A Simple Indirect ELISA using F(ab')2 Fragments of Immunoglobulin

By D. J. BARBARA* AND M. F. CLARK

East Malling Research Station, Maidstone, Kent ME19 6BJ, U.K.

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SUMMARY

An indirect ELISA is described in which (i) virus is trapped by F(ab')2 fragments of specific IgG immobilized on a solid phase support, (ii) trapped virus is detected by intact IgG (from the same or a different antiserum) and (iii) positive reactions are identified using an enzyme conjugate specific for the Fc portion of IgG. Pepsin digestion of the Fc portion of the trapping antibody permits the use of a general purpose enzyme conjugate to discriminate between trapping and detecting antibody. Consequently, the assay requires only a single virus-specific antiserum which is often all that is available to the plant virologist. The assay was at least as sensitive for detecting small amounts of antigen as the standard double-antibody sandwich procedure and, for some viruses, two- to fourfold more sensitive. The improvement in performance resulted largely from lower and more consistent background reactions. Both assays were equally effective in revealing heterologous reactions when optimized for detecting homologous antigen. However, increased cross-reactions were obtained in the F(ab')2 procedure by the use of higher concentrations of detecting antibody. The assay is considered particularly suited for comparing antisera from different sources or of different bleeds from the same source, and for investigations involving so few tests that the effort or expense of preparing individual virus-specific conjugates is not justified.

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) is now well-established in plant pathology as a sensitive and practical method for detecting and assaying viruses and other pathogens (Clark, 1981a, b; Bar-Joseph & Garnsey, 1981). Its widespread acceptance is due in part to the general applicability of the standard double-antibody sandwich (DAS) protocol (Clark & Adams, 1977) to a variety of macromolecular antigens of diverse morphology and antigenic characteristics. This form of ‘direct’ assay necessitates the production of individual, virus-specific immunoglobulin–enzyme conjugates for each virus or antiserum to be tested. The considerable time and expense involved in producing suitable individual conjugates may be difficult to justify, particularly if relatively few tests are to be performed. Moreover, it has been reported that indirect assays such as those using virus-specific immunoglobulins from two different animal species and an enzyme–immunoglobulin conjugate specific for the second antibody are more sensitive (Bar-Joseph & Malkinson, 1980) and able to detect a wider range of related virus strains (van Regenmortel & Burckard, 1980) than the DAS procedure (direct ELISA). This paper reports the development of an ELISA that has the main advantages of an indirect procedure yet requires only a single virus-specific antiserum. This has been achieved by trapping antigen (virus) with F(ab')2 immunoglobulin fragments. Trapped virus is detected with whole immunoglobulin (as the second antibody) and a conjugate reacting only with the Fc portion of the immunoglobulin. Nomenclature for the various immunoglobulin fragments is taken from Campbell et al. (1970).
METHODS

Viruses and antisera. Arabis mosaic virus (AMV), plum pox virus (PPV) and apple mosaic virus (ApMV) were as described previously (Clark & Adams, 1977). Potato virus Y (PVY) was isolated from an infected potato plant and propagated in Nicotiana tabacum cv. White Burley. Hop latent virus (HLV) was originally obtained from mosaic-free hops from which the virus was transferred by grafts to hops cv. Eastwell Golding grown in the glasshouse. Mild (F) and severe (B) isolates of barley yellow dwarf virus (BYDV; Plumb, 1974) were kept in oats, cv. Blenda, and serially transferred using Sitobion avenae and Rhopalosiphum padi aphids respectively, raised on virus-free oats in the glasshouse.

