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Short running title: Purification of virus-like particles expressed in silkworm

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ABSTRACT

A three-stage chromatography protocol for the purification of human papillomavirus-like particles (HPV-LPs) from the silkworm-based *Bombyx mori* nucleopolyhedrovirus bacmid expression system was developed. For host cell DNA separation, anion exchange chromatography was used after screening for a suitable stationary phase. Using the two separation principles of cation exchange chromatography and metal affinity of ceramic hydroxyapatite (CHT) as a second stage, the amount of baculovirus in the sample was reduced to less than the detection limit of qPCR. The CHT separation was optimized with respect to the elution buffer used; 150–600 mM sodium phosphate, pH 7.2, resulted in the highest recovery of HPV-LPs. Using heparin chromatography, it was possible to reduce the sample volume and to thus highly concentrate the target protein during the separation of contaminating proteins. During the second purification stage, over 99.3 % of the DNA was removed, and no infectious baculoviruses remained. After concentration by heparin column chromatography, over 99.9 % of the DNA and protein had been removed. The purity achieved by this method exceeds that obtained by DDDDK-tag-based affinity chromatography and sucrose gradient ultracentrifugation, which were used as comparative purification methods. The 3-stage purification of HPV-LPs from silkworm fat bodies described here was a proof of concept and is a scalable method, but the overall yield remains to be improved.

Keywords:[†]

Virus-like particle, Silkworm, BmNPV bacmid, Purification, Ion-exchange chromatography, Human papillomavirus

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[†] Abbreviations: HPV-LPs - papillomavirus-like particles; CHT - ceramic hydroxyapatite; HPV - Human papillomavirus; VLPs - virus-like particles; AIEX - anion- exchange; phosphate-buffered saline (PBS); TBS - Tris-buffered saline; PES - polyether sulfone; PVDF - polyvinylidene fluoride; HRP - horseradish peroxidase; TCID50- Tissue culture infective dose for 50 %; qPCR - quantitative PCR; TEM – transmission electron microscopy

1. Introduction

Viruses are generally known as infectious agents that require living cells for their replication. Many viral genomes encode additional necessary enzymes that are not present in their host cells. Viruses cause a myriad of diseases in animals and humans; in most cases, such as that of the common cold, these diseases are relatively mild, but they can also be fatal. Human papillomavirus (HPV) is ambivalent; infection with this virus can be symptomless, may cause genital warts and may even cause various types of cancer. HPV is a non-enveloped, double-stranded DNA virus, of which more than 170 different types are already known [1]. HPV infects skin or mucous membrane cells and may elicit uncontrolled cell growth leading to warts or malignant growth leading to penile or cervical cancer [1].

Viruses can also be used for pharmaceutical applications in the form of virus-like particles (VLPs). Like the viruses from which they originate, VLPs possess a capsid or can also have an additional membrane, but they contain no genetic material and thus are non-infectious [2]. VLPs can be used as vaccines and can elicit strong immune responses even without modification [3]. If certain antigens are displayed on the surfaces of VLPs, they are also usable as multi-unit vaccines [2]. Specific antibody-displayed VLPs can target specific types of cells, for example, cancer cells. Furthermore, VLPs offer the potential for use as a drug delivery system if their inner void is filled with a drug or a drug is conjugated to their surface [4–6].

The production of VLPs often utilizes classical systems such as *Escherichia coli*, insect or mammalian cell-based systems. The silkworm *Bombyx mori* has been used as an expression system for eukaryotic proteins because the posttranslational modifications that occur in cells of this species are similar to those of mammalian cells, and this system offers a very high production rate of the target recombinant protein at very low cost [7,8]. We developed the *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid system for rapid and

efficient expression of eukaryotic genes [9]. The desired gene is introduced into the BmNPV bacmid, which is then amplified in *E. coli* as a large plasmid; in this system, approximately 1-2 weeks are sufficient for the entire DNA manipulation process [10].

Conventional purification of VLPs is still often achieved using sucrose density gradient centrifugation or affinity chromatography, although the use of ion-exchange chromatography for this purpose is increasing [11,12]. The former methods are difficult to scale up, making them less suitable for industrial use and mass production [12]. Despite the advantages of the silkworm expression system as a production system, the purification of VLPs still remains problematic. Although sucrose density gradient centrifugation or affinity chromatography is often used for this purpose, the yield, purity and protein quality obtained using these methods remain low [13,14]. Chromatography methods on the other hand have not yet been investigated for the VLP purification. Because there are difficulties in the separation of VLPs from their original viruses and in availability of suitable column materials for large biomolecules. Recently, newer column materials are launched on the market, which seem to have a potential [15]. Therefore it is needed to develop a new purification system for the purification of VLPs, in particular from silkworm, because VLPs are larger biomolecules which can result in a different behavior in standard chromatography materials and their stability can be also more critical [16]. On the other hand, the knowledge from virus purification can be used for their respective VLPs, even if this aspect has to be more explored yet. We already published a review which shows in detail the actual state of the VLP purification from the silkworm [17].

In this study, we attempted to establish a new purification protocol using only chromatographic methods to purify VLPs expressed in silkworms. The VLPs from HPV L1 protein were expressed in silkworm larvae and self-assembled to HPV-LPs. The protocol consists of 3 different chromatography stages in series. The first stage consisted of an anion-

exchange (AIEX) system for the separation of DNA. For the separation of baculovirus, a ceramic hydroxyapatite (CHT) column was used because of its dual principle of metal affinity and cation exchange. Finally, a heparin affinity column was used to concentrate the product of the 2nd stage-elution. Purification of HPV-LPs containing a DDDDK tag and purification of HPV-LPs by sucrose gradient centrifugation was used for comparison.

2. Material and methods

2.1. Biological materials

A previously constructed BmNPV/HPV6b L1 DDDDK-tagged bacmid [18] was used in this study. Bm5 cells were maintained in Sf-900II medium (Thermo Fisher Scientific K. K., Yokohama, Japan) supplemented with 10 % fetal bovine serum (FBS, Sigma Aldrich Japan, Tokyo, Japan) and 1 × Antibiotic-Antimycotic (Thermo Fisher Scientific K. K.). The silkworms and artificial diet Silkmate S2 were purchased from Ehime Sansyu (Ehime, Japan) and Nosan (Yokohama, Japan), respectively.

2.2. Rearing, feeding and injection of silkworm larvae

Fifth-instar silkworm larvae were reared on an artificial diet of Silkmate S2 in a chamber (Nosan, Yokohama, Japan) at 25°C. The silkworms were injected on the second day of the fifth stage with 50 µl of a phosphate-buffered saline (PBS) solution containing 10 µg recombinant BmNPV/HPV6b L1 DDDDK-tag bacmid per silkworm and 10 % (v/v) DMREI-C reagent (Invitrogen, Carlsbad, CA, USA) using a 1-ml syringe (26G, 0.45 × 13 mm, Terumo, Tokyo, Japan). At 5 days post-infection, the hemolymph was collected and 5 mM 1-phenyl-l-2-thiourea added, and 0.1 µg of fat body tissue was collected in 1 ml of 0.1 % Triton

X-100 in Tris-buffered saline (TBS); the latter suspension was on ice for 5 minutes sonicated (Vibra Cell VC 130PB (Sonics & Materials Inc., Newtown, USA)) in 20 seconds intervals with 10 seconds break and centrifuged (Kubota 3700, Tokyo, Japan) at 12,000 × g, 4°C for 10 min. After centrifugation, the liquid layer between the membrane on the liquid surface and the sediment was transferred to a micro tube. The fat body extracts were stored at -30°C until use.

2.3 Reagents

If not else stated, all reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Three-stage purification

Anion and cation exchange chromatographies were tested as the first step for separating host and baculovirus DNAs. Hereafter, CHT column was used for the baculovirus separation. At least a heparin column served to concentrate the sample. Between this stages the samples were frozen and after thawing filtered again before loading on the next column.

2.3.1. Ion exchange column screening as 1st stage purification

All chromatography experiments were conducted using a BioLogic Duo-Flow system (Bio-Rad Laboratories, Inc.) at room temperature (25°C). The fat body extract was diluted with PBS or saline to 1 mg/ml and passed through a 0.45-μm syringe filter (Millex – HA 0.45 μm (Merck, Darmstadt, Germany)). TOYOPEARL GigaCaps S-650M and Q-650M (Tosoh Corporation, Tokyo, Japan) were used as cation- and anion-exchange resins, respectively, and were packed in stainless steel columns (column size: ϕ 4.6 mm × 35 mm, Sugiyama Shoji Co., Ltd., Tokyo, Japan). Loading and washing were performed with 20 ml of 10 mM sodium

phosphate buffer (pH 7.2) at a flow rate of 1 ml/min; the loading amount was 9.6 mg of protein/column, and elution was performed using a linear gradient (20 ml) up to 1.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.2). Two-milliliter fractions were collected during sample loading and column washing, and 1-ml fractions were collected during gradient elution; the fractions were stored at -80°C. In addition, Bio-Scale Mini Cartridge UNOsphere Q (Bio-rad, Hercules, CA, USA) was also used (column size: ϕ 12.6 × 40 mm; 5 ml). Loading and washing were performed with 172.5 ml of 10 mM sodium phosphate buffer (pH 7.2) at a flow rate of 7.5 ml/min and a loading amount of 37.5 mg of protein/column. Elution was done with 172.5 ml of a linear gradient from 0 to 1.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.2). As a check of reproducibility, purification with GigaCap Q-650M was performed 6 times using a standard-size column (35 mm × ϕ 4.6 mm) and 7 times using a large column (35 mm × ϕ 16 mm). Flow rates of 1 ml/min for the standard column and 10 ml/min for the large column were investigated. The loaded sample volumes ranged from 4.9 ml to 60 ml; the higher volumes were loaded on the larger columns. Sample loading and washing was performed with 10 mmol/l sodium phosphate (pH 7.2) buffer; elution was performed using a gradient of NaCl from 0 to 1.5 mol/l with 20 ml on the small column and 240 ml on the bigger column.

