Evaluation of Indirect Enzyme-Linked Immunosorbent Assay for the Detection of Plant Viruses

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ABSTRACT

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Indirect enzyme-linked immunosorbent assay (ELISA) was compared to double-antibody sandwich ELISA for the detection of two plant viruses in carnations. For purified virus, indirect ELISA proved to be more sensitive than sandwich ELISA and for detecting virus in crude extracts. The main advantage was its simplicity for routine virus detection. Interference and enhancement phenomena were observed when indirect ELISA was used for virus detection in crude plant extracts. The relative merits of the sandwich and indirect tests for the indexing of plant viruses are discussed.

Additional key words: carnation mottle virus, carnation ringspot virus, cross-absorption.

The use of the enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses is well documented (1,4,5,10,11,14). All of these report use of the double antibody sandwich ELISA method (16), which has proved to be a very valuable detection tool for plant viruses. The disadvantage of sandwich ELISA, however, is that the immunoglobulin (Ig) of each test serum must be purified and coupled to enzyme. In addition, the specificity of the test can preclude detection of even closely related strains of the same virus (3,9). Indirect ELISA uses an enzyme-labeled anti-Ig as a second antibody to detect the antigen-antibody complex on the solid phase (8). This avoids the necessity of making specific enzyme conjugates for each antigen to be tested and eliminates the extreme specificity, thus allowing for quantitative evaluation of strain relationships (6,7). Indirect ELISA has not been widely applied to the study of plant viruses. Recently, Bar-Joseph, et al (2) and Van Regenmortel and Burchard (15) described a modified type of indirect ELISA in which an enzyme coupled anti-Ig was used to detect antibody produced in one animal species bound to virus that was bound to the solid phase by an antibody produced in a second animal species. Their results clearly identified the advantage of indirect ELISA for detecting virus strains, but the test has the added complication of requiring two antigen-specific antisera produced in different animals.

Our concern was to evaluate ELISA as a method that would permit reliable indexing and a survey of carnation viruses in California. Constraints on the use of sandwich ELISA were defined by the limited quantities of antiserum available for producing conjugated antibodies for all of the viruses to be detected. Indirect ELISA, where the antigen is bound directly to the solid phase from the plant extract without the assistance of a bound antibody, was evaluated as the method of choice.

In this paper we have compared the use of sandwich and indirect ELISA with carnation mottle virus (CaMoV) and carnation ringspot virus (CRSV) and have defined some of the limitations of the indirect test.

MATERIALS AND METHODS

Virus preparation. The CaMoV isolate was obtained from commercially grown carnation stocks collected in California. The CRSV isolate was kindly supplied by R. I. Hamilton, Agriculture Canada, Vancouver. CRSV and CaMoV cultures were maintained under greenhouse conditions in seedling carnations *Dianthus caryophyllus* (L.) 'English Giants' (Geo. W. Park, Seed Co. Inc., Greenwood, SC 29647).

CRSV was purified for ELISA and serum production as follows: One hundred grams of infected carnation leaves were homogenized with 200 ml of 0.2 M sodium acetate buffer, pH 5.0, in a Waring Blendor. The extract was expressed through two layers of cheese cloth and centrifuged at 7,700 *g* for 20 min in a Sorvall GSA rotor. Four volumes of supernatant were mixed with one volume of 40% polyethylene glycol (PEG 6000) in 1 M NaCl, stirred for 1 hr at 0 C and was then centrifuged at 7,700 *g* for 20 min. The pellet was resuspended in 20 ml of 0.02 M sodium acetate buffer, pH 5.0, and further purified by one cycle of differential centrifugation and fractionation on linear log sucrose gradients in 0.02 M sodium acetate, pH 5.0. This same procedure was used when purifying CaMoV, except that 0.01 M Tris-HCl buffer, pH 7.3, was used to resuspend the pellets after the initial PEG precipitation step.

All carnation tissue samples were treated in the same manner for ELISA experiments. One gram of carnation leaf tissue was minced with 1 ml of water by using a sterile applicator stick in a 1.5 ml microfuge tube. The sample was then centrifuged for 90 sec in a Beckman Microfuge B. The supernatant was drawn off and used as the crude tissue extract. In some experiments, the tissue was minced in various buffers and clarified by the addition of organic solvents.