The antiserum to PVY was supplied by Dr S. Hill (ADAS, Cambridge, U.K.) and the antiserum to rose virus 1 (RV1, an isolate of the apple serotype of prunus necrotic ringspot virus) was a gift from Dr R. Casper. Donkey antiserum to whole rabbit immunoglobulin G (IgG) was a gift from the Scottish Antibody Production Unit and that to the Fc portion of rabbit IgG (raised in goat) from Miles Laboratories. The donkey antiserum was rendered specific to the Fc region of rabbit IgG (as indicated by gel diffusion tests) by treatment with an immunoadsorbent gel consisting of F(ab’)2 fragments of rabbit IgG with bovine serum albumin as a carrier protein. The two components were polymerized with 2% (v/v) glutaraldehyde at 4 °C. Before use, the immunoadsorbent gel was thoroughly washed by alternately homogenizing the gel in phosphate-buffered saline/Tween (PBS/Tween) and 0.2 M glycine–HCl pH 2.8 to remove excess glutaraldehyde and unbound protein. Protein A (from Staphylococcus aureus) was purchased from Pharmacia.

Conjugate preparation. IgG was prepared and conjugated to alkaline phosphatase (ALP) as described by Clark & Adams (1977). Conjugation of IgG to horseradish peroxidase (HRP) was by a periodate oxidation method (Boorsma & Streefkerk, 1979) which links protein to HRP via carbohydrate moieties on the enzyme. HRP (4 mg/ml in distilled water) was activated by the addition of freshly prepared 0.1 M sodium periodate (0.2 ml/ml HRP solution). After shaking for 20 min at room temperature ethylene glycol was added (1 drop/ml) and the whole dialysed against 1 M sodium acetate pH 4.4 (three changes of buffer). The activated HRP was then mixed with IgG in 0.01 M sodium carbonate pH 9.5 (0.5 mg HRP to 1 mg IgG in 1 ml) and 0.2 M sodium carbonate pH 9.5 added dropwise to raise the solution to approx. pH 9.5. After shaking for 2 h at room temperature freshly prepared sodium borohydride solution (4 mg/ml in water) was added (0.1 ml/ml solution). After 2 h at 4 °C the conjugate was either dialysed extensively against PBS or precipitated with an equal volume of saturated ammonium sulphate, the precipitate collected by centrifugation, redissolved in 1 ml PBS and dialysed extensively against PBS. Protein A was conjugated to HRP and to bovine serum albumin (BSA; added as a carrier protein) simultaneously using the same procedure (0.5 mg protein A, 1 mg BSA, 0.75 mg HRP). All HRP conjugates were stored at 4 °C after the addition of glycerol (1:1, v/v).

Preparation of immunoglobulin fragments. F(ab’)2 and Fab’ fragments of IgG were prepared by pepsin digestion and cysteine reduction (Campbell et al., 1970). IgG (1 to 5 mg/ml in 0.07 M sodium acetate pH 4 with 0.05 M sodium chloride) was digested by the addition of pepsin (Sigma, 1:10000) in distilled water (45 mg/mg IgG) and incubation overnight at 37 °C. When Fab’ fragments were required the digestion mixture was brought to 0.05 M-cysteine by the addition of a 0.1 M solution in water. Originally F(ab’)2 was separated from enzyme and other digestion products by exclusion chromatography using Sephadex G-100. However, it was found subsequently that rapid de-salting on Sephadex G25 or dialysis against three changes of PBS was equally effective.

ELISA tests. DAS ELISA was performed as described previously (Clark & Adams, 1977). The substrate for HRP, prepared immediately prior to use, consisted of 0.5 g/l ortho-phenylenediamine in 0.025 M sodium acetate buffer pH 5.5 with 0.06% hydrogen peroxide.
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peroxide. Reactions were stopped after 30 min in the dark with 50 μl 3 M-sulphuric acid and absorbance was measured at 490 nm.

