ELECTRON MICROSCOPIC OBSERVATION OF POTATO VIRUS A USING MURINE MONOCLONAL ANTIBODIES

T. MORAVEC, N. ČEŘOVSKÁ, A. PAVLÍČEK

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

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Summary. – Six monoclonal antibodies (MoAbs) against potato virus A (PVA) were prepared and used in enzyme-linked immunosorbent assay (ELISA), immunoblot analysis and electron microscopic study of the virus. Four MoAbs, 151, 290, 328 and 634, reacted with purified virus preparation in dot blot test and showed strong reaction also with virus coat protein (CP) denatured by sodium dodecyl sulphate (SDS), while two MoAbs, 534 and 187, gave significantly weaker reaction with denatured CP than with purified virus. On electron micrographs, MoAb 534 effected binding only on few separate locations of the virus surface after prolonged storage. We presume that this MoAb recognized a conformation-dependent epitope.

Key words: potato virus A; monoclonal antibodies; immunoblot analysis; electron microscopy

Introduction

PVA (genus *Potyvirus,* family *Potyviridae*) belongs to economically important potato pathogens. At present a large number of potato samples are routinely checked by means of ELISA for the presence of various viruses including PVA. For effective diagnosis, a set of 6 MoAbs has been recently prepared. These have been characterised by various ELISA and competition binding tests and their cross-reactivity with some other potyviruses (Čeřovská and Filigarová, 1992) as well as their reactivity with proteolytically cleaved PVA CP have been examined (Šubr, 1996).

MoAbs reacting with viruses can be generally divided into 3 groups. The first group consists of MoAbs that do not bind to virus CP in double-antibody sandwich ELISA (DAS-ELISA) or plate-trapped antigen ELISA (PTA-ELISA). They can recognise only virus particles bound to PVA antibody pre-coated microtitre plate wells. The epitopes that are recognised by these MoAbs arise from three-dimensional structure of the virus particle and are called neotopes (Van Regenmortel, 1982). MoAbs of the second group recognise both virus particles and CP in ELISA. In this case the target epitopes are present on both virus particles and CP and are called metatopes (Van Regenmortel, 1982). MoAbs of the third group recognise CP in both DAS-ELISA and PTA-ELISA but are unable to bind to virus particles in antibody-coated wells. These MoAbs react with continuous stretches of amino acid residues of the CP sequence, which are buried during the assembly of the virus particle. These MoAbs are thus specific for cryptotopes, i.e. epitopes that are present in the isolated protein but absent in the assembled virus particle. The fact that these MoAbs react with virus directly adsorbed on solid phase can be explained by partial degradation, which takes place on the plastic due to high pH (Altschuh *et al.,* 1985).

Abbreviations: BSA = bovine serum albumin; CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; DIECA = sodium N, N-diethyldithiokarbamate; EDTA = ethylene diamine tetraacetate; ELISA = enzyme-linked immunosorbent assay; MoAb = monoclonal antibody; PAb = polyclonal antibody; PBS = phosphate-buffered saline; PMSF = phenylmethylsulfonyl fluoride; PVA = potato virus A; PTA-ELISA = plate-trapped antigen ELISA; SDS = sodium dodecyl sulfate; SWAM-AP = swine anti-mouse alkaline phosphatase

Materials and Methods

MoAbs 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 (IgGs) were isolated from the ascitic fluid by caprylic acid precipitation according to the method of Steinbuch and Audran (1969).

Virus isolates 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 plants were mechanically inoculated and the leaves were harvested 3 – 8 weeks after inoculation.

Virus purification was carried out by high speed centrifugation on 30% sucrose cushion at 27,000 rpm for 3 hrs (Beckman rotor 50.2) according to Čeřovská *et al.* (1991). The purified virus was used for production of rabbit antiserum against PVA (polyclonal antibody, PAb) and also for the production of MoAbs. Virus concentration was estimated spectrophotometrically (A_{260} = 2.8 for 0.1% solution) (Stace-Smith and Tremaine, 1970). Such a virus preparation is further described as purified virus. Denatured virus was prepared from the purified virus by boiling for 5 mins in 2 x sample buffer for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (2% SDS, 5% ß-mercaptoethanol, 0.06 mol/l Tris, 10% glycerol, and proteinase inhibitors 1 mmol/l phenylmethyl sulfonyl fluoride (PMSF), 2 mmol/l sodium N,N-diethyldithiokarbamate (DIECA), 1 mmol/l ethylenediamine tetraacetate (EDTA), 5 µg/ml aprotinin and 1 µg/ml pepstatin (Smith, 1997).

