A Dot Immunobinding Assay for the Detection of Tobacco Mosaic Virus in Infected Tissues

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SUMMARY

A dot immunobinding assay (DIBA) was modified for the rapid detection of plant viruses in infected tissues by avoiding non-specific reactions and minimizing the amounts of antibodies used. With this method, less than 1 ng of tobacco mosaic virus could be detected in several milligrams of infected tobacco leaves. This simple, rapid and sensitive assay should prove to be a useful and practical diagnostic technique for plant virus diseases.

Enzyme-linked immunosorbent assay (ELISA), especially the double antibody sandwich form (Clark & Adams, 1977), is a routine method for detecting and assaying plant viruses because it is very sensitive. However, the method is rather laborious and time-consuming and needs some special equipment. Recently, the technique of immunoblotting described by Towbin et al. (1979) was simplified to a dot immunobinding assay (DIBA) by Hawkes et al. (1982) in order to assay monoclonal antibodies. Techniques essentially the same as DIBA, named Dot-ELISA and NC-ELISA were reported by Pappas et al. (1983) and Bode et al. (1984), respectively. The principles of DIBA are almost the same as those of ELISA, differing only in that antigen or antibody is bound to nitrocellulose and that the product of the enzyme reaction is insoluble. In this paper we describe experiments with purified tobacco mosaic virus (TMV) and an extract of tobacco leaves infected with the virus, which show that DIBA is simpler and more rapid than ELISA.

The reagents and solutions were: 20 mM-Tris HC1 containing 0.5 M-NaCl, pH 7.5 (TBS); 0.05 °, o Tween 20 in TBS (TBS–TWEEN); 2% polyvinylpyrrolidone (PVP, mol. wt. 40000, Sigma) and 2% bovine serum albumin (BSA, fraction V, Sigma) in TBS–TWEEN (blocking buffer); 2% PVP and 0.2% BSA in TBS–TWEEN (antibody buffer); 0.1 M-Tris–HCl containing 0.1 M-NaCl and 5 mM-MgCl₂, pH 9.5 (AP buffer). All buffers contained 0.02% sodium azide as a preservative. Colour development solution for alkaline phosphatase was AP buffer containing 0.33 mg/ml nitro blue tetrazolium (NBT, grade III, Sigma) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP, Sigma), prepared just before use as follows (Leary et al., 1983). One mg NBT was dissolved in 3 ml AP buffer by mixing vigorously for 1 to 2 min, then centrifuged for 5 min at 10000 r.p.m. One-half mg BCIP was dissolved in 10 μl N,N-dimethylformamide by mixing with a vortex mixer for 1 min and added dropwise with gentle mixing into the NBT solution.

Virus antigen was either a purified preparation of TMV (OM strain, Nozu & Okada, 1968) diluted in TBS–TWEEN or a clarified extract of tobacco leaves (Nicotiana tabacum cv. Xanthi) infected with TMV-OM. The extract was made by grinding about 25 mm² (about 5 mg) of leaf in 40 μl TBS–TWEEN and, after removal of the debris, centrifugation for 5 min at 10000 r.p.m. Freeze-dried tobacco powder was prepared from about 250 g of healthy tobacco leaves (cv. Xanthi). These were frozen at -70 °C and ground in 250 ml 0.1 M-phosphate buffer pH 7.2, containing 0.1% thioglycollate. After centrifugation at 8000 r.p.m. for 15 min, the supernatant
fluid was filtered through cheesecloth, made 4% (w/v) in polyethylene glycol (mol. wt. 6000) and 0.2 M in NaCl and kept on ice for 30 min. The precipitate was collected by centrifugation at 8000 r.p.m. for 15 min and resuspended in 100 ml 0.1 M-phosphate buffer pH 7.2, containing 10 mM-EDTA. After centrifugation at 10000 r.p.m. for 10 min, the supernatant fluid was centrifuged at 28000 r.p.m. for 1 h, and finally the pellet was freeze-dried in vacuo overnight, pulverized with a grinder, and stored at -70 °C.