The F(ab')2 assay was essentially similar to the DAS procedure: (i) polystyrene microtitre plates were coated with virus-specific F(ab')2 (in sodium carbonate buffer pH 9-6, 2 to 4 h at 30 °C); (ii) test samples in PBS containing 0.5 ml/l Tween 20 (PBS/Tween) or in PBS/Tween containing 20 g/l polyvinylpyrrolidone (mol. wt. 44000) and 2 g/l ovalbumin (PVP/ov) were allowed to react with the immobilized F(ab')2 (overnight at 4 °C); (iii) virus-specific intact IgG was added to react with trapped antigen (3 h at 30 °C); (iv) enzyme conjugate reactive only to the Fc portion of IgG was added (3 h at 30 °C) (IgG and conjugate both in PBS/Tween or PBS/Tween plus PVP/ov); (v) immobilized enzyme was visualized by adding enzyme substrate (usually 20 min at 30 °C in the dark). Reactions were stopped with 50 μl 3 M-sulphuric acid and absorbance measured at 490 nm. Plates were washed between each stage with PBS/Tween.

RESULTS

Characteristics of the F(ab')2-based assay

Antigen trapping

The abilities of Fab' and F(ab')2 fragments of IgG to trap antigen were compared with that of whole IgG for several viruses. For these tests the DAS protocol was employed, trapped antigen being detected with virus-specific conjugates. Fab' fragments trapped very little virus and were not considered further. F(ab')2 preparations trapped similar amounts of virus as untreated IgG from the same source. However, it was necessary to use the F(ab')2 at a higher concentration (normally 2 μg/ml) than that determined as optimal for the equivalent whole IgG (0.3 to 1 μg/ml) in the DAS ELISA (Fig. 1). Such preparations were effective in trapping both isometric (BYDV and AMV) and filamentous (PVY, PPV and HLV) viruses.

Second antibody

Best results were obtained when the second (detecting) antibody was used at a concentration equivalent to 0.5 to 1 μg/ml IgG. Either partially purified IgG or unfractionated whole serum was suitable, similar results being obtained with both types of antibody preparation when applied with filamentous (PVY) and isometric (BYDV) viruses.

Conjugates

We have previously evaluated virus-specific HRP conjugates produced by the one-step glutaraldehyde (GTA) and periodate oxidation (P) procedures for their comparative effectiveness in DAS assays (Clark, 1981b). Both procedures produce complex molecules containing several globulin and enzyme moieties, but P conjugates can usually be used at greater dilutions than GTA conjugates, often with reduced non-specific reactions. Sensitivity of virus detection using P conjugates was similar to or better than that using GTA conjugates, with an increase in sensitivity of up to threefold being recorded for the P conjugates with some combinations of virus and antibody. Accordingly, further tests and comparisons between the DAS and F(ab')2-based assays were made using only P conjugates.

The three types of Fc-specific conjugates were compared for their suitability for use in the F(ab')2-based assays. All three gave good detection of trapped antigen (Fig. 2) but the two immunoglobulin-based (i.e. goat anti-rabbit Fc and donkey anti-rabbit Fc) conjugates gave unacceptably high reactions in the absence of specific virus antigen. The strength of these non-specific reactions was related to the concentration of the F(ab')2 used for coating. By
Fig. 1. Relative effectiveness of whole IgG and F(ab')2 fragments in DAS ELISA tests for trapping (a) BYDV and (b) PVY from plant extracts. Trapped virus was detected with virus-specific IgG–ALP conjugates. ●, ○, IgG; ▲, △, F(ab')2 fragments; ●, ▲, infected plants; ○, △, healthy plants.

Fig. 2. Relative effectiveness of three Fc-specific conjugates for detecting PPV in F(ab')2 ELISA. ●, ○, Protein A/BSA/HRP; ▲, △, goat anti-rabbit Fc; ■, □, donkey anti-rabbit Fc; ●, ▲, ■, infected plants; ○, △, □, healthy plants. Virus was trapped with 2 µg/ml PPV-specific F(ab')2 and detected with whole IgG at 1 µg/ml.

contrast, the protein A/BSA/HRP conjugate gave good detection of antigen at high dilutions with low non-specific reactions.