2.3.2. Optimization of CHT chromatography

For CHT chromatography (ϕ 4.6 mm × 35 mm, CHT Type II, Bio-Rad, Hercules, CA, USA), 10 ml of eluate from the GigaCap Q-650M column was used. Washing was performed with 10 mmol/l sodium phosphate buffer (pH 7.2), and elution was performed twice with 15 ml of a sodium phosphate gradient (10 mmol/l to 600 mmol/l) at pH 6.4, 7.2 or 8.2 at a flow rate of 1 ml/min. From the flow-through and wash, 2-ml fractions were collected, and 1-ml fractions were collected during gradient elution. All fractions were stored at -80°C. Under the

same conditions, three additional concentrations of NaCl (0.5, 1.0, and 1.5 mol/l) were added to the elution buffer (15 ml, pH 7.2). Five additional elution buffers (gradients of 10 to 600 mmol/l sodium phosphate (15 ml), 80 to 600 mmol/l sodium phosphate (30 ml), 150 to 600 mmol/l sodium phosphate (26 ml), 200 to 600 mmol/l sodium phosphate (23 ml), and 250 to 600 mmol/l sodium phosphate (20 ml) were also tested.

To determine the reproducibility of the method, the purification was conducted three times under optimized conditions at a flow rate of 1 ml/min. The loaded sample amounts were 5, 6, and 5.8 mg of protein/column. Sample loading and washing were performed with 10 mmol/l sodium phosphate (pH 7.2) buffer, and elution was performed using a gradient of 26 ml sodium phosphate extending from 150 mmol/l to 600 mmol/l.

2.3.3. Optimized purification protocol

For the three-stage protocol involving GigaCap Q-650M, CHT and heparin-sepharose chromatography, 47 ml of the fat body sample was diluted with saline to 1 mg/ml and filtered through a 150-ml polyether sulfone (PES) 0.45-μm 45-mm bottle-top filter (Thermo Fisher Scientific). At the first stage, two portions of protein (210 mg and 222 mg) were loaded on a GigaCap Q-650M column (ϕ 32 mm × 35 mm, Vantage VL-32, Millipore, Tokyo, Japan). Washing was performed with 150 mmol/l sodium phosphate (pH 7.2) buffer, and elution was performed with a gradient of NaCl from 0 to 1.5 mol/l within 960 ml. Each fraction contained 48 ml.

In the 2nd stage of purification, 639 ml of the GigaCap Q-650M elution was loaded onto the CHT column (ϕ 16 mm × 35 mm). Washing was performed with 10 mmol/l sodium phosphate buffer (pH 7.2), and elution was performed with 312 ml of a sodium phosphate gradient extending from 150 to 600 mmol/l. Each fraction contained 12 ml.

As the 3rd stage of purification, a 1-mL HiTrap Heparin HP column (ϕ7 mm × 25 mm; GE Healthcare Japan, Tokyo, Japan) was used at a flow rate of 1 ml/min. The previously collected fractions were sonicated and filtered through a 0.45-μm filter (Millex – HA 0.45 μm, Merck); a total volume of 155 ml was loaded. The column was washed with 10 mmol/l sodium phosphate buffer (pH 7.2) and eluted with a gradient of 0 to 1 mol/l NaCl (25 ml); 1-ml fractions of the eluate were collected.

2.4. Purification by DDDDK-tag-based affinity chromatography and sucrose density gradient centrifugation

HPV 6b L1 VLPs from fat body samples were purified using Anti-DDDDK-tag gel (MBL, Nagoya, Japan) according to the manufacturer's instructions. Ten milliliters of the fat body sample were filtered through a 0.45-μm filter (Millex – HA 0.45 μm, Merck). Elution was performed three times using 1 ml of 0.1 mg/ml DDDDK-tag peptide in 50 mmol/l Tris-HCl (pH 7.4) (Star Chemicals, Nagoya, Japan) each time followed by three sequential elution with 1 ml glycine buffer (0.17 M glycine-HCl, pH 2.3); 1-ml fractions were collected.

For sucrose density gradient centrifugation, a 30 % sucrose solution was placed in a tube as a cushion; 4 ml of sample solution was then carefully added, and the tube was centrifuged at 122,000 g for 1 h at 4°C (Himac CS 120 GX II Micro Ultracentrifuge, Hitachi, Tokyo, Japan). The resulting pellet was washed in 1 ml PBS and resuspended in 1 ml PBS. A discontinuous density gradient consisting of 800 μl of 60 % sucrose, 700 μl of 50 % sucrose, 700 μl of 40 % sucrose, 700 μl of 30 % sucrose and 700 μl of 20 % sucrose was carefully layered in a centrifuge tube. The suspended pellet was carefully applied at the top, and the tube was centrifuged at 122,000 g for 3 h at 4°C. The entire volume of the gradient was then collected in 500-μl fractions.

2.5. Analytical methods

2.5.1. DNA concentration

DNA quantification was performed using the Quant-iT PicoGreen ds DNA Assay Kit (Thermo Fisher Scientific K. K.) according to the manufacturer's instructions.

2.5.2. SDS-PAGE and western blot analysis

The sample was diluted with an equal amount of sample buffer (Nacalai Tesque, Kyoto, Japan) and heated for 5 min at 99°C (DryThermoUnit DTU-1CN (Taitec Corporation, Koshigaya, Japan) before electrophoresis (Bio-Rad). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 12 % acrylamide. The gels were analyzed via Coomassie Blue staining or the silver staining II kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions.

For western blotting, the proteins were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) (Immobilon-P, Merck) membranes using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Blocking was performed for 1 h with 5 % skim milk in TBS containing 0.1 % Tween 20. After three 5-min washes with TBS and incubation for 1 h with mouse anti-DYKDDDDK antibody (1:10000, Wako Pure Chemical Industries), the blots were again washed three times for 5 min each time with TBS and incubated with a secondary antibody (sheep anti-mouse IgG-horseradish peroxidase (HRP), 1:10,000, GE Healthcare Japan, Tokyo, Japan) for 1 h. Immunoreactive bands were visualized using the Immobilon ECL Ultra Western HRP Substrate (Merck K. K., Tokyo, Japan). To detect glycoprotein GP64 from the baculovirus, rabbit anti-BmGP 64 (Bio Gate, Gifu, Japan) and

anti-rabbit IgG-HRP (Santa Cruz Biotechnology, California, USA) were used as primary and secondary antibodies, respectively. Band intensity was quantified by densitometry using the VersaDoc Imaging System (Bio-Rad).

2.5.3. Virus infectivity test

Tissue culture infective dose for 50 % ($TCID_{50}$) was used to measure the virus titer of BmNPV according to a previously reported method [19]. Nine hundred microliters of Sf-900 II medium (Thermo Fisher Scientific) containing 10 % FBS and antibiotic-antimycotic (Thermo Fisher Scientific) was mixed with 100 µl of virus sample, and the mixture was diluted 10-fold. The virus solution was diluted serially in medium to 10^7 dilution. Bm5 cells were cultivated at 1×10^4 viable cells/well in a 96-well plate, and 110 µl of virus solution was added to each well. As a negative control, Bm5 cells were cultivated in the absence of BmNPV. The cells were cultured at 27°C for 7 days, after which they were observed using a stereoscopic microscope. The Bm5 cell proliferation rate was compared with that of the negative control, and the titer of the baculovirus was determined from the obtained results using $TCID_{50}$. In short, the TCID50 calculation was done using a ratio of infected/non-infected cells and then a linear interpolation like stated in the section virus methods endpoint-titration of the Baculovirus expression vectors manual [20]

2.5.4. Baculovirus DNA measurement

Baculovirus DNA measurement was performed by quantitative PCR after extraction of viral DNA from the sample as previously described [21]. Viral DNA was extracted from 200 µl of each sample solution using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Tokyo, Japan) according to the manual provided with the kit. THUNDERBIRD® SYBER®

qPCR Mix (Toyobo, Osaka, Japan) was used as a reaction reagent for PCR. Bm ie-1 forward (5'-CCCGTAACGGACCTTGTGCTT-3') and Bm ie-1 reverse (5'-TTATCGAGATTTATTTACATACAACAAG-3') were used as the primers (FASMAC, Atsugi, Japan); the cycling conditions were heating at 95°C for 5 min followed by 60 cycles of 95°C for 10 sec and 60°C for 30 sec. The composition of the reaction mixture was as follows: 3 µl sample, 10 µl Thunderbird Syber qPCR mix, 1 µl of each primer (10 µM) and 5 µl demineralized water. If necessary, the PCR products were subjected to agarose gel electrophoresis, and their sizes were estimated by comparison with a DNA size ladder.

2.5.5. Morphological observation

Morphological observation was conducted using a transmission electron microscope (TEM: JEM-1400PLUS, JEOL Ltd., Tokyo, Japan). Twenty microliters of sample solution were placed on a grid, adsorbed, and dried; the grids were then stained three times with 2 % phosphotungstic acid (TAAB Laboratories Equipment, Aldermaston, UK). The grids were dried on filter paper at room temperature, and the samples were observed using TEM.

