Techniques

Improved Detection of Potato Leafroll Virus in Plant Material and in Aphids

J. F. J. M. van den Heuvel and D. Peters

Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands.
Accepted for publication 10 February 1989 (submitted for electronic processing).

ABSTRACT

van den Heuvel, J. F. J. M., and Peters, D. 1989. Improved detection of potato leafroll virus in plant material and in aphids. Phytopathology 79:963-967.

To improve detection of potato leafroll virus (PLRV) in plant material and viruliferous aphids, the enzyme-linked immunosorbent assay (ELISA) was modified by simultaneous incubation of sample and conjugate (cocktail ELISA) and by amplification of the enzyme reaction. Absorbance values of PLRV-containing samples in the cocktail ELISA were higher than those of comparable samples in a sandwich ELISA procedure in which sample and conjugate were incubated sequentially. Addition of sodium diethyldithiocarbamate or ethylenediaminetetraacetic acid to the sample buffer in the cocktail ELISA significantly increased the absorbance values of infected plant material, while the background signals were reduced. Amplification of the enzyme reaction, in which dephosphorylated substrate catalytically triggered an enzyme-mediated redox cycle, further increased sensitivity. Using this technique, 50–100 pg of PLRV could be detected per sample, and the virus could be detected in highly diluted leaf sap and in single *Myzus persicae* nymphs after a 12-hr acquisition access period on PLRV-infected *Physalis floridana* plants. In addition, when coated plates were used, the total assay time for PLRV detection in plant material could be reduced to 20 min.

Additional keywords: enzyme amplification.

Potato leafroll virus (PLRV), a member of the luteovirus group, causes a widespread and economically damaging disease in potatoes. The virus is transmitted in a persistent manner by aphids and occurs in low titers in infected plants and in its vectors. To study virus-vector relationships for PLRV, a highly sensitive method for detecting the virus is therefore required.

The microplate method of the sandwich form of enzyme-linked immunosorbent assay (ELISA), introduced by Voller et al (14), has been adapted by Clark and Adams (4) for use in routine testing of plant viruses. Although hybridization techniques are developing rapidly, ELISA is still considered a major assay for the detection of potato viruses, especially when small numbers of tests are involved, no labeled probes are available (2), or a quantitative interpretation of results is required.

Since the introduction of ELISA, numerous reports have been published on improvements to the assay conditions for many host-virus combinations (4,7). Modifications were made to maximize the sensitivity of the assays and to reduce nonspecific background signals. In most cases the improvements have involved only slight modifications of the original ELISA procedure.

Little attention has been paid to the "cocktail" ELISA technique, in which sample and conjugate are incubated simultaneously in the wells of a microtiter plate. Van Vuurde

and Maat (13) demonstrated the potential of the cocktail method in the routine testing of lettuce mosaic virus (LMV) and pea early-browning virus (PEBV). Furthermore, apple chlorotic leaf spot virus (CLSV) could be detected only by using simultaneous incubation of sample and conjugate (5).

Recently, amplification of the alkaline phosphatase reaction in ELISA (10) has been applied to detect barley yellow dwarf virus in oat plants and in individual vector aphids (12). Using the sandwich method, assay time could be reduced to less than 2 hr if coated and blocked microtiter plates were used. Whether simultaneous incubation of sample and conjugate followed by amplification of the enzyme reaction can increase both sensitivity and efficiency of ELISA has not been tested.

This paper illustrates first of all the advantages of cocktail ELISA over the sandwich method for the detection of PLRV in infected plant material and in viruliferous aphids. Second, the addition of chelating agents to the sample buffer was shown to reduce background signals. Finally, we showed that sensitivity was enhanced by combining cocktail ELISA with a procedure in which the bound enzyme was visualized by amplification of the enzyme reaction.