Antisera. The CaMoV antiserum was kindly supplied by J. H. Tremaine, Agriculture Canada, Vancouver. Antiserum against sucrose gradient purified (132S) CRSV was prepared in a white New Zealand rabbit. Virus in 0.85% NaCl (400 μl at 1.4 mg/ml) was initially injected intravenously into the ear (day 1) and intramuscularly into the thighs after emulsification with adjuvant on days 10 and 20. The rabbits were bled on days 20, 35, 46, 63, and terminally on day 70. All serum used in this report was from day 70 which had a double diffusion titer of 1:15, 625 (0.7% Ionagar, 0.02 M sodium acetate, pH 5.0).

Sera cross-absorption. Antisera were cross-absorbed with healthy plant protein prepared as follows: 10 g of seedling carnation tissue was homogenized in 10 ml of phosphate-buffered saline (PBS) and clarified by lowspeed centrifugation at 7,700 *g* for 20 min. The protein was concentrated to 1 ml by precipitation with 50% saturated ammonium sulfate and then dialyzed against PBS. Quantitative measurements of carnation tissue protein were made by the Bio-Rad Protein assay (Bio-Rad Laboratories, Richmond, CA 94804). Protein standards were made by using bovine serum albumin.

Antisera were cross-absorbed by mixing 1 ml of the concentrated plant protein with 0.2 ml of serum. The mixture was incubated for 1 hr at 37 C and then overnight at 4 C. The precipitate was removed

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by centrifugation and the cross-absorbed antibody was stored at −20 C in 50% glycerol.

Immunoglobulin preparation. The immunoglobulin (Ig) fraction was purified from antisera as described by Morris et al (12). DEAE Affi-Gel blue (Bio-Rad Laboratories) was used for Ig fractionation on a 1 × 7-cm Bio-Rad Econocolumn.

Enzyme antibody conjugation. Alkaline phosphatase (Type VII; Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178) was coupled to purified immunoglobulin by the glutaraldehyde method as described by Clark and Adams (5). Goat anti-rabbit IgG antiserum for the indirect ELISA was obtained from Miles Laboratories (P.O. Box 2000, Elkhart, IN 46514).

Sandwich ELISA. All ELISA tests were performed on the PR-50 EIA automatic analyzer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074) by using polystyrene cuvette plates containing 50 wells. Three 30-sec washes were performed between each step of the tests. The rinse solution was 0.15 M NaCl containing 0.1% Tween-20 and 0.01% sodium azide. Incubation for all steps was for 1 hr at 37 C at 60 rpm on a rotary shaker. Coating of the Ig for both CaMoV and CRSV was at a 1/5,000 dilution (1 μg/ml) in 200 μl of 0.05M carbonate buffer, pH 9.6, with 0.01% sodium azide. After washing, the antigens were added to the Ig-coated wells, either as pure virus or as crude tissue extracts in "sandwich ELISA buffer" (SEB: 0.01 M PBS, pH 7.4, 0.05% Tween-20, 2% polyvinylpyrrolidone-10, and 0.2% ovalbumin). The incubation with the enzyme-conjugated Ig was at a 1/100 dilution for CaMoV (5 μg/ml) and a 1/200 dilution for CRSV (5 μg/ml) in SEB. Reactions were recorded 45 min after the addition of substrate (300 μl of 1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine pH 9.8) at $A_{405\ nm}$ in the PR-50 analyzer.

Indirect ELISA. The indirect ELISA conditions were as described for the sandwich ELISA procedure except the initial coating was with antigen in 200 μl of 0.05 M carbonate, 0.01% sodium azide, pH 9.6, for hr at 37 C. After washing, the antiviral antibody or cross-absorbed Ig was then added for 1 hr at 37 C. Both CRSV Ig and CaMoV Ig were used at a 1/500 dilution in "indirect ELISA buffer," (IEB: 0.01 M PBS, pH 7.4, 0.05% Tween-20, 2% polyvinylpyrrolidone-10, and 0.05% bovine serum albumin). The goat anti-rabbit IgG, coupled with alkaline phosphatase was then added at a 1/3,000 dilution in IEB buffer for 1 hr prior to washing and substrate addition.

