https://doi.org/10.18321/ectj1131

Purification of *Tomato Bushy Stunt Virus* Particles by One-Step Hydroxyapatite Column Chromatography

Zh. Tleukulova[1], Z. Stamgaliyeva[1], A. Dildabek[2], G. Mukiyanova[1]*, R.T. Omarov[1]

[1]L.N. Gumilyov Eurasian University, 2 Satpayev Str., Nur-Sultan, Kazakhstan
[2]School of Sciences and Humanities, Nazarbayev University, 53 Kabanbay Batyr Ave., Nur-Sultan, Kazakhstan

Article info:

Received:
15 July 2021

Received in revised form:
17 September 2021

Accepted:
28 October 2021

Keywords:
Virus particles,
Tomato bushy stunt virus,
Purification,
Hydroxyapatite column chromatography.

Abstract

The main aim of this work was to develop a time-saving and cost-effective purification method of infectious plant viral nanoparticles. Virions of *Tomato bushy stunt virus* (TBSV), which is a member of *Tombusvirus* genus, were purified by one-step Bio-gel HT Hydroxyapatite (HA) column chromatography. Extracts from *Nicotiana benthamiana* plants infected with TBSV were directly loaded onto the HA column and eluted by 10 mM sodium phosphate buffer (pH 6.8). A specificity of virions has been confirmed by immunoblotting and electron microscopy. Homogeneity of virions was tested by SDS-PAGE, where only 41 kDa polypeptide bands referring to the capsid protein of TBSV were detected by Coomassie staining. The biological infectious activity of a purified material was demonstrated by observing TBSV-specific symptoms observed in *N. benthamiana* plants at 7–10 days of post-inoculation (dpi). Moreover, purified virions were used for immunization of the BALb/c mouse to raise primary antibodies against the TBSV virus. Our results show that in low concentrations of sodium phosphate buffer total proteins extracted from infected plants adsorb to HA sorbent, while viral particles do not adsorb to the HA matrix and flow throw column due to Ca^{2+} ions implicated in TBSV virions' structure. This highly effective and simple virus purification protocol can also be used for the isolation of other plant virions.

1. Introduction

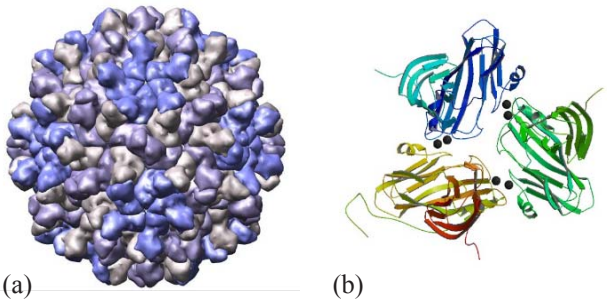
*Tomato bushy stunt virus (*TBSV) is a member of the family *Tombusviridae*, genus *Tombusvirus*, a type of important plant pathogenic viruses, with a single-stranded RNA(+) genome, ~ 4800 nt in length [1]. Genomic RNA of TBSV is wrapped by 180 identical capsid proteins (Mr = 41 kDa) and the self-assembled viral particles (virions) get 30–33 nm in diameter with triangulation number T = 3 (Fig. 1a). TBSV is the first among viruses, with an icosahedral virion shape, which X-ray crystallography was determined in 2.9A resolution [2–4].

Early deep physical and genetic analysis on TBSV (Cherry and BS3 strains) brought insights into the understanding of the structural arrangement of its virion in space [5]. Capsomer of TBSV virion consists of 3 polypeptide chains marked as A, B, C. Each capsomeric unit consists of three sides: P- domain, which projects outside, S- domain, which forms the internal part of the shell, and R- domain (tail) that is, directly linked with viral RNA and also is linked with S-domain. Positively charged amino acids are concentrated in the inner part (S- and R-domains), while polar and unipolar, uncharged amino acids can mostly be found in the outside part of the virion. Moreover, divalent calcium Ca^{2+} ions, as small ligands, are implicated in the virion structure [6]. Particularly, calcium ions are bound to asparagine (glutamine, serine R in the S domain) residues of each A-,B-,C-polypeptide chain (Fig. 1b). The total number of calcium ions implicated in the TBSV virion structure is 360 (Worldwide protein data bank, PDB ID: 2TBV).