MATERIALS AND METHODS

Aphid rearing. *Myzus persicae* (Sulzer) was maintained on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a greenhouse compartment at 20 C under a regime of 16 hr of light and 8 hr of darkness. To ensure a regular supply of aphids and to produce cohorts of similar-aged aphids, mature apterae, confined to leaf cages, were transferred daily to leaves of oilseed rape plants. Nonviruliferous nymphs used in the experiments were deposited onto *Vicia faba* L. 'Drie × Wit' by mature apterae derived from a cohort.

Virus sources. *Physalis floridana* Rydb. plants were inoculated in their seedling stage by single viruliferous nymphs of *M. persicae* in a 2-day inoculation access period. For symptom development the seedlings were kept in a glasshouse (16 hr light, 8 hr darkness) at 25 C. PLRV-infected and virus-free potatoes (*Solanum tuberosum* L. 'Bintje') were raised from tubers and were kept under the same conditions as the *P. floridana* seedlings.

Purification and enzyme conjugation of γ-globulins. Antiserum was kindly provided by D. Z. Maat. The γ-globulin fraction was partially purified by ammonium sulfate precipitation as described by Clark and Adams (4). γ-Globulins at 1 mg/ml were conjugated with 2,000 units of alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline, pH 7.4, as described by Avrameas (1). Conjugate was not further purified. γ-Globulin and conjugate were stored with 0.05% sodium azide at 4 C.

ELISA procedures. The following ELISA procedure (sandwich method) was used as a reference. Wells of Nunc-Immunoplate IF plates (Nunc, Denmark) were sensitized by adding 250 μl of 2 μg/ml γ-globulin in coating buffer (0.05 M sodium carbonate, pH 9.6). After a 3-hr incubation at 37 C, wells were vigorously rinsed with tap water and filled with samples (200 μl per well) triturated in 0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 2% polyvinylpyrrolidone (mol wt about 44,000), 0.05% Tween 20, and 0.2% ovalbumin (sample buffer). The samples were incubated overnight at 4 C. The wells were then flushed with tap water, filled with conjugate (diluted 1,000-fold in sample buffer [200 μl per well]), and incubated for 3 hr at 37 C. After the plates were washed, 200 μl of 2.7 mM *p*-nitrophenyl phosphate disodium salt (p-NPP) in 10% diethanolamine, pH 9.8, was added to each well. The immobilized enzyme concentration was measured by monitoring substrate conversion to colored end product.

The same kinds of plates and buffers used in the sandwich method were used in the cocktail ELISA, but sample and enzyme-conjugated γ-globulins were added together and incubated overnight at 4 C. The total volume of sample and conjugate was 200 μl when alkaline phosphatase concentration was determined using p-NPP and 100 μl when the enzyme reaction was amplified.

The amplification of the enzyme reaction was performed following Stanley et al (10). All chemicals used, unless otherwise stated, were purchased from Sigma Chemical Co., St. Louis, MO. We added 100 μl of 0.2 mM nicotinamide-adenine dinucleotide