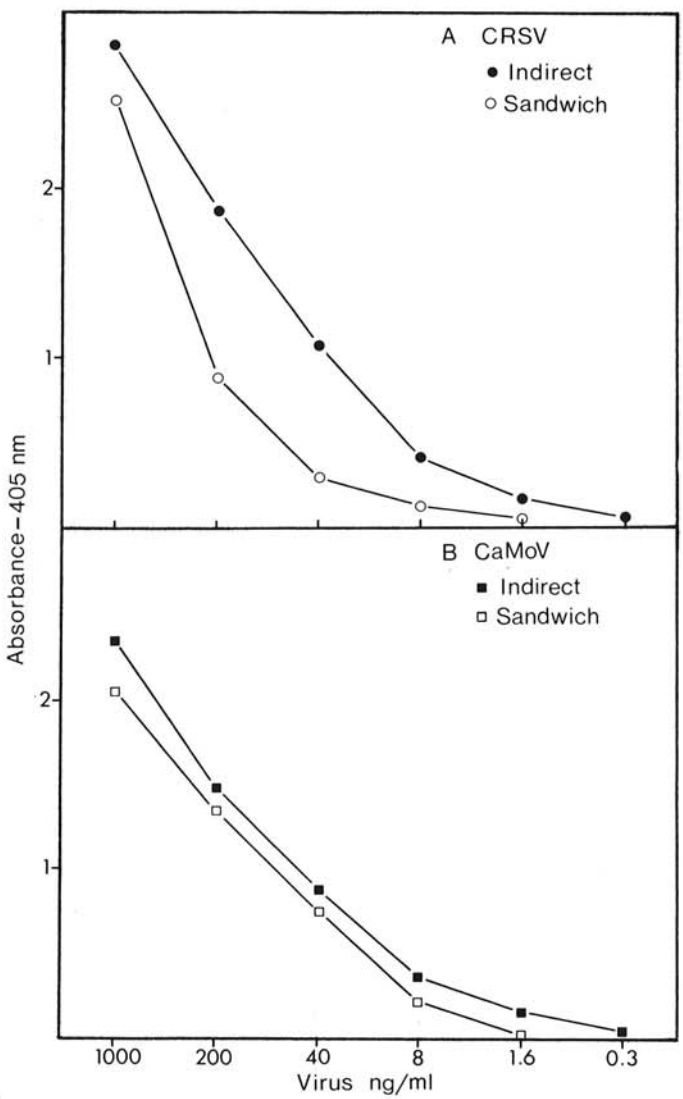

Fig. 1. A comparison of the relative sensitivity of indirect and sandwich ELISA. **A,** Detection of purified carnation ringspot virus (CRSV) by ● indirect ELISA and by O sandwich ELISA. **B,** Detection of purified carnation mottle virus (CaMoV) by ■ indirect ELISA and by □ sandwich ELISA. Each point is the mean of five replications with a maximum standard deviation of 0.01.

RESULTS

Sensitivity. The relative sensitivities of antigen detection were compared for indirect and sandwich ELISA using purified CaMoV and CRSV (Fig. 1). For the indirect test, the viruses were incubated in carbonate buffer in uncoated plates at concentrations ranging from 0.3 to 1,000 ng/ml. For the sandwich test, the plates were precoated with Ig near saturation (1 μg/ml) and the antigens were then added over the same range of concentrations in SEB. Both tests were developed as indicated in the methods section. The indirect and sandwich tests both showed a quantitative response over the concentration range tested for both viruses. The indirect test was sufficiently sensitive to detect both viruses at a concentration of 1.6 ng/ml (0.32 ng of pure virus) in contrast to the detection limit of the sandwich test at 8 ng/ml (1.6 ng of virus). It is evident from these results that the indirect test had a greater absolute sensitivity than the sandwich test for the detection of both viruses. Controls in these experiments consisted of extracts of healthy carnations or the heterologous antigen. Absorbance readings of control wells at $A_{405\ nm}$ were consistently below 0.02 for

