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DEVELOPMENT OF ENZYME LINKED IMMUNOSORBENT ASSAY FOR POTATO VIRUS X IN PAKISTAN

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Abstract

Potato virus X (PVX) was purified in substantial quantities and used for the production of antiserum with a homologous titre of 1: 256 in double immunodiffusion test. Immunoglobulins (IgG) were separated from the antiserum, conjugated with alkaline phosphatase and ELISA kit was prepared locally at a much cheapeer cost (<50%) than the imported one. Appropriate conditions for specific reactions i.e. dilutions, incubation time and precipitatioon were determined. The results were highly comparable and large number of potato samples tested against PVX with great rapidity and sensitivity.

Introduction

Different serological techniques are used for the identification of plant viruses, detection of viral infection and for determining close and remote serological relationships among viruses (Regenmortel, 1982). The techniques such as tube precipitin and microagglutination, gel immunodiffusion or latex require adequate quantities of good quality, high - titre antisera and high concentration of antigens so that the antibody/antigen reactions are clearly depicted. Some of the conventional techniques are simple,specific and can be standardized easily and comparable to biological and physical assays. However, these have limitations for large scale application and indexing of many important viruses because of low virus concentration in host, unsuitable particle morphology and use of specialized equipment. Moreover, they are costly, time consuming and laborious .

Voller *et al.,* (1976) and Clerk & Adams (1977) have developed Enzyme-linked immunosorbent Assay (ELISA) which is based on enzymatic reaction of the immunoglobulins (IgG) with antigen. This system combines rapidity and sensititivity of the serological virus assay with enhanced biological enzymatic reaction. Recent adoption of this technique created new interest in serodiagnosis of viruses in plants. It can detect extremly low concentration (1 ng/ml) of viruses in the host (Bar-Joseph & Garnsey, 1981). It has also been used to detect viruses in seeds (Lister, 1978), crude extracts and aphids vectors (Gera *et al.,* 1978, Gugerli, 1984), and recently its major application has been recognized in large scale assay of potato viruses in breeding lines, production of virus -free material and for seed certification programmes. (Bar-Joseph & Garnsey, 1981).

ELISA is highly specific and sensitive technique and requires immunoglobulins and conjugate which are of good quality, high -titre and free of antihost substances. Most of

the viruses are often not detected in infected plants due to extreme strain specificity of this technique with immunoglobulins and conjugates prepared for virus strain of foreign origin. These virus strains could easily be detected if immunoglobulins and conjugates prepared against these strains are used. Attempts were made to produce antiserum of the PVX locally and prepare lgG and enzyme conjugate for PVX -detection on a large scale in potato.

Materials and Methods

Virus Purification: PVX was propogated in *Nicotiana rustica* plants which were raised in an insect-free glass house and inoculated at 4-5 leaf stage. Leaves with well developed symptoms were havested after 3 weeks and after removing their midribs, were chilled and homogenized in 0.1 M Tris citric acid (TCA) buffer, pH 9.0, containing 0.15% thioglycollic acid (1 : 2 w/v). The homogenate was emulsified with one half volume each of carbon tetrachloride and diethyl ether and emulsion was broken at low speed centrifugation. The virus was precipitated by adding to the supernatant 5% polyethylene glycol (PEG, mol. wt. 6000) and 2% NaCl, the pellet was collected by centrifugation at 10,000 rpm for 10 min and suspended in 0.1 M TCA buffer. After clarification, the virus was sedimented by ultracentrifugation at 27,000 rpm for 75 min and pellet was resuspended in 0.1 M TCA buffer and stirred for 90 min at 4°C. Clarified suspension was passed through 20% sucrose pad at 30,000 rpm for 90 min and then layered on top of 10-40% linear sucrose density gradients and centrifuged for 1.5 h at 24,000 rpm in a SW-25 rotor. Virus zone was collected, dialysed against 0.01 M TCA buffer and concentrated at 25,000 rpm for 2.5 h. Purified virus preparations were used for electron microscopic examination and production of antiserum. Virus concentration was determined by spectrophotometry.

