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ION-EXCHANGE MEMBRANE CHROMATOGRAPHY AS AN ALTERNATIVE METHOD OF SEPARATION OF POTATO Y VIRUS

ABSTRACT

Procedures of separation of virus particles from a plant material are multistage. Furthermore often they are difficult in terms of methodology and require use of expensive, highly specialist equipment and yield of separation is often low. The antigen obtained is often degraded and contains admixtures of other proteins. Therefore, generation of high quality and specificity antibodies based on such antigen is very difficult and quality of the antibodies has impact on reliability, sensitivity and unambiguity of results of immunodiagnostic tests (e.g. ELISA) that are currently conventionally used to detect vegetable viruses. In this study three conventionally-performed methods of separation of potato virus Y (PVY) were compared and a method of separation based on membrane chromatography, as an alternative separation technique, has been presented. It has been demonstrated that in proper process conditions good quality virus preparation can be obtained.

Key words: , antigen, membrane techniques, immunodetection, virus purification

INTRODUCTION

The ion-exchange membrane chromatography allows for rapid and efficient separation of biological particles, requires no use of specialist equipment and the buffers used in the process have no negative impact on separated bioparticles (Zhou and Tressel, 2006; Zhou *et al.,* 2006). Thanks to high porosity of membranes and low height of the bed, big macromolecules, such as virus particles, can freely diffuse to inside of the membrane and combine with bed's functional groups. The process of separation in mem-

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branes runs very quickly, this is important in work with capsides composed of protein subunits that can easily be split (Ghosh, 2002).

The possibility to apply membrane chromatography for separation of viruses to generate antigens was earlier confirmed for animal viruses (Karger *et al.*, 1998, Downing *et al.*, 1992, Walin *et al.,* 1994). It was demonstrated that this technique is efficient and viral antigens received through its application had better quality than those purified in a standard way (Karger *et al.,* 1998). Due to improvement of antigen quality and increase of yield of its preparation, the process of generation of antibodies can be significantly simplified – this, in turn, can increase sensitivity and specificity of diagnostic tests based on immunological reactions. Currently, ELISA (Enzyme-Linked Immunosorbent Assay) diagnostic tests are conventionally used to detect vegetable viruses in production of seed potatoes (Wróbel, 2014). One of the most dangerous potato viruses, causing the highest losses in potato production worldwide, is PVY virus (Valkonen 2007). According to Hühnlein *et al.*, (2013), significance of PVY virus has been considerably increasing during the recent 30 years. This can be associated with climatic changes and more and more early dates of plants colonisation by virus carrying aphids, analteration that has occurred in the spectrum of strains of PVY (Chrzanowska 1991, Singh *et al .,*2003) or lower effect of insecticides on PVY transmission (Zahn 2004). Therefore, quick and certain diagnostics of affected plants is one of the best methods of prevention of this dangerous virus propagation.

Improvement in antigens production and betterment of quality of manufactured antibodies, may improve diagnostics of economically important potato viral diseases thus influencing improvement of quality of seeds. For this reason, an attempt was made in this study, to apply membrane chromatography for purification of potato virus Y.

MATERIALS AND METHODS

Potato tubers of Claustar variety that is susceptible to being affected by PVY virus, were being infected, planted in boxes, located in a greenhouse – the plants were being observed during the vegetation period. Based on macroscopic symptoms that are specific for PVY virus occurring in leaves, plants were singled out wherein PVY infection was confirmed using commercially available ELISA test (Neogen). The affected plants were cut, weighed in portions of 200 g, ground in liquid nitrogen and frozen at - 80° C. Such prepared material was then used for virus separation.

At the first stage of this research work, two methods of separation of PVY virus from affected material, using conventionally applied techniques described in literature (Čeřovská *et al.,* 1997, Hammond and Lawson, 1988), and a simplified technique consisting in application of phosphate

buffer for separation were compared. Then, virus was separated using S and Q ion-exchange membranes.