Traditionally, ultracentrifugation is a commonly used method for separating and purifying plant viral particles [7]. However, the method is

*Corresponding author.
E-mail: g.mukiyanova@gmail.com

© 2021 Eurasian Chemico-Technological Journal.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).



(a) (b)

Fig. 1. Modern representation of TBSV virion provided by Worldwide protein data bank, PDB ID: 2TBV. (a) – Spherical model of TBSV. (b) – Tree polypeptide chains in capsomer of the TBSV virion (A, B, C chains blue, green, and yellow). Two black dots in the central part of the capsomer are calcium ions.

time-consuming and does not provide the material in preparative amounts. It is very important to design a convenient and simple purification technique and HA column chromatography could be served as a foolproof technique in getting high pure biomolecules.

Hydroxyapatite (HA) is a hydroxylated calcium phosphate microcrystalline or ceramic compound with a chemical formula of $Ca_{10}(PO_4)(OH)_2$, and anionic and cationic exchangers or static attraction is involved in the adsorption of the biomolecules [8–10]. Ca^{2+} sites of HA particles interact with carboxylate residues of the acidic protein surface, whereas PO_4^{2-} group can bind with the amine functional group of basic protein surface [9, 11]. The adsorption of the proteins usually occurs through a low concentration of phosphate buffer, and elution of the biomolecules can occur with the mixed-buffer system or by increasing the concentration of the PO_4^{2-} group in phosphate buffer [11]. Initially, HA column chromatography was used to purify recombinant proteins. Now, it is used for the purification of nucleic acids [11], polypeptides [12], proteins [9], human serum high-density lipoproteins [13], also human viruses [14].

Nowadays few scientific works with human viruses and filamentous bacteriophage purification in HA column chromatography are known, but there is no plant virus purification in the HA column. In this article, we describe the one-step purification method of TBSV virions by HA column chromatography.

2. Experimental

2.1. Preparation of plant materials

30–35 days old *N. benthamiana* plants were used as laboratory hosts of TBSV for systemic multiplication of the virus. The seeds of *N. benthamiana* plants were grown in enriched black soil in the growth room. The condition in the growth room was a long-day photoperiod with 16 h of light, which lighted up with 2700 K and 6400 K spectrum lamps and 8 h of the dark, average temperature was 280 C and 75–80% humidity.

2.2. Virus multiplication

Wild type TBSV cDNA constructs were linearized by SmaI restriction enzyme (Thermofisher, EU) and used for *in vitro* transcription by High yield in vitro transcription kit (Thermofisher, EU) according to the protocol as described by the manufacturer. Healthy, 30–35 days old *N. benthamiana* plants were inoculated by *in vitro* obtained TBSV transcripts mixed with phosphate buffer and carborandrum.

2.3. Hydroxyapatite column chromatography

Bio-gel HT hydroxyapatite was used for purification. Bio-gel HT material is convenient for use with its large particle size and shipped in liquid form in 10 mM Na PB containing 0.02% NaN_3 (130-0150, Biorad, USA). After 10 days of post-inoculation, 1 g plant leaves were harvested, mechanically homogenized in 10 mM phosphate buffer (pH 6.8) in 1:2 proportion on ice-cold mortars. Samples were centrifuged twice for 20 min at 20.000 × g at 4 °C, using a centrifuge 5804R (Eppendorf, Germany) with a F45-30-11 rotor. The column with 2.5 cm in diameter and 30 cm in height (BioRad, USA) was loaded with Bio-Gel HT hydroxyapatite (130-0150, Biorad, USA) and equilibrated by washing with 10 mM phosphate buffer. Centrifuged samples from infected plants were loaded into the column and washed with 10 mM phosphate buffer (pH 6.8). Flow-throw material was collected into 1.5 ml microcentrifuge tubes (1 ml per 5–7 min) and frozen at -20 °C.

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was used for the visualization of purified virions. Sample preparation for TEM was performed in accordance with the methodology described in [15].

