Use of $F(ab')_2$ Antibody Fragment in ELISA for Detection of Grapevine Viruses

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A modification of the enzyme-linked immunosorbent assay using the $F(ab')_2$ antibody fragment [$F(ab')_2$ ELISA] was developed for the detection of grapevine fanleaf (GFLV) and grapevine leafroll (GLRV-type III) viruses. The protocol was sensitive and rapid. Sensitivity of the test was further enhanced by pre-mixing the detecting antibody (probe) and the enzyme-conjugated protein A (anti-probe). Due to the acidic nature of grapevine tissues, different extraction buffer compositions were also evaluated.

KEY WORDS: grapevine fanleaf virus, grapevine leafroll virus, ELISA, $F(ab')_2$ ELISA, grapevine viruses

Different methods are available for the detection and identification of plant viruses. Among them, serological techniques are frequently preferred because of their speed, specificity, and simplicity. The Enzyme-Linked Immunosorbent Assay (ELISA) is a serodiagnostic test which has been widely adopted for the detection of plant viruses (3), especially for the handling of large numbers of samples. "Direct"-ELISA (D-ELISA) uses antiviral antibodies to both *(1)* recover virus from plant samples by binding them onto an ELISA plate and *(2)* detect bound virus. Detection is quantitated by a colorometric reaction catalyzed by an enzyme (*e.g.,* peroxidase, alkaline phosphatase, or other alternate enzymes) which is conjugated to the detecting antibody, which is the second antibody, applied after the virus is bound by the first antibody. "Indirect"-ELISA (I-ELISA) has the advantage of higher sensitivity and lower background levels (6). This is accomplished by the use of a different animal (*e.g.,* chicken, instead of rabbit) as a source of the second antibody. The benefits of I-ELISA must be weighed against the need of raising antiserum against the same virus in two separate animals, which is especially disadvantageous when dealing with large numbers of viruses, *i.e.,* different grapevine pathogenic viruses. However, it is possible to retain the benefits of I-ELISA, while avoiding the necessity for two sources of antiserum, by the use of the $F(ab')_2$ technique (2,6). This paper describes the application of this technology to the ELISA analysis of grapevine fanleaf and grapevine leafroll viruses.

Materials and Methods

Grapevine fanleaf virus (GFLV) was obtained from *Vitis rupestris* cv. St. George, increased in *Chenopodium quinoa,* and purified (5). Grapevine leafroll virus (GLRV) was extracted from an infected *Vitis vinifera* cv. Italia (LR101). Antiserum for GFLV was prepared at the University of California, Davis, and the antiserum for GLRV-type III was produced in cooperation with Dr. Dennis Gonsalves, Cornell University, Geneva, NY.

Sample preparation: Coating buffers (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3 per liter, pH 9.6); PBS (8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, and 0.2 g KCl, pH 7.4); 0.1 *M* borate buffer pH 9.6; 0.1 *M tris*-HCl, pH 9.6 and PBS containing 2% nicotine, pH 9.8 were evaluated as ELISA extraction buffers for grapevine tissues infected with GFLV and GLRV. All extraction buffers contained two percent w/v polyvinylpyrrolidone (PVP 40), 0.2% w/v bovine serum albumin (BSA), and 0.05% v/v Tween 20. When comparisons were made between D- and I-ELISA, all tissues were extracted using a polytron homogenizer (Brinkman) in the carbonate buffer plus additives (1 g tissue: 9 mL buffer).

Preparation of antibody and enzyme-conjugate: Antibody (Ab) was purified on a protein A-sepharose column (Sigma Chemical Co.). One-half gram of protein A-sepharose was rehydrated in 0.02 *M* sodium phosphate buffer, pH 7.3, and packed into a small column by running several bed volumes of buffer through the column. Then, 1 to 3 mL of antiserum was passed through the bed, permitting the binding of Ab onto the sepharose beads, and the column washed with 200 mL of phosphate buffer. Elution of Ab was with 0.1 *M* glycine buffer, pH 3.0. One-milliliter fractions were collected, immediately neutralized with 40 µL of 2 *M tris*-HCl pH 8.5, and fractions were scanned at 280 nm. The fractions containing purified Ab were pooled, mixed with glycerol (1:1; v:v) and stored at -20°C. The $F(ab')_2$ fragment was prepared by a modification of the method of Barbara and Clark (1). Briefly, 5 mg of purified Ab was adjusted to 0.1 *M* sodium acetate, pH 4.5, then 50 µL of 1 mg/mL pepsin in 0.1 *M* sodium acetate, pH 4.5 (Sigma Chemical Co.) was incubated with Ab at 37°C for 16 to 20 hours. Prior to loading onto a second protein A-sepharose column, the pH of the solution was raised to 8.0 to 8.4 (tris). After discarding the initial bed volume, a few mL of 0.02 *M* sodium phosphate buffer pH 7.3 was layered onto the column (at this pH the Fc region of an antibody binds to the sepharose beads while the $F(ab')_2$ fragments pass through the column.) $F(ab')_2$ fractions were detected by the absorption at 280 nm, as described above. The concentration of $F(ab')_2$ was adjusted to 1 mg/

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mL, and solutions stored at -20°C with glycerol (1:1).