PVY virus separation

Preparation I – Separation according to Hammond and Lawson (1988)

Ground in liquid nitrogen leaves (200g) were blended in 400 ml of PKS buffer (0.5 M K_2H/KH_2PO_4 with 0,5%Na_2SO_3, pH=8.4) for 30 minutes. The homogenate was pressed through cloth and thus received filtrate was subjected to centrifugation at speed of 3400 × g at 4°C for 20 min. To supernatant slowly 13 ml Triton x-100 was being added and mixed for 30 min. at room temperature (RT). PVY was precipitated by addition of 6 g NaCl and then 40 g PEG 6000. The preparation was being dissolved for 30 min. at RT and then for1h at 10°C. The mixture was centrifuged for 20 min. at speed of 11000 × g, the sediment was being suspended in 60 ml of a buffer containing 0.1 M boric acid with 0.1 M KCl at pH=8.0, shaken for 1.5 h at RT and clarified 11000 × g for 15 min. The supernatant was being applied on a pad with 30% saccharose and centrifuged for 2.5 h at speed of 85600 × g. The sediment was being suspended in buffer of 0.1 M boric acid with 0.1 M KCl at pH=8.0 for the night at 4°C. Then the virus preparation was being clarified by 10 min. centrifugation at speed of 3300 × g.

Preparation II – Separation according to Čeřovská et al.(1997)

200g portions of affected leaves ground in liquid nitrogen were homogenised in 300 ml of buffer solution containing 0.03% ascorbic acid, 0.3% mercaptoethanol, + 0.01 M diethylpyrocarbonate (DEPC), 0.0025 % phenylmethylsulfonyl fluoride (PMSF), at pH 5.3. After 45-minute incubation, the homogenate was pressed through cloth, centrifuged for 20 minutes at 10000 × g, the sediment was discarded and to obtained supernatant Triton x-100 was added up to 2% volume and incubated at RT for 15 minutes. In next step polyethyleneglycol (PEG, MW 6000, 8% w/v) was added and mixture was incubated for 1h at 4°C and than centrifuged for 10 min at 10 000g at 4°C. Obtained sediment was dissolved in 40 ml 20 mM boric buffer pH = 7.8 by 1 h and homogenized in Polter homogenizer. The samples were shaken at 3000 RPM on BioSan Shaker overnight at 4°C and next clarified by centrifugation 10000 × g for 15 min at 4°C. The supernatant was applied on a pad containing 30% saccharose (3.5 ml/test tube) in boric buffer and centrifuged for 2h at 88000 × g for 15 minutes. The sediment was dissolved in 8 ml of boric buffer for several hours and clarified for 15 min/5000 × g/15°C. The obtained sediment was being dissolved in 20 mM boric buffer at pH=7 throughout the night at 4°C.

Preparation III – Separation through direct extraction in 20 mM phosphate buffer (KPi).

Virus PVY affected leaves ground in liquid nitrogen and homogenised in 20 mM phosphate buffer at pH = 7.4 containing 0.1% DEPC, 1mM PMSF, 0.05% Tween 20, 0.5% Na_2SO_3. The homogenate was shaken for 30 minutes, pressed through cloth and centrifuged at 10000 × g by10 min at 4°C. Thus obtained supernatant was centrifuged at speed of 15000 × g for10 min at 4°C, then precipitated by addition of 40 g PEG 6000. The preparation was being dissolved throughout the night at 4°C. The mixture was centrifuged for 20 min. with speed of 11000 × g, the sediment was suspended in 60 ml of phosphate buffer at pH= 7.4 and clarified by centrifugation at 11000 × g for 15 min. The obtained supernatant was transferred to solution of on a pad of 30% saccharose and centrifuged for 2.5 h at 85600 × g. The sediment was again suspended in 20 mM phosphate buffer throughout the night at 4°C. Finally the virus preparation was clarified by 10-minute centrifuging at speed of 3300 × g.

Preparation IV – Separation in ion-exchange membranes.

