

Rapid Magnetic Microsphere Enzyme Immunoassay for Potato Virus X and Potato Leafroll Virus

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ABSTRACT

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A magnetic microsphere enzyme-linked immunoassay was developed for detection of potato virus X (PVX) and potato leafroll virus (PLRV) in potatoes. Analyte, microspheres with covalently coupled antibody and antibody-enzyme conjugate, were mixed, incubated together for 10 min, magnetically separated from sap, and washed with buffer three times; finally, substrates containing a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium or p-nitrophenyl phosphate were added. Color development (A_{562}) or (A_{405}) occurred in positive samples within 15–20

min. Detection sensitivity for PVX was 1–3 ng of purified virus diluted into buffer or healthy leaf sap or PVX-infected potato sap diluted $\times 1,000$ in healthy potato sap. Detection sensitivity for PLRV was $\cong 10$ ng of purified virus diluted into healthy sap or PLRV-infected sap diluted $\times 1,000$ in healthy potato sap. This assay can be completed within 30–45 min and provides assay sensitivities comparable to double antibody sandwich enzyme-linked immunosorbent assays on polystyrene or nitrocellulose solid phase carriers for these viruses.

Enzyme-linked immunosorbent assays (ELISAs) for plant viruses often require relatively long processing times, with the results being read at 6–24 h (1,2,4). Since most of these ELISAs utilize microtiter plates or filter membranes as the immunosorbent surfaces, they suffer from slow binding kinetics and small surface areas (8). In contrast, enzyme immunoassays (EIAs) that utilize small beads (1–10 μ m diameter) have larger binding surfaces and faster binding kinetics (8).

Magnetizable beads have the added advantage of utilizing a separation step that is uniquely suited to efficiently extracting virus from samples (1–2 ml) that contain particulate debris (5). These beads are coated with antibody and mixed with analyte samples containing virus, whereupon the virus binds to the antibody-sensitized magnetic beads. Then a magnetic field is applied that pulls the beads and bound virus to the sides of a test tube, and the remaining soluble and particulate components are decanted or aspirated. The bound virus is subsequently quantitated by the addition of an antibody-enzyme conjugate using procedures similar to those employed by other double-antibody sandwich EIAs (1,2,4).

This report describes a rapid and sensitive magnetic microsphere EIA (MM-EIA) for potato virus X (PVX) and potato leafroll virus (PLRV) with sensitivity equal to that observed for parallel samples analyzed by DAS-ELISA in polystyrene plates or dot-ELISA on nitrocellulose membranes (1).

MATERIALS AND METHODS

PVX and PLRV were maintained in greenhouse and in field potato plants (*Solanum tuberosum* L., cvs. Kennebec and Russet Burbank). PVX was purified using the techniques of VeneKamp and Mosch (7).

PVX antibody was prepared by injecting each of four New Zealand white rabbits subcutaneously and intramuscularly with 1 ml of an emulsion of Freund's complete adjuvant (FCA) with 0.8 mg of purified PVX in phosphate buffered saline (PBS; 0.15 M NaCl, 0.01 M K_2HPO_4 , pH 7.4). The rabbits were further

boosted at 3-wk intervals by immunizations with 0.8 mg of PVX/FCA and later with 0.5 mg PVX (Freund's incomplete adjuvant).

PLRV was purified according to methods developed by Rowhani and Stace-Smith (6). New Zealand white rabbits were immunized with five weekly intra-muscular injections of 1 ml of PLRV emulsified in 1 ml of Freund's incomplete adjuvant.

PVX and PLRV antibodies (IgGs) were precipitated from serum with 33% ammonium sulfate before coupling to magnetic microspheres or alkaline phosphatase as described below.

Magnetic microsphere EIA (MM-EIA). Magnetizable beads (Bio-Mag 4100) were purchased (Advanced Magnetics, Inc., Cambridge, MA), and anti-PVX and anti-PLRV IgGs were covalently immobilized to the beads with glutaraldehyde according to methods supplied by the manufacturer. PVX and PLRV antibodies were immobilized at loading densities of 150 mg IgG/g of beads.