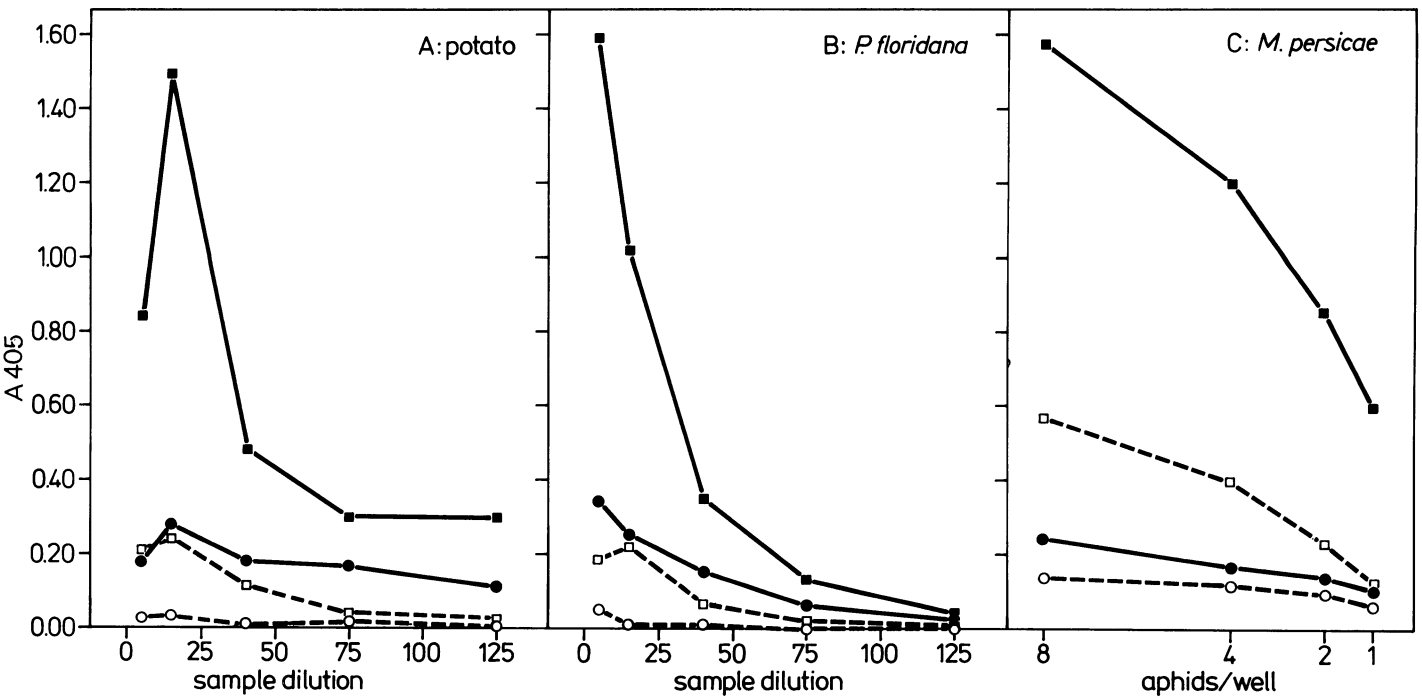

Fig. 1. Absorbance values at 405 nm (A405) obtained with healthy (open symbols and dotted lines) and PLRV-infected (solid symbols and solid lines) **A**, potato leaf material, **B**, *Physalis floridana* leaf material, and **C**, *Myzus persicae* aphids in the cocktail (□,■) and sandwich (○,●) ELISA procedures. Samples of leaf material and aphids were prepared and diluted in sample buffer. The sandwich procedure consisted of overnight sample incubation (200 μl per well) at 4 C and 2-hr conjugate incubation (1 μg per milliliter of sample buffer; 200 μl per well) at 37 C. In the cocktail procedure, 200 μl of sample and 0.2 μg of conjugate per well were incubated simultaneously overnight at 4 C. Absorbance values of A, B, and C were measured after 4, 0.5, and 2 hr of substrate incubation at room temperature, respectively. Each point in the graphs is the average of five absorbance values.

phosphate monosodium salt (NADP) in 0.05 M diethanolamine buffer, pH 9.5, to each well. After incubation at 20 C for 30 min, remaining alkaline phosphatase activity was blocked by adding 15 μl of 0.05 M ortho-nitrophenyl phosphate disodium salt (o-NPP) (BDH Chemicals Ltd., Poole, England) in 0.025 M phosphate buffer, pH 7.0, to each well (8). Subsequently, we added 150 μl per well of the amplification reaction mixture. The stock amplification mixture consisted of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase (type VI), 3% (v/v) ethanol, and 1 mM *p*-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer, pH 7.0.

In both enzyme assays, color development was allowed to proceed at room temperature. Absorbance values were read on a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland) at 405 nm when p-NPP was used and at 492 nm when NADP was used. The plates were blanked against wells that did not contain sample.