Frozen leaves taken from infected plants (200g) were ground in liquid nitrogen, then, suspended in extraction buffer (20 mM phosphate buffer at pH 7.4, 0.1% DEPC, 1mM PMSF, 0.05% Tween 20). Solids were removed from the extract by centrifugation 10000 × g/10 min. Obtained supernatant was divided in two portions – one of each was applied, using a peristaltic pump, on S75 membrane and the other one on Q75 membrane at a flow rate of 3.3 ml/min. The membranes were previously equilibrated with 20 mM phosphate buffer pH 7.4. After extract application the membranes were rinsed with buffer solution until complete washing out of the unbound proteins. Then the proteins were eluted from the membranes using of NaCl step gradient (0.1M, 0.25M, 0.5M and 1M). After application of each of the used NaCl concentrations, protein was monitored and subsequent salt concentration was applied until complete elution of protein at given salt concentration. Following completion of separation the membranes were regenerated with 1M KCl.

At each stage of performed separation, protein content was determined in particular fractions using Bradford or BCA test (in accordance with manufacturer's protocol) and presence of the virus was monitored testing samples based on DAS-ELISA (Neogen) test . All tests were performed in three repetitions.

Determination of quantity and purity of obtained preparation.

Purity of the virus preparation was evaluated spectrophotometrically and by analysis of protein composition in electrophoretic separation in SDS-PAGE sys-

tem. Specyfic PVY proteins were identified by immunodetection applying Western Blotting technique.

DAS-ELISA

The DAS-ELISA test was performed using Clark and Adams (1977) procedure. Microplate wells (Nunk) were coated with 250 ml of solution containing 1mg/ml γ-globulin in coating buffer (0.05M sodium carbonate, pH 9.6). After 3-hour incubation at 37°C the wells were rinsed with washing buffer (0.02 M sodium phosphate pH 7.4, 0.05% Tween 20, pH 7.6) and filled with 200ml of tested sample. The microplates were shaken throughout the night at 4°C. On the next day, the microplates were rinsed with washing buffer, filled with alkaline phosphatase solution in conjugate buffer (0.02 M sodium phosphate, pH 7.4, 0.15 M sodium chloride, 2% PVP, 0.05% Tween 20 and 1% gelatin). The microplates were incubated for 3 h at 37°C, rinsed with washing buffer and to each well 200 ml of substrate was added (0.1 % p-nitrophenyl phosphate disodium salt (p-NNP) in 1M diethanoloamine, pH 9.8). After 4-hour incubation at room temperature, level of absorbance was measured at 405 nm wavelength.

SDS Electrophoresis and protein transfer

Separation of proteins under denaturing conditions was performed in 12% polyacrylamide gel according to Leammli (1970) procedure. Transfer of separated proteins onto a nitrocellulose membrane was performed using buffer containing 25 mM Tris, 192 mM glycine, 20% v/v methanol at pH 8.3 in Mini PROTEAN, Tetra Cell, Bio-Rad, (350 mA/100 V/1 h).

Western Blotting

Following completion of the transfer, the membrane was rinsed with distilled water 3×25 ml for 5 minutes, dried and incubated in 25 ml of 1xTBST solution containing 5% skimmed powdered milk (SPM). After 1-hour incubation at 37°C the membrane was rinsed thrice in 25 ml 1% SPM in 1xTBST for 5 min. and incubated for 2h at 37°C in 10 ml of buffer 1xTBST+ PVP, $NaS0_3$, $MgCl_2$ x 6 H_2O containing secondary antibodies anti-PVY diluted in proportion of 10 µl conjugate PVY for 10 ml of the buffer (1:1000). After incubation the membrane was rinsed 5 × 5 min in 25 ml of 1xTBST and incubated in the substrate buffer with alkaline phosphatase (Sigma BCIP/NBT – B5655). The reaction was stopped by membrane transfer to a dish with distilled H_2O.