3. Results and discussion

HPV 6b L1 protein was expressed in the fat bodies of silkworms but not in the hemolymph (Supplementary Figure S1). The apparent molecular mass of the protein as determined by western blotting is 56 kDa. Based on this result, fat body samples were collected and used for purification of HPV 6b L1 protein.

3.1. Ion-exchange chromatography screening and reproducibility

HPV-L1 DNA and protein did not bind to the cation-exchange GigaCap S-650M column but were found in the flow-through (Supplementary Figure S2A). On the AIEX GigaCap Q-650M, the negatively charged DNA bound to the column and was eluted later than the bound VLPs (Supplementary Figure S2B). UNOsphere Q (Bio-Rad) separated DNA and proteins poorly compared to GigaCap Q-650M (Supplementary Figures S3A and B). Therefore, the anion exchanger GigaCap Q-650M was chosen as the 1st stage column in the subsequent experiments.

To confirm the reproducibility of GigaCap Q-650M column chromatography, after processing five different batches of HPV6b L1 expressed in silkworm fat bodies were passed over the column. Similar elution patterns of DNA and protein were observed in each case (Supplementary Figure S4). Upscaling on a 12-fold larger GigaCap Q-650M (35 mm × ϕ 16 mm) column was performed 6 times under the same conditions, and reproducibility was confirmed (Supplementary Figure S5). The elution behavior of DNA and HPV L1 on the larger column was almost the same as that shown in Supplementary Figure S4.

3.2. CHT chromatography buffer conditions and gradient analysis

After purification by AIEX, CHT chromatography was used as the 2nd stage in the purification. During buffer screening, were no changes in the elution position of HPV L1 when the buffer pH was changed; however, elution of the baculovirus protein BmGP64 occurred earlier with increasing pH (Supplementary Figure S6). Similar changes in the elution positions of viruses such as influenza virus and dengue virus in CHT column chromatography have been reported [22, 23]. After adding NaCl (0.5, 1 and 1.5 mol/l) to the elution buffer, it was confirmed that HPV L1, DNA and BmGP64 were all eluted (Supplementary Figure S7) at the same time, as indicated by SDS-PAGE and western blotting (data not shown). When 1-1.5 mol/l NaCl was added to the elution buffer, the elution of HPV L1 and BmGP64 overlapped; therefore, no NaCl was added in the optimized protocol. With increasing pH, HPV L1 eluted later in the linear gradient and BmGP64 eluted earlier. Therefore, the separation efficiency improved with increasing pH. Generally, when the salt concentration of the buffer is increased, the elution of DNA is delayed. In this case, the elution position of DNA did not change significantly, and HPV L1 and BmGP64 were eluted together with DNA.

In an attempt to improve the purification by changing the sodium phosphate concentration of the elution buffer, elution using five different sodium phosphate gradients at pH 7.2 was tested (Figure 1). At higher sodium phosphate concentrations, the contaminants were separated from the target protein and appeared in the flow-through fraction. However, when sodium phosphate gradients of 200 to 600 mmol/l and 250 to 600 mmol/l were used, HPV 6b L1 was also found in the flow-through fraction. Therefore, we decided to proceed with the elution using a 150-600 mmol/l sodium phosphate gradient.

<< Figure 1>>

The reproducibility of CHT column chromatography as a second chromatographic separation stage was confirmed. After pooling the fractions from the AIEX purification, CHT column chromatography was performed three times by elution with a 150-600 mmol/l sodium phosphate gradient (pH 7.2). All chromatograms showed similar UV peaks, and the elution position of HPV L1 did not change (Supplementary Figure S8).

3.3. Three-stage column chromatography

We examined the AIEX chromatography procedure, investigated the buffer used in CHT column chromatography, and attempted a 3-stage column chromatography purification protocol of HPV 6b L1 expressed in silkworm fat bodies under optimized conditions.

3.3.1. GigaCap Q-650M column chromatography

For DNA removal, AIEX chromatography was used as the first stage. Figure 2 shows the results of GigaCap Q-650M chromatography using a large column (ϕ 32 × 35 mm). Column chromatography on GigaCap Q-650M was performed twice under similar conditions; in both runs, VLPs and DNA showed elution behavior almost identical to that observed on the small column (ϕ 16 × 35 mm) (Supplementary Figure S5). VLPs were eluted prior to DNA, and effective DNA removal was confirmed (Figure 2A). The western blot results associated with this purification are shown in Figure 2B. The recovery of HPV 6b L1 in this stage was calculated to be approximately 31.4 %, based on densitometry comparing the amount of the loading fraction with the amount of the elution fraction.

<< Figure 2>>

3.3.2. CHT column chromatography

As an intermediate stage in the separation of baculovirus, CHT chromatography was performed on a larger column (ϕ 16 × 35 mm) (Figure 3). The UV pattern and HPV 6b L1 protein elution positions obtained using this column were the same as those obtained using the smaller column (ϕ 4.6 × 35 mm) (Supplementary Figure S7). The western blot analysis results are shown in Supplementary Figure S9A. A major portion of the BmGP64 appeared in the flow-through fraction without binding to the CHT stationary phase (Supplementary Figure S9B). Quantification of band intensity by densitometry indicated that the recovery of HPV 6b L1 protein at this stage was approximately 22.7 %, based on densitometry.

<< Figure 3>>

3.3.3. Heparin column chromatography

At the 3rd stage of purification, heparin affinity chromatography was performed to concentrate the HPV 6b L1. The CHT elution fraction was first loaded on HiTrap Heparin HP without desalting; as judged by western blotting, HPV 6b L1 was present in approximately 200 ml of the overall volume, which is in the middle of the main UV absorption peak (Figure 4B). HPV 6b L1 was eluted in fractions 23-32 (Supplementary Figure S10A), but no BmGP64 protein was detected in those fractions (Supplementary Figure S10B). This indicates that contaminating protein and BmGP64 were removed. The calculated approximate recovery of HPV 6b L1 in this stage was 86.9 %, based on densitometry.

<< Figure 4>>

3.3.4. Analysis of purified HPV L1

To investigate the rate of removal of baculovirus at each purification stage, HPV 6b L1 fat body samples and pooled fractions from GigaCap Q-650M column chromatography, CHT column chromatography and heparin column chromatography were investigated using virus titration by the $TCID_{50}$ method and quantitative PCR (qPCR) for baculovirus DNA (Table 1). Virus titer measurement by $TCID_{50}$ showed that the infectious baculovirus was present at 1.07×10^4 pfu/ml in the HPV 6 b L1-expressing fat body samples; however, after GigaCap Q-650M column chromatography, no infectious baculovirus was detected (Table 1). By qPCR, baculovirus DNA was present at levels equivalent to 4.00×10^6 pfu/ml in the fat body sample; after the 1st purification stage, the amount of baculovirus DNA decreased to 8.85×10^3 pfu/ml, a reduction of more than two-fold. The 2nd stage of purification using the CHT column did not remove significantly more baculovirus DNA. The qPCR products were subjected to agarose gel electrophoresis to determine whether the qPCR results reflected the presence of baculovirus DNA. Only one band with the expected size (200 bp) of the baculovirus DNA could be confirmed (data not shown). This result suggests that baculovirus DNA may bind to HPV L1, because the baculovirus DNA remaining after the third purification stage could not be removed.

To investigate the removal of DNA that occurred at each purification stage, DNA concentration measurements were performed. More than 80 % of the DNA present in the sample were removed during the first stage of purification on GigaCap Q-650M (Table 1). After CHT in the 2nd stage, much of the DNA had been removed. After purification by heparin column chromatography in the 3rd stage, 99 % or more of the DNA had been removed. The DNA concentration measurement results obtained for samples purified by DDDDK-tag-based affinity chromatography and sucrose density gradient centrifugation are also presented in Table 1.

The individual recovery of HPV L1 at the three purification stages was 31.4 %, 22.7 % and 86.9 %, and the overall recovery was 5.5 % based on densitometry analysis. However, the theoretical calculated recovery would be 6.19 % using the individual recoveries of each stage. Although the reason for the low yield could not be certainly determined, there are two possibilities. First, during the development of the method, the samples were frozen and thawed several times. This increases the possibility of protein denaturation. The second possible reason for the low yield is also associated with the freeze/thaw cycle. A precipitate appeared in the samples after thawing, and it was not possible to completely resuspend the precipitate. Therefore, the samples had to be filtered before they were applied to the next column. During this procedure, further loss of HPV L1 may have occurred. We showed both effects with an unprocessed fat body sample, which was several times frozen, thawed, filtered and then again frozen (Supplementary Figure S11 and Supplementary Table S1). Even with a high variance due to the low number of repeats we showed that there is a trend of HPV L1 lost during the freezing and thawing. The main loss of VLP is during the initial filtration with a specific pore size. This was for 0.8 µm 64.4 % loss and for 0.45 µm filter additional 27.6 % loss. The effect of the freezing is not definitely because of the variability, but the effect of the filtration is clear; on the last day alone a loss of 57.79 %. This aspect of the method requires further investigation to improve the protocol and the overall yield of the target VLP.

<< Table 1>>

3.4. Comparison with other purification methods

Purification of HPV 6b L1 from silkworm fat bodies was compared using three different methods. In the 3-stage purification, HPV 6b L1 was found in elution fractions 23–26 by silver staining (Figure 5A). After anti-DDDDK-tag purification, most of the target protein was found in elution fraction four, but other impurities were still present (Figure 5B).

Fractions three and four were used as a reference for the purification. An overall yield of 350 μg of L1 protein eluted in a volume of 2 ml from the fractions three and four could be obtained from 10 ml of fat body sample.

When the HPV 6b L1 protein was purified using sucrose density gradient centrifugation, the protein was found in fractions 5-10 together with many contaminating proteins (Figure 5C).

