Increased Sensitivity of ELISA for Potato Viruses S, X, and Y by Polystyrene Pretreatments, Additives, and a Modified Assay Procedure

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ABSTRACT

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The virus-specific absorbance (A_{405}) of enzyme-linked immunosorbent assay (ELISA) for potato viruses S, X, and Y (PVS, PVX, and PVY) was increased an average of 14.8% by pretreating new polystyrene cuvettes with 5.3 M NaOH:EtOH. Sodium diethyldithiocarbamate (0.01 M) in phosphate-buffered saline plus Tween 20 (PBS-T) increased the PVY-specific absorbance by 37.3% over that using a PBS-T control. Alkaline degradation of PVX by 0.2 M diethanolamine, pH 10.5, and 6–12% sodium sulfite increased the PVX-specific absorbance by 20.6% over that using PBS-T alone. Enhanced sensitivity was further characterized by regression analysis of the relationship between log A_{405} and log/sap dilution. Dilution end points for virus detection were predicted from regression equations and corresponded to increases in the virus-specific absorbance using undiluted infected sap. A modified double-antibody sandwich ELISA increased the dilution end point for PVS detection by 30–32%, which was less than that previously reported with other plant viruses.

A major application of serological assays in plant pathology is the large-scale assay of potato viruses, both for certification and in breeding for resistance (2). Enzyme-linked immunosorbent assay (ELISA) is one of the most sensitive serological methods available to detect viruses, with the ability to detect 1 ng/ml of certain plant viruses (2). The objective of this research was to examine and evaluate the effects several modifications have on the sensitivity of ELISA for potato viruses S, X, and Y. These modifications consisted of the use of agents that clean polystyrene cuvettes for reuse in ELISA (1), additives to the virus extraction buffer, and a modified form of double-antibody sandwich ELISA (DAS-ELISA) (3).

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MATERIALS AND METHODS

Virus sources. Healthy (virus-free) and virus-infected plant sap was obtained from leaves excised from greenhouse-grown, virus-free and PVS- and PVX-infected potato plants (*Solanum tuberosum* L. 'Norland,' 'Kennebec,' and 'Russet Burbank') and from virus-free and PVY-infected tobacco (*Nicotiana glutinosa* L. and *N. tabacum* L.).

Immunoglobulin preparation and enzyme antibody conjugation. The gamma globulin fractions of antisera produced in rabbits against potato viruses S, X, and Y were partially purified by ammonium sulfate precipitation and dialysis according to the procedure of Clark and Adams (4) but without passage through a DE22 cellulose column. Partially purified gamma globulins were coupled to alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO) according to the one-step glutaraldehyde method described by Clark and Adams (4). The goat anti-PVS gamma globulin used in the modified DAS-ELISA for PVS was kindly donated by Boehringer-Mannheim (Boehringer-Mannheim Gmbh., Mannheim, West Germany).

ELISA. The DAS-ELISA procedure of Clark and Adams (4) was used in these experiments. The assays were processed and analyzed with a Gilford PR-50 EIA automatic analyzer (Gilford Instrument

Laboratories, Inc., Oberlin, OH). All absorbance readings were taken within the linear range of absorbance increase and at the same time interval after addition of substrate.

For each treatment, the virus-specific absorbance values were calculated by computing the mean of the virus-infected samples minus the mean of the healthy plant sap plus two standard deviations of the mean of healthy plant sap. Three replicated experiments with four wells of virus-infected sap and four wells of healthy sap were used for each treatment unless otherwise stated. Analysis of variance (ANOVA) and Duncan's multiple range test were used to analyze the data (7).

Pretreatment of polystyrene cuvettes. A 5.3 M sodium hydroxide:ethanol solution (NaOH:EtOH) was made by adding 1,000 ml of absolute ethanol to a concentrated 680-g/L solution of NaOH in distilled water. After mixing overnight, an additional 1,200 ml of distilled water was added to form the pretreatment solution. Solutions were also made of 5.3 M NaOH, 15 M NaOH, 5.3 M potassium hydroxide (KOH), and 17 M KOH in distilled water. Polystyrene cuvettes (Gilford EIA cuvettes) were soaked for 48 hr in a pretreatment solution, then rinsed five times with tap water, five times with deionized water, and five times with distilled water. The cuvettes were drained and dried before use in DAS-ELISA.

