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BRIEF COMMUNICATION

Effect of formaldehyde and glutaraldehyde fixation on the immunochemical reactivity of potato virus A with monoclonal antibodies

T. MORAVEC and N. ČEŘOVSKÁ

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ-160 00 Praha 6, Czech Republic

Abstract

Potato virus A (PVA) was treated with different glutaraldehyde concentrations at a range of different pH values, and its immunochemical reactivity and integrity was tested in various types of ELISA using a panel of six monoclonal antibodies. Glutaraldehyde fixation was compared with formaldehyde fixation, and there was no significant difference in reactivity between PVA non-treated and treated with formaldehyde, but almost the entire immunochemical activity was lost after treating with glutaraldehyde.

Additional key words: ELISA, polyclonal antibodies, *Solanum tuberosum*.

The potyvirus potato virus A (PVA) causes heavy economical losses in most potato growing areas. At present a large number of potato samples are checked by means of ELISA for presence of various plant viruses including PVA. One of the obstacles connected with immunochemical testing is the instability of the virus, and so we tried to prolong its storage stability. The immunochemical reactivity of some plant viruses can be enhanced by stabilising their capsids with either formaldehyde or glutaraldehyde (Hollings and Stone 1962, Hajimorad and Francki 1991). In this paper we report the effect of formaldehyde and glutaraldehyde fixation on stability and antigenic reactivity of PVA by using six murine monoclonal antibodies raised against native virus preparations.

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Abbreviations: DAS-ELISA - double antibody sandwich ELISA; IgG - immunoglobulins G; MAbs - monoclonal antibodies; PAbs - polyclonal antibodies; PBS - phosphate buffer saline; PTA-ELISA - plate trapped antigen ELISA; SWAM-AP - swine-anti-mouse antibody conjugated to alkaline phosphatase.

PVA isolated from potato (*Solanum tuberosum* L. cv. Lichte Industrie) (isolate LI) was obtained from the Institute of Potato Research and Breeding, Havlíčkův Brod, Czech Republic, and was maintained in *Nicotiana tabacum* cv. Samsun. The leaves of infected plants were harvested three to eight weeks after inoculation.

The virus was purified by high speed centrifugation followed by equilibrium density gradient centrifugation in caesium chloride according to Čeřovská *et al.* (1991). The purified virus was used for rabbit anti-PVA antiserum production and also for the production of mouse monoclonal antibodies (MAbs). Virus concentration was estimated spectrophotometrically (A_{260} = 2.8; Stace-Smith and Tremaine 1970).

Monoclonal antibodies were prepared in collaboration with Dr. F. Franěk from the Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, Prague according to the method of Galfré and Milstein (1981). The immunoglobulins G (IgG) were isolated from the ascitic fluid by precipitation using caprylic acid according to the method of Steinbuch and Audran (1969). Their concentration was estimated spectrophotometrically (A_{280} = 1.4).

Freshly isolated IgG were diluted to a concentration of 1 mg cm^{-3} by PBS and were mixed with 2 000 units of alkaline phosphatase (*Boehringer*, Mannheim, Germany). Then they were dialysed against 0.05 % glutaraldehyde (Avrameas 1969) and than three times against 200 cm^3 of PBS.

Rabbit polyclonal antiserum was obtained after immunisation with purified virus in complete Freund adjuvans (1:1, v/v). Immunisation doses of 50 to 200 mg of purified virus each were applied subcutaneously. IgG isolated from the rabbit antiserum were conjugated the same way as the MAbs.

Double antibody sandwich ELISA (DAS-ELISA) was performed according to the method of Clark and Adams (1977). The wells of microtitre plates (*UMG*, Czech Republic) were coated with PAbs (2 mg cm^{-3}). The antibody solution was incubated for 4 h at 37 °C. After incubation the wells were washed four times with PBS containing 0.05 % Tween. After washing, the wells were incubated with antigen solution for 4 h at 37 °C. Bounded antigen was detected using MAb-alkaline phosphatase conjugate at a final concentration of 2 mg cm^{-3}.

For the plate-trapped antigen, a form of indirect ELISA (Mowat 1985) was performed in three steps, which were separated by washing the wells as previously described. For the first step the wells contained purified virus or fixed virus preparation (100 and 200 ng cm^{-3}) in a coating buffer (0.03 M carbonate buffer, pH 9.6), for the second step this was replaced by MAbs (5 mg cm^{-3}) and for the third step the linked MAbs were detected using SWAM-AP conjugate at a concentration of 1 - 2 mg cm^{-3}.