2.5. SDS-PAGE electrophoresis

After HA column chromatography, purified

samples and 4x loading buffer for SDS-PAGE were mixed, then heated to 90 °C for 10 min. Samples 12 µl in volume were loaded into 12% SDS polyacrylamide gel and separated by vertical electrophoresis. The resulting gel was incubated in Coomassie staining buffer for 3 h in a shaker. The gel was washed in destaining buffer (45 ml of ethanol, 10 ml of acetic acid, 45 ml of double-distilled water) until getting clear visible bands.

2.6. Immunoblotting

For detection of purified virions, 12 µl fractions were separated on 1% agarose gel with ethidium bromide for 50 min in TBE (Tris/Boric acid/EDTA) buffer. Detection of viral particles in agarose gel was performed under UV light. Further, capillary transfer onto nitrocellulose membrane was performed. The membrane was immune-stained with TBSV virion-specific polyclonal antibodies. Visualization of phosphatase activity after incubation with secondary antibodies was performed as described in [16].

2.7. Isolation of genomic RNA from virions

The mixture of 300 µl purified fractions, 140 nuclease-free water, and 60 µl of 10% SDS was heated at 600 C for 30 min in a water bath, then treated by 500 µl phenol-chloroform, then centrifuged at 10000 rpm (12.000 × g) for 25 min. The resulting supernatant was incubated at -200 C for 40 min with 8M LiCl and 96% ethanol. Further, the samples were centrifuged at 10000 rpm (12.000 × g) for 15 min and the precipitate was washed twice with 70% ethanol. The sediments were resuspended with nuclease-free water. Isolated RNA samples were separated on 1% agarose gel with ethidium bromide for 50 min in TBE buffer.

3. Results and discussion

3.1. Purification of TBSV virions in HA column chromatography

HA column chromatography was successfully used for the purification of virions from TBSV infected *N. benthamiana* plants. For the collection of pure homogeneous viral particles, 30–35 days old plants are rub-inoculated with in vitro generated TBSV transcripts. After 10 days of post-inoculation (dpi), 1 g of leaves were homogenized by 10 mM sodium phosphate buffer (pH 6.8) in 1:2 proportions on ice, and then centrifuged twice. The resulting supernatant was loaded into the column (BioRad, USA) previously filled with HA. After the extract was immersed in the matrix, the column was washed by 10 mM sodium phosphate buffer (pH 6.8). Collected purified samples were separated on 1% agarose gel electrophoresis and visualized under UV light.

TBSV virus particles contain capsid protein and genomic RNA, approximately 4800 bp in length. Therefore, purified virion fractions were easily detected in 1% agarose gel with ethidium bromide. As it is shown in Fig. 2a, purified virions were fluorescent due to the presence of encapsulated viral genomic RNA.

To test the purity and homogeneity of purified virion fractions, samples were resolved on 12% denaturing SDS-PAGE. Resulting gel stained in Coomassie Brilliant R solution. The separation of purified virions on SDS-PAGE, allowed demonstrating the 41 kDa capsid protein of the virus (Fig. 2b). Figure 2b clearly illustrates protein profiles of protein standard (1, molecular marker), samples from non-purified infected samples (2) and purified fractions (3). Only one polypeptide band referring to the capsid protein of TBSV compared to a non-purified sample was detected (Fig. 2b, well 3). Therefore, gel separation demonstrates the high purity and homogeneity of purified virion fractions.

