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A new application of monolithic supports: The separation of viruses from one another

J. Ruščić[a,*], I. Gutiérrez-Aguirre[b], M. Tušek Žnidarič[b], S. Kolundžija[a], A. Slana[a], M. Barut[c,1], M. Ravnikar[b], M. Krajačić[a]

[a] Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia
[b] Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia
[c] BIA Separations d.o.o., Ajdovščina, Slovenia

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ABSTRACT

The emergence of next-generation "deep" sequencing has enabled the study of virus populations with much higher resolutions. This new tool increases the possibility of observing mixed infections caused by combinations of plant viruses, which are likely to occur more frequently than previously thought. The biological impact of co-infecting viruses on their host has yet to be determined and fully understood, and the first step towards reaching this goal is the separation and purification of individual species. Ion-exchange monolith chromatography has been used successfully for the purification and concentration of different viruses, and number of them have been separated from plant homogenate or bacterial and eukaryotic lysate. Thus, the question remained as to whether different virus species present in a single sample could be separated. In this study, anion-exchange chromatography using monolithic supports was optimized for fast and efficient partial purification of three model plant viruses: *Turnip yellow mosaic virus*, *Tomato bushy stunt virus*, and *Tobacco mosaic virus*. The virus species, as well as two virus strains, were separated from each other in a single chromatographic experiment from an artificially mixed sample. Based on $A_{260/280}$ ratios, we were able to attribute specific peaks to a certain viral morphology/structure (icosahedral or rod-shaped). This first separation of individual viruses from an artificially prepared laboratory mixture should encourage new applications of monolithic chromatographic supports in the separation of plant, bacterial, or animal viruses from all kinds of mixed samples.

1. Introduction

With further advances in sequencing technology, it is becoming apparent that viruses are ubiquitous and that mixed virus infections are probably a common occurrence [1,2]. Different virus–virus interactions can have variable effects on disease development and can have great economic impact in the case of plant viruses [3], as well as an influence on human health where human viruses are concerned [4]. Furthermore, different strains of the same virus species can have devastating impacts on their hosts, and therefore, fast detection and identification of mixed infections/different virus strains is of great importance. In a recent review article, Boonham et al. [5] have addressed the advantages and disadvantages of current diagnostic techniques.

Monolithic supports allowed chromatography to be applied in virus purification and concentration. Unlike classical particle-based chromatographic supports where mass transfer is based on diffusion and pores are relatively small, monoliths are characterized by mass transfer greatly enhanced by convection and channels that are several microns in size [6]. They have been used successfully in virus purification and concentration from diluted samples [7–18], for monitoring virus titre during bacteriophage [19] and adenovirus-based vector production [20], as well as in the purification of virus-like particles (VLP) and other virus vectors [21–25]. So far, only two plant viruses, the rod-shaped *Tomato mosaic virus* (ToMV) and the filamentous *Potato virus Y* (PVY), have been purified using monolithic supports, and they were shown to be infective after the purification process [12,17]. Therefore, the first aim of this work was to extend the range of plant viruses purified in that way and to prove the applicability of this approach by dealing with three new models: *Turnip yellow mosaic virus* (TYMV; *Tymovirus*, *Tymoviridae*),

* Corresponding author at: Marulićev trg 9a, 10000 Zagreb, Croatia. Tel.: +385 1 489 8096.
E-mail address: jelena.ruscic@biol.pmf.hr (J. Ruščić).
[1] Present address: Sandoz Biopharmaceuticals, Mengeš, Slovenia.

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Tomato bushy stunt virus (TBSV; *Tombusvirus*, *Tombusviridae*), and *Tobacco mosaic virus* (TMV; *Tobamovirus*, *Virgaviridae*). All three viruses have pI values under pH 5.0 [26]; therefore, it is reasonable to assume that at pH > 5.0 they will be negatively charged and that they will bind to an anion exchanger.

TYMV and TBSV are small icosahedral stable plant viruses with diameters between 30 and 35 nm that reach high titres in experimental hosts and can be purified in high amounts with classical purification protocols employing ultracentrifugation. They have had a significant role in our understanding of the virus icosahedral structure [27], both of their detailed structures are known [28–30], and they are used as models in the research of various aspects of virus life cycle and cellular pathways [31,32]. The two viruses are interesting because of their biotechnological applications: TYMV-based bionanoparticles have been reported [33–36] as well as TBSV-based VLPs [37–39]. TYMV isolates are divided into two strain groups: TYMV-1 and TYMV-2 [40]. The serological relation between these two groups is one of the most distant reported among tymoviruses [41]; strains with that degree of serological difference with a serological differentiation index (SDI) of 3.5, can be considered different viruses [41,42]. Nevertheless, because of the similarities in their basic chemical composition, host range, and symptomatology, they are still considered strains of the same virus. The majority of reported isolates, including the type isolate that has been thoroughly analyzed, belong to the TYMV-1 group. We believe that, by investigating the chromatographic properties of different TYMV isolates, we could establish a basis for their separation and for monitoring the presence of different strains in mixed infections. This would provide a good model for the separation of strains and isolates of other virus species. Rod-shaped TMV, certainly the most famous plant virus, was the first virus discovered. It holds the first position among the top ten plant viruses in molecular plant pathology [43] and is still an interesting research model today as an expression vector in the context of plant-derived vaccines [44].

