Reuse of Coated Enzyme-Linked Immunosorbent Assay Plates

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ABSTRACT

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The dissociation reactions of four plant viruses: citrus tristeza virus (CTV), carnation mottle virus (CarMV), carnation yellow fleck virus (CYFV), and tobacco mosaic virus (TMV) and their respective γ-globulin alkaline phosphatase conjugates, sandwiched to antibody microplates, were examined. Treatment with 0.2 M glycine-HCl buffer pH 2.2 for 60 min caused the double antibody sandwiches of CTV and CarMV to dissociate from the antibody-coated microplates. In similar treatments CYFV was eluted less efficiently. The TMV double-sandwich remained undissociated by acidification but it could be partially dissociated under alkaline conditions (pH 12.1). The application of microplate recycling for economizing routine large-scale screening of CTV is described.

Additional key words: plant viruses, ELISA tests.

The microplate enzyme-linked immunosorbent assay (ELISA), based on the double-antibody sandwich method used by Voller et al (14) and Clark and Adams (6), is a reliable and highly sensitive method to detect several plant viruses (6,7,11,13). Recently we reported that citrus tristeza virus (CTV) can be detected by ELISA, and by using mechanical homogenization and bulk sampling of bark collected from fruit pedicels, field detection of CTV was achieved. The technique is routinely used in the CTV suppression program currently operating in Israel (1).

Use of ELISA in large-scale field tests imposes several economic problems associated with sample preparation and test costs. This paper examines the dissociation reactions of the microplate bound antibody-complexes of four plant viruses after acidification, and describes a simple technique that enables reusing microtiter plates for routine CTV diagnosis.

MATERIALS AND METHODS

Viruses and antisera. Tobacco mosaic virus (TMV) common strain was increased in tobacco (*Nicotiana tabacum*) and purified from tobacco leaves by polyethylene glycol (PEG) precipitation (9) and sucrose density gradient centrifugation (4). A rabbit was immunized by two intravenous injections of 2 mg virus at 1-wk intervals, followed by a single intramuscular injection at the 10th day with 2 mg of virus emulsified in Freund's complete adjuvant.

Carnation mottle virus (CarMV) was increased in *Chenopodium quinoa* Willd. leaves and purified according to Hollings and Stone (10) by PEG precipitation and sucrose density gradient centrifugation. A rabbit was immunized by intravenous injection of 5.8 mg of virus, followed by three intramuscular injections, spaced 1 wk apart, of 6.0, 5.0, and 2.5 mg virus emulsified with Freund's complete adjuvant. Carnation yellow fleck virus (CYFV) antiserum was used as serum to whole virus (CYFV-C) described recently (3).

The CTV antisera were (i) serum to fixed whole virus (CTV-FW) described by Gonsalves et al (8) and (ii) serum to fixed whole virus (ST strain) purified from Egyptian lime (*Citrus aurantifolia* (Christm.) Swing.) leaves and bark (2). A rabbit was immunized with CTV-ST by four intramuscular injections spaced 1 wk apart, each one consisting of about 0.2–0.3 mg of partially purified virus. Conjugates of enzyme (alkaline phosphatase, P-4502), and the purified γ-globulin of each antiserum were prepared by the glutaraldehyde binding technique (6).

Sample preparation. Control extract for CYFV and CarMV consisted of 1 g of healthy carnation leaves homogenized in 15 ml of extraction buffer (PBS-Tween + 2% polyvinylpyrrolidone (PVP 40) with the aid of an Ultra-Turrax (Janke & Kunkel, KG Staufen, West Germany) equipped with a T-18 shaft and generator.

For TMV and CTV controls, 2 g of tobacco and 1 g of Egyptian lime leaves were prepared similarly. Extracts of CarMV- and CYFV-infected carnation (*Dianthus caryophyllus* L.) leaves (1 g) and CTV (ST isolate)-infected Egyptian lime leaves (1 g) were prepared as the respective controls. The TMV-containing extract was prepared by adding purified TMV solution to a final concentration of 0.1 mg/ml in extract from a healthy plant.

Reagents. Microplates were from Dynatech (M 29 ARE) or Linbro Scientific Co. (IS MRC-96). Bovine serum albumin (A-4378), alkaline phosphatase (E.C. 3.1.3.1) P-4502, polyvinylpyrrolidone (PVP 40), and *p*-nitrophenylphosphate (104-0) were from Sigma Chemical Company, St. Louis, MO, USA. Glutaraldehyde (NC 5003) was from Polaron Equipment, Watford, England.

