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RAPID DETECTION OF CUCUMBER MOSAIC VIRUS BY A SIMPLIFIED F(ab')$_2$ ELISA USING PEG

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INTRODUCTION

After the introduction of the enzyme-linked immunosorbent assay (ELISA) in plant virology, the method has been widely used for the detection of many plant viruses as being a method suitable for large-scale disease-indexing programs. However, it is laborious and time-consuming to analyze large numbers of samples. To overcome this, several modified forms of ELISA have been developed. Flegg and Clark (1979) described a modified ELISA based on simultaneous incubation of sample and enzyme conjugate for the detection of apple chlorotic leafspot virus. This procedure has been successfully applied to diagnosis of some other plant viruses, too[2, 7, 8, 12].

In this report we describe the comparison of direct and indirect procedures of ELISA with simultaneous incubation of sample and conjugate in relation to their suitability for rapid detection of cucumber mosaic virus (CMV).

MATERIALS AND METHODS

1. *Virus and Antiserum*

The yellow strain of CMV[11] was propagated in *Nicotiana tabacum* cv. White Burley and was purified by the method described previously[10]. Polyclonal antiserum[10] to the virus was prepared in a rabbit by four intramuscular injections of purified virus emulsified with an equal volume of Freund's complete adjuvant. The titer of the antiserum was 1:256 in an agar-gel double diffusion test. Before use the antiserum was absorbed with insolubilized proteins of healthy tobacco leaves[5].

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2. *Preparation of Immunoglobulins and Conjugates*

The γ-globulin fraction (IgG) from rabbit antiserum was purified by ammonium sulfate precipitation followed by DEAE-cellulose column-chromatography. $F(ab')_2$ fragments of IgG were obtained by digestion of IgG with pepsin (1:60,000, Sigma)[1, 9].

Anti-CMV IgG was conjugated with horseradish peroxidase (HRP) (Type I-C, 3,000 u/mg, RZ:3.29, Toyobo, Osaka) by a periodate-oxidation method[1, 9]. Protein A (Nakarai Pure Chem. Co., Kyoto) was conjugated simultaneously with HRP and bovine serum albumin (BSA) at a ratio of 0.5:1:0.75 (w/w) by the same method. Initial concentrations of IgG-HRP and Protein A-HRP conjugates were adjusted to 1 mg IgG/ml and 1.25 mg Protein A/ml, respectively. HRP-conjugates were stored at 4℃ after addition of 0.5% BSA and 0.02% sodium merthiolate (w/v).

3. *Simplified ELISA*

Simplified direct ELISA (D-ELISA) and indirect ELISA (I-ELISA) were essentially based on DAS ELISA[4] and $F(ab')_2$ ELISA[1], respectively. Wells of polystyrene microtiter plates (Nunc-Immuno Plate UII, Inter Med) were incubated for 3 hr at 30℃ with IgG (2 μg/ml, D-ELISA) or $F(ab')_2$ (2 μg/ml, I-ELISA) in coating buffer (0.05 M carbonate buffer, pH 9.6). After washing the wells with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST), 75 μl each of sap and IgG-conjugate (final dilution: 1/800) (D-ELISA) or the mixture of native IgG (final concentration: 2 μg/ml) and protein A-conjugate (final dilution: 1/2,000) (I-ELISA) were added to the wells at the same time, and then incubated at 30℃ or 37℃. The sap was prepared from infected and healthy tobacco plants by homogenizing leaf tissue in PBST containing 0.2% BSA and 2% polyvinyl pyrrolidone. After washing of the plates the enzyme substrate (0.5g/l *o*-phenylenediamine in 0.025 M sodium acetate buffer, pH 5.5, containing 0.06% hydrogen peroxide) was added and incubated for 20 min at 30℃ in the dark. The reaction was stopped by the addition of 50 μl of 3 M sulphuric acid to each well and the absorbance of the diluted reaction mixture was measured at 490 nm using a spectrophotometer.