Antiserum Production: Antiserum to PVX was produced in an Australian Chinchilla rabbit which was given 3 intramuscular injections, each at 10-day intervals, followed by booster injection at one month interval. The antigen consisted of 1 mg/ml purified virus mixed with an equal volume of Freund's complete adjuvant. The blood was collected by puncturing ear vein of the rabbit, allowed to clot at room temperature and serum was separated by centrifugation at 6000 rpm for 30 min. Sodium azide was added to the antiserum as a preservative at final concentration of 0.01% and titre and purity were determined in gel immunodiffusion (Noordam, 1973). Serial dilutions of the antiserum were made in 0.14 M sodium chloride. Purified virus used as antigen was sonicated in a Titertek Ultrasonic cleaner for 10 min., to make it diffusible in gel (Tomlinson & Walkey, 1967).

Preparation of Immunoglobulins (lgG) for ELISA: Immunoglobulins were separated by ammonium sulphate precipitation method (Regenmortel, 1982). The antiserum

was diluted with distilled water (1 : 9) and 10 ml of 3 M ammonium sulphate was added drop wise while stirring. After incubation for 1 h at room temperature (25°C), precipitate was collected by centrifugation at 8,000 rpm for 10 min. and dissolved in 2 ml of half strength phosphate buffer saline (1/2 PBS). The suspension was dialysed 3 times against 500 ml of 1/2-PBS and passed through G-25 Sephadex column. Fractions were collected and their optical density (OD) was measured at 280 nm in a Beckman spectrophotometer Model 34 coupled with recorder. All fractions showing OD above 1.4 were combined and adjusted at 1.4 OD with 1/2-PBS.

Enzyme Conjugation of lgG: Alkaline phosphatase (E.C. 3.1.3.1. VII, Sigma) was centrifuged for 10 min at 8,000 rpm. Supernatant was discarded and the pellet was dissolved in 2 ml of purified lgG and dialysed 3 times against 500 ml of 1/2-PBS. The dialysates were fixed with 0.06% glutaraldehyde (Avrameas, 1969) and incubated at room temperature for 4 h. Excess glutaraldehyde was removed by dialysis against 1000 ml of 1/2-BPS. Finally, 5 mg/ml bovine serum albumin (BSA) and 0.02% sodium azide were added and the conjugate was kept in glass vials at 4°C. Locally produced 1gG and IgG-conjugate were compared with commercial preparations (Bioreba, Switzerland).

ELISA test procedure: It was performed as described by Clark & Adams (1977) with the exception that the plates were incubated at 25-30°C (McLaughlin *et. al.*, 1981). Polystyrene ELISA- plates were coated with IgG, antigen, conjugated IgG and then with enzyme substrate. After every step, plates were incubated for specific period and thoroughly washed with PBS Tween 20.

Different dilutions of IgG and enzyme conjugate were made in coating buffer (0.05 M sodium carbonate, pH 9.6) and conjugate buffer (PBS-Tween containing 2% Polyvinylpyrrolidon (PVP) and 0.2% ovalbumin), respectively, to determine the best dilution. Crude infected sap or viral antigen was prepared in extraction buffer (PBS containing 0.05% Tween-20) and 2% PVP. Sample dilutions from fresh leaves were maintained at 1 : 10 (w/v). Substrate (p-nitropheny phosphate) was used at 0.7 mg/ml in substrate buffer (10% diethanolamine adjusted to pH 9.8 with HC1). In each plate, positive and negative controls were also included. Reaction was observed and extinction values were measured at 405 nm in a Titertek Multiscan Model MC 340. Samples giving double the value of negative control were considered as positive (Singh & Rojas, 1983).