Antibody-alkaline phosphatase enzyme conjugates (AP-IgG) were prepared by coupling IgGs to alkaline phosphatase (AP, 3,200 units/mg) according to methods reported by Clark and Adams (3). The conjugates were stored at 4 C, with 2 mg/ml bovine serum albumin (BSA) added to stabilize the conjugates.

Following optimization of individual assay parameters, a three-step format (standard protocol) was found to produce maximum sensitivity with minimal assay time. First, 500 μ l of virus-infected or healthy sap samples and 100 μ l of buffer A (1% w/v glycine + 0.1% Tween-20 pH 9.6) for PVX or 100 μ l of buffer B (1% w/v glycine + 0.85% NaCl + 0.1% Tween-20 pH 7.4) for PLRV were added to 12 \times 75 mm polystyrene tubes. Then 100 μ l each of IgG-coated beads and 100 μ l of AP-IgG (10–15 μ g each, based on IgG content) were added to each sap mixture and the tubes were shaken on a vortex mixer. The mixtures were incubated at room temperature for 10 min. Then the beads were magnetically separated by placing the test tubes in a Corning Magnetic Separator Unit (Advanced Magnetics, Inc.) and washed three times with 1-ml aliquots of buffers A or B for PVX and PLRV, respectively. After each magnetic separation, the beads were resuspended by vortex mixing. Finally, 0.2 ml of BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium) (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) or 0.2 ml p-nitrophenyl phosphate (Sigma, St. Louis, MO) was added and

incubated 15–20 min. To prepare the BCIP/NBT substrate, one part each of concentrates of BCIP and NBT were mixed with 10 parts of 0.1 M Tris, 0.1% w/v Tween-20, pH 9.6. The p-nitrophenyl phosphate substrate at 0.6 mg/ml was prepared in diethanolamine buffer pH 9.8. The reactions were stopped by addition of 60 μ l of 1N HCl, and absorbances (A_{562}) or (A_{405}) of the supernatants were read using a spectrophotometer.

RESULTS

Optimizing MM-EIA for PVX. Assay parameters were optimized using a range of 10–1,000 ng of purified PVX for each experiment. The basic protocol, as previously described, was used; however, some individual parameters were modified as described below. First, buffer A and PBS were compared for the sample incubation steps, with buffer A giving the best signal to noise (S/N) ratio. Adding 1% protein (ovalbumin or BSA) increased the background and decreased the S/N ratio with both buffers. Buffer A was tested at pH 8, 8.5, 9, and 9.6, with pH 9.6 producing the lowest background (absorbance in the absence of virus). The addition of 0.1% Tween-20 to buffer A improved the S/N ratio equally, whereas buffer A without Tween-20 produced nearly a twofold decrease in S/N ratio. These results show that buffer A (without added proteins such as BSA), which included 0.1% Tween-20, produced the best assay sensitivity.

Next, the antibody incubation step was varied by first incubating the IgG-coated beads with the purified virus samples (diluted into either healthy sap or buffer) and then washing before adding the AP-IgG conjugate. This additional washing step was expected to improve the assay sensitivity by: 1) maximizing the binding of virus to the beads (when added at the same time, the AP-IgG might compete with and reduce binding to the IgG on beads) and 2) maximizing the specific binding of AP-IgG to immobilized virus. However, when purified virus was diluted into buffer A, this alternative format produced S/N ratios similar to that obtained with the standard protocol. Also, adding ovalbumin during the virus plus IgG bead incubation step did not improve assay sensitivity. These results show that optimum sensitivity for assaying PVX was obtained when IgG-coated beads, AP-IgG conjugate, and virus were incubated together in the first step.

The sample incubation time was also varied to optimize sensitivity. The sensitivity improved with increasing incubation time, with maximum sensitivity occurring at ≥ 10 min. Therefore, 10 min was selected as the optimal incubation time.

The number of washing steps was then varied from one to four to optimize the sensitivity and reproducibility. Two and three washing steps gave the best assay sensitivity, but three washing steps were chosen because the results were more consistent.