<< Figure 5>>

These findings are supported by the result of the DNA assay that illustrate the higher DNA concentration achieved by purification on sucrose density gradients compared to the DDDDK-tag-based purification; with the latter method, the amount of impurities are also higher than those achieved using the 3-stage protocol. Although these methods produce higher yield than the 3-stage chromatography protocol, the final product contains more host cell proteins. Thus, the multistage chromatographic process resulted in more effective DNA removal than the DDDDK-tag-based purification, at a cost to the overall yield (Table 1), despite the fact that it was originally intended to produce a comparable or higher yield.

To investigate the morphology of HPV L1 purified by the 3-stage chromatography method, TEM was performed (Figure 6). Particles with diameters of approximately 10-50 nm were observed, suggesting that HPV L1 formed VLPs in silkworms. Moreover, no baculovirus particles were visually observed (Figure 6A-1 and A-2). Although the anti-DDDDK-tag affinity method results in relatively good purification, parts of baculovirus still remain in the eluted material (Figure 6B). Purification by sucrose density gradient centrifugation showed less separation of baculovirus, and baculovirus were also present in the eluted material (Figure 6C). It can be clearly seen in the TEM images that many impurities and sucrose crystals are present in the purified sample (Figure 6C). The analysis showed that these purification methods are only semi-usable for the separation of the target protein. The

L1 protein forms a pentamer, and one virus particle is formed by the assembly of 72 pentamers. The size of one pentamer is approximately 10 nm, and the size of a complete particle is 50 nm. However, it has been reported that when L1 VLPs are expressed in *E. coli*, yeast or mammalian cells, not only large 50-nm particles but also 20-40 nm particles are formed [24, 25]. Therefore, it was suggested that when the L1 protein is expressed in silkworm larvae, the particle size is not uniform, and VLPs of various sizes are formed [18].

<< Figure 6>>

4. Conclusion

In this study, we established a purification method for non-enveloped HPV L1 VLPs from silkworm fat bodies. To our knowledge this is the first study to report the VLP purification from the silkworm fat body aiming high purity without using affinity chromatography or sucrose gradient ultracentrifugation. The 3-stage chromatography protocol was designed and optimized the operating conditions for purification of HPV L1 VLP expressed in silkworm: Complete removal of the host cell and baculovirus DNAs and the infectious baculovirus from the silkworm fat body. Using anion-exchange chromatography GigaCap Q-650M with a 150 mmol/l sodium phosphate (pH 7.2) buffer and elution with a gradient up to 1.5 mol/l NaCl, all infectious baculovirus was removed; 99.5 % of the remaining baculovirus DNA was subsequently removed by CHT purification using a 10 mmol/l sodium phosphate buffer (pH 7.2), and elution up to 600 mmol/l sodium phosphate. Heparin column chromatography was used to concentrate the sample at the end of the process. The removal of impurities through this 3 stage chromatography protocol exceeded those of the widely used affinity tag-purification and sucrose density gradient ultracentrifugation purification methods. This process also displayed high reproducibility. The only serious drawback of this method is the unsatisfying yield of the target VLP, particularly considering

that the actual HPV L1 recovery was with 5.5 % partially due to the repeating freezing and filtration. This is due to the instability of the protein and definitely necessitates more research on VLP purification. It should be taken into account that the aim of this study was only to show the possibility of purification of VLPs from the silkworm fat body using chromatographic methods as a proof of concept.

Acknowledgments

This work was supported by the Japan Society for the Promotion of Science KAKENHI (grant nos. 16H02544 and 16K14900).

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Table 1. Separation and purification results (each value besides the TCID50 was measured at least twice)

Sample	Volume	Densitometry		TCID50	qPCR	dsDNA (Pico Green)	
	Volume	Gp64	Gp64 Removal	Virus titer	Virus DNA	DNA	Removal
	ml		%	Pfu/ml	Pfu/ml	µg	%
3-Stage purification							
Fat body × 9.2 dil.	380	25.89	---	1.07×10^{4}	4.00×10^{6}	670	---
Stage 1: Q pool	672	17.41	48.06	N.D.	8.85×10^{3}	83	86.4
Stage 2: CHT pool	168	1.46	94.33	N.D.	8.75×10^{3}	2.9	99.5
Stage 3: Heparin pool	6	0.02	99.92	N.D.	5.82×10^{3}	0.02	99.997
Sucrose gradient ultracentrifugation							
Fat body 0.45 µm filtered	4				6.17×10^{7}	125.23	---
Fraction 7	0.5				9.66×10^{7}	2.95	97.64
Anti-FLAG-tag affinity purification							
Fat body 0.45 µm filtered	10				6.17×10^{7}	313.07	---
Elution fraction 3	1				1.53×10^{4}	0.05	99.98
Elution fraction 4	1				1.49×10^{4}	0.33	99.89

N.D.: Not detected.

FIGURE LEGENDS

Fig. 1. Investigation of the use of various sodium phosphate gradients to elute proteins in CHT column chromatography. The flow rate was always 1 ml/min. Sodium phosphate (pH 7.2) gradient elution was conducted from 10 to 600 mmol/l (linear gradient 15 ml) (**A),** from 80 to 600 mmol/l (linear gradient 30 ml) (**B),** from 150 to 600 mmol/l (linear gradient 26 ml) (**C),** from 200 m to 600 mmol/l (linear gradient 23 ml) (**D)** and from 250 to 600 mmol/l (linear gradient 20 ml) (**E).** The sample load was for (A) to (C) 8 ml and for (D) and (E) 10 ml, because of a batch change. Blue lines: absorbance at 280 nm; green lines: absorbance at 260 nm; dotted lines: conductivity (mS/cm); purple lines: DNA concentration (ng/ml). The pink and purple areas indicate the elution positions of HPV L1 and BmGP64, respectively, as confirmed by western blotting.

Fig. 2. Purification of HPV 6b L1 by GigaCAP Q-650M column chromatography using a large column (ϕ 32 mm × 35 mm) with a flow rate of 1 ml/min. (A) GigaCap Q-650M column chromatography. Sample was loaded 7 times in 30 ml batches. The linear gradient was done within 960 ml of 0 – 1.5 mol/l NaCl in a 150 mmol/l sodium phosphate (pH 7.2) buffer. FT: flow-through; other lines and symbols are the same as those used in Figure 1. (B) Western blot analysis of each fraction from GigaCap Q-650M-column chromatography. The HPV L1 and BmGP64 proteins in each fraction were detected by western blotting using anti-DYKDDDDK tag monoclonal antibody and anti-BmGP64 antibody, respectively, as the primary antibody. M: Magic Mark XP; FB: HPV 6b L1-expressing fat bodies (9.2 dil + 0.45-μm filter). The red arrows in (A) and (B) indicate the positions of HPV L1 and BmGP64, respectively.

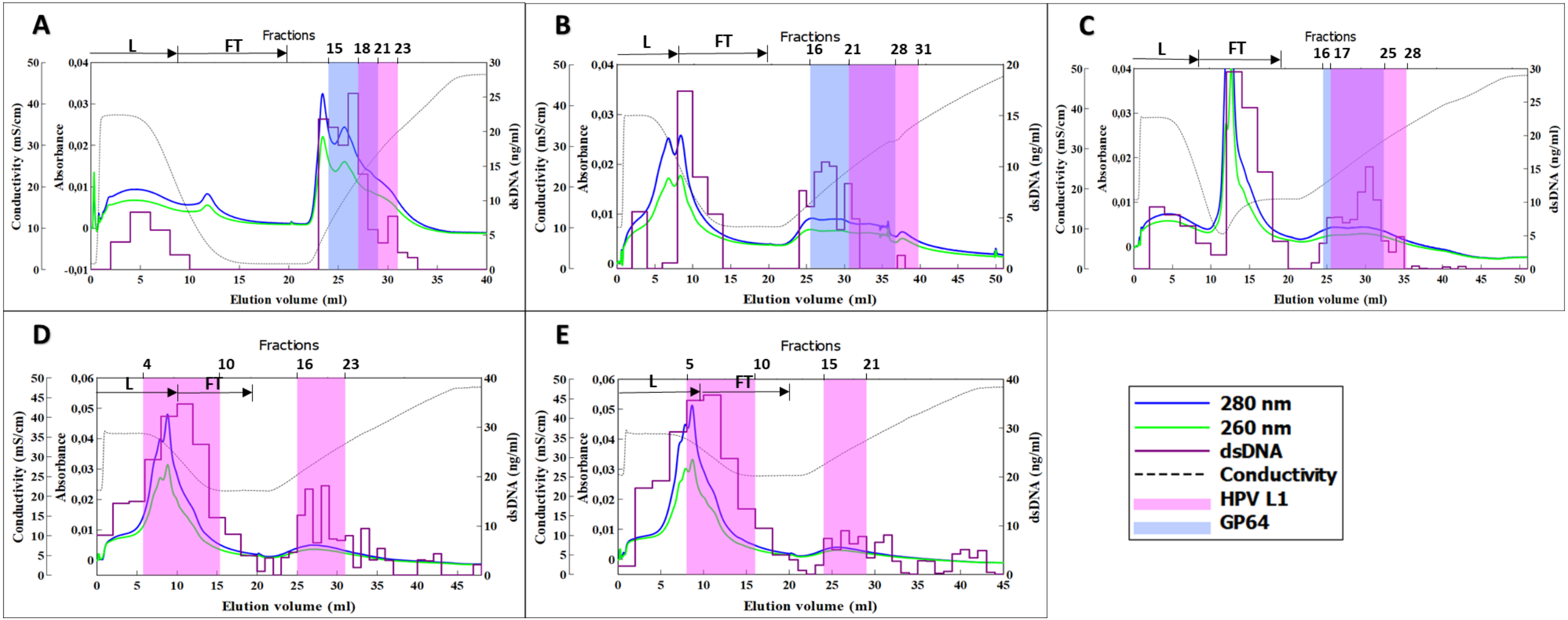
Fig. 3. CHT column chromatography purification using a large column (ϕ 16 × 35 mm) (stage 2). The chromatogram shows an example of the process of the purification on the CHT column. Flow rate was 1 ml/min and the sample was loaded 21 times in 30 ml batches.