RESULTS

Detection of PLRV with cocktail ELISA. Cocktail ELISA was compared with the sandwich method in sensitivity tests with PLRV-infected potato and *P. floridana* leaf tissue, PLRV-carrying *M. persicae* adults, and appropriate virus-free controls. A purified PLRV suspension was included as a positive control. Extracts were prepared by grinding either 1 g of leaf material with 4 ml of sample buffer or 100 aphids with 1 ml of sample buffer.

All dilutions prepared from PLRV-infected leaf tissue and virus-containing aphids had higher absorbance values in the cocktail ELISA than in the sandwich method (Fig. 1). With most sample dilutions, absorbance values were four to seven times higher in the cocktail ELISA. The improvement in values in PLRV-infected potato and *P. floridana* at five- to 40-fold dilutions was remarkable (Fig. 1A and 1B).

Background absorbance values were higher in cocktail ELISA than in the sandwich method when the absorbance values of a particular sample were measured in both procedures after the same period of color development (Fig. 1). However, when the absorbance values of potato, *P. floridana*, and *M. persicae* in sandwich ELISA were allowed to develop to values comparable to those obtained with cocktail ELISA after 4, 0.5, and 2 hr, respectively, background values were higher in the sandwich method.

With purified PLRV, 1 ng per well could readily be detected in the cocktail ELISA. An absorbance value of 0.14 was obtained after 2.5 hr of substrate incubation at room temperature, while the virus-free buffer gave a value of 0.01. Under the same conditions, 8 ng of virus per well was necessary to obtain similar values in the sandwich method. On the other hand, at PLRV concentrations above 50 ng per well, the sandwich method yielded higher readings than the cocktail ELISA. In a direct comparison of potato and *P. floridana* leaves, with purified PLRV in both

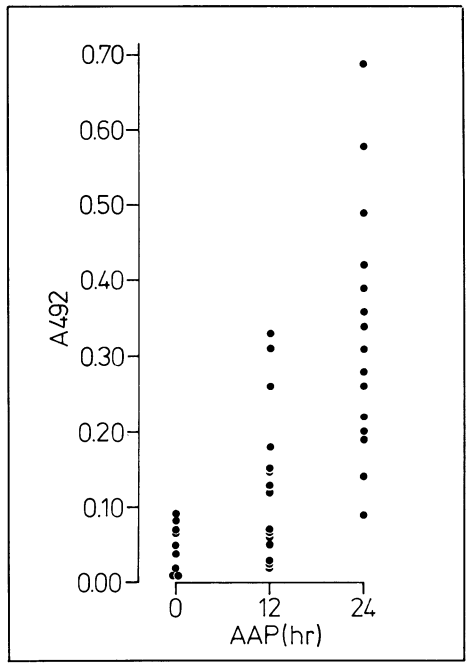

Fig. 2. Absorbance values at 492 nm (A492) of individual 6-day-old *Myzus persicae* nymphs, obtained with amplification of the enzyme reaction after simultaneous incubation of sample and conjugate. Nymphs, which were placed for 0, 12 and 24 hr (AAP) on infected *P. floridana* plants, were individually homogenized in 100 μl of sample buffer containing 12.5 mM sodium diethyldithiocarbamate and 1 μg/ml conjugate. The cocktail was incubated overnight at 4 C. The amplification of the enzyme reaction was done at room temperature by sequentially adding 100 μl of 0.2 mM nicotinamide-adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer (30-min incubation), 15 μl of 0.05 M ortho-nitrophenyl phosphate disodium salt in 0.025 M phosphate buffer (5-min incubation), and 150 μl of the amplification reaction mixture. A492 was measured after 25 min of color development.