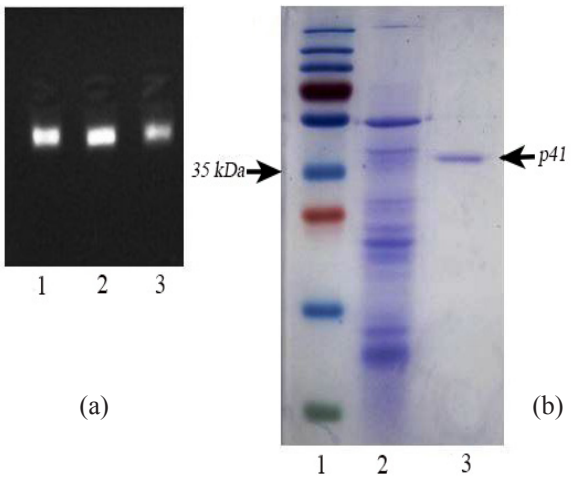


Fig. 2. (a) Visualization of purified TBSV virions on 1% agarose gel. 1 – TBSV fraction, 2 – TBSV fraction, 3 – TBSV fraction. (b) – SDS-PAGE analysis of purified samples. 1 – molecular marker, 2 – crude extract from TBSV infected plants (non-purified), 3 – purified virion fraction. Obviously, in resulting gel it is detected only one polypeptide band (p41), which is appropriate to TBSV p41capsid protein.

3.2. Morphology of virion shape and isolation of genomic RNA from TBSV virions

Most representatives of the Tombusviridae family form icosahedral (T = 3) virion shape. The inner part of the virions is packed with genomic RNA. After detecting the viral RNA on 1% agarose gel and capsid protein on 12% SDS-PAGE, we decided to check the structural entity of purified virions. Purified virion samples were analyzed by conventional TEM. Our data obtained by TEM demonstrated the highly structured spherical shape of the virus particles in samples purified by HA (Fig. 3).

The vast majority of plant virus particles are extremely tiny. Earlier it was shown that some environmental conditions, such as high pH level and EDTA can bring to the structural deformations of TBSV virions [6]. Transmission electron micrograph of virion structure of TBSV stained by 1% uranyl acetate is demonstrated in Fig. 3. Our data revealed the presence of spherical particles with an approximate size of about 30–35 nm. It means that hydroxyapatite column chromatography does not affect negatively the virion shape of TBSV. Probably, it is due to elution with low concentrated 10 mM sodium phosphate buffer (pH 6.8).

Furthermore, genomic RNA was isolated from purified virion fractions and was separated in 1% agarose gel (Fig. 4). Isolation of genomic RNA was performed to check the entity of encapsulated genomic RNA. In nature, genomic RNA serves as an infectious agent for the virus. Capsid proteins protect genomic RNA from degradation and help to transmit a genomic material during infection. Genomic RNA was isolated from purified virions by denaturing with 10% SDS and precipitating

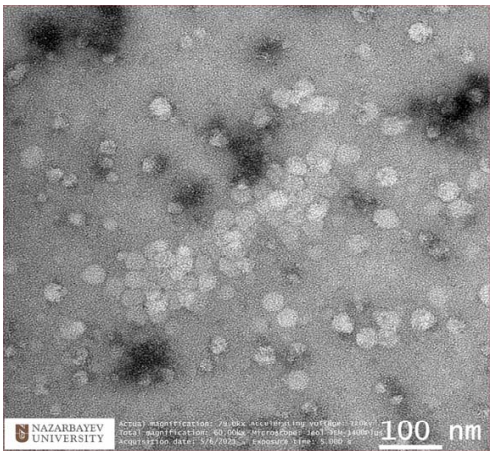


Fig. 3. Electron microscopy of purified virions by HA column chromatography

Fig. 4. Viral genomic RNA isolated from virions by 8M LiCl. 1 – TBSV linear cDNA, as control, 2 – genomic RNA of TBSV isolated from virions by 8 M LiCl.

with 8M LiCl. Isolation of entire genomic RNA confirms the accuracy of the developed method.

Microscopic observations and isolation of genomic RNA from purified particles demonstrate the entity of purified virion particles and their genomic RNA, respectively. Altogether, data shows that this purification protocol is quite accurate and does not affect the structural content of particles.

3.3 Testing for virions infectivity and biological activity

Further, purified virions were tested for infectivity. Plants were rub-inoculated with a mixture of 25 µl of virions and 75 µl of 10 mM sodium phosphate (pH 6.8). As a result, we observed symptoms typical of TBSV infection, such as apical necrosis and collapse of the plants infected by purified virions at 10 dpi. Figure 5 shows typical severe virus-mediated symptoms such as stunting, stopping of growth parameters, necrosis of apical shoots and stems. Virus infection was tested by western blotting for TBSV encoded p19 suppressor protein detection (data not shown).