Assays. Enzyme-linked immunosorbent assays were done as described by Clark and Adams (6) and Bar-Joseph et al (1). Microplates were coated with 1.5, 6.0, 3.0, and 2.5 μg/ml of purified γ-globulin of CarMV, CYFV, TMV, and CTV-ST, respectively, and incubated for 3 hr at 33-35 C. The wells in each plate were filled, as shown in Fig. 1-a, with 0.2 ml per well of extracts of healthy or infected leaves prepared as described, and then incubated overnight at 6 C. After the extracts were removed, the plates were washed and filled with γ-globulin enzyme conjugate (0.2 ml per well) at a concentration of 1, 2, 3.5, and 3 μl/ml for CarMV, TMV, CYFV, and CTV-FW, respectively, and incubated for 3 hr at 33-35 C.

The conjugates were removed and the plates were washed three times with PBS-Tween. Enzyme substrate, *p*-nitrophenylphosphate (P-NPP), was added and after 30–90 min of incubation the contents of each of the two wells were carefully transferred to tubes that contained 0.1 ml of 3 M NaOH. Absorbance measurements at 405 nm (6) were performed in a Varian Techtron 635 spectrophotometer. After being washed, the plates were used for the dissociation tests.

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RESULTS AND DISCUSSION

Double-antibody sandwich dissociation tests. For many antigen-antibody complexes, dissociation can be accomplished by adjusting the pH to 2.0–3.2 (5). We examined the possible elution of CTV, CarMV, CYFV, and TMV antigens and their respective conjugates from the plate-bound antibodies by acidification.

Plates used previously in the ELISA test were washed twice with PBS-Tween, rinsed with distilled water, and finally shaken dry. Glycine-HCl buffer 0.2 M, pH 2.2, 2.5, and 2.8, was added for 2, 30, and 60 min according to the scheme in Fig. 1b. After removal of the glycine buffer, the plates were washed three times with PBS-Tween and 0.2 ml of healthy or virus-containing extracts added to each well as shown in Fig. 1c. The plates were incubated overnight at 6 C, washed three times with PBS-Tween. Then γ-globulin enzyme conjugates, at the concentrations described, were applied for 3 hr at 33–35 C. The plates were washed again and 0.2 ml of P-NPP substrate was added. The ensuing reactions were stopped by adding 3 M NaOH. The ELISA values for the four virus-elution combinations are summarized in Figs. 2, 3, 4, and 5. The dissociation efficiency (DE) of various treatments was calculated using the formula

$$DE = (1 - \frac{Aa - Ac}{Ab - Ac}) \times 100$$

in which

Aa = ELISA value ($A_{405\ nm}$) in wells that contained infected sap at first and healthy sap after elution

Ab = infected sap both at first and after elution

Ac = healthy sap both at first and after elution with 0.2 M glycine-HCl at pH 2.2.

Elution for 60 min with 0.2 M glycine-HCl pH 2.2 buffer almost completely dissociated CTV (DE 94%) and CarMV (DE 99%) antigens and their conjugates from their plate-bound antibodies (Figs. 2 and 3). Similar treatments for CYFV double-sandwich (Fig. 4) gave only DE 75%, whereas the TMV double-sandwich (Fig. 4) was only slightly affected (DE 7%). These results were reproduced in five tests for each virus combination carried out with Dynatech M 29 ARE plates and in one test of a Linbro IS-MRC-96 microplate. Similar treatments applied to Dynatech M 129 A plates were less successful, possibly indicating that the quality of the plate affects the elution reaction. The dissociation also proved ineffective for TMV-reacted plates when 0.2 M, glycine-HCl at pH 2.0, or 0.01 M acetic acid was applied up to 60 min (Fig. 5). The pH of glycine-HCl markedly influenced DE. Elution for 60 min at pH 2.5 gave satisfactory results for CarMV (DE 94%) but similar treatment for CTV (DE 60%) was unsatisfactory. Low DE values of 29% and 37% were obtained with CTV and CarMV-reacted plates eluted at pH 2.8. For both CTV and CarMV 2 min of elution gave slightly lower DE values of 85% and 93%, respectively, compared with a 60-min elution treatment.

