岡大資生研報 (Bull. Res. Inst. Bioresour. Okayama Univ.) 5 : 23-30 (1997)

Rapid and Sensitive Detection of Cucumber Mosaic Virus by a Simplified ELISA Using Two Monoclonal Antibodies

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To detect cucumber mosaic virus (CMV), virus samples and conjugate were incubated together in a simplified double-antibody sandwich ELISA. The use of the same monoclonal antibody (MAb) as trapping (coating) and detecting antibodies resulted in considerable decrease of ELISA values and sensitivity due to the competition for antigen between trapping and detecting antibodies. The simplified ELISA using two MAbs which recognize different epitopes of CMV proved to be a rapid and sensitive method for virus detection.

Key words : Cucumber mosaic virus, Monoclonal antibody, Simplified ELISA

INTRODUCTION

Monoclonal antibodies (MAbs) have been used successfully in ELISA for detection of various plant viruses and MAb-based ELISA has proved to have some advantages over polyclonal antibodies (PAb)-based ELISA with regard to specificity, sensitivity and reproducibility of virus detection (Sander and Dietzgen 1984 ; Halk and De Boer 1985). Previously (Maeda *et al.* 1988), we demonstrated that the MAb conjugate to cucumber mosaic virus (CMV) could be used at higher dilutions (up to 8-fold) without decreasing the specific activity than PAb conjugate. Moreover, we showed that the sensitivity of ELISA with MAb conjugate for virus detection was also superior to that with PAb conjugate when PAb was used as a trapping (coating) antibody in the standard double-antibody sandwich ELISA (DAS ELISA). However, the

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Received January 22, 1997
This study was supported in part by a Grant-in-Aid for Developmental Scientific Research (No. 61860005) from the Ministry of Education, Science, Sports and Culture of Japan
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use of the same MAb for trapping and detecting antibodies in this system resulted in a considerable decrease of sensitivity.

In this study, we evaluated a simplified ELISA employing two MAbs which recognize different epitopes for detection of CMV in purified preparations and in crude extracts of infected plants.

MATERIALS AND METHODS

1. Virus and purification

The yellow strain of CMV (CMV-Y, Serotype Y) (Tomaru and Hidaka 1960) and CMV-Z (a strain obtained from *Zinnia elegans*, Serotype P) (Maeda *et al.* 1983) were propagated in *Nicotiana tabacum* cv. White Burley and purified by the method previously described (Maeda *et al.* 1983).

2. Polyclonal and monoclonal antibodies

Polyclonal antiserum to CMV-Y was prepared in a rabbit by intramuscular injection of purified virus emulsified with Freund's complete adjuvant (Maeda *et al.* 1983). The antiserum had a titer of 1 : 256 in an agar gel double-diffusion test. The antiserum was cross-absorbed with insolubilized healthy tobacco proteins before use (Clark and Bar-Joseph 1984).

The production of MAbs to CMV-Y was described previously (Maeda *et al.* 1988) and MAb-2 and MAb-4 were used in this study. The isotypes of MAb-2 and MAb-4 are IgG_{2a} and IgG_1, respectively, and both MAbs recognize the epitopes common to Y (Subgroup I) and P (Subgroup II) serotypes of CMV found in Japan. The competition tests using indirect ELISA revealed that the two MAbs used in this study recognize different epitopes of CMV.

3. Preparation of immunoglobulins and enzyme conjugate

The IgG (γ-globulin fraction) from rabbit antiserum was purified by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography. MAbs were affinity-purified from ascitic fluid by using Affi Gel-Protein A and MAb Purification Buffer (Bio-Rad). Rabbit polyclonal antibody (PAb) and MAbs were conjugated to alkaline phosphatase (Type Ⅶ-S, Sigma) by the glutaraldehyde one-step procedure as described by Clark and Adams (1977), using 2,500 units enzyme/mg immunoglobulin.

