential candidates for vectors in recombinant DNA experiments with plant cells (14). Rapid and sensitive quantitation of such viruses in plant tissues would be an asset not only to recombinant DNA research, but also to the development of an understanding of the biology of these viruses. The caulimoviruses, of which cauliflower mosaic virus (CaMV) has been most often studied (11,12), contain double-stranded DNA. Quantitation of CaMV and other caulimoviruses has been performed by infectivity assays, by the appearance of radioactivity in viral particles separated by sucrose density gradient centrifugation, and by traditional serological techniques (2,5,10).

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Infectivity assays have been performed by limiting dilution (2,10), a procedure which requires large numbers of plants. The virus is reported to produce local lesions on leaves of some species (9), but even with a local lesion assay, several weeks are required before infectivity can be quantitated. The measurement of radioactivity at the position of virus in sucrose gradient centrifugation (5) is limited to viruses that can be radioactively labeled, and is complicated by considerations of precursor pool specific activity and extraction yield. Moreover the number of samples that can be assayed simultaneously is limited. Serological estimation of caulimoviruses has been difficult because nonspecific precipitates form when antiserum is reacted with plant saps and because the amount of free virus in sap is limited (2,10). The main intracellular location of caulimoviruses is within inclusion bodies. Thus, the bulk of the virus probably is not accessible unless the inclusion bodies are disrupted. In an effective purification scheme of CaMV the inclusion bodies are disrupted by stirring with detergent and 1 M urea (7).

RIA has been used in the quantitation of other plant viruses, although not extensively. Turnip yellow mosaic virus has been assayed by RIA with a sensitivity of 0.1 μg (4). A solid-phase RIA using antibody-coated tubes has been developed for several viruses (1) and can detect about 0.5 μg/ml of these viruses. An RIA based upon ^{3}H-labeled antibody to soybean mosaic virus also has been developed (Hill, *personal communication*).

We have sought to develop a radioimmunoassay (RIA) that would be rapid, sensitive, and capable of handling many samples simultaneously.

MATERIALS AND METHODS

Preparation of virus. The CM4-184 isolate of CaMV was obtained from Dr. R. J. Shepherd (Davis, California) and propagated in turnip plants (*Brassica rapa,* 'Just Right') by spreading a suspension of it on carborundum-abraded leaves of 3-wk-old plants. Virus was purified from the infected leaves 4–5 wk after inoculation by the procedure of Hull et al (7). Yields measured by absorbance at 260 nm (12) were typically 15–20 mg/kg leaves. DNA was extracted from the virus by the procedure of Hull and Shepherd (6) and periodically analyzed by gel electrophoresis of Eco RI and Hind III restriction endonuclease digestion products to assure strain homogeneity.

Preparation of antiserum. Purified virus (0.2 mg in 1.0 ml of 0.15 M NaCl) was injected into an ear vein of a rabbit. The initial injection was followed 1 wk and 3 wk later with an injection of the same amount of virus emulsified in 1.0 ml complete Freund's adjuvant into the rabbit's footpad. The rabbit was bled 2 wk later, sodium azide was added to a concentration of 0.1 mM and the serum (RACaMV) was stored at −20 C. An antiserum raised in rabbits against whole mouse serum (obtained from David Hart, Dallas, TX) was used as a source of carrier rabbit antibody. A goat antiserum against rabbit antibody Fc fragments (GARFc) was obtained from the same source. Heat-killed *Staphylococcus aureus* for use as an immunoadsorbent was prepared according to Kessler (8).

Iodination of virus. Virus was radiolabeled with ^{125}I by mixing CaMV (0.05 mg) with 5 nmoles of NaI, 10 μg lactoperoxidase, and 20 μCi Na^{125}I in 0.50 ml of phosphate-buffered saline (0.15 M NaCl, 0.015 M sodium phosphate, pH 7.0) (PBS). The reaction was initiated by the addition of 12.5 μl 0.03% hydrogen peroxide. A second aliquot of 12.5 μl 0.03% hydrogen peroxide was added after 5 min of incubation at room temperature. After 10 min, the sample was chilled and transferred to a dialysis bag. After overnight dialysis against PBS at 4C, labeled virus was separated from labeled lactoperoxidase by centifugation in a sucrose density gradient (7). The virus band, which was identified by its light scattering, was removed through the side of the tube with a needle, dialyzed against water to remove sucrose, and stored at −20 C after the addition of an equal volume of glycerol. The removal of sucrose and storage in glycerol was necessary to prevent irreversible virus aggregation. The specific activity of the final virus preparation was 1.0–1.5 × 10^{5} dpm/μg. Radioactivity was counted in a Packard Autogamma solid scintillation counter at an efficiency of ~70%.