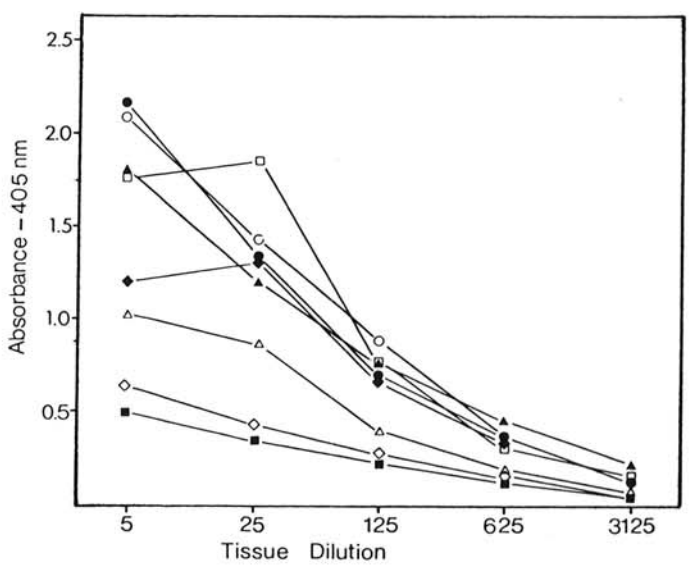

Fig. 2. The effect of extraction medium on the detection of carnation mottle virus (CaMoV) in homogenates of infected carnation tissue by indirect ELISA. Leaf tissue (1 g) was minced at 1 ml of medium, clarified by centrifugation for 1 min and 0.05 ml was serially diluted in wells containing 0.2 ml of 0.05 M carbonate buffer, pH 9.6. The serological activity was measured at 405 nm for tissue extracted with: ● dH_2O, o dH_2O and 0.1% mercaptoethanol, ▲dH_2O and 0.1% diethyldithiocarbamic acid (DIECA), △ PBS and DIECA, ■ PBS and chloroform-butanol (5:1), □ sodium acetate, pH 5.0, ◆ sodium carbonate, pH 9.6, ◇ sodium carbonate, pH 9.6 and chloroform-butanol (5:1). Each point is a mean of five determinations with a maximum standard deviation of 0.01.

the indirect test and 0.05 for the sandwich test.

Optimization of extraction buffer for indirect ELISA. The most critical step in detection of antigens by the indirect test is the adsorption onto the polystyrene plates. We, therefore, evaluated various extraction buffers and clarification methods in order to optimize the detection of both CaMoV and CRSV in crude extracts. Equivalent 1-g samples were minced in 1 ml of the following: distilled water (dH_2O), dH_2O and 0.1% mercaptoethanol, dH_2O and 0.1% diethyldithiocarbamate (DIECA), PBS, and 0.1% DIECA, 0.2 M sodium acetate, pH 5.0, 0.05 M carbonate, pH 9.6 and the same buffer extracts clarified with an equal volume of chloroform-butanol (5:1, v/v). The effect of the extraction medium on the ELISA activity detected in serial dilutions of the crude extracts for CaMoV-infected tissue is illustrated in Fig. 2. The results were essentially the same for CRSV and are not reported. The highest absorbance values were consistently obtained when distilled water (with or without added reducing agents) was used for tissue extraction. All of the buffers tested gave poorer results which was even more evident when chloroform-butanol was included during extraction. Consequently, water was used for tissue homogenization in all subsequent tests.

Quantitative detection by indirect ELISA. Indirect and sandwich ELISA were directly compared for ability to quantitatively detect both viruses in water extracts of infected tissue. Serial dilutions of the virus extracts and equivalent dilutions of purified virus and purified virus supplemented with concentrated carnation protein were evaluated by both tests. The effect of antigen dilution on sandwich and indirect ELISA reactivity is compared for CRSV (Fig. 3) and CaMoV (Fig. 4) in the presence and absence of host proteins. Artificially constructed mixtures with an initial concentration of virus at 5 μg/ml and host protein at 200 μg/ml (a ratio of 40:1) produced dilution curves which most closely resembled those of the infected tissue extracts. In the sandwich tests, the slopes of the plots of $A_{405\ nm}$ versus dilution (virus concentration) were relatively equivalent. This indicated that quantitative detection of the viruses was unaffected by the presence of contaminating host proteins in the sandwich test. However, when the same samples were evaluated in indirect tests, there was a depression of absorbance at the lowest dilution (highest concentration of host proteins) and an apparent enhancement of absorbance at the highest dilution compared to samples containing only pure virus. These results indicated that the indirect test would