The linear gradient was done within 312 ml of 150 to 600 mmol/l sodium phosphate (pH 7.2). FT: flow-through; other lines and symbols are the same as those used in Figure 1.

Fig. 4. Chromatogram showing the results of heparin column chromatography (ϕ 7 × 25 mm) (stage 3). Both chromatograms show an example of the process of purification on the heparin column. One shows the whole process **(A);** a more detailed and enlarged version is presented in **(B)**. Flow rate was 1 ml/min and 155 ml sample was loaded 6 times. The elution was done within 25 ml of a 0 to 1 mol/l NaCl gradient in a 10 mmol/l sodium phosphate buffer (pH 7.2). Lines and symbols are the same as those used in Figure 1.

Fig. 5. Comparison of purified proteins. **(A)** Silver stain analysis of SDS-PAGE of fractions from the heparin column chromatography. M: Magic Mark XP; FB: HPV 6b L1 expressing fat body (× 10); (+): CHT Fr. 4-17 pool + 0.45-μm filter; Q: Large Q pool; 1 – 29: Fr. No. **(B)** CBB staining of anti-DDDDK-tag gel purification results obtained for HPV6b L1. M: Protein Marker; FB: fat body extract expressing HPV 6 b L1 VLP; FB fil: filtered fat body sample; FT: flow-through fraction; W1-2: washing fraction; E: equilibration fraction; 1-8: elution fractions. **(C)** CBB staining showing the results of sucrose density gradient centrifugation purification of HPV6b L1. M: Protein Marker; FB: fat body extract expressing HPV 6 b L1 VLP; 1-10: fraction numbers. The volume of each fraction was 500 μl. The red arrows indicate HPV 6 L1.