As shown in Fig. 5, host plants inoculated with purified virion fractions were effectively infected and demonstrated acute systemic disease symptoms.

The maintained biological activity of purified virions and their accumulation level in plants show that the developed method is highly reproducible. Moreover, virions were used as an antigen to obtain primary mouse antibodies, and these antibodies were tested in immunoblotting assays. Primary mouse antibodies against TBSV particles were raised according to the standard protocol of

Fig. 5. Symptom detection at 10 dpi on *N. benthamiana* plants infected by purified virions. On the left – infected plant, on the right – control plant.

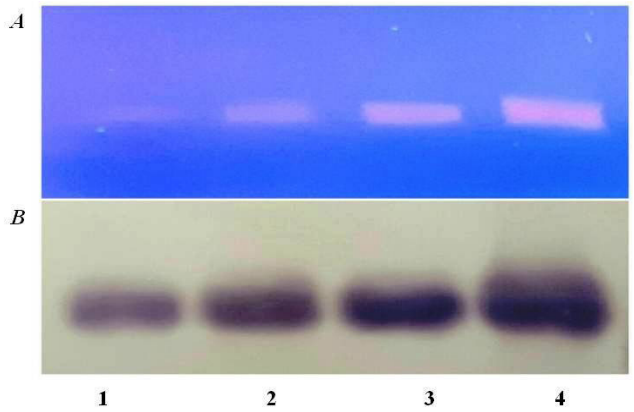


Fig. 6. Detection of purified TBSV virions: (a) – Purified TBSV virions separated on 1% agarose gel electrophoresis; (b) – Virions detected on the nitrocellulose membrane. 1, 2, 3 – purified virions, 4 – sample from TBSV infected plant (non-purified).

immunization and were used in testing TBSV virion accumulation in plants as described in [16]. This helped to couple the purification step with a rapid immune-detection assay. Therefore, this data shows a high potential of the developed method in virus diagnosing with immunological methods.

3.4. Detection of TBSV virions

To detect purified TBSV virions, the samples were separated in 1% agarose gel electrophoresis and followed by an immune-detection assay. Earlier this detection method was described in [16]. As it is shown in Fig. 6 (A panel), 1–3 wells were loaded with virion fractions. An unpurified extract from infected plant leaves was used as a control. After visualization under UV light, probes were transferred by capillary into a nitrocellulose membrane. The membrane was blocked in 1% casein added 1x TBS-Tween 20 solution for one hour, then incubated with primary antibodies raised against TBSV virions. Secondary antibodies specific for mouse antibodies and conjugated with alkaline phosphatase were used to detect the formation of complexes of antibodies with virions (Fig. 6 B panel).

Figure 6 (B) clearly shows the forming of effective immune complexes between purified virion fractions and raised antibodies as demonstrated by a well released signal from secondary antibodies conjugated with alkaline phosphatase. Raised antibodies were able to detect virions in non-purified infected samples (Fig.6 (B, line 4)). Overall, this data provided us with our own resilient virus detection system [17]. Moreover, virus detection in agarose electrophoresis shows a high yield of purified virions.

4. Conclusions

In our current work, we have developed an easy and effective purification method of plant virions. The virion specificity and morphology were tested by immune-detection, quality analysis of TBSV genomic RNA, and electron microscopy. Moreover, the biological ability to infect plants was tested by inoculation of *N. benthamiana* laboratory host of TBSV.

Purified particles were pure, kept their native structure and plant infecting ability. As aforementioned, calcium ions are implicated in self-assembled TBSV virions, on the other hand, hydroxyapatite poses positively and negatively charged groups. We suggest that TBSV particles do not adsorb to the HA matrix, because they lose their calcium ligands. Calcium ions could be hung on the HA surface and pure proteinaceous virions flow throw the column. The practical value of the developed method is that purified material can be used to rise TBSV virion-specific polyclonal antibodies and use them in virus diagnosis. Purified TBSV virions can be used as nanocarriers of vaccines and medical drugs. The method is economically useful from the point of time and labor consumption and can be used at a big manufacturing level. Moreover, the method can be used for the purification of other plant viruses, however, it needs further investigations.