Preparation of additives. All additive solutions were made with 0.15 M phosphate-buffered saline plus 0.05% Tween 20, pH 7.4 (PBS-T), unless otherwise stated, and mixed 1:1 with healthy, PVS-, PVX-, or PVY-infected sap. PBS-T and distilled water were also combined 1:1 with sap as controls. Distilled water and 0.1 M Tris-HCl (2-amino-2(hydroxymethyl)-1,3-propanediol), pH 7.4, plus 0.85% NaCl were also used with sodium sulfite. PBS-T plus 0.01 M sodium diethyldithiocarbamate (Na DIECA) was used in experiments on pretreatment of polystyrene cuvettes.

Additives tested were as follows: 1) viral degrading agents—0.04 M urea, 6% pyridine, 0.6% pyridine, 6% pyridine plus 0.02 M disodium ethylenediaminetetraacetate (Na EDTA), 0.5% pyrrolidine, and 0.2 M diethanolamine; 2) reducing agents—6% sodium sulfite, 12% sodium sulfite, 0.2% cysteine HCl, and 0.4 M 2-mercaptoethanol; 3) a detergent—3% Igepon T-73 (sodium-*N*-methyl-*N*-oleoyl taurate); 4) a chelating agent—0.01 M Na DIECA; 5) a viral precipitating agent—0.1% polyethylene glycol (PEG); and 6) 0.02 M caffeine.

Determination of dilution end points. Sap from virus-infected potato or tobacco leaves was serially diluted in healthy potato or tobacco leaf sap, respectively. An aliquot of 0.1 ml of virus-free or virus-infected sap was added per well and mixed with 0.1 ml of PBS-T, PBS-T plus 0.01 M Na DIECA, PBS-T plus 0.2 M diethanolamine, or 0.1 M Tris-HCl plus 0.85% NaCl plus 12% sodium sulfite. Final sap dilutions (after mixing sap with buffer plus additive) are reported in Results. The mean absorbance of the healthy sap plus two standard deviations was not subtracted from the mean absorbance with diluted virus-infected sap but was used to predict the dilution end point of virus detection from a regression equation.

Modified DAS-ELISA for PVS. A modified DAS-ELISA procedure (or heterologous double-antibody ELISA) was adapted from Bar-Joseph and Malinkson (3). Polystyrene cuvettes were coated with goat anti-PVS gamma globulin diluted to 0.3 ml of gamma globulin per 20 ml of bicarbonate buffer, pH 9.6, according to the instructions of the supplier, Boehringer-Mannheim. The coated cuvettes were incubated for 4 hr at 36 C, then washed seven times with PBS-T. Virus-infected sap was serially diluted in healthy sap, and an aliquot of 0.1 ml of sap was added per well and mixed with

Table 1. Effect of NaOH:EtOH pretreatment of new polystyrene cuvettes on absorbance (A_{405}) values of an enzyme-linked immunosorbent assay for potato viruses S, X, and Y (PVS, PVX, and PVY)

	Absorbance[a]		
Virus	No pretreatment	NaOH:EtOH pretreatment	Difference[b,c]
Healthy	0.031	0.046	0.015
PVX	1.808	2.160	0.352*
Healthy	0.095	0.114	0.019*
PVS	1.655	1.832	0.177*
Healthy	0.126	0.156	0.019*
PVY	1.698	1.935	0.237*

[a] Each number represents the mean absorbance of three experiments, 10 paired cuvette strips, four wells per strip. Virus-specific absorbance equals mean absorbance of infected sap minus mean absorbance of healthy sap +2 SD of mean absorbance of healthy sap.
[b] Difference = absorbance with NaOH:EtOH pretreatment minus absorbance without pretreatment.
[c] Means of pretreated and untreated cuvettes followed by an asterisk were significantly different according to a paired t test ($P = 0.05$).