RESULTS

1. *Comparison of Simplified D-ELISA and I-ELISA*

In preliminary experiments the optimum concentrations of antibodies and conjugates for the detection of CMV in sap from infected plants by both simplified procedures of ELISA were determined (see

materials and methods).

Both procedures were compared for their ability to detect CMV in serially diluted extracts from infected tobacco leaves and for the production of nonspecific reactions with healthy controls. As shown in Fig. 1, similar dose-response curves with a depression of the absorbance at the lowest dilution (at the highest virus concentration) of infected samples were obtained for both procedures. Although CMV could be reliably detected by both procedures, I-ELISA gave higher positive ELISA values and lower backgrounds than D-ELISA. The results of these experiments indicated that I-ELISA is superior to D-ELISA for detecting CMV, and therefore in subsequent experiments only I-ELISA was tested.

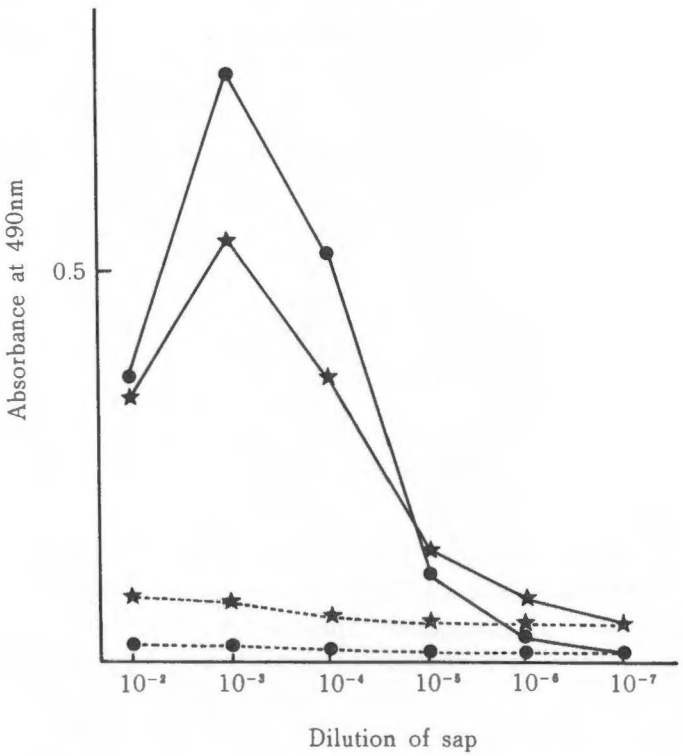

FIG. 1. Comparison of simplified D-ELISA and I-ELISA with simultaneous incubation of samples and conjugates for the detection of cucumber mosaic virus. D-ELISA and I-ELISA were based on standard DAS ELISA and F(ab')$_2$ ELISA, respectively.
★ : D-ELISA, ● : I-ELISA,
—— : infected, ····· : healthy.

2. *Effect of PEG in Simplified F(ab')$_2$ ELISA*

It is well known that immune reactions can be accelerated in the presence of polyethylene glycol (PEG). Various concentrations (1 to 6%) of PEG 6,000 were included in the reaction mixtures containing sample (plant extract diluted 1:100), IgG and protein A-conjugate, and the mixtures were incubated for 2 hr at 30℃. The ELISA values of infected extracts considerably increased by adding PEG to the reaction mixtures. The positive ELISA values increased with increasing PEG concentrations up till 3% PEG and decreased somewhat at higher concentrations. On the other hand nonspecific reactions markedly increased with increasing PEG concentrations (TABLE 1). Including 2 to 3% PEG in the reaction mixtures seemed to be optimal for the detection of CMV by simplified I-ELISA.

TABLE 1. Effect of addition of PEG in reaction mixtures in simplified F(ab')$_2$ ELISA for the detection of cucumber mosaic virus[a].