TABLE 1. Influence of sodium diethyldithiocarbamate (NaDIECA) on the detection of potato leafroll virus (PLRV) in leaf material[t] and in aphids[u] by cocktail enzyme-linked immunosorbent assay[v]

NaDIECA concentration (mM)	*Physalis floridana*[w]		Potato[x]		*Myzus persicae*[y]	
	PLRV-infected	Healthy	PLRV-infected	Healthy	PLRV-carrying	Healthy
0	0.817 (0.039) a[z]	0.049 (0.038) a	1.315 (0.110) b	0.038 (0.012) a	0.821 (0.235) a	0.216 (0.017) a
6.25	0.970 (0.082) ab	0.029 (0.018) b	1.501 (0.052) a	0.043 (0.013) a	0.544 (0.102) ab	0.117 (0.046) b
12.5	1.023 (0.066) b	0.018 (0.014) bc	1.404 (0.093) ab	0.035 (0.014) a	0.514 (0.090) b	0.098 (0.042) b
25	0.623 (0.107) c	0.012 (0.008) c	0.856 (0.118) c	0.027 (0.006) a	0.351 (0.042) b	0.063 (0.013) bc
50	0.216 (0.080) d	0.014 (0.011) c	0.430 (0.075) d	0.023 (0.006) a	0.042 (0.013) c	0.038 (0.007) c
Standard deviation	0.102	0.008	0.060	0.014	0.121	0.023

[t] For each NaDIECA concentration and type of leaf material, 30 samples from two different extracts were divided into six samples per plate. Ground *P. floridana* leaf material was diluted 100-fold (w/v); ground potato leaf material was diluted 18-fold (w/v).

[u] For each NaDIECA concentration and type of aphid (PLRV-carrying and virus-free), 18 samples from two different extracts were divided into six samples per plate. The aphids were given an acquisition access period of 3 days on PLRV-infected *P. floridana*. Aphids were homogenized, and each sample corresponded to two aphids.

[v] γ-Globulins and conjugate were used at concentrations of 2 and 1 μg/ml, respectively. Samples and conjugate were incubated together overnight at 4 C.

[w] Absorbance at 405 nm after 2 hr of substrate incubation; values are the average absorbance values of six wells of five microtiter plates tested ($N = 5$); standard error in parentheses.

[x] Absorbance at 405 nm after 4 hr of substrate incubation; values are the average absorbance values of six wells of five microtiter plates tested ($N = 5$); standard error in parentheses.

[y] Absorbance at 405 nm after 3.5 hr of substrate incubation; values are the average absorbance values of six wells of three microtiter plates tested ($N = 3$); standard error in parentheses.

[z] Values within a column not followed by the same letter are significantly different ($P = 0.05$) according to Tukey's test.

ELISA systems, the virus concentration in leaf sap diluted 20- and 40-fold, respectively, is about 50 ng per well.

Influence of chelating agents on the enzyme reaction. To reduce background signals in the cocktail ELISA, sodium diethyldithiocarbamate (NaDIECA) or ethylenediaminetetraacetic acid (EDTA) was added to the sample buffer in concentrations of 6.25, 12.5, 25, and 50 mM. NaDIECA significantly diminished absorbance values of healthy *P. floridana* leaf tissue and virus-free *M. persicae* (Table 1). The absorbance values obtained with healthy potato leaf tissue were slightly but not significantly ($P = 0.05$) affected by NaDIECA. The values with PLRV-infected potato and *P. floridana* leaf tissue were significantly enhanced when 6.25 or 12.5 mM of this chelating agent was used; higher NaDIECA concentrations inhibited color development. Although the values for virus-carrying aphids decreased with increasing NaDIECA concentrations, the discriminative capacity of the system was improved overall because the absorbance values of virus-free aphids fell more than those of virus-carrying aphids.

When applied in the sandwich procedure, NaDIECA reduced the absorbance values of PLRV-containing samples without affecting background signals.

The effects of adding EDTA were analogous to those demonstrated for NaDIECA.

Amplification of the enzyme reaction. The enzyme reaction was amplified to enhance the detection of PLRV in single viruliferous aphids. Six-day-old *M. persicae* nymphs were individually triturated in small Elvejhem-Potter tubes in 100 µl of sample buffer containing 12.5 mM NaDIECA, after an acquisition access period of 12 or 24 hr on PLRV-infected *P. floridana*. The triturates were centrifuged for 15 min at 10,000 rpm in an Eppendorf centrifuge. After incubation of the cocktail of conjugate and supernatant at 4 C overnight, amplification of the enzyme reaction was applied.