Anti-TMV-OM rabbit antiserum solution (titre of 1/1024 in tube precipitin tests) diluted 1/200 to 1/2000 with antibody buffer was mixed with 10 mg/ml freeze-dried powder for 2 h at 37 °C. The mixture was centrifuged at 15000 r.p.m. for 10 min to remove the powder and the procedure was repeated twice using 5 mg/ml powder. The final absorbed antiserum solution (first antibody solution) was stored at 4 °C. The second antibody solution was anti-rabbit IgG goat IgG-F(ab')2 conjugated with alkaline phosphatase (enzyme concentration 9852 units/ml, Tago, Burlingame, Ca., U.S.A.), diluted 1/2000 in antibody buffer.

For the following DIBA procedures, all steps were performed at room temperature. A nitrocellulose sheet (15 × 9.2 cm, pore size 0.45 μm, Bio-Rad) was cut to an appropriate size, marked with a grid of 1 × 1 cm squares with a soft pencil and immersed in TBS so as not to entrap air, left for 15 min and dried on filter paper for 5 min. Sheets were always handled using forceps and vinyl gloves. Antigen solutions (1 μl) were then dotted onto each square and allowed to dry for 5 min. The sheet was then put in blocking buffer for 30 min, blotted briefly with filter paper, transferred onto a glass plate and covered completely with about 25 μl/spot of the first antibody solution. The sheet together with the glass plate was incubated either in a moisture chamber or in a sealed plastic bag for 1 h. It was then rinsed briefly in distilled water, shaken gently for 20 min in each of two changes of antibody buffer, transferred onto a glass plate and incubated with the second antibody solution as above for 1 h. The sheet was again rinsed and washed as above, and then washed twice in AP buffer for 20 min with gentle shaking. It was then put on a glass plate, covered with about 25 μl/spot of the colour development solution, and incubated in the dark in a moisture chamber. Positive reactions began to appear as purple spots after 5 to 10 min, but the sheets were incubated for 2 to 4 h to complete the reaction and then washed for 10 min in 10 mM-Tris-HCl, 5 mM-EDTA, pH 7.5. The wet sheets were observed in transmitted light and a well-defined purple spot was regarded as positive. For quantitative assessment the reflectance of the spots was determined at 400 nm using a Shimadzu CS-910 chromatoscanner equipped with a Shimadzu C-R1B data processor. The sheets could be stored dry and re-wetted in distilled water to restore the colour intensity.

Fig. 1 shows a DIBA of purified TMV at concentrations of 1 μg/μl to 10 μg/μl using the first antibody solution diluted either 1/200 or 1/2000. The minimum amounts of TMV detected were 100 pg and 1 ng, respectively. One experiment, in which the purple spots were scanned and the integrated spot densities were plotted against the amounts of dotted antigens (Fig. 2), showed that the spot densities were proportional to the amounts of antigens dotted. Fig. 3 shows a result of DIBA to detect TMV in infected tobacco leaves using the first antibody solution diluted 1/2000. With the infected leaf extracts, positive reactions were observed up to an antigen dilution of 1/8000. The very faint purple spots in healthy samples were observed only up to a dilution of 1/80. The visible spot for a healthy extract diluted 1/8 in Fig. 3 is green from sap and not purple. When the first antibody solution was used at a dilution of 1/200 instead of 1/2000 in the same assay, the dilution endpoint of detection increased 10-fold, but the non-specific reactions of healthy extract also increased 10-fold. Thus for practical diagnosis, the optimum dilutions of the first antibody solution and the plant extracts were 1/2000 and between 1/80 and 1/800, respectively. When purified TMV was added to a 1/80 dilution of healthy sap to give concentrations of 1 μg/μl to 10 pg/μl, the minimum amount of TMV detected by DIBA was 100 pg, determined from the lowest concentration at which the intensity of the spot was darker than that of healthy sap without TMV (data not shown).