DAS-ELISA was performed according to the method of Clark and Adams (1977). The coating with PAbs (2 µg/ml) proceeded at 37°C for 4 hrs. The same time and temperature were used for the binding of the antigen. The latter was detected with a MoAb-alkaline phosphatase conjugate at a final concentration of 1 µg/ml. A result was regarded as positive when its absorbance was at least equal to the twofold of the absorbance of the negative control.

PTA-ELISA, a form of indirect ELISA (Mowat, 1985), was performed in three steps. In the first step, the wells contained the purified virus (0.1 – 1 µg per ml) in a coating buffer. In the second step, the virus was replaced by MoAbs at a concentration of 0.5 –5 µg per ml. In the third step, the bound MoAbs were detected with swine anti-mouse alkaline phosphatase (SWAM-AP) conjugate at a concentration of 1 µg/ml. The positivity of a result was evaluated in the same way as by DAS-ELISA.

SDS-PAGE. Virus purificates in the sample buffer were resolved by SDS-PAGE according to Laemmli (1970) in 1.92 mol/l glycine – 0.25 mol/l Tris buffer. The bands were visualised by Coomassie Brilliant Blue or silver staining (Marcinka *et al.,* 1992).

Immunoblot analysis. Proteins resolved by SDS-PAGE were electroblotted on PVDF or nitrocellulose membrane (Sigma, 0.45 µm) using a semidry Omni-Bio (Czech Republic) apparatus according to the manufacturer's instructions. The membrane was then incubated at room temperature for 1 hr in 4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and then washed four times with PBS. Then the membrane was either cut into stripes, which were incubated individually in plastic bags with different concentrations of various antibodies, or directly incubated with various concentrations of primary antibodies in a Mighty Small® cell (Hoefer). This device allows simultaneous incubation of one membrane with different antibodies and/or with various concentrations of the same antibody. The bound antibody was detected with a SWAM-AP conjugate (overnight incubation, 4°C) and visualised with BCIP/NBT tablets (Sigma) according to supplier's instructions.

Dot blot test. Samples of virus purificates were denaturated, applied to a membrane (1 – 2 mg of virus per dot) and further processed in a standard manner.

Electron microscopy. Samples were prepared essentially as described by Milne (1975). Drops (20 µl) of rabbit PAbs (10 µg/ml) in the coating carbonate buffer pH 9.6 were deposited on microscopic grids. After 1 hr of incubation, the grids were covered with drops (10 ml) of the purified virus (1 – 10 µg/ml) in PBS for the 1 hr at room temperature. Then the grids were incubated with different dilutions of MoAbs for an additional hour. The bound mouse MoAb was visualised with a goat anti-mouse antibody conjugated to particles of colloid gold (15 nm, BioCell). After each step, the grids were washed with a drop (20 ml) of PBS 4 times for 1 min. The grids were examined after staining with 1% uranyl acetate.

Results and Discussion

ELISA

At first we examined the MoAbs for their reactivity with purified virus and denatured viral CP in ELISA. Four of them (MoAbs 151, 290, 328 and 634) showed the same reactivity with both the antibody-trapped (DAS-ELISA) and the plate-trapped antigen (PTA-ELISA) (Table 1). Regarding these MoAbs we can postulate that their target epitopes are metatopes, i.e. epitopes that are present on both the PVA particle and the PVA CP subunit (Regenmortel, 1982). MoAbs 534 and 187 reacted with purified virus particles only in DAS-ELISA. They showed no reactivity with denatured CP in either type of ELISA. The strength of the reaction decreased with the time of sample storage. It is known that during the direct binding of virus particles to polystyrene surface of microtitre plate a disruption of virus particles and an unfolding of proteins take place (Dore *et al.*, 1988). Thus we can suggest that the target epitope of this antibody is conformation-dependent.