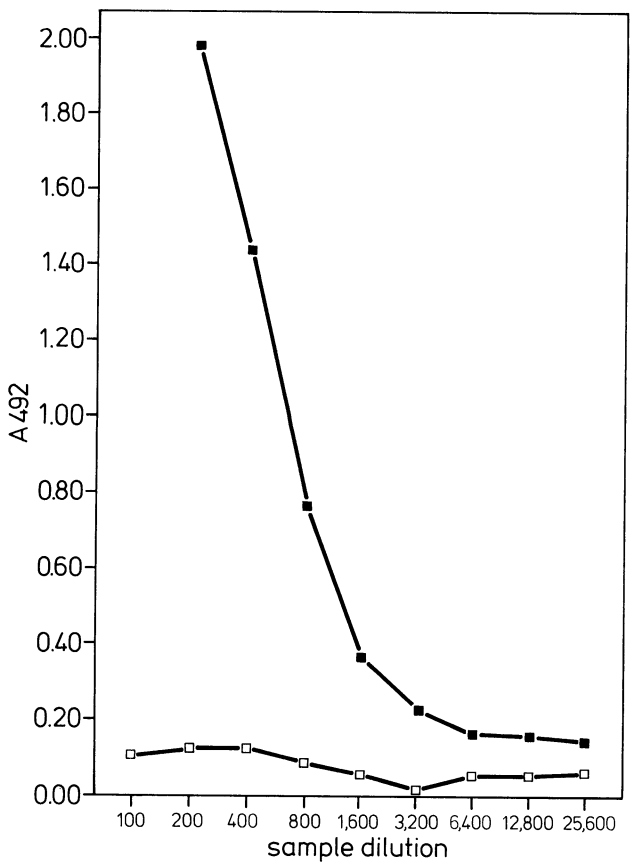

Fig. 3. Absorbance values at 492 nm (A492) of highly diluted PLRV-infected (■) and healthy (□) potato leaf material, obtained with amplification of the enzyme reaction after the simultaneous incubation of sample and conjugate. Leaf material was ground and diluted in sample buffer containing 12.5 mM sodium diethyldithiocarbamate and 1 µg/ml conjugate. The cocktail (100 µl) was incubated overnight at 4 C. The amplification of the enzyme reaction was done at room temperature by sequentially adding 100 µl of 0.2 mM nicotinamide-adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer (30-min incubation) and 150 µl of the amplification reaction mixture. A492 was measured after 15 min of color development. Each point in the graph is the average of three absorbance values.

The absorbance values (Fig. 2) show that PLRV antigen could be detected even in nymphs after an acquisition access period of 12 hr. Individual nymphs varied widely in the efficacy with which they acquired PLRV during an acquisition access period.

The amount of purified PLRV that could be detected using the amplification of the enzyme reaction following cocktail incubation was between 50 and 100 pg of virus per well. The enhanced sensitivity of ELISA obtained with amplification, in comparison with p-NPP, enabled us to detect PLRV in highly diluted leaf material samples (Fig. 3) and to develop a faster assay (Table 2). For the latter purpose, disks (5 mm in diameter) were punched out of healthy and PLRV-infected potato leaves and individually ground in 100 µl of extraction buffer containing 12.5 mM NaDIECA and 0.1 µg of conjugate. These homogenates were incubated for 1, 5, 10, or 15 min in the wells of a coated microtiter plate, followed by 5, 10, or 15 min of NADP incubation and 10 min of color development. With a total assay time of 16 min—1 min of cocktail incubation, 5 min of NADP incubation, and 10 min of color development—an absorbance value at 492 nm of 0.125 (blank: 0.038) was obtained (Table 2). Values over 0.1 could be scored as positives by visual evaluation. The o-NPP step could be omitted because of rapid color development. The amplification procedure enabled us to perform the assay in less time than the procedure using p-NPP as substrate. When p-NPP (100 µl, 2.7 mM, in 10% diethanolamine, pH 9.8) was used after short incubation periods of the cocktail, absorbance values at 405 nm obtained after 6 hr of color development were comparable to those obtained with enzyme amplification after about 20 min.