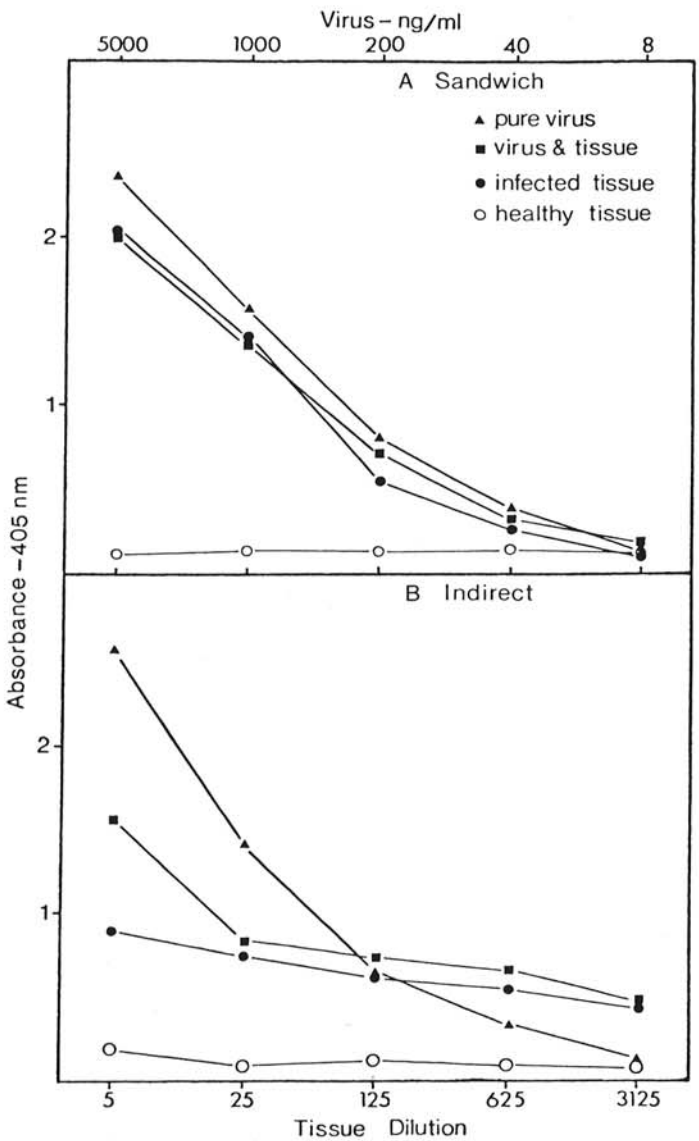

Fig. 3. A comparison of the quantitative detection of carnation ringspot virus (CRSV) by **A,** sandwich ELISA and **B,** indirect ELISA at different stages of purity. The following samples were serially five-fold diluted in the plates: ▲ pure CRSV at 5 μg/ml, ■ CRSV at 5 μg/ml with carnation protein added at 200 μg/ml, ● CRSV-infected carnation tissue, and O healthy carnation tissue. Each point is the mean of five replications with a maximum standard deviation of 0.01.