Two methods of resuspending the beads after the magnetic separation step were compared: 1) vortexing individual tubes and 2) hand shaking a rack that contained several test tubes. Hand shaking was faster and more convenient; however, vigorous vortexing gave more reproducible results. The hand-shaken test tubes produced a higher rate of false positive results. Such false positives might be attributed to bead clumping, which prevented adequate washing of the beads. In an attempt to improve bead dispersion, polyethylene glycol 600, polyethylene glycol 3350, polyvinylpyrrolidone, CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and deoxycholate were individually added to the assay buffer. None of these reagents were successful in reducing variability. It therefore appears that vortexing is necessary for adequate and reproducible mixing of the beads.

After mixing, the supernatant washes were removed either by aspirating the supernatant with a Pasteur pipette or by decanting the rack with the magnetic portion in place for 10 seconds. Decanting was faster and gave reproducible results.

The optimal amount of IgG needed per assay for each preparation of IgG-magnetic beads and IgG-alkaline phosphatase conjugate was systematically determined by varying the concentrations used and then selecting the IgG concentration that gave the best S/N ratio. The bead concentration and conjugate concentration used per assay varied from 10 to 15 μ g each (based on

IgG concentration).

In conclusion, the final optimized magnetic microsphere assay format for PVX consisted of: 1) mixing the assay reagents (beads, conjugate, and sample) and incubating for 10 min; 2) magnetically separating the beads and washing three times (the supernatants were removed by aspiration, and the beads were vortexed to produce complete dispersion and resuspension); and 3) adding the enzyme substrate and incubating for 15–20 min. The bead and conjugate concentrations varied from 10 to 15 μ g each (based on IgG concentration).

Optimizing MM-EIA for PLRV. Most of the format developed for PVX assays was also applicable for PLRV assays; however, some modifications were incorporated. The glycine sample buffer pH 9.6 (A) resulted in weak signals, possibly because of PLRV instability in the buffer at that pH. Therefore, several buffer-additive combinations were compared, including 1.0% glycine + 0.85% NaCl + 0.1% Tween-20, (T20) pH 7.4; 0.1 M Tris-HCl + 0.1% T20, pH 7.4; 0.01 M PBS (KH_2PO_4 + Na_2HPO_4 + 0.85% NaCl) + 2.0% polyvinyl-pyrrolidone-10 (PVP-10) + 0.5% BSA, pH 7.4; and 0.01 M Tris-HCl + 0.85% NaCl + 0.05% T20 + 2.0% PVP-10 + 0.5% BSA, pH 7.4. The glycine buffer + 0.85% NaCl + 0.1% Tween-20 at pH 7.4 was incorporated into the PLRV assay format.

Magnetic microsphere concentrations of $\times 1$ (8.3 μ l/92 μ l buffer B), $\times 2$ (16.6 μ l/83.7 μ l buffer B), and $\times 3$ (24.9 μ l/75.4 μ l buffer B) were tested. Maximum signal for infected samples was obtained with the $\times 1$ concentration that was not significantly improved by using a $\times 2$ or $\times 3$ concentration of microspheres. Substrates BCIP/NBT and p-nitrophenyl phosphate were compared in assays of PLRV-infected and healthy potato sap using MM-ELISA. DAS-ELISA in polystyrene plates and dot-ELISA on nitrocellulose membranes were included for comparison (Table 1).

Sensitivity of MM-EIA. Figure 1 shows typical dilution curves for infected PVX sap serially diluted into healthy potato sap or into sample buffer A. In both cases, the assays were positive at dilutions of 1:1,000. In order to determine the actual concentration of PVX that may be detected in potato sap, purified PVX was serially diluted into healthy leaf sap and assayed with MM-EIA (Fig. 2A). A 10-ng PVX/ml detection limit was attainable. Also, a side-by-side study was performed where sap from potato leaves infected with PVX (Fig. 2B) was serially diluted into healthy potato sap. This comparison shows that infected leaf sap diluted 10,000-fold produced respective assay signals comparable to 10 ng/ml of purified virus.