Fig. 6. Morphological observation by TEM. **(A-1 and A-2)** Purified VLP sample after 3-stage column chromatography purification. **(B)** Purified VLP sample after DDDDK-tag purification. **(C)** Purified VLP sample after sucrose density gradient purification. The bars in (A-1), (A-2) and (B) indicate 200 nm; those in (C) indicate 100 nm. The red arrows indicate baculovirus particles.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B
C
D
E
L
FT
Fractions
Conductivity (mS/cm)
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Elution volume (ml)
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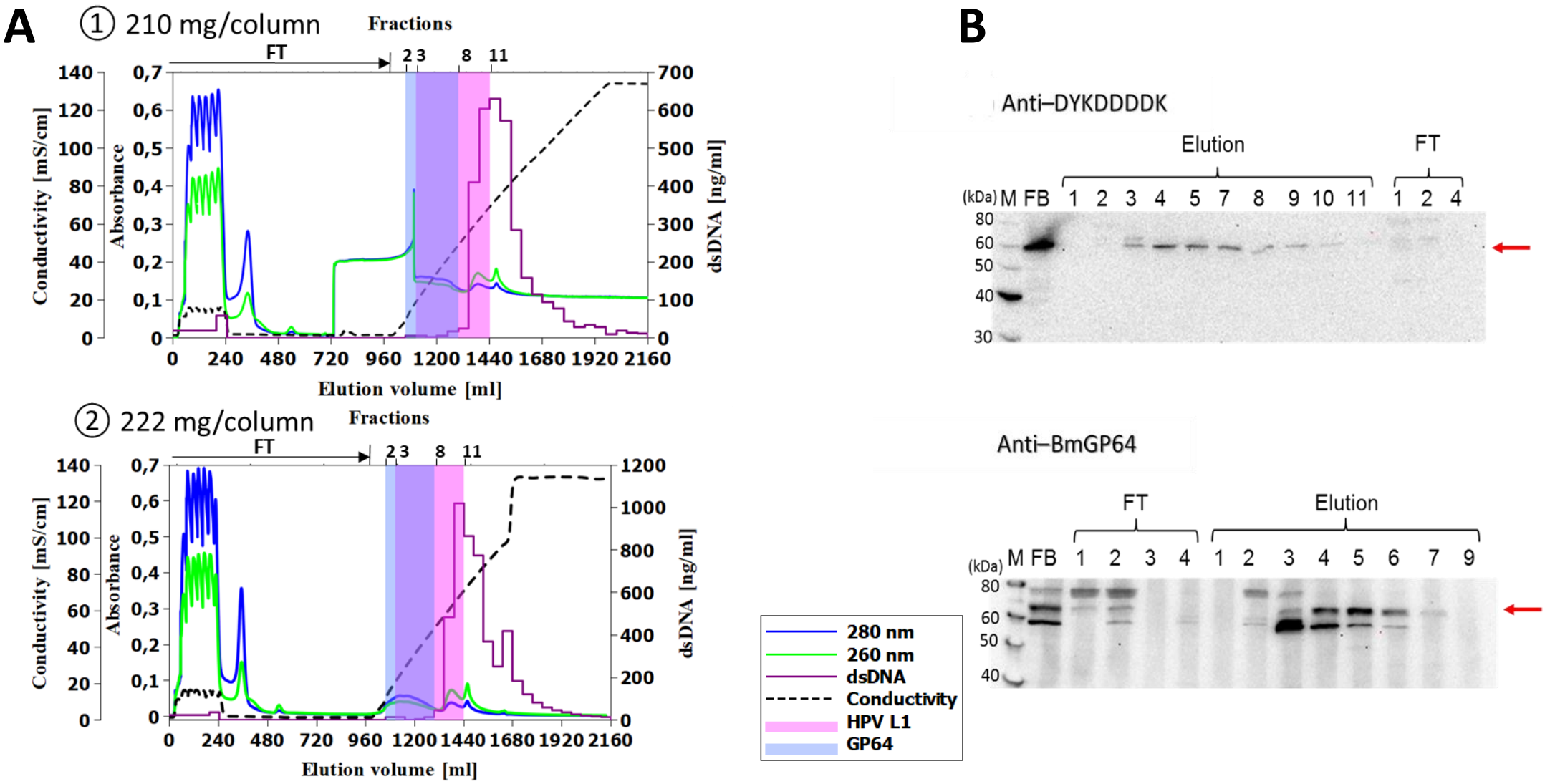
260 nm
dsDNA
Conductivity
HPV L1
GP64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FT
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8
11
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Absorbance
dsDNA [ng/ml]
Elution volume [ml]
② 222 mg/column
Fractions
FT
2 3
8
11
Conductivity [mS/cm]
Absorbance
dsDNA [ng/ml]
Elution volume [ml]
280 nm
260 nm
dsDNA
Conductivity
HPV L1
GP64
B
Anti–DYKDDDDK
Elution
FT
(kDa) M FB 1 2 3 4 5 7 8 9 10 11 1 2 4
80
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50
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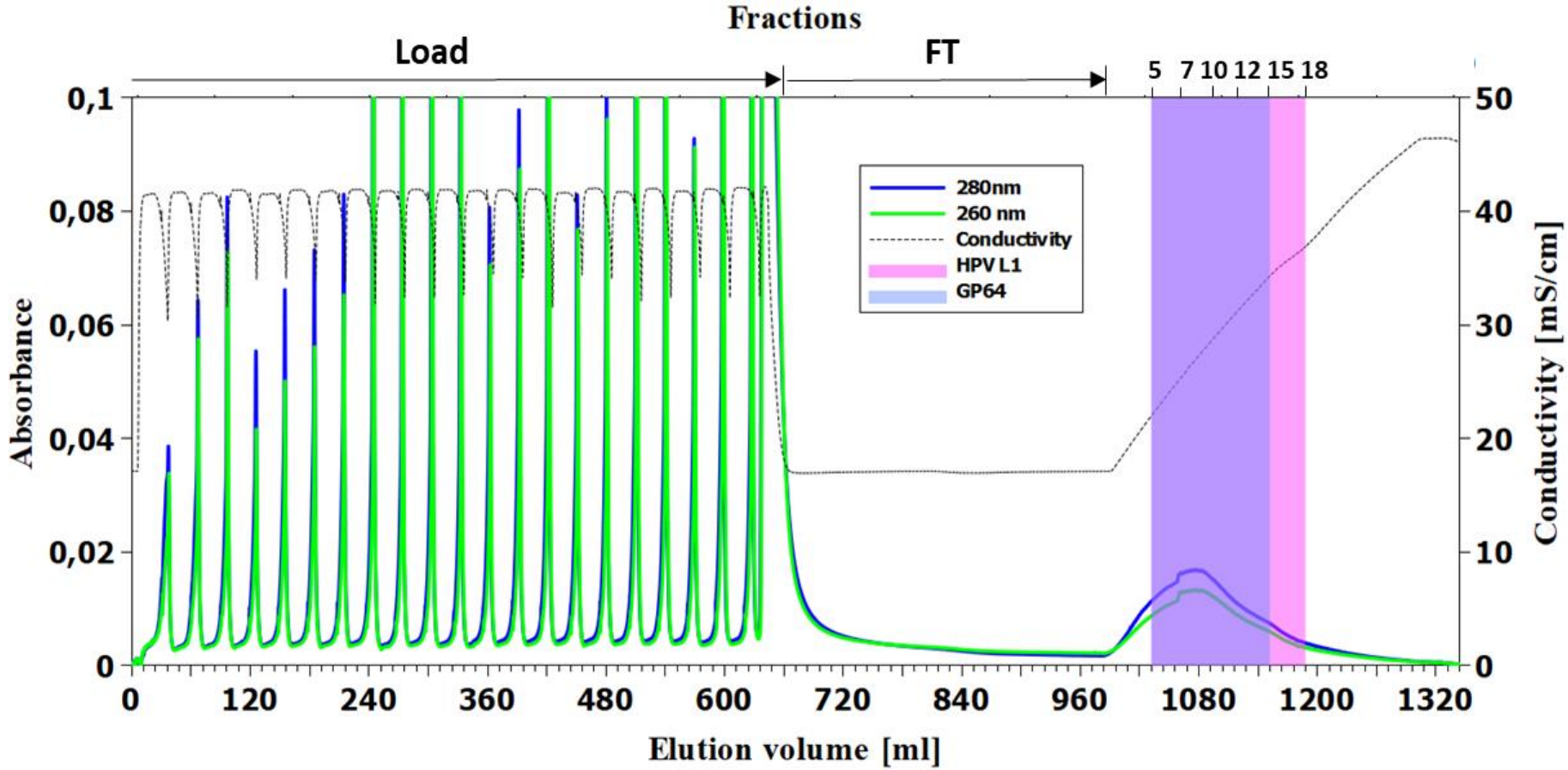
30
Anti–BmGP64
FT
Elution
(kDa) M FB 1 2 3 4 1 2 3 4 5 6 7 9
80
60
50
40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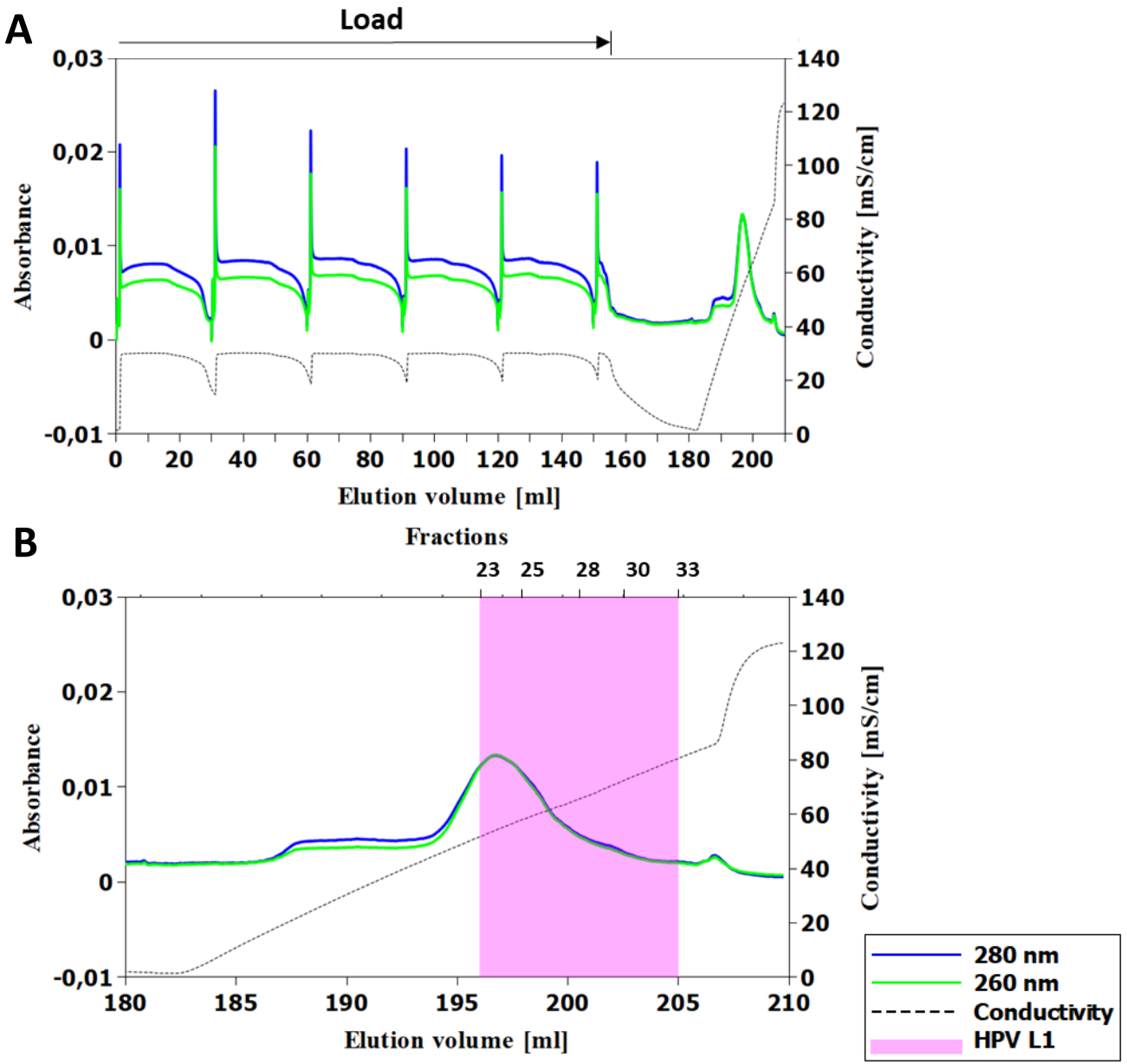
0,08
0,06
0,04
0,02
0
Absorbance
50
40
30
20
10
0
Conductivity [mS/cm]
280nm
260 nm
Conductivity
HPV L1
GP64
0 120 240 360 480 600 720 840 960 1080 1200 1320
Elution volume [ml]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Conductivity [mS/cm]
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Elution volume [ml]
B
Fractions
23 25 28 30 33
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0,02
0,01
0
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Absorbance
140
120
100
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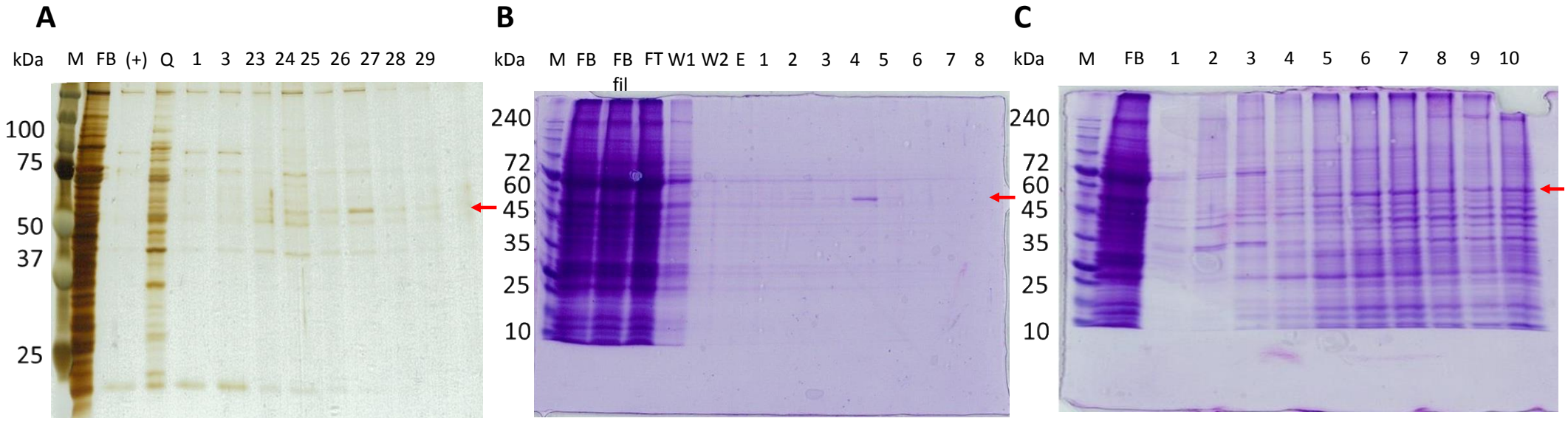
Conductivity
HPV L1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B
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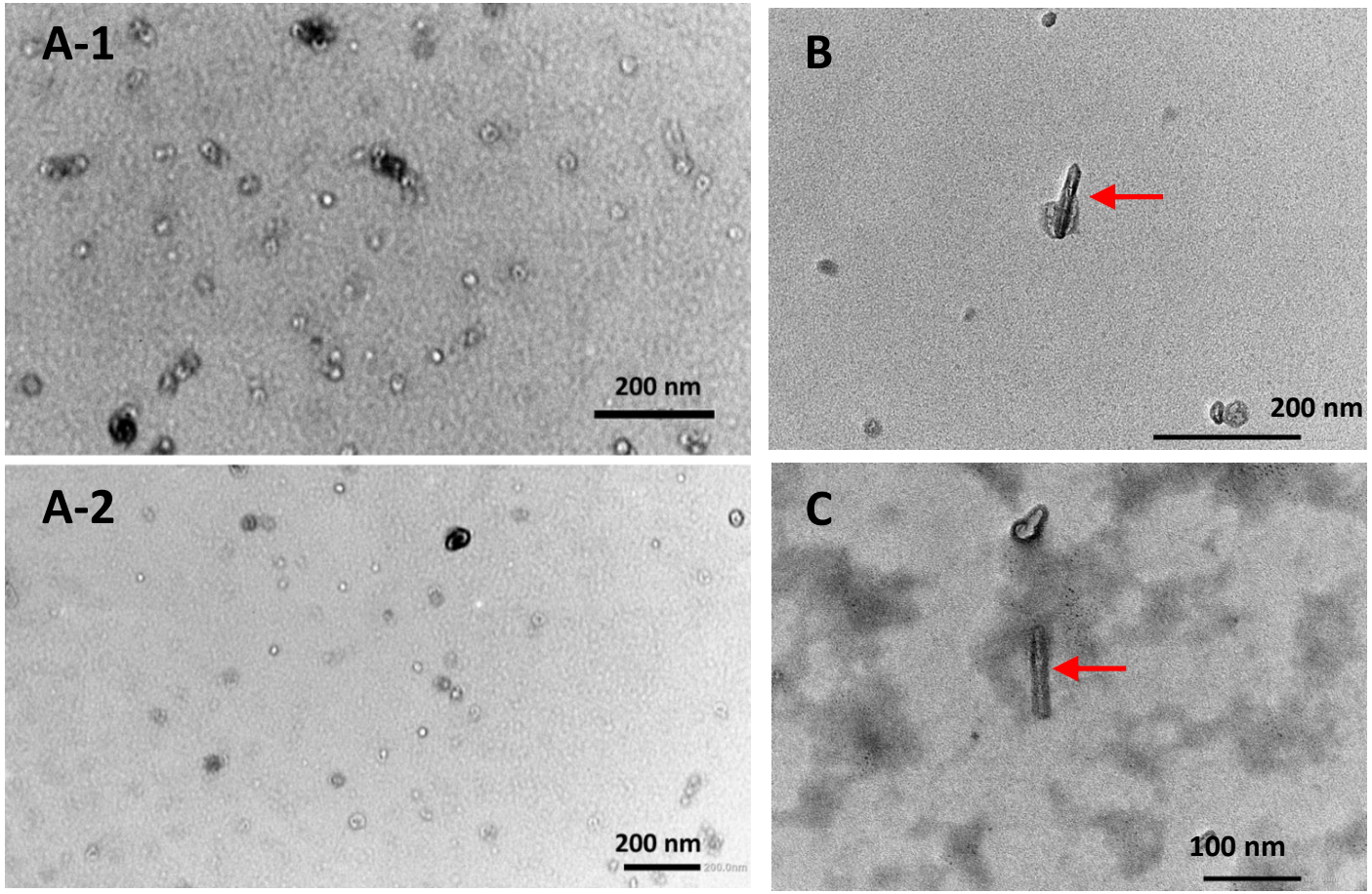
A-1
200 nm
B
200 nm
A-2
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C
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Journal of Chromatography B