DISCUSSION

We used the recently developed technique of the amplified alkaline phosphatase reaction in ELISA (10) to detect the enzyme immobilized in the simultaneous incubation of samples and conjugate (cocktail ELISA). Cocktail ELISA was more sensitive than sandwich ELISA and less time-consuming, as one step could be omitted. The absorbance values of virus-containing samples were higher for all sample dilutions prepared from extracts of both potato and *P. floridana* leaves and of *M. persicae* vector

TABLE 2. Effect of amplification of the enzyme reaction following cocktail incubation for short periods on the detection of potato leafroll virus (PLRV) in crushed potato leaf disks[a] by enzyme-linked immunosorbent assay

Cocktail incubation[b] (min)	NADP incubation[b] (min)					
	5		10		15	
	PLRV	Healthy	PLRV	Healthy	PLRV	Healthy
1	0.125[c]	0.038	0.204	0.046	0.280	0.051
5	0.127	0.016	0.223	0.018	0.312	0.015
10	0.169	0.000	0.302	0.015	0.429	0.023
15	0.183	0.018	0.353	0.036	0.516	0.029

[a] Samples were made by grinding single potato leaf disks in 100 µl of extraction buffer containing 12.5 mM sodium diethyldithiocarbamate and 1 µg/ml conjugate.

[b] The simultaneous incubation of sample and conjugate (cocktail) and the incubation of nicotinamide-adenine dinucleotide phosphate monosodium salt (NADP) solution (0.2 mM NADP in 0.05 M diethanolamine buffer; 100 µl per well) were done at room temperature.

[c] Values (absorbance at 492 nm) are the averages of five potato leaf disks. Absorbance values were measured 10 min after the amplification reaction mixture was added to the NADP solution.

aphids (Fig. 1). This effect was also observed for CLSV in petals, leaves, and fruit of various apple cultivars (5).

The improved detection may be caused by immobilization of a larger proportion of antigen-conjugate complexes on the solid phase, binding of more conjugate molecules to PLRV antigen, or the fact that the omission of one washing step reduces "leakage" of antigen-antibody complexes from the solid phase. Application of the cocktail ELISA also resulted in reduced background reaction. This reduction could be demonstrated when the absorbance values in the sandwich ELISA were allowed to develop to levels similar to those obtained with the same samples in the cocktail ELISA (results not shown). Antigenic sites on plant proteins that compete with immune complexes for binding to the solid phase may be obstructed by the conjugate molecules in the solutions during simultaneous incubation.

The view that the cocktail method can be applied only with smaller antigens (3) seems at least questionable in view of previous results with CLSV and apple stem grooving virus (closteroviruses), PEBV (tobravirus), and LMV (potyvirus) (13). Moreover, good results were also obtained in the detection of beet necrotic yellow vein virus (furovirus) in our laboratory (*unpublished data*).

Reduced sensitivity of the cocktail ELISA in concentrated plant extracts compared to dilute ones, as found by Flegg and Clark (5), was also observed for extracts of PLRV-infected potato leaf material (Fig. 1A) and purified PLRV suspensions at concentrations over 50 ng per well. *P. floridana* leaf material, on the other hand, did not react in this way (Fig. 1B), although virus yield after purification was almost equivalent to that of potato leaf material (*unpublished data*). The question arises whether this reduced sensitivity when a relatively high virus concentration was tested was caused by an unfavorable conjugate-antigen ratio (3) or by negative influences of components in the leaf extract of potato. Sample composition can also affect virion stability or interfere with the formation of immune complexes or with binding of those complexes to the solid phase.