4. Simplified DAS ELISA

Simplified ELISA was performed according to the standard DAS ELISA described by Clark and Adams (1977) with some modifications. Wells of

polystyrene plates (Nunc Immuno-Plate II, Inter Med) were coated with immunoglobulins (2 μg/ml) in coating buffer for 3 hr at 37℃. The wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Then 75 μl each of virus samples and conjugate were added to the wells at the same time, and sample and conjugate were thoroughly mixed. The crude extracts were prepared from infected and healthy tobacco plants by homogenizing leaf tissues in PBST containing 0.2% BSA and 2% polyvinylpyrrolidone. After incubation of the plates at 37℃, the enzyme substrate was added and incubated at 30℃. The reaction was stopped by the addition of 50 μl of 3M NaOH to each well and the absorbance was measured at 405 nm using an immunoreader.

RESULTS

1. Comparisons of activities of two MAb-conjugates in simplified ELISA

Activities of alkaline phosphatase labeled MAb-2 and MAb-4 conjugates were compared for the detection of purified virus in a simplified ELISA. The wells of polystyrene plates were coated with PAb, and then serially diluted purified CMV, and MAb-2 or MAb-4 conjugate diluted 1 : 3200 were added at the same time. PAb-conjugate diluted 1 : 800 was also included in the experiments as the standard system. Plates were incubated for 2 or 3 hr. As shown in Table 1, the MAb-2 conjugate gave higher ELISA values than the MAb-4 conjugate within the range of antigen concentrations tested in the two different experiments.

Table 1 Comparison of alkaline phosphatase labeled polyclonal (PAb) and monoclonal antibodies (MAb) for the detection of purified cucumber mosaic virus by simplified DAS ELISA[a)]

Virus concentration (ng/ml)	2hr incubation			3hr incubation		
	PAb	MAb-2	MAb-4	PAb	MAb-2	MAb-4
0	0.04[b)]	0.03	0.02	0.01	0.02	0.02
1	0.05	0.02	0.02	0.04	0.04	0.02
10	0.14	0.11	0.05	0.24	0.20	0.08
100	0.55	0.50	0.20	0.77	0.72	0.34
1000	0.86	0.80	0.47	1.40	1.42	0.87

a) PAb (2 μg/ml) was used as trapping (coating) antibody. PAb and MAb conjugates were used at dilutions of 1 : 800 and 1 : 3,200, respectively.
The conjugate and purified virus were incubated together for 2 or 3 hr.

b) The average of ELISA values for two experiments.

2. Effect of combination of MAbs on detection of CMV in simplified ELISA

Since the two MAb conjugates diluted 1 : 3200 had relatively higher activities in simplified DAS ELISA, the sensitivities of virus detection by simplified ELISA using one MAb or two MAbs in combination were compared. The wells of plates were coated with MAb-2 or MAb-4, and then serially diluted purified virus and MAb conjugate were incubated together. Regardless of MAb species the use of the same MAb as trapping and detecting antibodies markedly decreased ELISA values and markedly decreased the sensitivity of virus detection. In contrast, the ELISA in which two MAbs were used in combination gave higher sensitivity (Fig. 1). Although using the two MAb conjugates we could detect 1 ng/ml of purified virus when another MAb was used as trapping antibody, the ELISA value was higher for the MAb-2 conjugate than for the MAb-4 conjugate at higher virus concentrations. ELISA values were lowered at the highest concentration of virus (10 μg/ml) in some combinations of MAbs .

As the detecting system employing MAb-4 as trapping and MAb-2 as detecting antibodies gave satisfactory results, this system was adopted in subsequent experiments. By this system, we could detect efficiently the