Supplementary file

Purification of human papillomavirus-like particles expressed in silkworm using a *Bombyx mori* nucleopolyhedrovirus bacmid expression system

Short running title: Purification of virus-like particles expressed in silkworm

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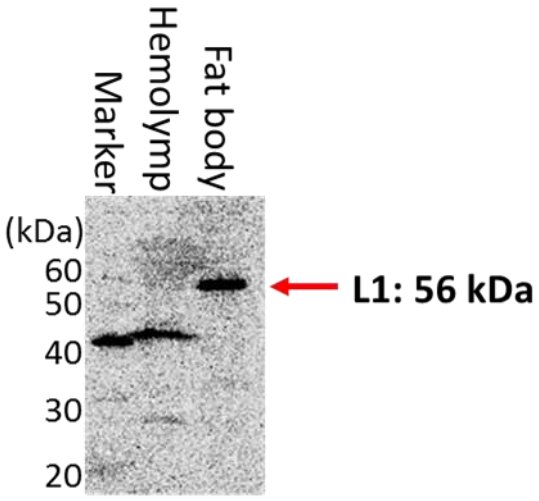
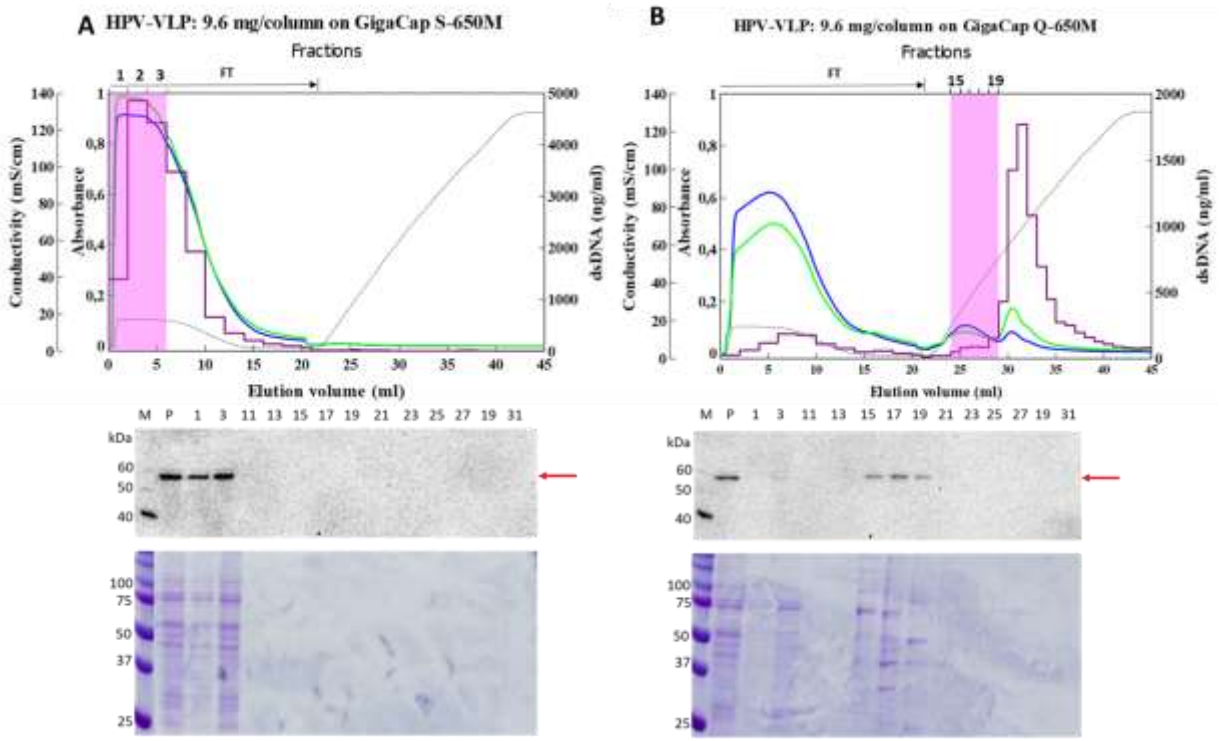
E-mail address: park.enoch@shizuoka.ac.jp (Enoch Y. Park).