Concentration of PEG (%)	Experiment 1		Experiment 2	
	Healthy	Infected	Healthy	Infected
0	0.01[b]	0.60	0.02	0.76
1	0.02	0.97	0.04	1.22
2	0.02	1.34	0.04	1.35
3	0.03	1.36	0.04	1.25
4	0.04	1.34	0.07	1.21
5	0.08	1.33	0.12	1.18
6	0.43	1.26	0.43	1.10

a) F(ab')$_2$-coated wells of microplates were incubated simultaneously with sap, IgG and protein A-conjugate for 2 hr at 30℃.

b) Absorbance values at 490 nm.

The use of PEG in D-ELISA caused severe nonspecific adsorption of conjugate on the solid phase (data are not shown).

The influence of incubation time on ELISA values for infected and healthy extracts was tested by incubating reaction mixtures containing 2% PEG at 30℃ for various times. The ELISA values of infected samples with PEG increased with incubation time and reached a maximum at 2 hr after initiating the reaction. On the other hand the positive ELISA values of reaction mixtures without PEG gradually increased up to 3 hr and were lower than those obtained with PEG at the various incubation times (FIG. 2). The results indicated that an incubation time of 2 hr in simplified I-ELISA with PEG is sufficient for a reliable detection of CMV.

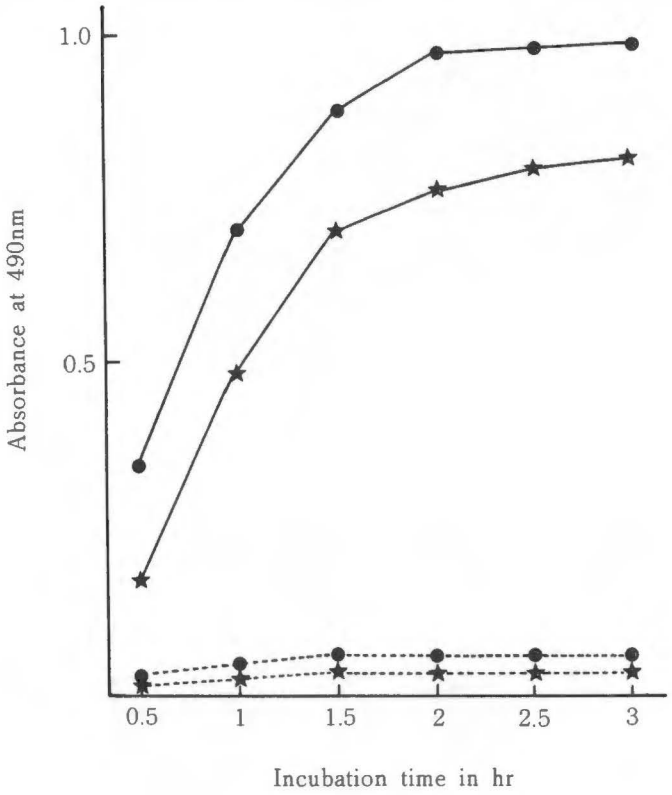

FIG. 2. Relationship between ELISA values and incubation time in simplified I-ELISA with or without PEG in the reaction mixtures. F(ab')$_2$-coated wells were filled with the mixture of sap (diluted 1:100), IgG and protein A-conjugate, and incubated at 30℃.
● : with PEG (3%), ★ : without PEG,
—— : CMV-infected, ------ : healthy.

3. *Detection of CMV by Simplified F(ab')$_2$ ELISA*

Nonspecific reactions in simplified I-ELISA with PEG were considerably decreased by addition of 1% Triton X-100 to the reaction mixtures without reducing specific ELISA values (data are not shown). The simplified I-ELISA described above was tested for the detection of CMV in infected tobacco plants. Serially diluted extracts from infected and healthy tobacco leaves were incubated with IgG and conjugate simultaneously in the presence of 2% PEG and 1% Triton X-100 for 2 hr at 30℃. The virus could be detected in extracts diluted 1:1,000,000 (FIG. 3).