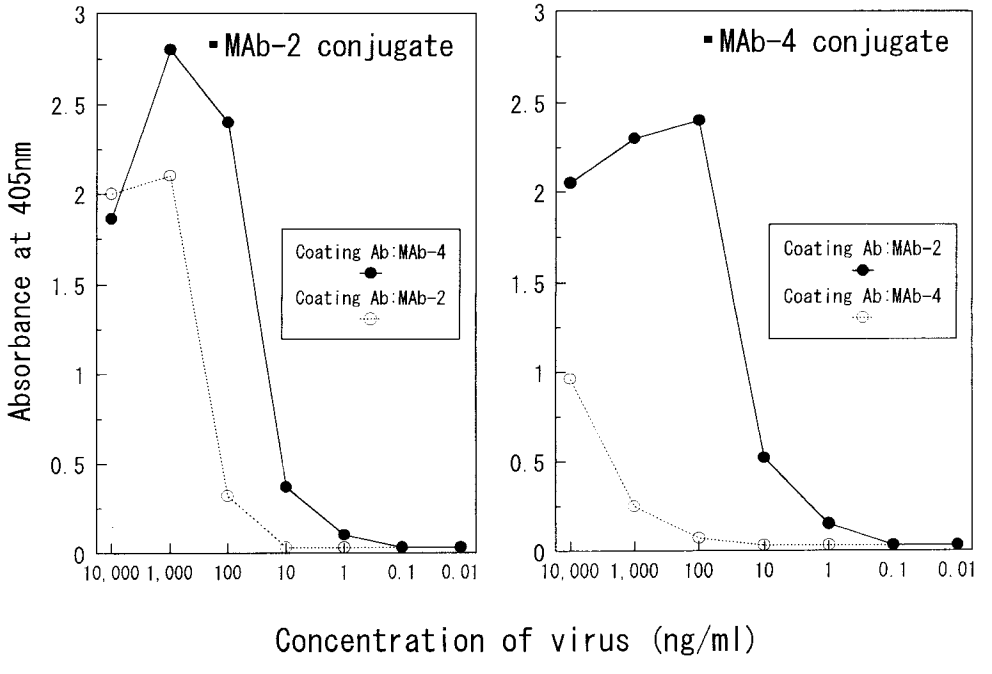

Fig. 1. Effect of combinations of monoclonal antibodies on detection of purified cucumber mosaic virus by simplified ELISA. The wells of plates were coated with MAb-2 or MAb-4, and then homologous or heterologous MAb conjugate and virus samples were incubated together for 3 hr at 37℃.

CMV-Z strain (P serotype) with ELISA values and sensitivity similar to those for CMV-Y (Y serotype) (data not shown).

3. *Detection of CMV from infected plants by simplified ELISA using two MAbs*

The simplified ELISA using MAb-2 as trapping and MAb-4 as detecting antibodies proved to be a sensitive and rapid system for detection of CMV. This system was applied to detect CMV from infected plants. The wells of plates coated with MAb-4 were loaded with extracts of CMV-infected *N. tabacum* cv. White Barley and MAb-2 conjugate simultaneously, and then incubated for 1, 2 or 3 hr at 37℃. The virus could be detected in extracts diluted 1 : 1,000,000 when the reaction mixture was incubated for 3 hr (Fig. 2). Although the reduction of incubation time gave lower ELISA values and sensitivity, the detection endpoint was 1 : 100,000 for 1 hr incubation time.