Results

Properties of Purified Preparation of PVX: The method adopted for the purification of PVX was highly satisfactory and routinely applicable. The virus sedimented as a single component in the gradient tubes. Using an extinction coefficient of 3 (Purcifull & Edwardson, 1981), virus yield of about 30 mg/kg infected material was consistently ob-

Fig. 1. Electron micropraph of purified preparation of Potato Virus X negatively stained with 2% PTA (Mag. 58,000).

tained. Purified virus preparations were opalescent, showed light scattering and had UV absorption spectrum typical of uncleoprotein i.e. maximum at 260 nm and minimum at 240 nm with 260 and 280 ratio of 1:1.2. Preparations were negatively stained with 2% PTA (Phasphotungstic acid), pH 6.8, examined under JOEL EM 100, CX-11 electron microscope and found to contain large number of unaggregated and unbroken elongated particles which measured 490 nm in modal length (Fig.1). These were also free from normal plant proteins as no reaction was observed in immunodiffusion tests against anti-host serum (Fig.2).

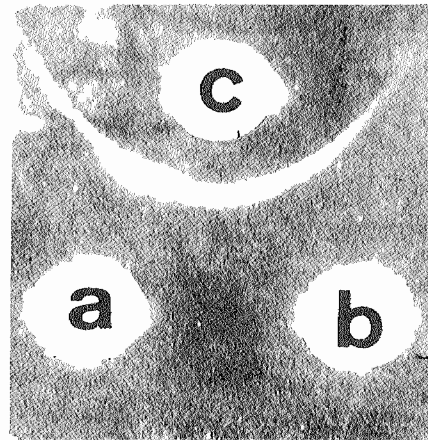

Fig. 2. Immunodiffusion test in agar gel between purified PVX and its antiserum. Wells a and b were charged with homologous antiserum and well c contained purified PVX (500 μg/ml) which was sonicated.

Properties of Antiserum Produced: PVX was found to be strongly immunogenic and in gel diffusion test antiserum had a homologous titre of 1 :128 after first bleeding and 1.256 after the booster injection. It gave a single precipitin line around the antigen well (Fig. 2) and did not react against healthy plant material which confirmed freedom of the antiserum from host antibodies.

Evaluation of PVX IgG and conjugate: Different dilutions of IgG and conjugated IgG were tried for coating the polystyrene plates and best results were obtained at a dilution of 1:1000 (Table 1). At dilutions lower than 1:800, extinction values did not increase but led to the formation of non-specific reaction due to intense colour. A minimum of 30 min., incubation was necessary to differentiate between positive and negative samples and clear reaction was observed with an incubation period of 45-60 min. Substrate at 0.7 mg/ml gave satisfactory results but at 1 mg/ml, reaction appeared faster without any significant increase in extinction values. Best hydrolysis activity was found within 30-45 min., of adding substrate and no significant change was noticed upto 90 min. Stability of conjugated IgG was not affected upto 16 months but was reduced to 50% after 20 months and to 20% after 24 months storage at 4°C (Table 1). Locally prepared ELISA-kit was as effective and efficient as the imported one and was used in testing large scale indexing of potato.

Discussion

Since the introduction of ELISA by Voller *et al.,* (1976) and Clark & Adams (1977), it has been adopted on a large scale (Bar Joseph & Garnsey, 1981), improved (McLaughlin *et al.,* 1981) and further modifications are anticipated (Gugerli, 1984). It has been successfully employed in the differentiation of closely related antigens, prevention of disease transmission through early detection of viruses in their respective vectors, controlling seed-borne diseases by sowing virus-free seed, large scale ELISA- base surveys, production of virus-free material, resistance breeding and in detection of latent virus infections. The multidimensional application of ELISA is derived from its simplicity, rapidity, sensitvity and economical use of reactants, not offered by any other serological technique. It does not involve use of elaborate equipments and antigens are easily detected in crude extracts. We attempted to develop an ELISA-kit locally and use it for potato improvement programme. PVX , a widely distributed and mechanically transmissible potato virus causing mosaic and latent infection in potato was selected for this study.