Microscopic analysis

Fractions obtained during purification process were analyzed by transmission electron microscopy using a nickel grid, which was incubated with a solution with viral particles at RT for 10 minutes, than washed and contrasted with 2% aqueous uranyl acetate solution for 2 minutes. Observations were made in the transmission electron microscope Morgagni 268D (FEI, The Netherlands). Photographic documentation was made using a digital camera Morada (SIS) and computer program iTEM (SIS).

Statistic

The significance of the effects on the analyzed factors were determined using analysis of variance, n-way ANOVA Statistica program 10. To test the difference between the average values at a significance level of $p < 0.05$ was used Tukey's test and Student t-test.

RESULTS AND DISCUSSION

Comparing efficiency of the methods of separation of PVY virus through application of conventional techniques. It was demonstrated that the highest level of signal in DAS-ELISA test at the homogenate stage (1) was obtained for separation with phosphate buffer KPi (Fig. 1). However, already from the second stage of purification, i.e. after precipitation with PEG (2), high signal was visible for the preparation separated by application of Čeřovská *et al.* (1997) method. Comparison of the level of absorbance at wavelength A=405 nm between the final preparations containing purified PVY has shown also the highest level of absorbance for preparation II obtained in this method. It was additionally demonstrated that an extra ultracentrifugation stage was not essential; it does not increase number of virus particles in obtained preparation. The level of absorbance for the preparation after a single and double ultracentrifugation remained the same (Fig. 1). Statistical analysis based on t-student test of PVY virus preparations obtained in three compared methods has shown significant differences between the preparations at $\alpha \leq 0.0006$, NIR=0.455 level.

The highest amount of protein was obtained in preparation I separated by application of the method described by Hammond *et al.* (1988), however, relative amount of PVY virus in this preparation was considerably lower than in the other separations. Preparation II (according to Čeřovská *et al.*, 1997) and preparation III (direct separation with phosphate buffer) contained smaller amount of protein (Table 1) but relative amount of separated PVY was almost twice as high as in preparation I. Analysis of obtained preparations by application of Tukey test, has shown that preparations II and III did not differ statistically in terms of virus content (PVY U/ml).

Significantly smaller amount of the virus obtained was shown in preparation I (Table 1).

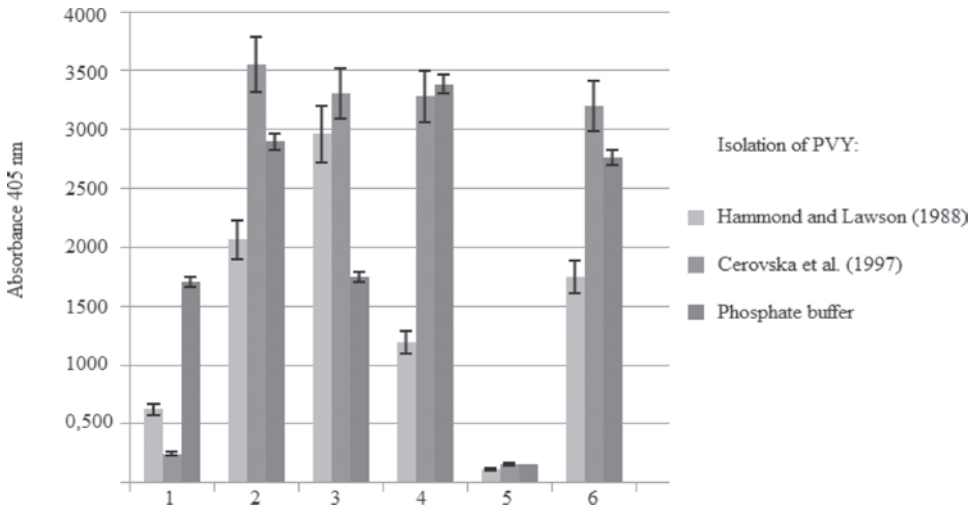


Fig. 1. The level of PVY virus (by ELISA assay) during subsequent purification steps by three methods (according to Hammond an Lawson (1988), Čeřovská *et al.,* (1997) isolation with phosphate buffer: 1) The homogenate after isolation of the infected plant material, 2) The precipitate after precipitation with PEG, 3) The supernatant from the first ultracentrifugation 4) The precipitate after first ultracentrifugation, 5) The supernatant from the second ultracentrifugation, 6) Formulation of PVY after second ultracentrifugation