Dot blot test

A similar pattern of reactivity of the MoAbs was observed in the dot blot test. Again, MoAbs 151, 290, 328 and 634 were able to react with purified virus and their reactivity was only slightly affected by denaturing the virus. The reaction with the denatured virus was significantly weaker but still clearly apparent (data not shown). This can be

Table 1. Reactivity of MoAbs in dot blot test, DAS-ELISA and PTA-ELISA

MoAb	Dot blot test		DAS-ELISA		PTA- ELISA	
	Purified virus	Denatured virus	Purified virus	Denatured virus	Purified virus	Denatured virus
151	+	+	+	+	+	+
187	+	–	+	+	–	–
290	+	+	+	+	+	+
328	+	+	+	+	+	+
534	–	–	+	–	–	–
634	+	+	+	+	+	+

(+), (–) = positive, negative reaction.

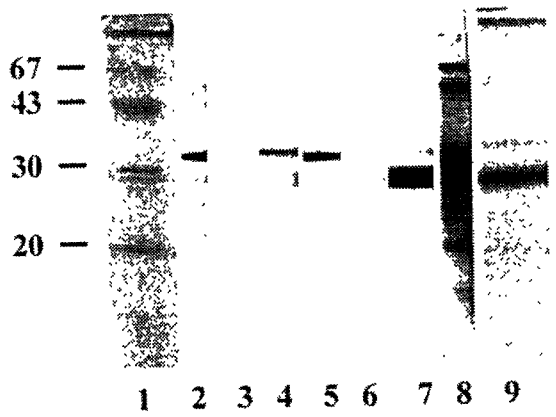

Fig. 1
SDS-PAGE and immunoblot analysis of PVA CP
Immunoblot analysis: MoAb 151 (lane 2), MoAb 187 (lane 3), MoAb 290 (lane 4), MoAb 328 (lane 5), MoAb 534 (lane 6), MoAb 634 (lane 7), PAb (lane 8). SDS-PAGE: size markers (lane 1, M_r values (K) on the left), PVA purificate (lane 9).

explained by a decreased affinity of the virus to the nitrocellulose membrane in the presence of SDS. In performing the dot blot and immunoblot tests we tried several combinations of different membranes, denaturing sample buffers, buffers used for antibody dilution and incubation times. As a result, we obtained a positive reaction with 4 of 6 MoAbs tested in comparison with only 3 MoAbs antibodies reacting in our earlier experiments (Šubr *et al.*, 1996). Namely MoAb 634 was able to give reaction only after optimisation. We assume that the addition of protease inhibitors to the sample buffer had the most significant impact on the reactivity of the MoAbs. This is in correlation with our results from synthetic peptide analysis (Čeřovská *et al.*, in press) which confirmed the continuity of the epitopes of MoAbs 634, 151 and 328. The synthetic peptide mapping also confirmed the location of the MoAb 634 epitope near the N-terminus of viral CP where it was easily accessible to the action of proteases.

As expected, MoAbs 534 and 187 did react only weakly or not at all with the denatured virus. Even the variation of conditions of denaturation or binding could not change their inability to recognise the denatured target epitopes.

SDS-PAGE and immunoblot analysis

Purified virus preparations consisted usually from 3 electophoretic components. Two major bands with electophoretic mobility of 29 and 33 K were observed (Fig. 1). The amount of the 33 K protein decreased while that of the 29 K protein increased with storage time of virus preparations. Thus we assume that the 29 K protein is a putative product of autoproteolytic cleavage of the PVA CP. Also in fresh virus preparations a great excess of this fragment was detected. With prolonged storage time an additional electrophoretic band appeared in the front of the lane. This can be regarded as another evidence of continuing autoproteolysis.

The tested MoAbs can be divided into 3 groups according to their reactivity in the immunoblot analysis. The first group consists of MoAbs 151, 290 and 328. They showed a very high affinity to the 33 K CP fragment but their reaction with the 29 K protein was weak. The reaction pattern of MoAb 634 was quite similar except of the fact that it reacted also with the 29 K fragment. The band corresponding to the smaller fragment was more intensive due to its excess in comparison with the 33 K fragment. The third group consists of MoAbs 534 and 187. Their reaction capability was heavily affected by antigen denaturation. The reaction with the 33 K protein was faint and often undetectable while that with the smaller 29 K fragment was none.