With the modified method of Francki & McLean (1971), it was possible to obtain substantial quantities of purified preparations of PVX which were free from host impurities (Fig. 1) and thus qualified specific antiserum production. The antiserum had a high homologous titre in gel immunodiffusion, complete freedom from antibodies to normal

Table 1. Activity and Characteristics of ELISA Kit of PVX (Extinction Values)

Treatment	Test sample (a)	Positive Infected control (b)	Healthy control	Observations
1. *Dilution of IqG and Conjugated IqG*				
1:400	Out of range	Out of range	0	Intense colour, non-specific reaction
1:600	"	"	0	" "
1:800	0.583	0.436	0	Moderate reaction
1:1000	0.589	0.436	0	Appropriate
(c) 1:1000	0.580	0.435	0	" "
2. *Incubation period of Coated plates*				
30 minutes	0.264	0.234	0	Difference visible
45 "	0.310	0.288	0	Differences distinct
60 "	0.553	0.431	0	Clear differences
2 hours	0.556	0.430	0	" "
3 "	0.553	0.433	0	" "
4 "	0.558	0.431	0	" "
3. *Addition of p-nitrophenyl phosphate*				
0.7 mg/ml	0.581	0.434	0	Reaction within 30 min.
1.0 mg/ml	0.580	0.434	0	Fast reaction
4. *Hydrolysis activity of substrate at 1 mg/ml*				
15 minutes	0.213	0.176	0	Initiation of reaction
30 "	0.310	0.248	0	Progressive reaction
45 "	0.552	0.432	0	Clear reaction
60 "	0.561	0.437	0	" "
75 "	0.561	0.439	0	" "
90 "	0.563	0.440	0	" "
4. *Viability/storability of IqG and conjugated IqG*				
6 months	0.554	0.430	0	Active
12 "	0.556	0.428	0	Active
16 "	0.530	0.419	0	Active
20 "	0.275	0.221	0	Activity reduced by 50%
24 "	0.121	0.107	0	Activity reduced by 80%

a) Fresh tobacco leaves from infected plant;
b) Standard infected control; maintained in freeze dried sample;
c) Kit imported from Bioreba, Switzerland.

host protein (Fig. 2) and did not produce any non-specific reaction. High titre antisera free from host antibodies are desirable for ELISA, but antisera with modest titre have also been used (Clark & Adams, 1977; Lister, 1978). Although we did not compare different concentrations of ammonium sulphate, but satisfactory results were obtained with 3 M $(NH_4)_2\ SO_4$ which agreed very well with Regenmortel (1982). Extinction values of IgG and conjugate of the locally prepared kit were comparable at 1:1000 with those of imported one (Table I).

The incubation period of IgG and conjugate is of less imprtance when only the negative and positive samples were to be detected. We were able to distinguish between negative and positive samples after 30-45 min., incubation, but prolonged incubation of conjugate gave higher level of reaction. Similar reports have been made by McLaughlin *et al.*, (1981) and Jankulova *et al.*, (1983). Selection of suitable combination of incubation time for IgG and conjugate also depended on the relative importance of the reaction level. In general, incubation of IgG at 30° C for 2-3 h and conjugate for 4 h has been reported to give satisfactory results. In this study, antigen was always incubated overnight at 4^0 C which was found to be convenient and produced precise and accurate results. Substrate p-nitrophenyl phosphate used at 0.7 mg/ml was suitable for routine tests and its higher concentration did not lead to any significant increase in extinction values. Hydrolysis reaction started appearing 15 min. after the addition of substrate, but colour intensity increased upto 45-60 min., and no significant increase was noticed till 1.5 h. Normally, results were read or visually evaluated 45 min., after the addition of substrate when the reaction was stopped with 50 µl of 3 M sodium hydroxide. Stability of conjugate appeared to be an important factor in ELISA. Its activity was not affected for 16 months but subsequently reduced to 50% and 20% after 20 and 24 months, respectively. However, negative and positive samples could be distinguished with lower level of reaction and colour intensity. Similar observations were made with the imported reactants.

The results suggest that the ELISA kits can be prepared locally at much cheaper cost than the imported ones. PVX kit was successfully used for commercial indexing of potatoes, selection of healthy propagative material and production of virus free seed.

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