Immunoprecipitation. Immunoprecipitation reactions generally were carried out in 0.5-ml volumes in 12 × 75 mm polystyrene test tubes. The buffer used contained 0.015 M Tris-HCl pH 8.0, 0.15 M NaCl, and 0.1% bovine serum albumin. To this ^{125}I-virus (from 3,000 to 5,000 dpm), 5 μl of rabbit anti-whole-mouse-serum sample or standard and the equivalent of 0.33 nl of RACaMV was added. Other additions were as specified in the text. After 10 min of incubation at 37 C, 50 μl GARFc was added and the samples incubated for an additional 10 min at 37 C. The amount of GARFc was predetermined to be optimal for the formation of antibody-antigen complexes. After storage for 1–2 hr on ice when flocculation had occurred, the samples were centrifuged 10 min at 1,000 *g*, and the supernatants were decanted. The precipitates were

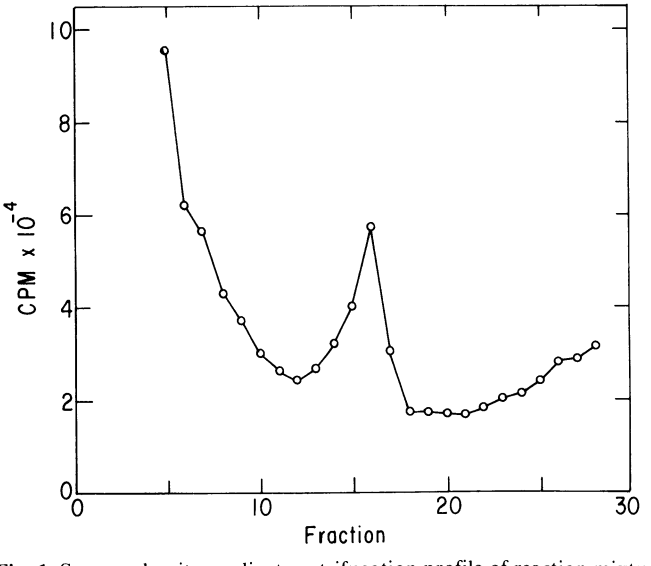

Fig. 1. Sucrose density gradient centrifugation profile of reaction mixture after iodination of CaMV with ^{125}I. In parallel experiments, unlabeled virus was found in fractions 15–17. Centrifugation was for 1 hr at 42,000 rpm in an SW50.1 rotor and a 10%–40% gradient of sucrose.

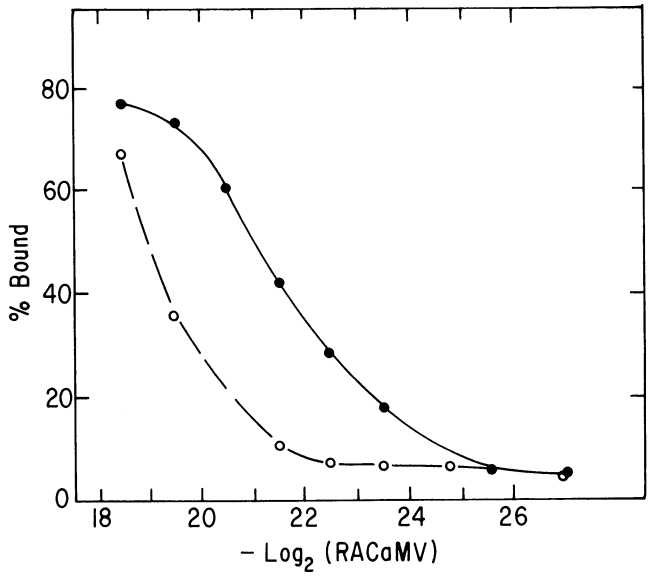

Fig. 2. Comparison of amounts of rabbit antiserum required for immunoprecipitation of radioactive CaMV by the second antibody (o—o) and staphylococcal (•——•)methods. ^{125}I-CaMV was incubated with varying amounts of rabbit antiserum to CaMV and a constant amount of an unrelated rabbit antiserum. The insoluble complexes that formed on subsequent addition of a goat antiserum to rabbit IgG or heat-killed *Staphylococcus aureus* were recovered by centrifugation and counted after several washes of the pellets.