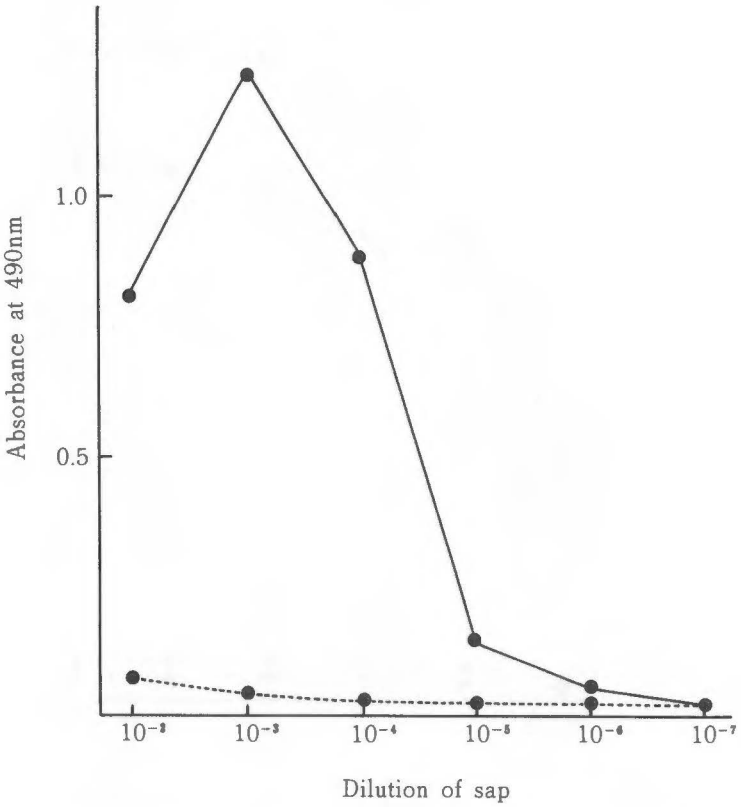

FIG. 3. Detection of cucumber mosaic virus by simplified I-ELISA. PEG (2%) and Triton X-100 (1%) were included in reaction mixtures and incubated at 30℃ for 2 hr.
—— : infected, ------ : healthy.

DISCUSSION

Many modified ELISA procedures have been described that reduce the assay time required for plant virus diagnosis. Simplified direct DAS ELISA with simultaneous incubation of virus samples and conjugates was first reported by Flegg and Clark (1979) to detect apple chlorotic leafspot virus which could not be detected by the standard procedure. This procedure has been successfully applied for the diagnosis of some plant viruses without reducing the sensitivity of the assay[2, 7, 8, 12].

In this study we compared direct and indirect procedures of simplified ELISA to evaluate their suitability for rapid detection of CMV. Reduced ELISA values in undiluted plant extracts as compared to di-

luted ones were obtained in both procedures. This phenomenon may be due to the reaction of antibodies with free virus particles which are present at a high concentration in those reaction mixtures, which in the end results in a decrease of conjugate (in D-ELISA) or IgG (in I-ELISA) molecules which bind to captured virus and immune complex on the solid phase[3].

I-ELISA showed better detection of CMV with less nonspecific reactions than the direct procedure. The higher background in D-ELISA seemed to be caused by the different type of conjugate used in that procedure. Barbara and Clark (1982) evaluated three types of Fc-specific conjugates for use in F(ab')$_2$ ELISA and demonstrated that the two immunoglobulin-based conjugates gave higher values with healthy sap than the protein A-conjugate. In our experiments a serially diluted IgG-conjugate (direct procedure) or a mixture of IgG and protein A-conjugate (indirect procedure) were tested. The ELISA values for infected sap increased with the conjugate concentrations in both procedures. However, the increase of nonspecific reactions with various conjugate concentrations was much less for the protein A-conjugate than for the IgG-conjugate. Furthermore, the protein A-conjugate could be used at higher dilutions than the IgG-conjugate (unpublished data).