Table 1

Purification of PVY virus using traditional techniques of separation

Parameters	PVY preparation		
	I	II	III
PVY (OD in 405)/0.1	17.6 ± 2.33	32 ± 1.48	27.6 ±0.42
Protein concentration [mg/ml]	0.4 ±0.01	0.3 ±0.07	0.2 ±0.01
Volume [ml]	2	2	2
Total protein [mg]	0.7 ±0.07	0.5 ±0.01	0.4 ±0.07
Relative PVY amount (OD/ml)	175.7 ±23.3	320 ±14.85	276 ±4.53
PVY U (OD/ml)/mg	499.3 ±19.3	1222.6 ±57.06	1343.8 ±106.39
PVY Total U × ml	998.6^{a} ±38.54	2445.2^{b} ±114.13	2687.7^{b} ±112.85

I – Hamond and Lawson, II - Čeřovská *et al.,* III – Separation with phosphate buffer

The obtained preparations were separated electrophoretically in denaturising conditions in 12% polyacrylamide gel. In order to confirm virus identity, transfer onto a nitrocellulose membrane and immunodetection by PVY specific antibodies was performed (Fig. 2). Preparations obtained by application of Čeřovská and Hammond technique contain proteins specific for PVY virus (Fig. 2, Line 2 and 3). Characteristic coat protein dominates therein (approx. 30 kDa), whereas proteins featuring size approx. 60 kDa can be precursor proteins or the viral helper compo-

nent protein (Hc-Pro, 58 kDa), which was earlier described as interacting with virus particles (Thornbury *et al.*, 1985, Roudet-Tavert *et al.*, 2002).

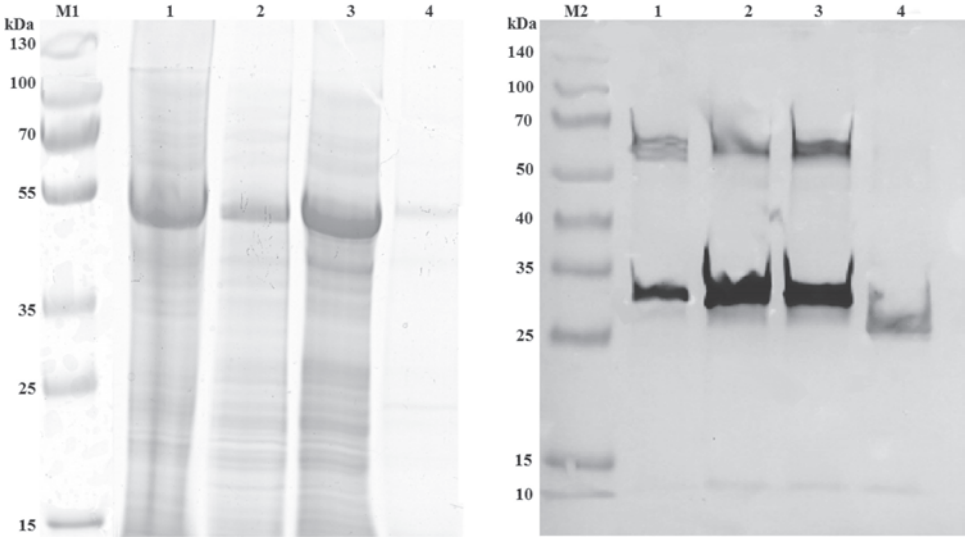


Fig. 2. Electroforetic profile (A) and immunoblotting (b) of virus formulation purified according to Hammond and Lawson (2), Čeřovská et al. (3), phosphate buffer (4), positive control (1), M1 – Protein marker (Page Ruler Plus Presteined Protein Ladder, Thermo Scientific), M2 - (Spectra Multicolor Broad Range Protein Ladder, Thermo Scientific, Cat. 26634)

In the preparation that was separated by application of the third method using KPi buffer (Fig. 3. Line 4) there occurred protein that was slightly smaller than 30 kDa but this protein was also recognised by specific antibodies. This is probably coat protein in degraded form. According to Sing (1978)location and intensity of bands of virus proteins separated in SDS electrophoresis can depend on the method of separation, virus strain and on the host plant. Paul (1975) demonstrated before that potato virus Y always produced more than one specific band.