Table 2. Effect of additives to plant sap on absorbance (A_{405}) values of an enzyme-linked immunosorbent assay for potato viruses S, X, and Y (PVS, PVX, and PVY)

	Absorbance[b]					
Additive[a]	Healthy[c]	PVS[c]	Healthy[c]	PVX[c]	Healthy[c]	PVY[c]
PBS-T (control)	0.215 ab	1.441 abc	0.055 a	1.674 cd	0.097 a	1.175 bc
Distilled water	0.200 ab	1.372 bc	0.080 a	1.545 cde	0.107 a	1.334 bc
0.01 M Na DIECA	0.132 a	1.637 a	0.054 a	1.674 cd	0.103 a	1.613 a
0.2 M Diethanolamine	...	...	0.062 a	2.019 a	0.103 a	0.855 d
6% Sodium sulfite	0.126 a	1.339 c	0.059 a	1.861 ab	0.125 a	1.131 c
0.5% Pyridine	0.320 bc	1.330 c	0.086 a	1.737 bc	0.144 a	1.366 b
6% Pyridine	0.248 abc	1.608 abc	0.190 b	1.438 efg	0.101 a	1.210 bc
6% Pyridine + 0.2 M Na EDTA	0.371 c	1.623 ab	0.217 b	1.334 g	0.102 a	1.354 b
0.5% Pyrolidine	...	...	0.054 a	1.546 def	0.088 a	0.655 e
0.04 M Urea	0.187 ab	1.412 abc	0.073 a	1.663 cd	0.104 a	1.255 bc
0.4 M Mercaptoethanol	0.201 ab	1.397 abc	0.056 a	1.383 efg	0.089 a	0.744 de
0.2% Cysteine HCl	0.188 ab	1.374 bc	0.057 a	1.509 defg	0.096 a	1.128 c
3.0% Igepon T-73	0.212 ab	1.380 bc	0.063 a	1.674 cd	0.124 a	0.923 d
0.02 M Caffeine	0.214 ab	1.393 abc	0.065 a	1.632 cd	0.127 a	1.363 b
0.1% PEG	0.179 ab	1.501 abc	0.092 a	1.670 cd	0.108 a	1.214 bc

[a] PBS-T = phosphate-buffered saline plus Tween 20, Na DIECA = sodium diethyldithiocarbamate, Na EDTA = disodium ethylenediaminetetraacetate, and PEG = polyethylene glycol.
[b] Each number represents the mean absorbance of three experiments, four wells per additive in each experiment. Virus-specific absorbance equals mean absorbance of infected sap minus mean absorbance of healthy sap +2 SD of mean absorbance of healthy sap.
[c] Means within a column and followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

0.1 ml of 12% sodium sulfite in 0.1 M Tris-HCl plus 0.85% NaCl. This buffer plus additive gave the greatest PVS-specific absorbance with the modified assay.

The cuvettes containing antigen were incubated overnight at 4 C, then washed nine times with PBS-T. Rabbit anti-PVS antibody (1.0 μg/ml) was added to each well and incubated at 22 C for 1.5 hr. Unbound antibody was removed by washing the cuvettes seven times with PBS-T, and a 1:600 dilution of goat antirabbit-IgG antibody conjugated to alkaline phosphatase (Sigma) was added.

Cuvettes containing enzyme conjugate were incubated at 22 C for 1.5 hr, then washed seven times with PBS-T, and substrate was added. Absorbances of hydrolyzed substrates were measured after identical time intervals for the modified and unmodified assays. Mean absorbance of healthy sap plus two standard deviations of the mean was not substracted from the mean absorbance of the virus-infected sap but was used to predict the dilution end point from a regression equation. Statistical significance of the predicted dilution end points was determined by linear calibration modified for population prediction (7).

RESULTS

Pretreatment of polystyrene cuvettes. Pretreating new Gilford EIA polystyrene cuvettes for 48 hr with 5.3 M NaOH:EtOH resulted in a significant increase in the virus-specific absorbance of PVS, PVX, and PVY (Table 1). Virus-specific absorbance equals the mean absorbance of the infected sap minus the mean absorbance of the healthy sap plus two standard deviations of the mean absorbance of the healthy sap. The average increase in absorbance for the three viruses was 0.255 (14.8%); the greatest enhancement in absorbance was 0.352 (19.5%) for PVX. Pretreating new cuvettes with 5.3 M NaOH without EtOH or EtOH without NaOH did not result in a significant increase in virus-specific absorbance. Pretreatment with 5.3 M KOH, 17 M KOH, or 15 M NaOH also did not significantly increase the virus-specific absorbance.