Dilution-end-point comparisons were made with PVX-infected potato sap diluted in healthy potato sap using MM-ELISA and DAS-ELISA in polystyrene plates and dot-ELISA on nitrocellulose membranes (Table 2). Positive reactions for MM-ELISA were obtained at 1:1,000 dilutions for MM-ELISA and marginally positive at 1:10,000 dilutions for DAS- and dot-ELISA.

The assay of purified PLRV (0.01 mg/ml) in 10-fold dilutions into healthy potato sap was positive at a 10 ng/ml but not at a further 10-fold dilution (Fig. 3A). Serial 10-fold dilutions of PLRV-infected sap in healthy potato sap assayed with MM-EIA

TABLE 1. Comparison of magnetic microsphere enzyme-linked immunosorbent assay (MM-ELISA) with DAS- and dot-ELISAs for detection of potato leafroll virus in infected potato^a

Potato sap dilution	MM-ELISA			
	BCIP + NBT A_{562}	p-nitrophenyl Phosphate A_{405}	DAS-ELISA A_{405}	dot-ELISA ^b
PLRV-infected sap				
Non-diluted	2.266	2.171	2.102	+++
1:10	1.044	1.079	1.128	++
1:100	0.445	0.263	0.199	+
1:1,000	0.017	0.069	0.019	+,-
Healthy sap	0.126	0.162	0.141	-

^aMean of five experiments. PLRV-infected potato sap dilutions in healthy potato sap. Absorbance (A_{405}) and (A_{562}) values = \bar{X} of PLRV infected sap (-) the \bar{X} of healthy sap + 2SD of the \bar{X} of healthy sap.

^bVisual assessment.

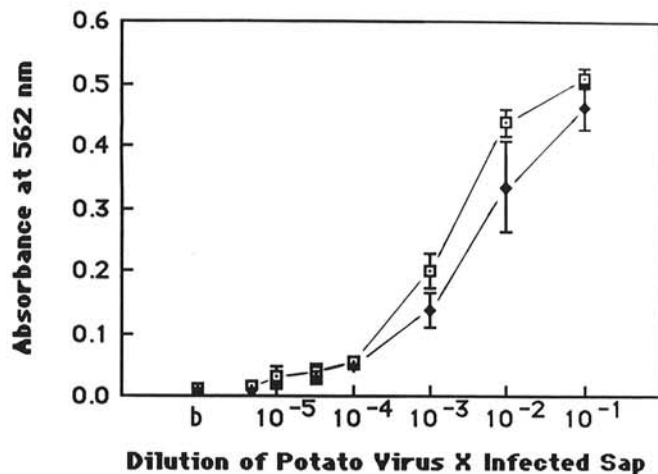


Fig. 1. PVX-infected potato sap diluted into buffer or healthy potato sap. The means and standard errors (vertical bars) were obtained from three assays. Assays performed in 0.5 ml of healthy sap plus 0.1 ml of 1% glycine, 0.1% Tween-20, pH 9.6 are denoted as □. Assays performed in 1 ml of 1% glycine, 0.1% Tween-20, pH 9.6 are denoted as ◆. Virus-free backgrounds, absorbance (A_{562}) of healthy plant sap, are indicated by "b".