Based on retention times obtained for different bacteriophages, Adriaenssens et al. [13] and Smrekar et al. [45] suggested that ion-exchange chromatography on monolithic supports can separate viruses present in the same sample as long as they have different charge properties. The latter group even demonstrated a virtual separation by overlapping chromatograms obtained from separate experiments with individual bacteriophages. To test if this hypothesis can be applied to plant viruses as well, we used our three model viruses. Furthermore, we included different TYMV isolates/strains in our separation experiments. Our model viruses had different pI values: TBSV pI 4.1, TYMV pI 3.7, and TMV pI 3.5 [26]. Therefore, we assumed that they would have variable degree of net charge and different charge distribution at certain buffer pH values and that different amounts of salt would be necessary to elute them from the column. Thus, the ultimate aim of this study was to achieve a real separation of viruses. By using ion-exchange chromatography on monolithic supports, we were able to separate individual plant viruses and virus strains from the laboratory-prepared mixed samples.

2. Experimental

2.1. Propagation and preparation of TYMV, TBSV and TMV

Several TYMV isolates were propagated in *Brassica rapa* L *var. rapa* 'Purple top' and 'Turnip Atlantic'. Two isolates, Edinburgh (TYMV-E) and Northumberland (TYMV-N), were obtained by Dr. Đorđe Mamula in 1978 from John Innes Centre (Norwich, UK) while the origin of the third isolate, the Yugoslavia isolate (TYMV-Y), is described elsewhere [46]. TBSV was propagated in *N. benthamiana* Domin. and TMV in *N. megalosiphon* Van Huerck and Müll. Arg. The latter two viruses (TBSV-Cro651, TMV-Cro510) are part of a plant virus collection maintained at the Department of Biology, University of Zagreb (Zagreb, Croatia). The infected tissues were collected after the onset of virus characteristic symptoms: bright yellow mosaic for TYMV, mottling and leaf distortion for TBSV, and deformation and vein clearing of the young leaves for TMV. The collected tissues were either frozen at −20 °C or immediately homogenized in universal extraction bags with a synthetic intermediate layer (Bioreba, Reinach, Switzerland) to pre-filter plant extracts. The plant tissue mass and homogenizing buffer volume ratio (w/v) was kept at 1/5 throughout the experiments and the homogenizing buffer was the chromatographic loading buffer used in the particular experiment. The pre-filtered plant homogenates were centrifuged for 10–15 min at 16,060 × *g* at room temperature and the virus containing supernatants were filtered through 0.45 μm HPLC certified filters (Spartan 13 mm Syringe Filter, Whatman, GE Healthcare, Uppsala, Sweden). The virus-containing filtrates were either frozen at −20 °C or loaded directly onto the column used in the particular experiment. The uninfected plant tissue used in the chromatographic experiments as a negative control was prepared in the same way.

Table 1
Chromatographic buffers.

Loading buffer	Elution buffer
20 mM sodium acetate pH 5.5	20 mM sodium acetate 2 M NaCl pH 5.5
20 mM MES pH 6.0	20 mM MES 2 M NaCl pH 6.0
20 mM MOPS pH 7.0	20 mM MOPS 2 M NaCl pH 7.0
20 mM HEPES pH 8.0	20 mM HEPES 2 M NaCl pH 8.0
20 mM Tris pH 9.0	20 mM Tris 2 M NaCl pH 9.0

2.2. Chromatography conditions for optimization and partial purification

All chromatographic experiments were performed on an Agilent series 1100 (Agilent Technologies, Santa Clara, CA, USA) HPLC system. ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was used to measure peak area and height for specific chromatographic peaks, and the A_{260}/A_{280} ratios were calculated. Unless otherwise indicated, the reported data was obtained from the peak height values. Two anion exchangers were used in the experiments: Convective Interaction Media (CIM) DEAE and CIM QA (BIA Separations, Ajdovščina, Slovenia), with a column volume of 0.34 mL, ID 12 mm, length 3 mm, 62% porosity, and an average channel size of 1.2–1.5 μm. Throughout the initial and optimization experiments, several buffers with different pH values were used (Table 1). In all the experiments, the flow rate was set at 2 mL/min and absorbance was monitored at 260 nm and 280 nm. Chromatographic runs were performed in triplicate, and fractions were collected manually from two runs.

The TYMV samples, prepared as described in Section 2.1, were loaded onto a weak anion exchanger (CIM DEAE) or a strong anion exchanger (CIM QA). Unbound macromolecules were washed out with 4 mL (12 column volumes) of loading buffer, and the bound macromolecules were eluted using linear gradient 0–75% elution buffer in 8 min (47 column volumes). The TBSV and the TMV samples, also prepared as described in Section 2.1, were loaded onto the CIM DEAE and CIM QA columns, respectively. In both cases, unbound macromolecules were washed out with 2 mL (6 column volumes) of loading buffer and the bound macromolecules were eluted using linear gradient 0–50% elution buffer in 7 min (41 column volumes).

After each run, the columns were equilibrated with 4–7 mL (12–20 column volumes) of 100% elution buffer followed by 3–4 mL