It is generally agreed that antigen-antibody complexes become increasingly stable with time (5). We have examined the efficiency of dissociation of the CarMV and CTV double-sandwiches formed 0.5, 24, and 48 hr before washing with 0.2 M glycine-HCl at pH 2.2 but found no differences in elution efficiency at the three time intervals. The TMV complex (when not bound to the solid phase as in the ELISA double-sandwich) is dissociable by acidification (12). Short treatment with cold 0.1 M glycine pH 2.5 was reported by Langenberg and Schlegel (12) to release γ-globulin from TMV. Although acidification usually reverses antigen-antibody combinations, this may not be a general rule. Strain-specific differences in DE were found recently in elution tests of microplate bound double-sandwiches of certain *Rhizobium* isolates (Bar-Joseph and Kishinevski, *unpublished*). Campbell and Weliky reported that 2,4-dinitrophenyl hapten and its specific rabbit antibody do not dissociate in dilute acid but apparently will dissociate under alkaline conditions (5). When alkaline conditions for TMV were used (0.2 M KCl-NaOH and 0.2 M NaCl-NaOH, both at pH 12.1), greater dissociation was accomplished, ie, DE 73% and 79%, respectively, (Fig. 5); but neither treatment enabled complete elution of the virus-antibody complex.

Because the antibodies coated on the plate remain bound after glycine-HCl dissociation, one-step affinity separation of antigens

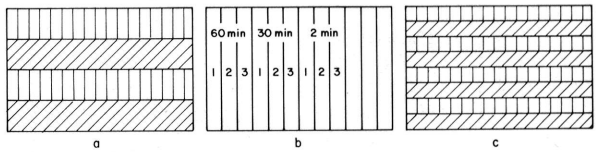

Fig. 1. A microplate scheme for enzyme-linked immunosorbent assay illustrating **a)** distribution of the wells in which extracts of healthy and virus-infected leaves were applied, **b)** dissociation washing for different times with (1) 0.2 M glycine-HCl, pH 2.2, (2) pH 2.5, (3) pH 2.8, and **c)** test sample distribution after the double-sandwich complexes were dissociated. ▥ = extracts of healthy leaves; ▨ = extracts of virus-infected leaves.

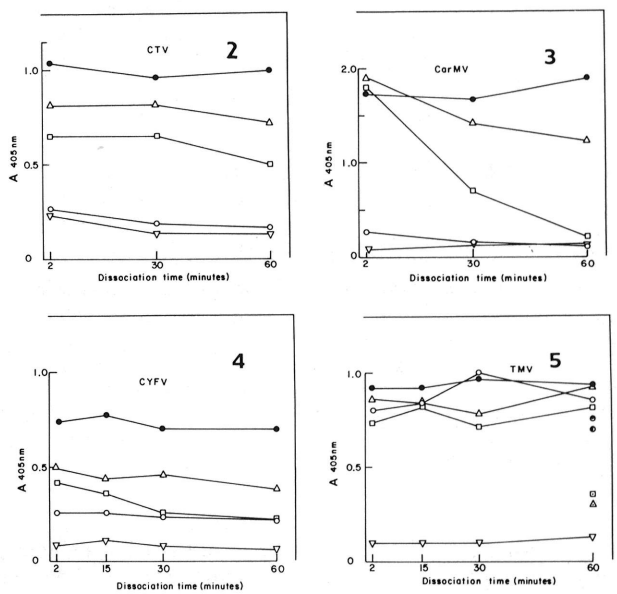

Fig. 2-5. Tests of double antibody-sandwich dissociation on enzyme-linked immunosorbent assay (ELISA) microplates. **2)** Dynatech M 29 ARE plates were coated with 2.5 μg/ml of anticitrus tristeza virus (CTV-ST) γ-globulin. Extracts of healthy and CTV-ST infected Egyptian lime leaves were placed in wells according to the scheme in Fig. 1-a. After the addition of CTV-FW/enzyme/conjugate (3 μl/ml), the plates were washed with PBS-Tween 20 and *p*-nitrophenylphosphate (in substrate buffer) was added for 30 min. The plates were washed with PBS-Tween, rinsed with distilled water, and shaken dry, and 0.2 M glycine-HCl pH 2.2, 2.5, and 2.8 was placed in wells for different times according to the scheme in Fig. 1-b. After the plates were washed with PBS-Tween, healthy sap, and CTV-infected sap were placed in wells according to the scheme in Fig. 1-c, and a repeated ELISA reaction occurred. Graph symbols: ELISA values obtained in wells that contained healthy sap both at first and after eluting with 0.2 M glycine-HCl buffer, pH 2.2 (▽); CTV-infected sap at first and healthy sap after eluting with 0.2 M glycine-HCl, pH 2.2 (o); pH 2.5 (□); pH 2.8 (△); infected sap at first and infected sap after elution with glycine-HCl at pH 2.2 (•). **3)** Antiserum to carnation mottle virus (CarMV) was coated 1.5 μg/ml and extracts of healthy and CarMV-infected carnation leaves were placed in wells according to the scheme in Fig. 1-a. After the addition of CarMV/enzyme/conjugate (1 μl/ml), plates were treated as described in the legend of Fig. 2. **4)** Antiserum to carnation yellow fleck virus (CYFV) was coated (6 μg/ml) and extracts of healthy and CYFV-infected carnation leaves were placed in wells according to the scheme in Fig. 1-a. After the addition of CYFV/enzyme/conjugate (2.5 μl/ml) plates were treated as described in the legend of Fig. 2. **5)** Antiserum to tobacco mosaic virus (TMV) was coated (3μg/ml) and extracts of healthy and TMV-containing sap (0.1 mg/ml) were placed in wells according to scheme 1-a. After the addition of TMV/enzyme/conjugate (2 μl/ml), plates were treated as described in the legend of Fig. 6. In addition, 0.2 M glycine-HCl pH 2.0 (◒), 0.01 M acetic acid (◐), 0.2 M NaCl-NaOH, pH 12.1 (◬) and 0.2 M KCl-NaOH pH 12.1 (⊡) were tested.