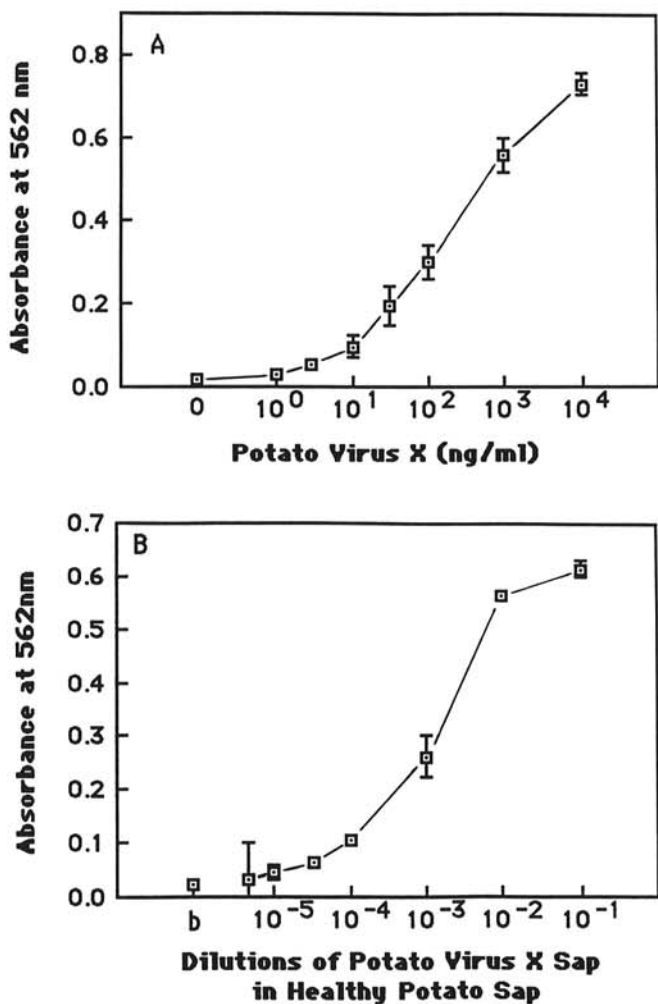


Fig. 2. Comparison of assay sensitivity of purified PVX, A, and PVX infected sap, B, diluted into healthy potato sap. Serially diluted purified PVX and serially diluted PVX infected leaf sap were assayed in 0.5 ml of healthy potato sap or in 0.5 ml of 1% glycine, 0.1% Tween-20, pH 9.6, using the standard PVX magnetic microsphere assay. The means and standard errors (vertical bars) of three experiments are given above. Virus-free backgrounds (A_{562}) are indicated by "b".

resulted in positive signals at 1:1,000 dilutions (Fig. 3B). A comparison of MM-ELISA and DAS-ELISA in polystyrene plates and dot-ELISA on nitrocellulose membranes for assay of PLRV in potato leaf sap showed that the sensitivity of these methods was comparable and produced marginally positive absorbance values at PLRV sap dilutions of 1:1,000 (Table 1).

Using dried antibody reagents for PVX assay. The antibody components (IgG beads and AP-IgG conjugate) were mixed with a stabilizer solution (Stablecoat, developed at Bio-Metric Systems,

TABLE 2. Comparison of magnetic microsphere enzyme-linked immunosorbent assay (MM-ELISA) with DAS- and dot-ELISAs for detection of potato virus X in infected potato^a

Potato sap dilution	MM-ELISA (A_{562})	DAS-ELISA (A_{405})	dot-ELISA ^b
PVX-infected sap			
Non-diluted	2.387	2.318	+++
1:10	2.669	2.245	+++
1:100	2.496	1.269	+++
1:1000	0.310	0.431	++
1:10,000	(-)0.012	0.092	+
Healthy sap	0.037	0.011	(-)

^aMean of three experiments. PVX-infected sap was diluted in healthy potato sap. Absorbance (A_{405}) and A_{562} values = \bar{X} of PVX-infected sap (-) the \bar{X} of healthy sap + 2SD of the \bar{X} of healthy sap.

^bVisual assessment.