Purified virus preparations were fixed with glutaraldehyde as described by Hajimorad and Francki (1991). Virus at concentrations 0.1 - 0.01 mg cm^{-3} was dialysed against 1 - 0.01 % formaldehyde or glutaraldehyde in different buffer systems with various pH values. For pH = 5.7 (0.05 M) acetate and phosphate buffers were used, for pH value 6.5 acetate, phosphate and carbonate buffers, and for pH 7.4 phosphate and carbonate buffers. Excess of the aldehyde was removed by overnight dialysis against buffer.

In the first set of experiments we determined the effect of various concentrations of stabilising agents (glutaraldehyde, formaldehyde) on the reactivity of virus particles with separate MAbs in PTA ELISA and/or in DAS-ELISA (Table 1). In the second set of experiments we investigated the effect of different pH values on the surface of the virus particle by its fixation with either glutaraldehyde or formaldehyde. There were no differences between individual buffer systems used and so we discuss activity dependence only on the pH value. We assumed that these monoclonals which are directed against continuous epitopes would be less sensitive to the conformational changes fixed by aldehyde stabilisation (Table 2).

Table 1. Effect of aldehyde fixation on the immunochemic activity of purified virus. The virus preparation was fixed in carbonate buffer, pH 9.6, and was then subjected to PTA or DAS ELISA. The binding affinity of rabbit polyclonal and six monoclonal (151, 187, 290, 328, 534, 634) antibodies to the fixed antigen were compared with their affinity to the similarly treated but not fixed antigen (control). A_{405} more than 80 (++++), 60 (+++), 40 (++) and 20 (+) % of control and between 0 and 20 % of control (0). Results of PTA experiments are in the numerator, while results from DAS-ELISA arrangement are in denominators.

Treatment	Concentration [%]	Antibody Polyclonal	151	187	290	328	534	634
Formaldehyde	0.50	++	++/+	+++/++	++/+	++/++	0/0	0/0
	0.20	++	+++/++	+++/++	+++/+	++/++	0/0	0/0
	0.10	++	+++/++	+++/+	++/++	+/++	0/+	0/0
	0 05	+++	+++/++	+++/++	++/++	+/+	+/+	0/+
	0.02	+++	++++/++	+++/+	+++/+	+/+	+/++	+/+
	0.01	+++	++++/++	++/+	+/++	+/+	+/++	+/++
Glutaraldehyde	0 50	+	0/0	0/0	0/0	0/0	0/0	0/0
	0.20	+	0/+	0/0	0/0	0/0	0/0	0/0
	0.10	++	0/+	0/0	0/0	0/0	0/0	0/0
	0.05	++	+/+	0/+	+/0	0/+	0/+	0/0
	0.02	+++	+/+	+/+	+/+	0/+	0/+	0/+
	0.01	+++	++/+	+/+	++/+	0/++	0/++	0/+

Table 2. Effect of pH during stabilisation of the virus with 0.05 % formaldehyde in 0.05 M acetate, phosphate or carbonate buffer. The fixed antigen was subjected to PTA/DAS ELISA and the A_{405} values were compared with appropriate absorbances of not fixed (control) wells. Symbols are identical as in Table 1.

pH	Antibody Polyclonal	151	187	290	328	534	634
5.7	+/+	+/0	0/0	+/0	0/0	0/+	0/0
6.5	+/+	+/0	0/0	+/0	0/0	0/++	0/0
7.5	++/++	++/++	+/0	+/0	0/+	0/++	0/+
8.0	++/++	++/++	++/0	++/+	+/+	+/++	+/+

The aldehyde fixation did not increase the immunochemical activity either with polyclonal antibodies or with monoclonals. In most cases the effect was adverse, especially when the bifunctional agent (glutaraldehyde) was used. The effect of glutaraldehyde was detrimental even at low concentrations in almost all conditions tested. Its ability to block the binding of antibodies was very intensive even when formaldehyde was used for prior saturation of free amino moieties in higher concentration. On the other hand, treating with formaldehyde was not so unfavourable for binding of selected antibodies, but it embodied no positive effect. Activity of stored stabilised material decreased at the same rate as that of the non-stabilised.

Glutaraldehyde may form covalent cross-links between free amino-groups (Richards 1968) and is also able to cross-link adjacent coat protein subunits, even the possibility of forming inter-viral cross-links is not impossible. With respect to long rod shaped virions, we may suppose that the creation of large cross-linked virus aggregates after glutaraldehyde fixation is the main reason for the loss of immuno-reactivity. This was confirmed by SDS-PAGE, where the fixed virus preparations were not able to enter the gel even when they were denatured by boiling in 2 % SDS under reduction conditions.

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