Additives to plant sap. For assays of PVX, addition of 0.2 M diethanolamine, pH 10.5, or 6% sodium sulfite gave a significantly higher virus-specific absorbance than that of the PBS-T control (Table 2). Enhancement in sensitivity caused by these additives appeared to be specific for PVX because they resulted in a lower virus-specific absorbance for PVS and PVY (Table 2). An increase in sodium sulfite concentration from 6 to 12% caused an additional significant increase in the virus-specific absorbance for PVX. The effectiveness of sodium sulfite was not significantly changed by using distilled water or 0.1 M Tris-HCl instead of PBS-T as the solvent. Best results were obtained with 12% sodium sulfite added to 0.1 M Tris-HCl plus 0.85% NaCl, which caused a 0.453 (32.9%) increase in the PVX-specific absorbance over the PBS-T control.

Addition of 0.01 M Na DIECA to PVY-infected sap gave a virus-specific absorbance significantly greater than those obtained with any other additives tested and the PBS-T control (Table 2). Na DIECA (0.01 M) increased the PVY-specific absorbance by 0.438 (37.3%) over that of the PBS-T control.

Although addition of 0.01 M Na DIECA resulted in the highest increase in virus-specific absorbance for PVS, it was not significantly different from the absorbance for the PBS-T control (Table 2). Data for 0.5% pyrrolidine and 0.2 M diethanolamine were not included because they produced highly variable results. Combining 0.01 M Na DIECA with 12% sodium sulfite resulted in a virus-specific absorbance lower than that with PBS-T or either additive alone. No single additive enhanced the sensitivity of ELISA for PVS, PVX, and PVY.

Effect of additives on the dilution end point for virus detection. To obtain a regression line for prediction purposes, the data were transformed to produce a plot of log of the absorbance (without the absorbance of healthy sap subtracted) versus log of the reciprocal of the sap dilution. This resulted in a linear relationship with a high R^2 value, indicating a good correlation between the transformed absorbance and sap dilution. The dilution end point of PVS-, PVX-, or PVY-infected sap was calculated from these lines and was defined as the infected sap concentration whose absorbance exceeded the mean absorbance of the healthy sap plus two standard deviations of the mean.

For PVS, the highest dilution where virus could be detected was 1:6,989 when infected sap diluted in healthy sap was mixed 1:1 with PBS-T (Table 3, Fig. 1).

Addition of 12% sodium sulfite tripled and diethanolamine doubled the PVX dilution end point over that of the PBS-T control (Table 3). This enhanced

Table 3. Relationship of absorbance (A_{405}) of enzyme-linked immunosorbent assay (ELISA) to dilutions of potato viruses S-, X-, and Y-infected sap combined with various additives and results of regression analysis and predicted dilution end points[a]

Potato virus	Additive + buffer[b]	Predicted dilution end point[c]	Mean A_{405} of healthy (+2 SD)	R^2	Regression equation[d]
PVS	PBS-T	1:6,989	0.191	0.894	$Y = 0.692 - 0.367X$
PVS	12% Sodium sulfite + 0.1 M Tris-HCl	1:4,913	0.169	0.828	$Y = 0.741 - 0.410X$
PVS	0.1 M Na DIECA + PBS-T	1:6,758	0.210	0.895	$Y = 0.835 - 0.395X$
PVX	PBS-T	1:3,448	0.097	0.966	$Y = 0.794 - 0.511X$
PVX	12% Sodium sulfite + 0.1 M Tris-HCl	1:10,985	0.059	0.978	$Y = 1.12 - 0.582X$
PVX	0.02 M diethanolamine + PBS-T	1:6,723	0.054	0.984	$Y = 0.983 - 0.588X$
PVY	PBS-T	1:2,715	0.154	0.839	$Y = 0.697 - 0.440X$
PVY	12% Sodium sulfite + 0.1 M Tris-HCl	1:1,474	0.261	0.803	$Y = 0.672 - 0.396X$
PVY	0.1 M Na DIECA + PBS-T	1:6,448	0.148	0.930	$Y = 0.963 - 0.470X$

[a] Regression equation and coeffient of correlation (R^2) for additives combined with PVS were derived from data in Figure 1. Figures used to derive regression equations for additives combined with PVX and PVY are not shown.