Also a possibility to use ion-exchange membrane chromatography was assessed during the tests performed as an alternative method of separation of viruses coming from vegetable material.

The ion-exchange chromatography technique was already successfully used in potato virus Y separation. In experiments performed by (Rupar *et al.,* 2013) comparison of the classic separation technique and purification by Convective Interaction Media (CIM) monoliths as ion-exchange chromatographic supports was done. Despite three-fold higher concentration of classically separated virus, the potato virus Y preparations obtained were of comparable purity, just like total yield. Additional procedure using CIM took half of the time needed for separation by application of classical methods (Rupar *et al.*, 2013). Membrane based anion-exchange chromatography was already previously been successfully used for purification of

rotavirus like particles (Vincente *et al.,* 2008) and plasmid purification (Grunwald and Shields, 2001; Nochumson *et al.,* 2002).

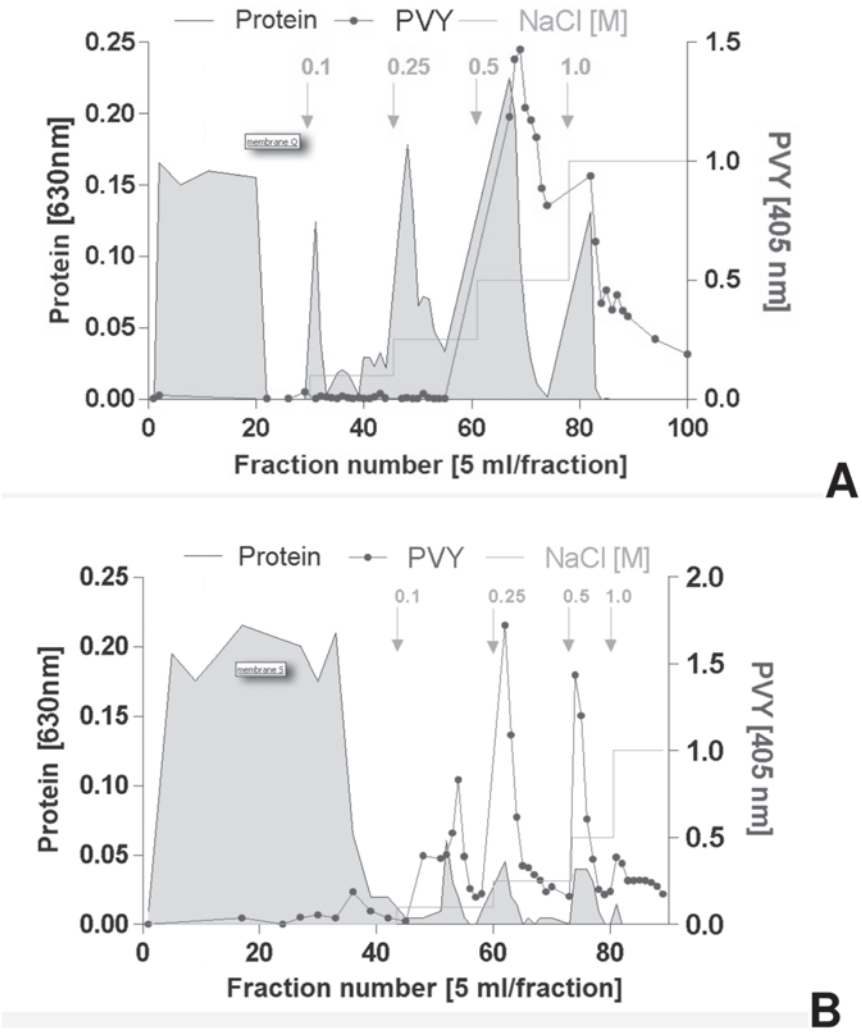


Fig. 3. Ion Exchange chromatography of PVY on 75Q (A) and 75S (B) membrane (Sartorius AG). Binding and elution buffer (20 mM phosphate buffer, pH = 7,4), elution buffer (20 mM phosphate buffer, pH = 7,4 + NaCl) NaCl in gradient (0,1M, 0,25M, 0,5M and 1M). Protein fractions were determinate by Bradford method, and the presence of PVY was tested by DAS ELISA

Occurrence of virus proteins was noted both in the fraction bound with the anion-exchange membrane S as well as cation-exchange Q (Fig. 3). This means, that after homogenisation of the infected material in buffer at pH=7.4 the virus

protein is separated into fractions of different charge. Three positively-charged fractions (alkaline) bound themselves with membrane S whereas one fraction featuring negative charge (acidic) bound itself to membrane Q. In order to find out if the observed forms resulted from degradation of PVY virions or they were the entire virus particles, those fractions that contained virus protein were collected and subjected to ultracentrifugation at 100000 × g. In such conditions non-degraded virus particles transferred to the sediment whereas coat proteins originating from decomposition of virions remained in the supernatant. It was demonstrated that the alkaline fractions, binding with membrane S, contain mostly degraded virus protein (Fig. 4B), whereas the acidic fraction bound with membrane Q (Fig. 4A) is composed both from complete virus particles as well as from products of their degradation. Occurrence of virus protein in sediments obtained from cation-exchange membrane Q proves that virions of the virus binding with that membrane did not decompose during the separation process.