Electron microscopy

Similarly to the immunoblot analysis, the tested MoAbs can be divided into 3 groups according to the pattern of their reactivity in immunosorbent electron microscopy. The first group consists of MoAbs 151, 187, 290 and 328. These four MoAbs were able to bind along the whole length of the virus particle, and the intensity of binding decreased with the time of storage of the virus preparation (Figs. 2 and 3). From these results we conclude that the number of free target epitopes decreases due to the autoproteolytic cleavage of CP. In contrast to this group, the target epitope of MoAb 634 was stable for 5 months. Experiments with virus

preparations stored for different time periods were performed under the same conditions and no significant decrease of binding was observed (Figs. 4 and 5).

Using a fresh virus preparation (Fig. 6), MoAb 534 was also able to bind along the whole virus particle but leaving some stretches of the particle vacant. With a prolonged storage these vacant stretches extended (Fig. 7) up to the binding pattern seen on Fig. 8, where the MoAb bound only at several distinct sites along the virus particle. We suggest that two independent processes play a role in this behavior. The first process is an autoproteolytic cleavage of the CP, which most probably takes place at random sites on the virus particle (see results with MoAbs 151, 187, 290 and 328). The second process is a conformational change of the CP, which takes place preferably on protein subunits adjoining already conformationally changed subunits. So we could observe extended stretches of virus particles not bound to MoAbs.

All the experiments were performed on several virus preparations stored for various periods using the same dilutions of virus and antibodies and the same incubation times to allow mutual comparison of the results.

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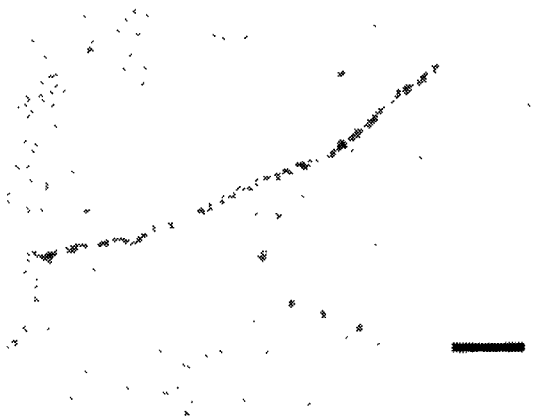

Fig. 2
Virus particle from a fresh purificate
Visualised with MoAb 187. Bar = 100 nm.

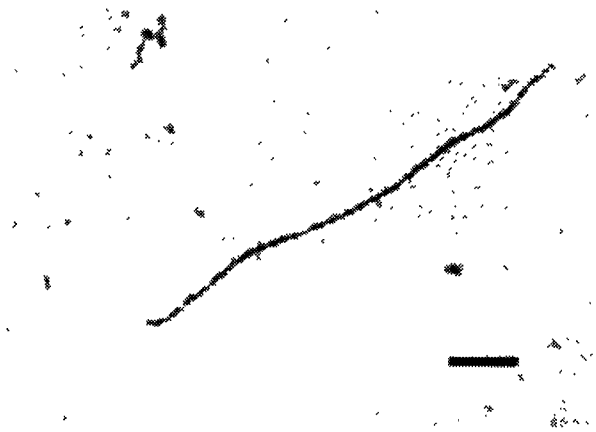

Fig. 4
Virus particle from a fresh purificate
Visualised with MoAb 634. Bar = 100 nm.

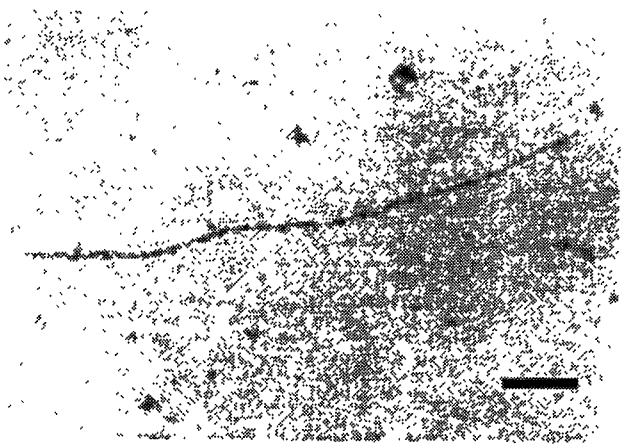

Fig. 3
Virus particle from a purificate stored for 5 months at 4°C
Visualised with MoAb 187. Bar = 100 nm.