Adding NaDIECA or EDTA to the sample buffer in the cocktail ELISA further improved the detection of PLRV; a concentration of 12.5 mM was most effective (Table 1). Absorbance values for PLRV-infected potato and *P. floridana* leaf samples were higher, and background signals in healthy aphids were suppressed significantly. Gibbs and Harrison (6) and Smith and Banttari (9) discuss the possible functions of these chelating agents in stabilizing virus nucleocapsid structure by reducing oxidized phenolic compounds or the release of viruses from cell organelles.

Amplification of the enzyme reaction increased the ultimate detection level of PLRV about 15-fold. This enabled the serological detection of PLRV antigen in single vector aphids within an hour, which included a 30-min NADP incubation and 25 min of color development (Fig. 2). Tamada and Harrison (11) also succeeded in detecting PLRV in single aphids, but in their experiments the most clear-cut results were obtained only after an overnight incubation with substrate.

A quantitative interpretation of absorbance values obtained from amplification of the enzyme reaction is possible because the blockage of remaining phosphatase activity with o-NPP excludes any interference from newly dephosphorylated NADP while color development is proceeding. Hence, the color development caused by the reduction of *p*-iodonitrotetrazolium violet is proportional to the NAD originally formed by alkaline phosphatase in the bound conjugate (8). This accurate and sensitive enzyme assay permits the analysis of various factors that play a role in the acquisition of PLRV by aphids, such as the determination of amounts of virus involved when PLRV is acquired from different virus sources.

For qualitative purposes, amplification of the enzyme reaction offers the possibility of a field test that can be completed within 20 min and the detection of PLRV in highly diluted samples. The o-NPP step can be omitted because of rapid color development. Moreover, the use of glutaraldehyde coupling of alkaline phosphatase with γ-globulin, without any further purification of the conjugate, makes the enzyme amplification assay economically attractive.

LITERATURE CITED

1. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry 6:43-52.
2. Boulton, R. E., Jellis, G. J., Baulcombe, D. C., and Squire, A. M. 1986. The application of complementary DNA probes to routine virus detection, with particular reference to potato viruses. Pages 41-53 in: Developments in Applied Biology: I. Developments and Applications in Virus Testing. R. A. C. Jones and L. Torrance, eds. Association of Applied Biologists, Wellesbourne, U.K.
3. Clark, M. F. 1981. Immunosorbent assays in plant pathology. Annu. 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. 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.
6. Gibbs, A., and Harrison, B. 1976. Plant Virology: The Principles. Edward Arnold (Publishers) Ltd., London. 292 pp.
7. McLaughlin, M. R., Barnett, O. W., Burrows, P. M., and Baum, R. H. 1981. Improved ELISA conditions for detection of plant viruses. J. Virol. Methods 3:13-25.
8. Self, C. H. 1985. Enzyme amplification—A general method applied to provide an immunoassisted assay for placental alkaline phosphatase. J. Immunol. Methods 76:389-393.
9. Smith, F. D., and Banttari, E. E. 1987. Dot-ELISA on nitrocellulose membranes for detection of potato leafroll virus. Plant Dis. 71:795-799.
10. Stanley, C. J., Johannsson, A., and Self, C. H. 1985. Enzyme amplification can enhance both the speed and the sensitivity of immunoassays. J. Immunol. Methods 83:89-95.
11. Tamada, T., and Harrison, B. D. 1981. Quantitative studies on the uptake and retention of potato leafroll virus by aphids in laboratory and field conditions. Ann. Appl. Biol. 98:261-276.
12. Torrance, L. 1987. Use of enzyme amplification in an ELISA to increase sensitivity of detection of barley yellow dwarf virus in oats and in individual vector aphids. J. Virol. Methods 15:131-138.
13. van Vuurde, J. W. L., and Maat, D. Z. 1985. Enzyme-linked immunosorbent assay (ELISA) and disperse-dye immuno assay (DIA): Comparison of simultaneous and separate incubation of sample and conjugate for the routine detection of lettuce mosaic virus and pea early-browning virus in seeds. Neth. J. Plant Pathol. 91:3-13.
14. Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165-167.