[b] PBS-T = phosphate-buffered saline plus Tween 20 and Na DIECA = sodium diethyldithiocarbamate.

[c] Dilution of infected sap, predicted from regression equation, whose ELISA absorbance equals mean absorbance of healthy sap +2 SD of mean.

[d] Y = log of absorbance, X = log of reciprocal of sap dilution.

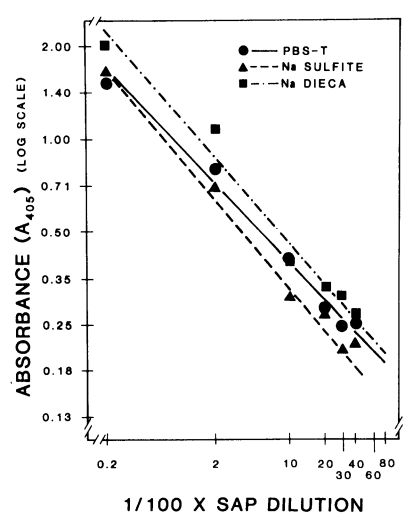

Fig. 1. Effect of additives on detection of potato virus S. Enzyme-linked immunosorbent assay absorbances of sap dilutions combined with either phosphate-buffered saline plus Tween 20 (PBS-T), 0.01 M sodium diethyldithiocarbamate (Na DEICA) in PBS-T, or 12% sodium sulfite in 0.1 M Tris-HCl plus NaCl. Each point is the mean of three experiments; regression equation and R^2 are determined from means of each experiment. Dilution end point for virus detection is at the right terminus of the regression line.

Table 4. Relationship of absorbance (A_{405}) of enzyme-linked immunosorbent assay (ELISA) to dilutions of PVS-infected sap using a modified and unmodified double-antibody sandwich (DAS) procedure and results of regression analysis and predicted dilution end points[a]

Assay type	Dilution end point[b,c]	Mean A_{405} of healthy (+2 SD)	R^2	Regression equation[d]
Modified DAS[e]	1:12,368 a	0.211	0.966	$Y = 1.28 - 0.477X$
DAS-2[f]	1:8,433 b	0.178	0.976	$Y = 1.38 - 0.543X$
DAS-1[g]	1:8,667 b	0.069	0.932	$Y = 1.01 - 0.551X$

[a] Regression equation and R^2 derived from data represented in Figure 2.
[b] Dilution of infected sap, predicted from regression equation, whose ELISA absorbance equals mean absorbance of healthy sap +2 SD of mean.
[c] Predicted dilution end points followed by same letter are not significantly different according to linear calibration ($P = 0.10$).
[d] Y = log of absorbance, X = log of reciprocal of sap dilution.
[e] Modified DAS-ELISA.
[f] Unmodifed DAS-ELISA whose maximum absorbances are similar to those of the modified DAS.
[g] Unmodified DAS-ELISA whose absorbance values were read at same time as modified DAS.

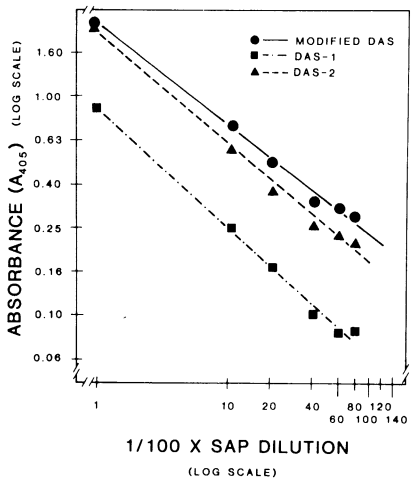

Fig. 2. Enzyme-linked immunosorbent assay (ELISA) absorbances of sap dilutions using a modified double-antibody sandwich (DAS) ELISA, an unmodified DAS assay (DAS-1) read at same time as the modified DAS, and an unmodified DAS assay (DAS-2) read at a comparable maximum absorbance as the modified DAS. Each point is the mean of four experiments; regression equation and R^2 are determined from means of each experiment. Dilution end point for virus detection is at the right terminus of the regression line.

sensitivity agreed with results with undiluted sap where addition of sodium sulfite and diethanolamine significantly increased the PVX-specific absorbance over that using a PBS-T control (Table 2).