washed twice with buffer by vigorous mixing with a Vortex mixer and centrifugation. Tubes were counted both before the initial centrifugation and after the final wash. In experiments in which heat-killed *S. aureus* was used as an immunoadsorbent, the components of the incubation mixtures were the same except that 10 μl of a 10% (v/v) suspension of bacteria was used in place of GARFc, and the addition of carrier rabbit antibody was omitted. The incubation regime was 10 min at 37 C and 2 hr at 0 C with RACaMV, then 30 min at 0 C with bacteria. All results were converted to fraction of the total radioactivity bound by the antiserum.

Preparation of leaf extracts. For routine analysis, the plant material was weighed and ground with a little sand and 2.0 ml of the immunoprecipitation buffer per gram of tissue in a mortar and pestle. The extract was passed through cheesecloth and adjusted to 1.0 M urea and 1% Triton X-100 by adding 8.0 M and 10% stock solutions, respectively. After 15 min of incubation at 0 C, aliquots were removed for radioimmunoassay. Modifications of this procedure are described in the text.

RESULTS

The virus was radioactively labeled by the lactoperoxidase-hydrogen peroxide method to specific activities of the order of 10^5 dpm/μg. The iodinated virus was freed from labeled soluble protein, presumably lactoperoxidase, by sucrose density gradient centrifugation. The profile of radioactivity from such a gradient is shown in Fig. 1. The radioactive virus band sedimented to the position that unlabeled virus sediments, indicating that the labeling procedure had not damaged the virus particle.

An antiserum, produced by injection of CaMV into a rabbit, had a titer of 1:512 when tested by double diffusion in agar. This antiserum was capable of binding to the virus at low dilutions of the antibody when either GARFc or *Staphylococcus aureus* were used as the precipitating agents (Fig. 2). With the latter assay 50% of the radioactivity was bound at a dilution of antiserum of approximately 2×10^6. When the second antibody method was used under the same conditions, slightly higher antiserum concentrations (a dilution of 5×10^5) were necessary to achieve the same degree of binding. This is probably due to the slower rate of reaction of the second antibody with the RACaMV compared to that of heat-killed *S. aureus* cells with RACaMV.

Since virus is best extracted from leaves of infected plants in media containing Triton X-100 and urea (7), it was anticipated that it would be necessary to perform the radioimmunoassay in the presence of these reagents. As can be seen in Table 1, the addition of Triton with or without urea did not affect the efficiency of precipitation. The background, nonspecific binding of labeled virus, which was fairly high in the absence of additions, was considerably reduced when both Triton and urea were added to the assay mixture. The level of competition was the same either with or without Triton. Because of this lower background (in many experiments as low as 4–6%), assays were routinely performed in the presence of 0.2% Triton X-100 and 0.2 M urea. When this was not feasible, care was taken to assure that standard curves and samples were all assayed under the same conditions of Triton and urea concentration.

In Fig. 3 (a typical standard curve) the percentage of radioactive virus bound in the immune precipitate is plotted as a function of the amount of unlabeled virus present during precipitation in the presence of Triton and urea. Approximately 10 ng virus per assay or, taking account of volume used and leaf weight used, 0.2 μg virus per gram of tissue can be detected. The assay becomes insensitive to small changes in virus concentration above 300 ng per assay. With unknown samples therefore, several amounts of samples (up to the equivalent of 50 mg of leaf tissue) are tested to assure that one of them is in the range of 10–300 ng CaMV. A buffer with a pH of 8.0 (tris buffer) was chosen for the RIA because it was found that at pH 7.0 (phosphate buffer), the addition of increasing amounts of unlabeled CaMV resulted in the binding of increasing amounts of radioactivity. This suggested to us that a concentration-dependent aggregation might be occurring at this pH. Similar results were seen

TABLE 1. Effect of Triton and urea on cauliflower mosaic virus (CaMV) radioimmunoassay parameters

Sample	Immunoprecipitable cpm (%)[a]		
	No addition	Triton[b]	Triton, urea[c]
No RACaMV[d]	23.5	19.3	10.7
Complete	76.5	80.1	78.7
Complete + 0.26 μg unlabeled CaMV	33.3	31.0	31.3

[a] By the *Staphylococcus aureus* method; 2,500 cpm per assay tube.
[b] In the presence of 0.2% Triton X-100.
[c] In presence of 0.2 M urea and 0.2% Triton X-100.
[d] Rabbit serum containing CaMV antibody.