The addition of PEG to the reaction mixtures in F(ab')$_2$ ELISA gave higher positive ELISA values and it reduced the time required to reach the highest ELISA values. It is not clear whether PEG accelerates the reaction between IgG and virus or/and between IgG and the protein A-conjugate. The addition of PEG in each reaction step in a standard procedure will eliminate these questions. Background values which increased in simplified F(ab')$_2$ ELISA using PEG considerably decreased by addition of Triton X-100. This may be due to Triton X-100 solubilizing green plant materials, which subsequently results in reduction of adsorption of IgG and conjugate to the solid phase.

The simplified ELISA described here has the big advantage that the same conjugate can be used for all viruses, and may therefore be useful for the rapid detection of other plant viruses, too.

SUMMARY

Direct and indirect procedures of double antibody sandwich ELISA with simultaneous incubation of sample and conjugate were compared for their suitability to detect cucumber mosaic virus in sap from infected plants. Although CMV could be reliably detected by both procedures, indirect F(ab')$_2$ ELISA using protein A-conjugate gave lower background levels than the direct procedure. In F(ab')$_2$ ELISA, includ-

ing 2% PEG in the reaction mixtures resulted in acceleration of the reaction and in higher ELISA values for infected samples. An incubation time of 2 hr was sufficient for reliable detection of CMV in sap infected plants by the procedure described.

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REFERENCES

1. Barbara, D. J. and Clark, M. F. 1982. A simple indirect ELISA using $F(ab')_2$ fragments of immunoglobulin. J. gen. Virol. 58 : 315-322.
2. Bar-Joseph, M., Garnsey, S. M., Gonsalves, D., Moscovitz, M., Purcifull, D. E., Clark, M. F. and Loebenstein, G. 1979. The use of enzyme-linked immunosorbent assay for the detection of citrus tristeza virus. Phytopathology 69 : 190-194.
3. Clark, M. F. 1981. Immunosorbent assays in plant pathology. Ann. Rev. Phytopathol. 19 : 83-106.
4. Clark, M. F. and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. 34 : 475-483.
5. Clark, M. F. and Bar-Joseph, M. 1984. Enzyme immunosorbent assay in plant virology. *In* Methods in Virology. 7 : 51-85.
6. Flegg, C. L. and Clark, M. F. 1979. The detection of apple chlorotic leafspot virus by a modified procedure of enzyme-linked immunosorbent assay (ELISA). Ann. appl. Biol. 91 : 61-65.
7. Franken, A. A. J. M., Maat, D. Z. and Kamminga, G. C. 1990. Detection of squash mosaic virus in seeds of melon (Cucumis melo) by enzyme-linked immunosorbent assay (ELISA). Neth. J. Pl. Path. 96 : 91-102.
8. Kawai, A., Kimura, S., Nishio, T. and Nagao, N. 1985. Detection for cucumber green mottle mosaic virus in cucumber seeds using enzyme-linked immunosorbent assay. Res. Bull. Pl. Prot. Japan 21 : 47-53.
9. Maeda, T. and Inouye, N. 1986. Detection of cucumber mosaic virus by enzyme-linked immunosorbent assay (ELISA). -Evaluation of various conjugates in double antibody sandwich method of ELISA-. Nogaku Kenkyu 61 : 119-127.
10. Maeda, T., Wakimoto, S. and Inouye, N. 1983. Serological properties of cucumber mosaic virus in Japan. Ann. Phytopath. Soc. Japan 49 : 10-17.
11. Tomaru, K. and Hidaka, Z. 1960. Strains of cucumber mosaic virus isolated from tobacco plants. III. A yellow strain. Bull. Hatano Tobacco Exp. Sta. 46 : 143-149.
12. Vuurde, J. W. L. van and Maat, D. Z. 1983. Enzyme-linked immunosorbent assay (ELISA) and disperse-dye immuno assay (DIE): comparison of some aspects of simultaneous and separate incubation of sample and conjugate for the routine detection of lettuce mosaic and pea early-browning viruses in seed. Neth. J. Pl. Path. 91 : 3-13.