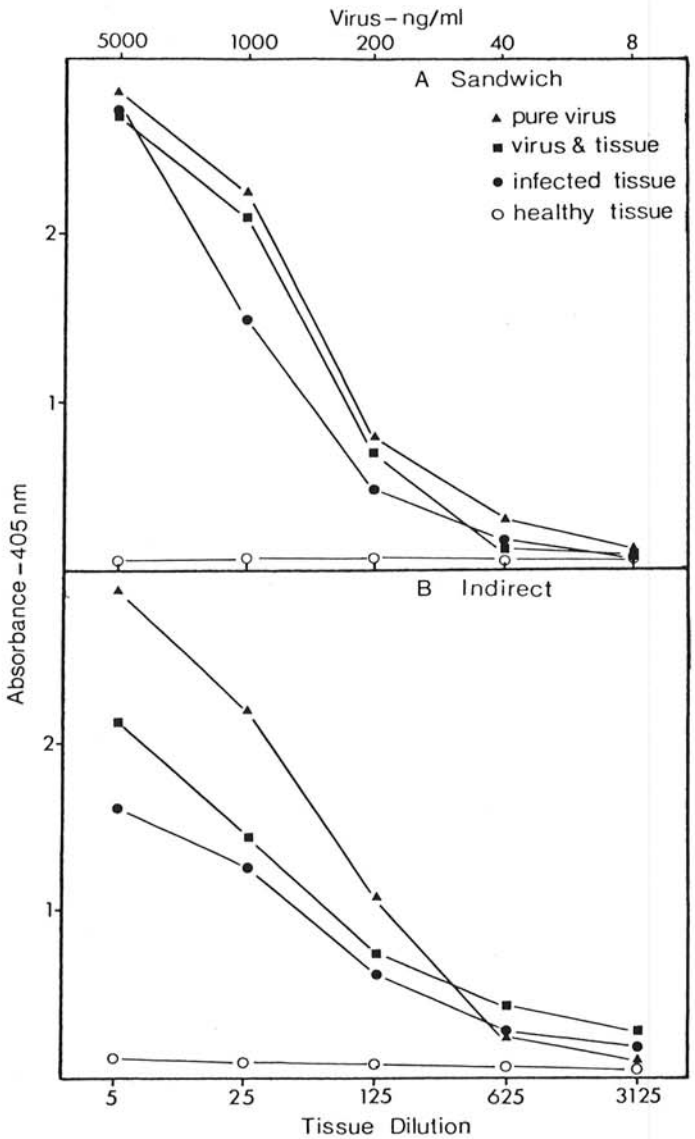

Fig. 4. A comparison of the quantitative detection of carnation mottle virus (CaMoV) by **A,** sandwich ELISA and **B,** indirect ELISA at different stages of purity. The following samples were serially five-fold diluted in the plates: ▲ pure CaMoV at 5 μg/ml, ■ CaMoV at 5 μg/ml with carnation protein added at 200 μg/ml, ● CaMoV-infected carnation tissue and O healthy carnation tissue. Each point is the mean of five replications with a maximum standard deviation of 0.01.

not be useful for accurate quantitation of virus in crude plant extracts.

The effect of nonviral protein on indirect ELISA. The interference by nonviral protein on the detection capabilities of indirect ELISA was further evaluated. An experiment was performed in which samples containing viral antigen at a constant concentration slightly under the binding capacity of the solid phase were supplemented with increasing concentrations of nonviral protein. Pure CaMoV at 1.25 μg/ml, in carbonate buffer, was coated onto plates for 1 hr in the absence and in the presence of an increasing amount of purified host protein or bovine serum albumin, BSA (Fig. 5). At a 200-fold excess of nonviral protein, there was a 52% reduction in the absorbance values, with a proportional decrease in the interference as nonviral protein was titrated. As the nonviral protein concentration was reduced, a point was reached where the concentration of viral antigen was in excess. This was in the region of the estimated adsorption capacity (5 μg/ml) of the polystyrene solid phase (see Fig. 4). Under these experimental conditions, the interference phenomenon most likely resulted from a competition between virus and nonviral protein for a finite number of binding sites on the solid phase. In addition, there was a slight (103%), but significant, enhancement of absorbance in samples when the concentration of added protein and CaMoV roughly equaled the binding capacity of the well.

To further define the enhancement phenomenon the following experiment was conducted. ELISA plates were coated with purified CaMoV in carbonate buffer over a range of concentrations from 5 to 0.008 μg/ml. Carnation protein or bovine serum albumin was added, either at the time of addition of virus to the plates or 60 min after the virus had been added at 50 μg/ml (10 μg to each well) for each concentration of virus. The reactivity of the protein supplemented samples was recorded as a percentage of the absorbance observed for CaMoV alone (Fig. 6). Thus, when 0.0008 μg/ml of CaMoV and 50 μg/ml of nonviral protein were added together the absorbance at 405 nm was approximately 180% of 0.0008 μg/ml of CaMoV alone. When a 180% enhancement was observed the absolute absorbance was less than 0.5A (Fig. 6). The enhancement, at very low virus concentrations, can also be seen in Figs. 3b and 4b, in which virus plus tissue was approximately 0.5 $A_{405\ nm}$ and pure virus alone was approximately 0.1 $A_{405\ nm}$.