Addition of Na DIECA doubled the dilution end point for PVY over that using a PBS-T control (Table 3).

Modified DAS-ELISA for PVS. Using the transformed log absorbance versus log of the reciprocal of the sap dilution, the predicted dilution end point of a modified DAS-ELISA was significantly greater than the dilution end point of the unmodified DAS-ELISA (Table 4, Fig. 2). Despite a higher PVS absorbance when the results of the unmodified DAS-ELISA were read later, the dilution end point remained virtually unchanged. This was probably due to a corresponding increase in the absorbance of the healthy controls. The rate of the enzymatic reaction with virus-infected and healthy sap was greater in the modified assay than in the unmodified assay.

DISCUSSION

Pretreating cuvettes with NaOH:EtOH may enhance the sensitivity of ELISA for PVS, PVX, and PVY by removing impurities on the surface of new polystyrene (6). These impurities are probably organic compounds with limited water solubility that NaOH:EtOH may help to solubilize. The NaOH may also cause a certain degree of base-catalyzed hydrolysis of the impurities. Impurities on polystyrene could reduce or weaken antibody binding so that antibody or antibody-antigen complexes elute from the surface during latter steps of the assay.

The sensitivity of ELISA for PVY was especially enhanced by the addition of Na DIECA, which may directly help stabilize the nucleocapsid structure of PVY and thereby enhance its serological reactivity.

Relatively high concentrations (6 and 12%) of sodium sulfite may dissociate viruses by reducing the disulfide bonds of viral proteins; diethanolamine and pyridine are alkaline viral degrading agents (5) and these compounds, when added to virus-infected sap, resulted in the greatest increase in the sensitivity of ELISA for PVX. It is possible that partial degradation of the virus into fragments enhanced its serological activity for ELISA.

In general, increases in virus-specific absorbance with undiluted sap agreed well with increases in the dilution end point for the additives tested. This indicated that the prediction of a dilution end point from a regression equation was a reasonable means of determining ELISA sensitivity.

The dilution end points predicted for PVS, PVX, and PVY detection were frequently beyond the range of the experimental results. One should be cautious in interpreting the predictions because the linear regression model may not be valid at those dilutions. There was, however, no evidence of any trend toward nonlinearity in the data nor was there evidence that the predictions were invalid. Some of the predicted dilution end points were within the range of experimental results. For instance, the dilution end point for PVX using PBS-T was predicted to be 1:3,448 (Table 3) and was found experimentally to be about 1:3,000. The dilution end point for PVY using 12% sodium sulfite was predicted to be 1:1,474 (Table 3) and was determined experimentially to be slightly less than 1:2,000.

ELISA results have not usually been interpreted by regression analysis. If no transformations of data are made, then one may conclude that alterations in the assay are normally ineffective because absorbance values differ by only small amounts at very high virus dilutions. When combined with data from lower dilutions to form a regression equation, these low absorbances can still indicate large differences in sensitivity. In a virus dilution series, all the data, rather than only that of the highest virus dilution, should be used to evaluate the effect of a modification on the sensitivity of ELISA.

Bar-Joseph and Malinkson (3) reported a threefold increase in sensitivity of a modified DAS-ELISA, but only a 30–32% increase was observed in our experiments. This enhancement was still relatively large and could be important for assays requiring the maximum possible sensitivity. Increasing the length of substrate reaction for the unmodified assay did not compensate for its slower reaction rate, and the virus-specific absorbance for the unmodified assay remained lower than that for the modified assay. The dilution end point or sensitivity of the assay was relatively constant during the period when the enzymatic rate of substrate conversion was linear.

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