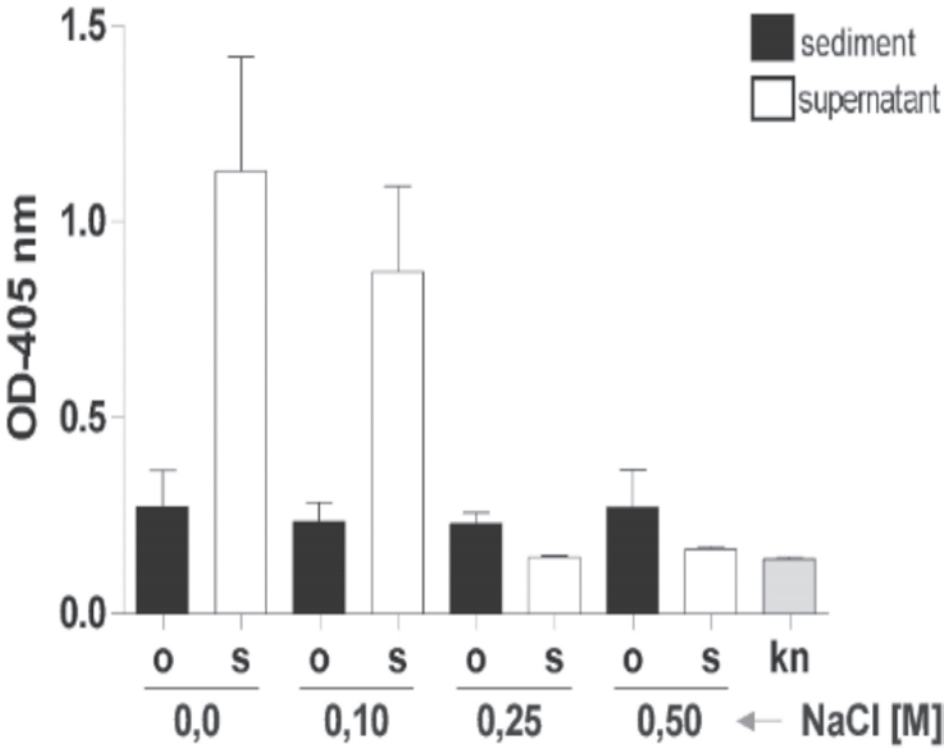
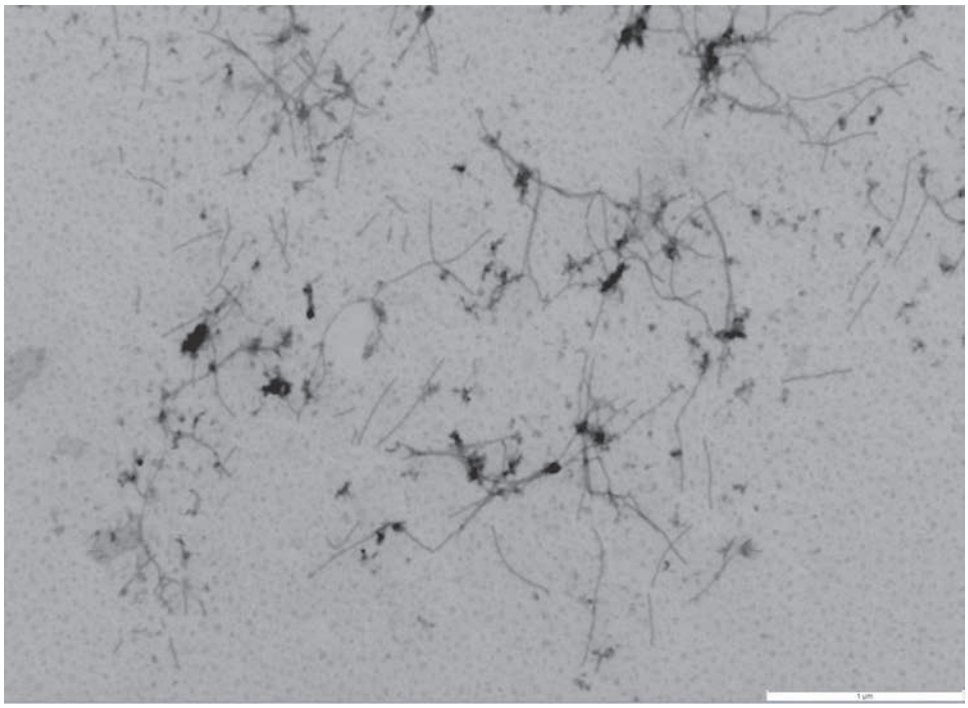


Fig. 4. Assessment of the integrity of PVY virions. The fractions were separated on a membrane Q (2A) and S (2B) at various concentrations of NaCl. The presence of PVY was detected by ELISA and fractions containing viral proteins were collected and ultracentrifugated for 2 h at 100 000 × g. The concentrations of the virus was estimated by the ELISA assay in the precipitates from the ultracentrifugation (p) and the supernatants (s)

Preparations obtained by separation in ion-exchange membranes Q were then assessed using a transmission electron microscope (Fig 5). Analysis of the images



obtained confirms that PVY virus preparations obtained from membrane Q did not degrade, in the image dominate particles with dimensions close to 700 × 11 nm.

Fig. 5. The structure of the viral particles obtained by isolating PVY the ion exchange membrane Q. The study was carried out in a transmission electron microscope Morgagni 268D (FEI, The Netherlands)

CONCLUSIONS:

The results obtained confirm the possibility to use ion-exchange membranes for separation of viruses from infected vegetable material. Thread-like shape of PVY virus definitely hampers free movement of virus particles in membrane pores. Such impediments are considerably smaller in the case of separation of spherical viruses (Kovac *et al.,* 2009, Burden *et al.*, 2011) or bacteriophages (Smrekar 2008). Additionally, presence of vegetable components can also have unfavourable impact on separation yield. Certainly, an advantage of the above presented method is curtailment of the separation time and the possibility of omission of the ultracentrifugation stage. To obtain high yields and repeatability of the presented method, further work on optimisation of the conditions of binding and elution of virus particles bound to ion-exchange membranes is necessary.

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