Purified Ab was conjugated with peroxidase enzyme as previously described (7). The protein A-peroxidase conjugate was purchased from Sigma Chemical Co.

ELISA procedures: The $F(ab')_2$ method of Barbara and Clark (1) was used with some modifications; the polystyrene microtiter plates (Costar, flat bottom) were coated with 200 µL of virus-specific $F(ab')_2$ at 0.5 µg/mL in coating buffer and pre-incubated. The plates were washed in PBST (PBS containing 0.05% Tween 20) and 200 µL of sample extract spotted in each well (duplicate wells used per sample) and incubated. After the plates were rewashed, 200 µL of purified Ab (at 1 µg/mL) mixed with protein A-peroxidase conjugate (at 0.35 µg/mL) in PBST containing 2% PVP40 and 0.2% BSA were placed into each well and incubated. All incubations were for 1.5 hours at 37°C. After a final wash, the presence of the immobilized enzyme conjugates was quantitated by the hydrolysis of *O*-phenlenediamine, 200 µL of 0.7 mg/mL per well, in substrate buffer (7.3 g Na_2HPO_4, 5.2 g citric acid and 0.4 mL of 30% H_2O_2/L, pH 5.0). The plates were incubated in the dark for 30 minutes at room temperature and the color intensity measured with an ELISA reader (Emax, Molecular Devices) at A_{450}. The D-ELISA procedure described by Clark and Adams (3) was used without modification.

Results

Sample and antibody preparation: Different buffers were compared for the preparation of samples from different grapevine tissues. Table 1 lists the mean values for two wells of A_{450} generated in these various extraction buffers. The numerical values were expressed as the ratio obtained for the infected (I) extracts divided by healthy (H) extracts. With GFLV, the borate, carbonate, and PBS + nicotine buffers were similar in preserving viral antigenicity. The virus particles apparently lost most, if not all, of their antigenicity in *tris*-HCl or in PBS buffers. With GLRV, all extraction buffers except PBS were similarly effective in preserving viral antigenicity.

Table 1. Effect of various buffers and grapevine tissue extracts in the detection of GFLV and GLRV by $F(ab')_2$ ELISA.

Buffer[a]	Extract dilution	pH of extract	ELISA readings (I/H)[b] GFLV	GLRV
Borate, 0.1 *M*	1:5	7.2	66	51
pH 9.6	1:25	9.1	62	61
	1:125	9.3	19	32
Carbonate 0.05 *M*	1:5	6.7	63	54
pH 9.6	1:25	9.1	35	72
	1:125	9.4	9	26
PBS + Nicotine (2%)	1:5	7.9	56	69
pH 9.8	1:25	8.7	28	120
	1:125	9.0	9	55
Tris-HCl, 0.1 *M*	1:5	8.0	22	65
pH 9.6	1:25	8.7	12	52
	1:125	8.9	4	29
PBS, pH 7.4	1:5	4.1	1	34
	1:25	6.4	1	40
	1:125	6.9	1	21
PBS with the pH	1:5	7.5	4	87
readjusted to	1:25	7.2	2	75
7.0 to 7.5	1:125	7.2	1	32

[a]All buffers alone contained 2% PVP40, 0.2% BSA, and 0.05% Tween 20.

[b]Value expressed as the ratio of I/H, where values of 2 and higher are positive extracts.

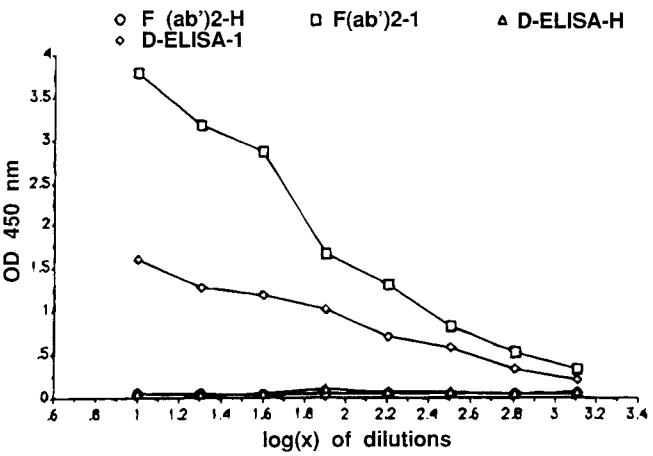

Fig 1. Comparison of direct- and $F(ab')_2$ ELISA using leaf extracts from GFLV-infected (I) and healthy (H) grapevines. Extractions and dilutions were prepared in carbonate buffer containing 2% PVP, 0.2% BSA, and 0.05% Tween 20 at 1:10 (w/v) dilution.