It seems that additional protein in solutions at very low concentrations of virus either promote binding or, in some other manner, protect the integrity of the antigenic sites. An interesting observation is that both effects were observed whether or not the nonviral protein was in the sample at the time of addition to the plate or added after the virus had preincubated for an hour (Fig. 6). These results suggest that either the binding of protein to polystyrene is reversible or there is a blockage of antigenic sites at high concentrations of protein and a protection of sites at low concentrations.

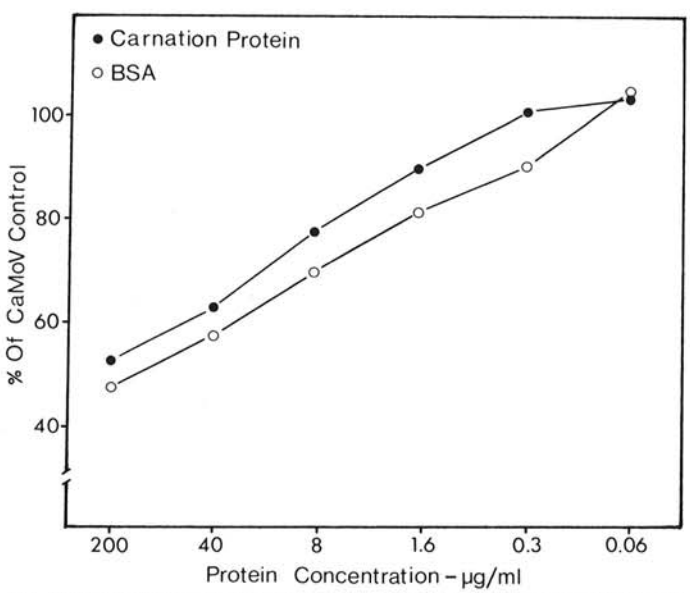

Fig. 5. Interference by an increasing concentration of nonviral protein on the detection of carnation mottle virus (CaMoV) by indirect ELISA. Samples of pure CaMoV at a constant concentration (1.25 μg/ml) were supplemented with increasing amounts of carnation protein ● or bovine serum albumin O and coated on ELISA plates in carbonate buffer. The data are expressed as the percentage of the absorbance recorded for pure virus alone. Each value is a mean of 10 replications.

DISCUSSION

We have evaluated and compared indirect and sandwich ELISA for the detection of two carnation viruses in plant sap. The indirect test had a greater absolute sensitivity than the sandwich test for the detection of purified antigen, and approached the sensitivity of the radioimmunoassay.

Indirect ELISA differs fundamentally from sandwich ELISA in that antigen is bound directly to the polystyrene surface. Quantitation is therefore, directly affected by the binding event. Consequently, because most proteins are bound to the polystyrene in the carbonate coating buffer, it is expected that there would be a competition for available sites by host proteins and viral antigens in crude plant extracts. This interference phenomenon was