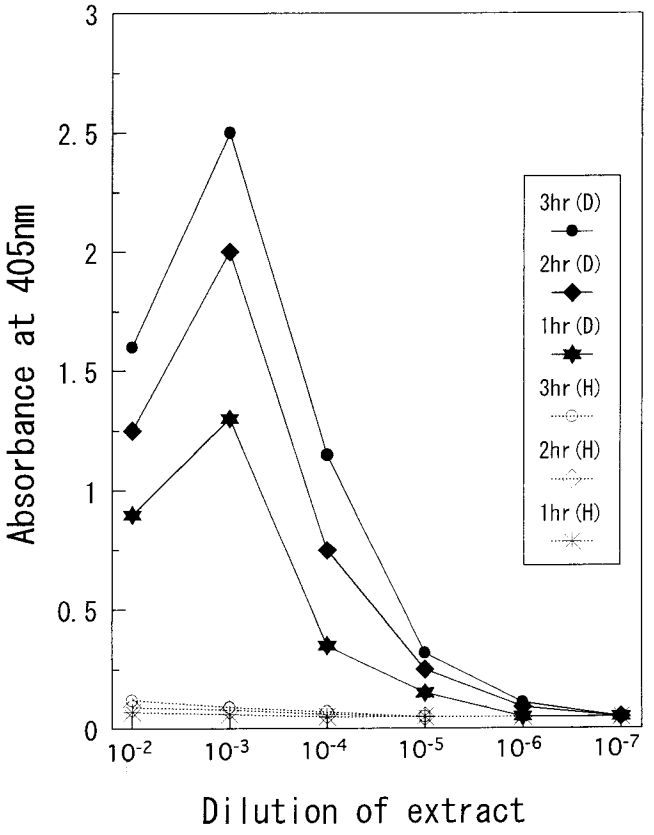

Fig. 2. Detection of cucumber mosaic virus in extracts of infected tobacco plants by simplified ELISA using two monoclonal antibodies. The wells of plates were coated with MAb-4 and then virus samples and MAb-2 conjugate were incubated together for 1, 2 or 3 hr at 37℃.

DISCUSSION

Enzyme-linked immunosorbent assay (ELISA) proved to be a powerful tool for detection of many plant viruses. However, it is laborious and time-consuming to analyze a large number of samples. To overcome this disadvantage, several modified procedures of ELISA have been developed. Flegg and Clack (1979) described a simplified ELISA based on simultaneous incubation of sample and conjugate for detection of apple chlorotic leaf spot virus. This procedure has been successfully applied to diagnosis of several other plant viruses, such as lettuce mosaic virus, pea early-browning virus (Van Vuurde and Mart 1983), peach rosette mosaic virus (Stobbs and Barker 1985) and rice stripe virus (Takahashi *et al.* 1987).

In this study, we evaluated the use of MAbs in simplified ELISA for detecting CMV. Previously (Maeda *et al.* 1988), we found that the use of the same MAb as trapping (coating) and preparation of enzyme conjugate (detecting antibody) in standard DAS ELISA for detecting CMV resulted in considerable decrease in ELISA values and sensitivity due to competition for antigen between trapping and detecting antibodies. This phenomenon was also prominent in simplified ELISA based on simultaneous incubation of virus samples and conjugate. In this study, two MAbs which recognize different epitopes on CMV were applied to a simplified ELISA to overcome this problem. In simplified ELISA using PAb as trapping antibody, the MAb-2 conjugate had higher activity than the MAb-4 conjugate although the indirect ELISA titer of MAb-4 was the same as that of MAb-2 (Maeda *et al.* 1988). In simplified ELISA using MAb, the combination of MAb species markedly affected the ELISA values and sensitivity for the detection of virus. The use of the same MAb as trapping and detecting antibodies resulted in considerable decrease in ELISA values and sensitivity. This is evidence that decrease in ELISA values is due to competition between two reaction phases. Hill *et al.* (1984) also reported that the use of the same MAb in double-antibody sandwich radioimmunoassay decreased in sensitivity compared with the use of two MAbs which recognize different epitopes for detection of soybean mosaic virus.

In this study we developed a rapid and sensitive simplified ELISA using two MAbs that recognize different epitopes on CMV antigens. Moreover the use of MAbs which recognize the same epitopes of two serotypes of CMV be useful for a wide range of detection of CMV strains in ELISA. This system will be a reliable and rapid method for diagnosis of CMV infections in various crops.

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2種のモノクローナル抗体を用いた簡易ELISAによるキュウリモザイクウイルスの迅速・高感度検出

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キュウリモザイクウイルス（CMV）に対するモノクローナル抗体（MAb）をコンジュゲートと試料を同時に反応させる簡易DAS-ELISAに適用したところ，同じMAbを捕捉抗体と検出抗体に使用した場合，ELISA値がかなり低くなり検出感度も低下した．一方，CMVの異なるエピトープ（抗原決定基）を認識する2種のMAbを用いた場合には，高感度な検出が可能であったことから，この検出システムはCMVの迅速で高感度な検出法であることが示された．

キーワード：キュウリモザイクウイルス，モノクローナル抗体，簡易ELISA