There are two possible explanations for the non-specific reactions produced by the immunoglobulin-based conjugates: (i) incomplete digestion of the IgG by pepsin leaving residual IgG in the F(ab')2 preparation used for coating; (ii) residual reactivity to F(ab')2 in the antisera. To test the first possibility, a preparation of PPV-specific IgG was treated with a higher concentration of pepsin (200 µg/mg IgG compared with 45 µg/mg IgG standard treatment). No significant reduction in the intensity of non-specific reactions was obtained when this preparation was compared with a standard F(ab')2 preparation, suggesting that incomplete digestion was not the cause of the non-specific reactions. An attempt was then made to remove residual anti-F(ab')2 activity in the conjugates by cross-absorption using
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Fig. 3. Comparison of the sensitivity of DAS and F(ab')₂ ELISA for detecting (a) PPV and (b) RV1.

Comparison with DAS ELISA

Sensitivity

The DAS and F(ab')₂ assays were compared for their sensitivity in detecting virus. In general, both permitted detection of virus to similar endpoints, especially when reactions were assessed visually. The comparison for PPV and for RV1 is illustrated in Fig. 3. Most other virus–antiserum combinations gave similar results although in some tests there was a slight (two- to fourfold) increase in sensitivity with the F(ab')₂-based assay, resulting from slightly lower and more consistent background reactions. Sensitivity was never less than with the DAS procedure.

Two situations were recorded in which the F(ab')₂-based assay offered a significant advantage. First, an antiserum raised to HLV performed only adequately, rather than well, in DAS assays, due to a tendency for the enzyme conjugate to bind non-specifically to the coating IgG, thereby producing high and variable control reactions. By contrast, the same antiserum performed well in a F(ab')₂-based assay, giving low non-specific reactions and more reliable detection of virus. Second, DAS assays of BYDV which occurs at low concentrations in plant extracts have given reliable detection, but the apparent titre of antigen has varied among tests. F(ab')₂-based assays were more consistent and in some comparisons with the DAS assay gave an increase in apparent titre of antigen of up to 25-fold.

Detection of heterologous antigens

The ability of the two assays to detect serologically related strains was investigated using ‘F’ and ‘B’ isolates of BYDV. An antiserum to an ‘F’ isolate, which reacted only weakly with the ‘B’ isolate in the DAS assay, gave only slightly better results in a F(ab')₂-based assay.
Fig. 4. Relative effectiveness of (a) F(ab')2 and (b) DAS ELISA for detecting 'F' and 'B' serotypes of BYDV using antiserum homologous for the 'F' serotype. For the F(ab')2 assay, 'F' and 'B' viruses were trapped using F(ab')2 at 2 μg/ml and detected with IgG at 1 μg/ml. For the DAS assay, 'F' and 'B' viruses were trapped using IgG at 0.5 μg/ml and detected with F-specific HRP conjugate at 1:1000 dilution. □, 'F' isolate; □, 'B' isolate; ■, healthy.

Fig. 5. Detection of 'F' and 'B' serotypes of BYDV by F(ab')2 ELISA using dilutions of antiserum homologous for the 'F' serotype. ○, 'F' isolate; ▲, 'B' isolate; □, healthy oat.

optimized for the detection of homologous antigen (i.e. to give greatest differentiation between 'F' virus and control values) (Fig. 4). In reciprocal tests the 'B'-specific antiserum did not react with the 'F' isolate in either the standard assay or a F(ab')2-based assay optimized for the detection of 'B' virus.

However, heterologous detection of either virus [immobilized by trapping with homologous F(ab')2] was obtained in the F(ab')2-based assay by using increased concentrations of detecting antibody. For example, an 'F' antiserum detected only 'F' virus when high dilutions of antiserum were used but detected both 'B' and 'F' viruses at low dilutions of antiserum (Fig. 5). Similar results were obtained in equivalent tests employing 'B' antiserum to detect homologous and heterologous viruses. In these experiments, the maximum strength of reaction was dependent on the amount of virus homologously trapped by F(ab')2 fragments. Thus, for either antiserum higher maximum absorbance values were sometimes observed with heterologous virus than with homologous virus. However, it is apparent that the extent to
which cross-reactions occur in this type of assay is a function primarily of the dilution of the antiserum preparation used as the detecting antibody.