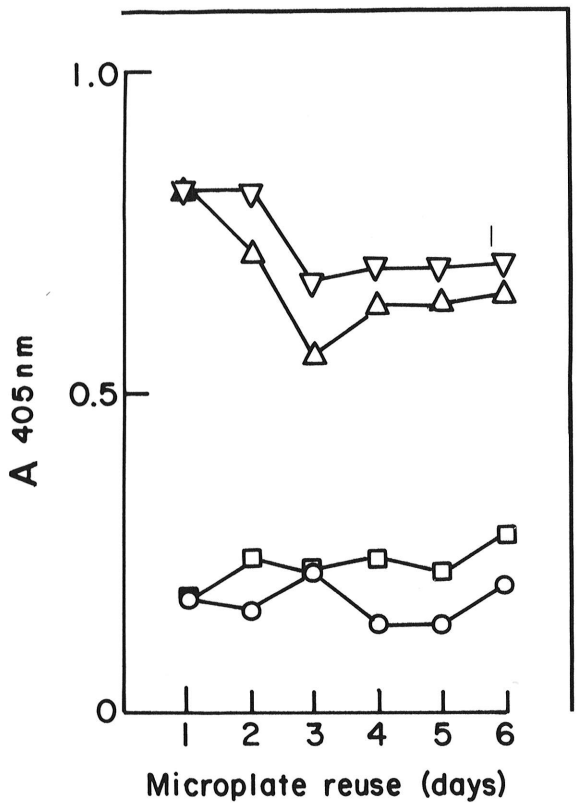

Fig. 6. ELISA values obtained after six repeated uses of a microplate that contained healthy and citrus tristeza virus-infected test samples. After completion of each cycle of the ELISA test, the plate was washed with PBS-Tween, rinsed with distilled water, and shaken dry, and 0.2 M glycine-HCl, pH 2.2, was applied for 60 min.Graph symbols: healthy sap both at first and after eluting by acidification, (o); CTV-infected sap at first and healthy sap after elution, (□); healthy sap at first and infected sap after elution, (△); CTV-infected sap both at first and after elution (▽).

in microplates can be performed. This process has been applied recently for separation of CarMV translation products (R. Salomon, et al, *unpublished*) and has enabled the separation of labeled coat protein from wheat-germ system components and from two other proteins synthesized in vitro.

Repeated use of microtiter plates for routine CTV diagnosis. During the last 2 mo microplate recycling was adopted for the daily indexing of 200–400 field samples tested for CTV. The recycling process consists of the following steps:

(i) coating with γ-globulin (1.5–3.0 hr at 33-35 C), (ii) test sample application (overnight at 6 C), (iii) conjugate application (1.5–3.0 hr at 33–35 C), (iv) substrate application and scoring results, (v) two 3-min washings with PBS-Tween followed by a short rinse with H_2O, (vi) 0.2 M glycine-HCl, pH 2.2, application for 60 min, and (vii) three washings with PBS-Tween.

Now the plate is ready for new test sample application. The results of five consecutive tests and glycine washing according to this scheme for CTV are summarized in Fig. 6. Clear positive results were obtained in rinsed wells to which infected sap was applied, whereas after dissociation these wells could be used for control sap. The ELISA values were only slightly higher than those obtained with wells to which both the first and the later application consisted of control sap.

Because the coating step used for new plates is not needed, the dissociation procedure does not involve extra work and the economic advantage (a single reuse means 50% less plate consumption) is obvious, especially for routine large-scale tests.

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