ELISA tests: In all experiments with crude extract of GFLV-infected tissue, the $F(ab')_2$-ELISA was superior to D-ELISA. The absorbance values were more than twice for $F(ab')_2$-ELISA (*i.e.*, A_{450} of 3.75) in comparison with D-ELISA values (A_{450} of 1.60) (Fig. 1). Healthy controls yielded absorbance values of 0.1 or less. With GLRV, the results of both protocols appeared similar (Fig. 2). The A_{450} values were 2.0 and above at extract dilutions of 1:10. Healthy tissue extracts were 0.1 and below. Overall, the optimal concentrations for the reagents used in the $F(ab')_2$-ELISA were 0.35 to 1.0 [$F(ab')_2$ fragment], 0.5 to 1.0 (probe), and 0.2 to 0.7 µg/

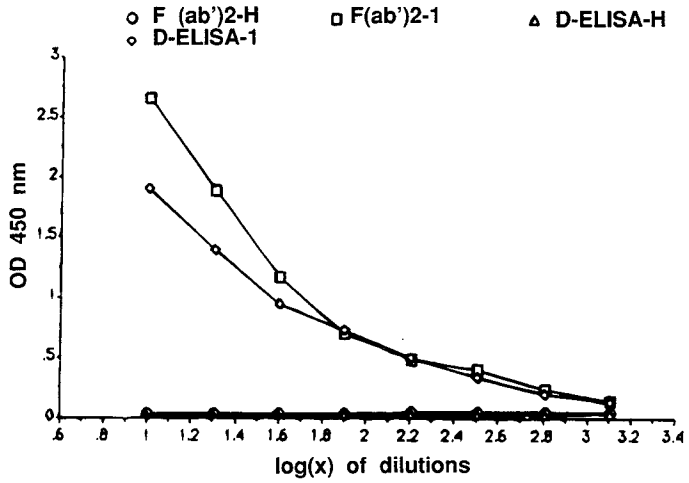

Fig. 2. Comparison of direct- and $F(ab')_2$ ELISA for GLRV-NY1. Leaf extract and dilutions were prepared as described in Figure 1.

mL (anti-probe enzyme conjugate) for GFLV and 0.5 to 1.0 [$F(ab')_2$ fragment and probe] and 0.2 to 0.7 μg/mL (anti-probe-enzyme conjugate) for GLRV.

Discussion

Different modifications of ELISA have been developed (2,6). They are generally divided into two categories: *i.e.,* D-ELISA and I-ELISA. In D-ELISA the probe antibody (Ab) is conjugated with peroxidase, while in I-ELISA a second Ab specific to the probe antibody, or in the system described here, protein A [in $F(ab')_2$ ELISA] is conjugated to peroxidase for the eventual quantitation of the specifically bound primary antibody.

By definition, $F(ab')_2$ ELISA is an example of I-ELISA because the primary bound antibody is detected by a separate enzyme-conjugated probe, in this case protein A conjugate (6). In this system, virus-specific Ab was produced only once. The ELISA plate was coated with the $F(ab')_2$ fragment derived from this Ab to trap the virus, then whole Ab is bound to the trapped virus in a second step. The virus is detected by a reaction with the second antibody, which is quantitated by a peroxidase-catalyzed colorometric reaction. The detection of the second antibody is by Protein A, which had previously been conjugated to peroxidase. The Protein A specifically binds to Fc region of an Ab; thus, in this system, no background can be generated by reaction of the Protein A with the primary antibody [the $F(ab')_2$ fragment] which coats the ELISA plate.

In general, the $F(ab')_2$ ELISA was found to be more sensitive than D-ELISA. The increased sensitivity is perhaps conditioned by the pre-mixing of the probe with protein A. This leaves the protein A free to react with several probe units, thus forming a multimeric complex (4). Overall, the $F(ab')_2$ ELISA protocol has the advantages over other ELISA systems in that it eliminates *(1)* the need to conjugate each separate source of virus-specific antibody and *(2)* the necessity to prepare antiserum from two different animal species as required in other modified I-ELISA systems.

Although grapevine leaf extract is acidic in nature, the antigenicity of GFLV, but not GLRV, was adversely affected if the buffering capacity of the extraction medium failed to hold around neutral (Table 1). With GFLV, readjustment of PBS extracts from pH 4.1 to 7.0-7.5 did not restore serological activity. Even though, PBS extractions of GLRV infected tissues were reactive at pH 4.1, raising the pH above 7.0 greatly increased the ELISA absorbance values. It is apparent that GLRV is stable at low pH, while GFLV appears to be less stable.

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