In order to compare the sensitivities of DIBA and ELISA, the same samples were assayed by DIBA and by ELISA according to the methods of Clark & Adams (1977) using alkaline phosphatase-conjugated γ-globulin of anti-TMV-OM antiserum. The minimum concentrations detectable by ELISA were 100 pg/ml for purified TMV and a dilution of 1/80000 for the leaf
Short communication

Diln. of 1st antibody | 1 µg | 100 ng | 10 ng | 1 ng | 100 pg | 10 pg |
1/200
1/2000

Fig. 1. DIBA of purified TMV. Samples were 1 µl of TMV at concentrations of 10^3, 10^2, 10, 1, 0.1 and 0.01 ng/µl. The first antibody solution was diluted either 1/200 or 1/2000.

![Graph showing calibration curve](image)

Fig. 2. Calibration curve plotted from data shown in Fig. 1 of the colour intensities of the spots against the amounts of dotted TMV.

Dilution

![Graph showing dilution](image)

Fig. 3. DIBA of TMV in infected leaves. Samples were 1 µl of extracts from TMV-infected or healthy leaves diluted 1/8, 1/80, 1/800, 1/8000, 1/80000 or 1/800000. The first antibody solution was diluted 1/2000.

extracts, respectively, by photometric measurement at 405 nm, and 1 ng/ml and 1/8000, respectively, by visual assessment. As the assay volume of a sample was 200 µl, the minimum amount of TMV detectable by photometric ELISA was 20 pg. Therefore the sensitivity of DIBA was five- to 10-fold lower than that of ELISA using photometric measurement but almost the same using visual assessment. When cucumber mosaic virus (CMV) was assayed by DIBA using the same methods as for TMV but using anti-CMV antiserum, the minimum amount of CMV
detectable was 100 pg in purified preparations and a dilution of 1/800 of an extract of CMV-infected tobacco leaf.

Non-specific reactions were the main problem with DIBA. In preliminary experiments, we tried to apply DIBA to detection of TMV in leaf extracts using the original methods described by Hawkes et al. (1982) in which the second antibody is conjugated to horseradish peroxidase. But this proved impracticable because high non-specific reactions were observed. Therefore we modified the method as described above so as to minimize non-specific reactions. Alkaline phosphatase was used as the conjugate enzyme to avoid reactions caused by the endogenous plant peroxidases. Adding both PVP and BSA in the antibody buffer decreased non-specific adsorption of the antibodies to some plant components. But the most important step was absorbing the antiviral antiserum with freeze-dried tobacco powder. Without absorption, the positive endpoint, as well as the intensity of the spot, of healthy leaf extracts were the same as those of infected ones. When the antiserum was absorbed with acetone-extracted powder of healthy tobacco leaves prepared as described by Otsuki & Takebe (1969), some non-specific reaction remained. Probably, with its great sensitivity, DIBA could detect even very small amounts of contaminating anti-tobacco antibodies. Using γ-globulin in place of antiserum as the first antibody gave no improvement. Similar results were obtained using chromatography paper (Whatman No. 1) as described by Esen et al. (1983) instead of the nitrocellulose sheet, but the sensitivity was 10-fold less, perhaps because the spot of antigen solution diffused more widely in the paper and also because the background became slightly purple in the final colour development step. Modification like that of ELISA to a double antibody sandwich procedure (Clark & Adams, 1977) so as to reduce non-specific reactions, was not possible for an indirect antibody method like DIBA. In order to reduce non-specific reactions further, it may be necessary to use either antibodies purified by the affinity chromatography or monoclonal antibodies. Adopting a biotin/avidin system may increase the sensitivity of DIBA.

In this paper, it is shown that DIBA is a valuable method because it is simple, economical, rapid and sensitive. Although some further technical improvements as mentioned above might be possible, DIBA appears to us to be a useful and practical diagnostic technique not only for TMV and CMV but also for other plant viruses.

REFERENCES


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