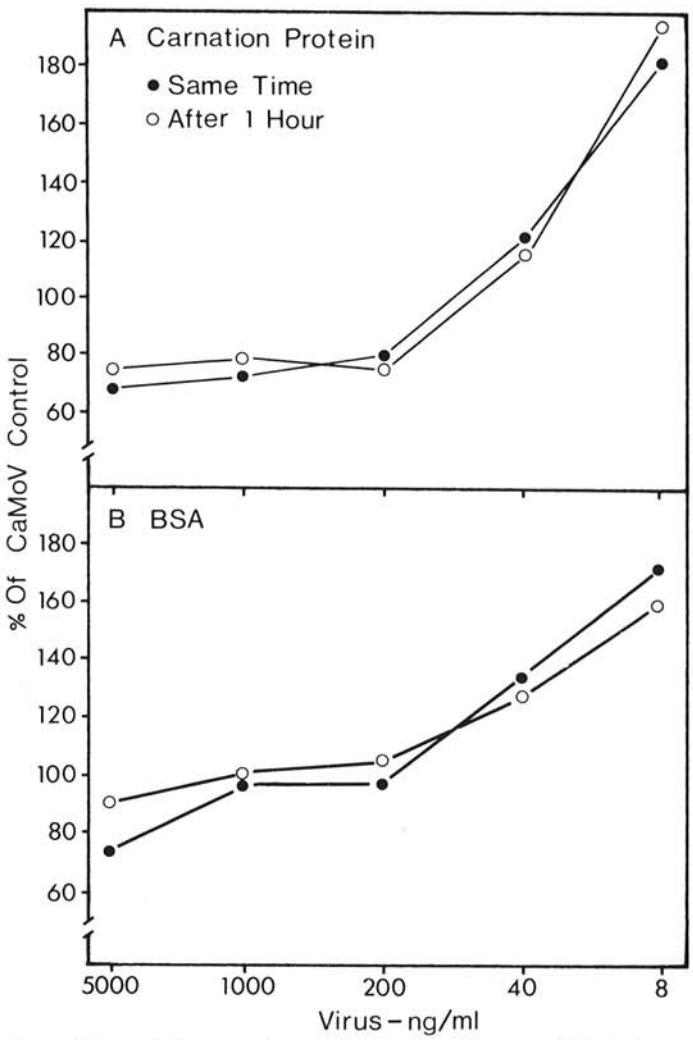

Fig. 6. Enhanced detection of carnation mottle virus (CaMoV) by indirect ELISA in the presence of nonviral protein. Pure CaMoV over a range of concentrations, was supplemented with additional protein (**A,** carnation protein or **B,** bovine serum albumin) at 50 μg/ml (total of 10 μg) at the time of addition of virus to the ELISA plates ● or 60 min after virus alone had been added to the plates O. All samples were coated in carbonate buffer for a total of 120 min. The data are expressed as the percentage of the absorbance recorded for pure virus alone. Each value is a mean of five replications.

demonstrated and precluded the use of indirect ELISA for accurate quantitation of viral antigens in crude extracts. Sandwich ELISA was clearly superior for quantitative virus detection in crude extracts.

In defining the optimal conditions for the detection of the two carnation viruses in plant extracts, we observed a depression of absorbance readings (interference) at the lower dilutions of plant sap and an increase of absorbance readings (enhancement) at high dilutions. Our data, at least partially, supports the contention that interference was due to a competition between viral and nonviral protein for a finite number of binding sites on the solid phase. However, because interference could also be demonstrated when nonviral protein was added after the virus had been bound, (Fig. 6), the possibilities of reversible binding of the proteins or the blocking of antigenic sites by bound protein should also be considered as possible explanations.

The enhancement phenomenon was most evident at low concentrations of virus (below 100 ng/ml) when the total amount of protein in the sample was about 5 μg/ml or less. This was close to the maximum amount of protein (Ig or virus) that could be bound by the well surface. Consequently, at low virus concentrations, nonviral protein may enhance detection by blocking unoccupied sites on the solid phase which would otherwise promote denaturation of the antigen (13) or otherwise affect the antigen-antibody reaction.

In conclusion, we feel that indirect ELISA could be very useful as routine plant virus detection tool for virus disease diagnosis and surveys where accurate quantitation is not of concern. It provides the following advantages over sandwich ELISA: 1. Sample processing is simplified in that the tissue need only be macerated in water and added to the plate. We have demonstrated this for carnation viruses, but it also applies for most other virus-host combinations tested to date (S. A. Lommel et al, *unpublished*). 2. Antisera for each antigen to be detected, which may only be available in limited quantity, need not be purified and coupled to enzyme. Crude antiserum is preferred, although it should be cross-absorbed to prevent spurious host reactions. 3. A single, commercially available second antibody conjugate is utilized, thus eliminating the problems of preparing and storing many different conjugated antisera.

Finally, there is one clear advantage of the indirect test for those interested in virus surveys or indexing programs. Several authors have observed that sandwich ELISA precludes the detection of virus strains because of the extreme specificity of the double antibody sandwich (3,15). This specificity is not a problem in the indirect test, which can be utilized as a sensitive serological test for defining strain relationships (3,5,6,7,15).

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