Acknowledgments

We deliver our thanks to Ms. Bayansulu Ilyasova, Nazarbayev University for taking TEM

images of TBSV virions. This work was financed by a research grant AP08957713 "Development of new spectrophotometric method for the detection of viral plant diseases" from the Ministry of Education and Science of the Republic of Kazakhstan.

References

[1]. K.-B.G. Scholthof, S. Adkins, H. Czosnek, P. Palukaitis, E. Jacquot, T. Hohn, B. Hohn, K. Saunders, T. Candresse, P. Ahlquist, C. Hemenway, G.D. Foster, *Mol. Plant Pathol.* 12 (2011) 938–954. DOI: 10.1111/j.1364-3703.2011.00752.x

[2]. S.C. Harrison, A.J. Olson, C.E. Schutt, F.K. Winkler, G. Bricogne, *Nature* 276 (1978) 368–373. DOI: 10.1038/276368a0

[3]. P. Hopper, S.C. Harrison, R.T. Sauer, *J. Mol. Biol.* 177(1984) 701–713. DOI: 10.1016/0022-2836(84)90045-7

[4]. A.J. Olson, G. Bricogne, S.C. Harrison, *J. Mol. Biol.* 171 (1983) 61–93. DOI: 10.1016/s0022-2836(83)80314-3.

[5]. P.Q. Hearne, D.A. Knorr, B.I. Hillman, T.J. Morris, *Virology* 177 (1990) 141–151. DOI: 10.1016/0042-6822(90)90468-7

[6]. A. Llauró, E. Coppari, F. Imperatori, A.R. Bizzarri, J.R. Caston, L. Santi, S. Cannistraro, P.J. de Pablo, *Biophys. J.* 109 (2015) 390–397. DOI: 10.1016/j.bpj.2015.05.039

[7]. H. Peyret, *J. Virol. Methods* 225 (2015) 59–63. DOI: 10.1016/j.jviromet.2015.09.005

[8]. E. Schröder, T. Jönsson, L. Poole, *Anal. Biochem.* 313 (2003) 176–178. DOI: 10.1016/s0003-2697(02)00567-5

[9]. L.J. Cummings, M.A. Snyder, K. Brisack, *Method Enzymol.* 463 (2009) 387–404. DOI: 10.1016/S0076-6879(09)63024-X

[10]. M. Niimi, T. Masuda, K. Kaihatsu, N. Kato, S. Nakamura, T. Nakaya, F. Arai, *Sensor. Actuat. B-Chem.* 201 (2014) 185–190. DOI: 10.1016/j.snb.2014.04.011

[11]. G.G. Markov, I.G. Ivanov, *Anal. Biochem.* 59 (1974) 555–563. DOI: 10.1016/0003-2697(74)90309-1

[12]. G. Bernardi, M.-G. Giro, C. Gaillard, *Biochimica et Biophysica Acta (BBA) - Protein Structure* 278 (1972) 409–420. DOI: 10.1016/0005-2795(72)90001-3

[13]. G.M. Kostner, A. Holasek, *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* 488 (1977) 417–431. DOI: 10.1016/0005-2760(77)90200-4

[14]. G.P. Smith, T.R. Gingrich, *BioTechniques* 39 (2018). DOI: 10.2144/000112032

[15]. PM 7/126 (1) Electron microscopy in diagnosis of plant viruses. EPPO Bulletin 45 (2015) 450–453. DOI: 10.1111/epp.12260

[16]. T.M. Yergaliyev, Zh. Nurbekova, G. Mukiyanova, A. Akbassova, M. Sutula, S. Zhangazin, A. Bari, Zh. Tleukulova, M. Shamekova, Zh.K. Masalimov, R.T. Omarov, *Plant Physiol. Biochem.* 109 (2016) 36–44. DOI: 10.1016/j.plaphy.2016.09.001

[17]. A.B. Dildabek, Z.B. Stamgaliyeva, B.B. Ilyasova, Z.B. Tleukulova, A.A. Madirov, S.M. Kassenova, R.T. Omarov, S.K. Naekova, A.Zh. Akbassova, *Russ. J. Plant Physiol.* 68 (2021) 883–889. DOI: 10.1134/S102144372104004X