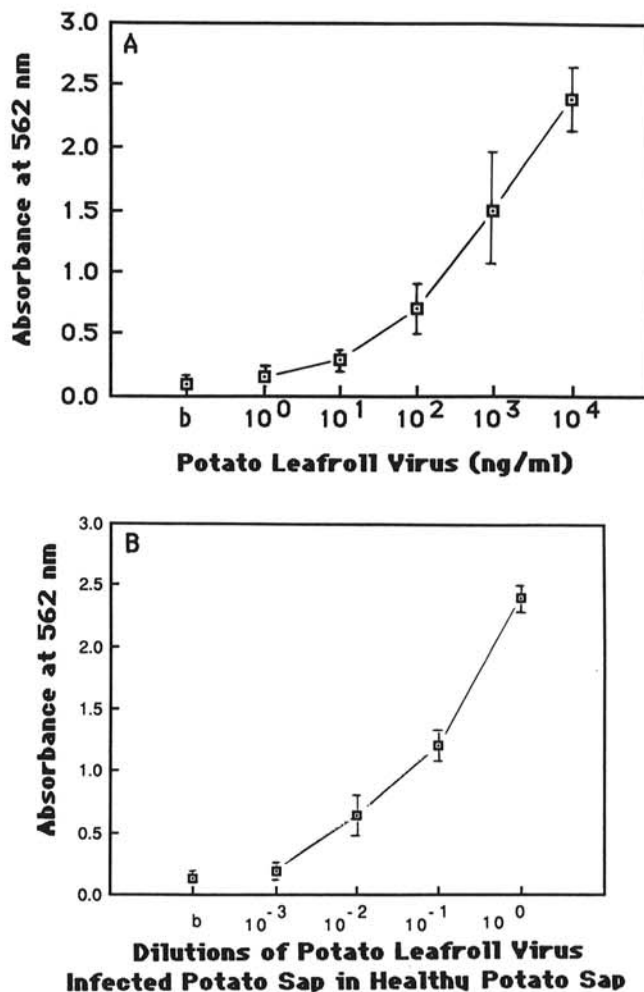


Fig. 3. Comparison of assay sensitivity of magnetic microsphere enzyme immunoassays (MM-EIA) for purified potato leafroll virus (PLRV), A, or PLRV-infected potato leaf sap, each in 10-fold dilutions in healthy potato leaf sap, B. The data are the means of four separate experiments each. Virus-free backgrounds are designated "b".

Inc.), aliquoted into 12- × 75-mm polystyrene tubes (in volumes required for individual assays), and vacuum-dried. The reagents were dried either individually or in combination and then used to detect purified PVX after 3 days of storage at room temperature. Reagents dried separately produced nearly the same assay sensitivity as did reagents stored in solution at 4 C. However, when the beads and conjugate were dried together, the assay sensitivity was reduced (data not shown). Beads and conjugate can each be dried separately with no loss of assay sensitivity and were stable at room temperature for at least 3 days. This approach has the potential for allowing an assay to be developed that utilizes reagents that are stored dry at room temperature.

DISCUSSION

A rapid and sensitive magnetic microsphere EIA for detecting PVX and PLRV was developed. The assay is a double-antibody sandwich EIA that requires two incubation steps of 10 and 15–20 min and has detection limits of 1–3 ng/ml for purified PVX and 10 ng/ml for PLRV (Figs. 2A and 3A, respectively), or dilutions of 1:1,000 each for PVX- or PLRV-infected leaf sap in healthy potato sap (Figs. 2B and 3B). This assay, therefore, has a sensitivity comparable to DAS-ELISA in polystyrene plates or dot-ELISA on nitrocellulose membranes (Tables 1 and 2) and is considerably faster. Both BCIP + NBT and p-nitrophenyl phosphate substrates produced approximately comparable absorbance readings.

Maximum sensitivity of the magnetic microsphere assay was observed with a three-step format that included two incubation steps of 10 and 15–20 min. Antibody-coated beads, analyte, and enzyme conjugate were mixed and incubated for 10 min; unbound material was removed during the wash steps; and, finally, enzyme substrate (chromogen) was added and incubated 15–20 min. The total time required for this assay is approximately 30–45 min.

Assays for PVX were made in sample buffer or plant sap (Fig. 1), with the same detection limit being observed in each dilution medium. Therefore, the sensitivity was unaffected by potentially interfering substances that are present in plant samples.

For an assay to be conveniently used, the reagents must be stable, preferably at room temperature. Both the antibody-coated beads and the antibody-alkaline phosphatase conjugate were stable for 7 mo (the longest storage time tested) when stored in solution at 4 C, and a preliminary experiment utilizing a stabilizing agent indicated the beads and conjugate can be dried and are stable at 4 C.

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