**DISCUSSION**

In developing this assay we have attempted to formulate a procedure which combines the advantages of an indirect assay with the requirement of the DAS ELISA for a single antigen-specific antiserum. To be successful it was necessary that the procedure should be kept as simple as possible and that it should match, in terms of sensitivity of antigen detection, the performance of the equivalent direct procedure. There is little additional effort involved in making the F(ab')₂ preparation other than the requirement to incubate the salt-precipitated antibody fraction with pepsin for some hours. The need to subsequently chromatograph or dialyse the hydrolysate to remove the smaller products of digestion, which would otherwise compete with the F(ab')₂ for adsorption sites, is equivalent to the dialysis step following salt precipitation in the standard procedure. The extra effort needed for the digestion step is, in any case, more than offset by eliminating the need to prepare antigen-specific enzyme–antibody conjugates for each antigen to be assayed. The use of a single protein A–enzyme conjugate for all tests also adds a significant measure of reproducibility to the technique. Moreover, the potential for using dilutions of unfraccionated antisera in place of partially purified IgG as the source of the detecting antibody is a distinct advantage when comparing antisera or evaluating their suitability for use in this type of assay.

The F(ab')₂-based assay compared well with the DAS ELISA procedure. In none of the tests was its sensitivity less than that of the best comparable standard assay, while in several it performed significantly better, mainly because of lower and more consistent background values. This observed improvement applied also to the detection of virus in plants naturally infected with BYDV and of HLV. For both these viruses, but particularly for HLV, we have adopted the F(ab')₂ procedure in preference to DAS ELISA as being significantly better and more reliable for the routine serodiagnosis of virus in field infections. However, it is probable that the F(ab')₂-based assay will be particularly useful in small scale tests for detecting antigens, which do not warrant the production of individual conjugates, and for making direct comparisons of antisera from different sources or of different bleeds from the same source. For the latter application, the virus selected may be trapped reproducibly by a single F(ab')₂ preparation, following which dilutions of the test antisera can be reacted with the virus, antibody attachment being subsequently assayed using the protein A–enzyme conjugate.

Indirect assays employing specific antisera from two animal species have been observed to exhibit lower specificity in discriminating among serologically related viruses or strains of virus than direct assay methods (Bar-Joseph & Malkinson, 1980; van Regenmortel & Burckard, 1980; Torrance, 1980). In the F(ab')₂ indirect assay once antigen had been immobilized the apparent specificity of the detecting, second antibody was highly dependent on its concentration. In practice, the overall specificity of the procedure was regulated by the efficiency with which virus was trapped by the F(ab')₂ coat and the need to keep control values as low as possible, the latter dictating the use of relatively high dilutions of second antibody. When optimized in this way for detecting homologous antigen the potential of the F(ab')₂ assay for eliciting heterologous reactions was similar to that of the equivalent direct ELISA. In direct ELISA, however, the amount of second antibody effective in detecting virus is normally very low, the conjugate concentration being governed more by the activity of the enzyme in the conjugate than by the possibility of optimizing the concentration of useful second antibody. Indirect ELISA procedures are less subject to this constraint, permitting the use of higher concentrations of second antibody when the degree of cross-reactivity among heterologous combinations of virus and antibody is being investigated (Fig. 5).
Although the assay requires higher concentrations of antibody for coating than the DAS ELISA it is still extremely economical in the use of immuno-reagents compared with classical precipitation procedures. Protein A itself is relatively expensive to purchase but the conjugate produced by periodate oxidation with peroxidase is very economical in use. The test is intended to complement the DAS ELISA procedure and as such should extend the versatility of sero-diagnostic techniques for detecting plant pathogens.

REFERENCES


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