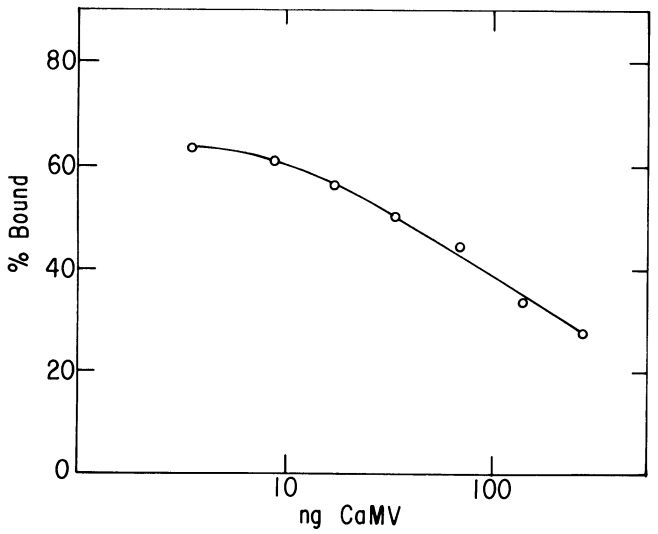

Fig. 3. Standard curve for RIA of CaMV. Constant amounts of ^{125}I-CaMV were incubated in the presence of Triton and urea with varying amounts of unlabeled CaMV (ordinate) and constant amounts of rabbit antiserum to CaMV and of an unrelated rabbit antiserum. Complexes were recovered by precipitation with goat antiserum to rabbit IgG.

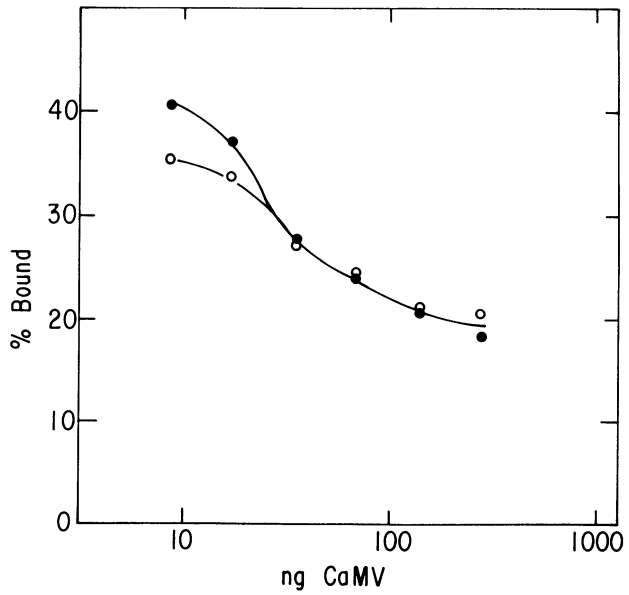

Fig. 4. Detection of standard amounts of nonradioactive CaMV in the presence (o——o) or absence (•——•) of extracts of leaves of turnip plants. Experimental details as in Fig. 3 legend except that in one set, aliquots of a leaf extract (equivalent to 50 mg of leaf tissue) were present in the incubation.

at pH 6.5 (phosphate), 6.0 (phosphate), and 5.5 (acetate). No such anomalous results were detected at pH 7.5 (phosphate), 8.0 (tris), and 8.5 (tris). As expected, it was found that strict adherence to the time and temperature regimes of the incubation scheme was necessary to obtain reproducible sensitivities from the standard curves.

In order to utilize the RIA in the quantitation of CaMV in tissue, the antiserum must not cross-react appreciably with tissue antigens. This was tested by incubating RACaMV and labeled virus with increasing amounts of nonradioactive virus either in the presence or in the absence of extracts from leaves of plants that had not been infected with CaMV. The results of such a determination are shown in Fig. 4. It can be seen that for most levels of virus there is no difference between assays done in the presence or absence of tissue extracts (0.1 ml of extract per assay; 0.5 g leaf tissue per milliliter). At 17 ng of virus and less, however, in the presence of extract, a reproducibly lower percent of the radioactivity was bound, suggesting that there was a slight cross-reactivity which could account for at most 0.34 μg of suspected virus per gram of leaf extracts. In most cases, this amount was negligible.