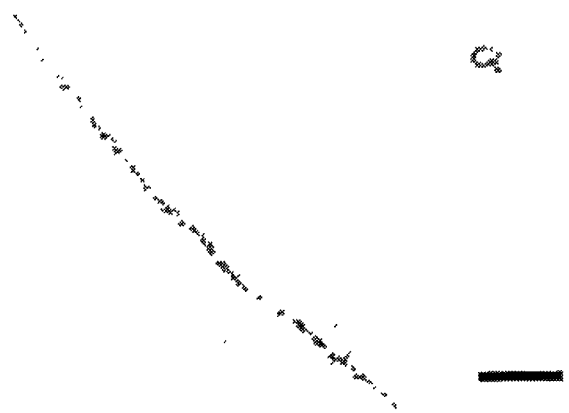

Fig. 5
Virus particle from a purificate stored for 5 months at 4°C
Visualised with MoAb 634. Bar = 100 nm.

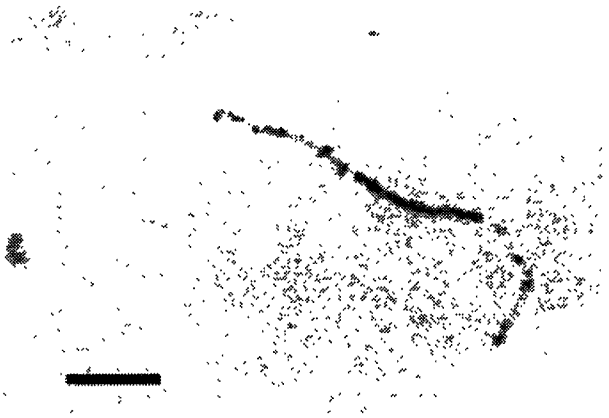

Fig. 6
Virus particle from a fresh purificate
Visualised with MoAb 534. Bar = 100 nm.

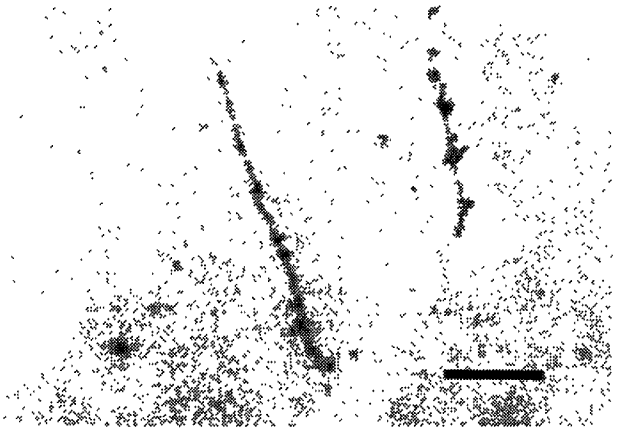

Fig. 8
Virus particle from a purificate stored for 5 months at 4°C
Visualised with MoAb 534. Bar = 100 nm.

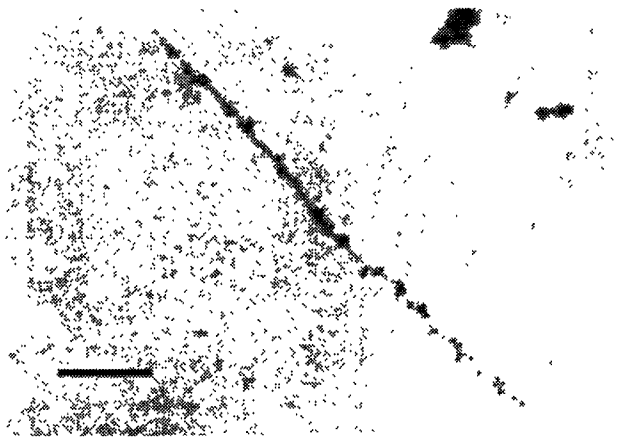

Fig. 7
Virus particle from a purificate stored for 3 months at 4°C
Visualised with MoAb 534. Bar = 100 nm.

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