Hull et al (7) demonstrated that the best yields of purified virus are obtained when the leaf extract is treated with Triton X-100 and urea prior to purification. These reagents presumably disrupt the inclusion bodies, freeing the virus for isolation. Since the virus is sequestered in these inclusion bodies, a direct RIA of leaf extracts would not detect all the virus present. We thus sought to determine what conditions were necessary for maximum accessibility of the virus within leaves of infected plants to antiserum under RIA conditions. Extracts were first adjusted to 1% Triton X-100 and 1 M urea and aliquots removed for RIA at various times. As little as 15 min of incubation at 0 C sufficed to produce maximum accessibility; longer incubations at 0 C did not increase the amount of virus detected. The question whether both Triton and urea were necessary for maximum generation of virus accessibility was investigated next. For this experiment, separate RIA standard curves were prepared for each treatment to assure that further release of virus was not determined by the conditions prevailing during RIA, but rather to those in the preincubation. Aliquots of the same extract were incubated alone at 0 C or with Triton or with both Triton and urea present. As can be seen from Table 2, considerable quantities of virus were detectable when the extract was not treated with either Triton or urea. The presence of urea alone did not alter the accessibility of the endogenous virus. The presence of Triton allowed over half of the virus to become accessible. Urea had no effect by itself, but it improved the accessibility of CaMV when Triton was present. For this reason, tissues for RIA were always disrupted with Triton and urea.

The viral antigen was stable to three cycles of freezing and thawing either in the presence or absence of Triton X-100 and urea, since the same amount of antigen was detected in frozen-thawed extracts as in extracts that had never been frozen.

TABLE 2. Effect of Triton X-100 and urea on the accessibility of virus in leaf extracts to radioimmunoassay (RIA)

Additions[a]	CaMV detected (μg/g of leaf)	Accessibility[b] (%)
None	46	27
1% Triton X-100	111	65
1 M Urea	40	23
1% Triton X-100, 1 M Urea	172	100

[a] Extracts of leaves of plants infected with CM4-184 strain of CaMV for 10 wk were adjusted to the indicated concentrations of detergent and/or urea. After 15 min on ice the apparent virus content was determined by RIA by using *Staphylococcus aureus*. Triton X-100 and urea, (if present in the extract) were present during RIA at 0.2% and 0.2M, respectively. Separate standard curves were prepared for each case.

[b] CaMV detected with Triton X-100 and urea present was set at 100%.

DISCUSSION

The limit of sensitivity of the RIA described here is about 0.3 μg per gram of leaf tissue or 20 ng/ml of RIA reaction. It is likely that by appropriate manipulation of experimental conditions the assay could be made substantially more sensitive. The virus can be labeled to a greater specific activity by the omission of carrier NaI during labeling. Longer incubations both at 37 and at 4 C very likely would increase the sensitivity as would preincubation of antibody with unlabeled virus before the addition of labeled virus. Since for our purposes, the sensitivity achieved was sufficient, these possibilities were not pursued. The sensitivity we obtained is similar to that of other RIA's of plant viruses (13).

The present assay is rapid in that it can be completed in less than 1 day. As many samples as can be centrifuged at one time can be processed simultaneously. Extracts of uninfected leaves when used in large amounts do reduce the maximum precipitation observed with low amounts of nonradioactive antigen. This may be due to a nonspecific interference with precipitation or to cross-reacting antigens in the extract. In either case, the error introduced from this source was negligible for most of the determinations.

It should be emphasized that the present assay detects viral antigen, presumably coat protein, and not necessarily infectious viral particles. Indeed the level of viral antigen detected in extracts of leaves (100–200 μg per gram of leaf) is several-fold higher than the amount of virus we routinely isolate from the same leaves by sucrose density gradient centrifugation (15–20 μg per gram of leaf). Electron microscopic observations of inclusion bodies reveal the presence of two kinds of particles in the size range of isolated virus particles (3). One is uniformly dense and resembles the purified virions, while the other appears as an electron-dense ring with an electron-translucent core. The latter are thought to represent empty viral particles, coat protein without DNA, although the possibility of inadequate stain penetration has not been ruled out. The RIA would detect empty virions as well as complete virions, perhaps accounting for the discrepancy between virus yield and viral quantitation by RIA. It is likely that unassembled coat protein also is detected in the RIA.

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