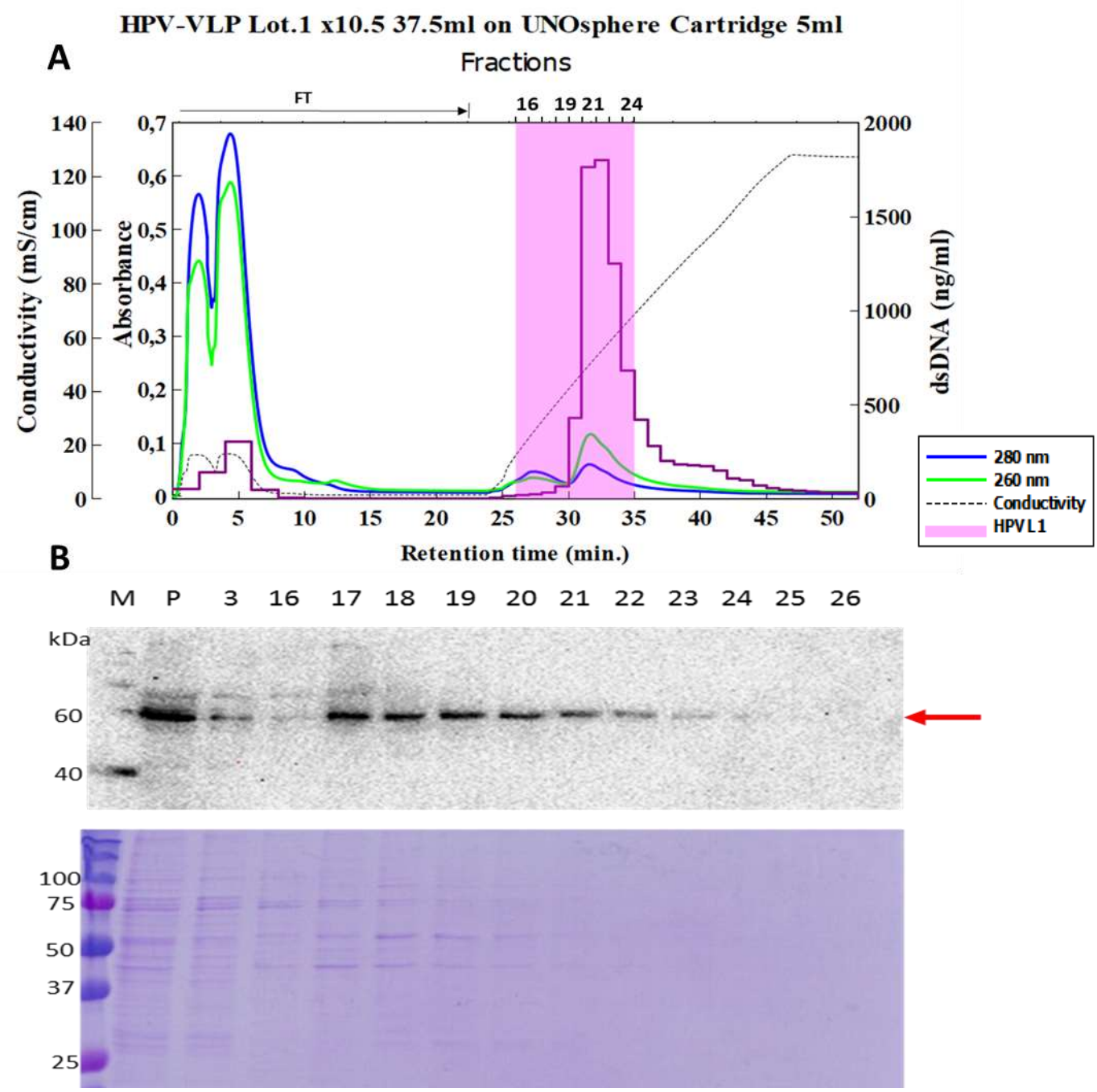


Supplement Figure S1 Confirmation of expression of HPV 6b L1 protein by western blotting. The size of the HPV 6b L1 protein was 56 kDa, and the expression of HPV 6b L1 protein was confirmed in the fat body sample. Primary antibody was a monoclonal anti-DYKDDDDK antibody and the secondary antibody was an anti-mouse IgG-HRP antibody. Red arrow indicates L1 protein.



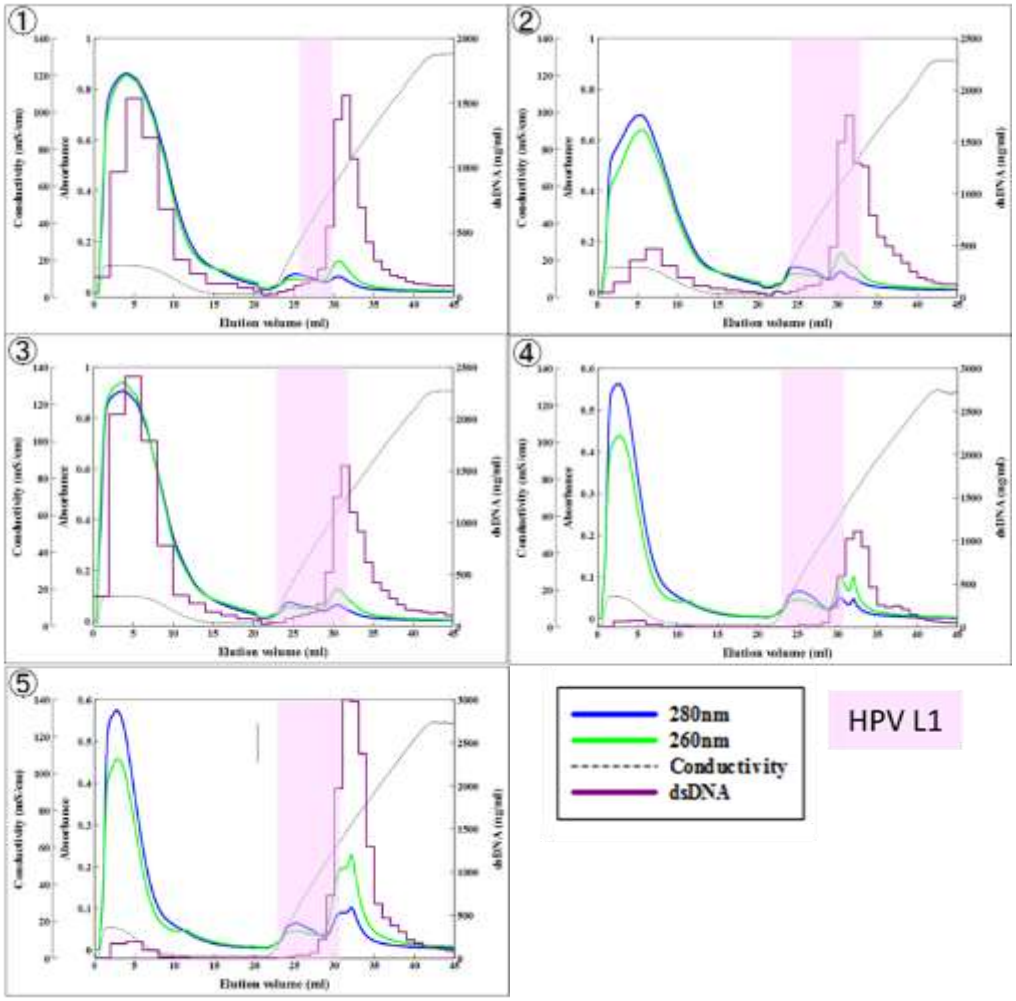
Supplement Figure S2 Chromatogram, the SDS-PAGE and Western blot results of the cation exchange column GigaCap S-650M (A) and the anion exchange column GigaCap Q-650M (B). Flow rate was 1 ml/min and in both cases 9.6 ml sample was loaded. The linear gradient was done within 20 ml up to 1.5 mol/l NaCl in 10 mM sodium phosphate buffer (pH 7.2). Upper panels show of the chromatogram and lower panels shows the SDS-PAGE and Western blot.

Blue line: absorbance at 280 nm; Green lines: absorbance at 260 nm; Dotted lines: conductivity (mS/cm); Purple lines: DNA concentration (ng/ml); Pink areas: elution position of HPV L1 confirmed by western blotting; M: molecular marker; P: load. Numbers indicate fractions. Flow through and elution fractions are 1–12 and 13–35, respectively. Red arrows indicate L1 protein.

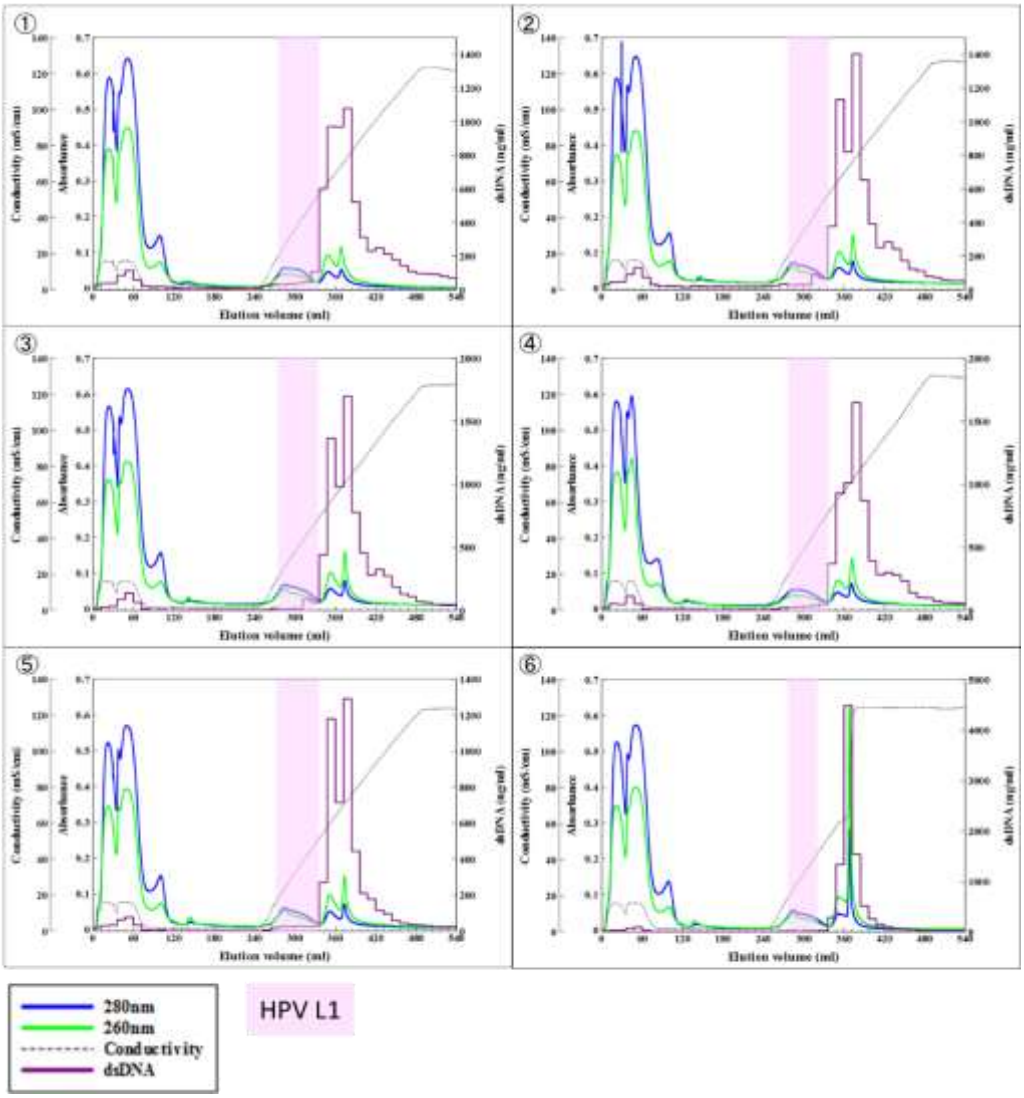


Supplement Figure S3 Chromatogram of the anion exchange column (A) and SDS-PAGE and Western blot (B) of UNOsphere Q column. Flow rate was 7.5 ml/min. The linear gradient was done within 172.5 ml up to 1.5 mol/l NaCl in10 mM sodium phosphate buffer (pH 7.2).

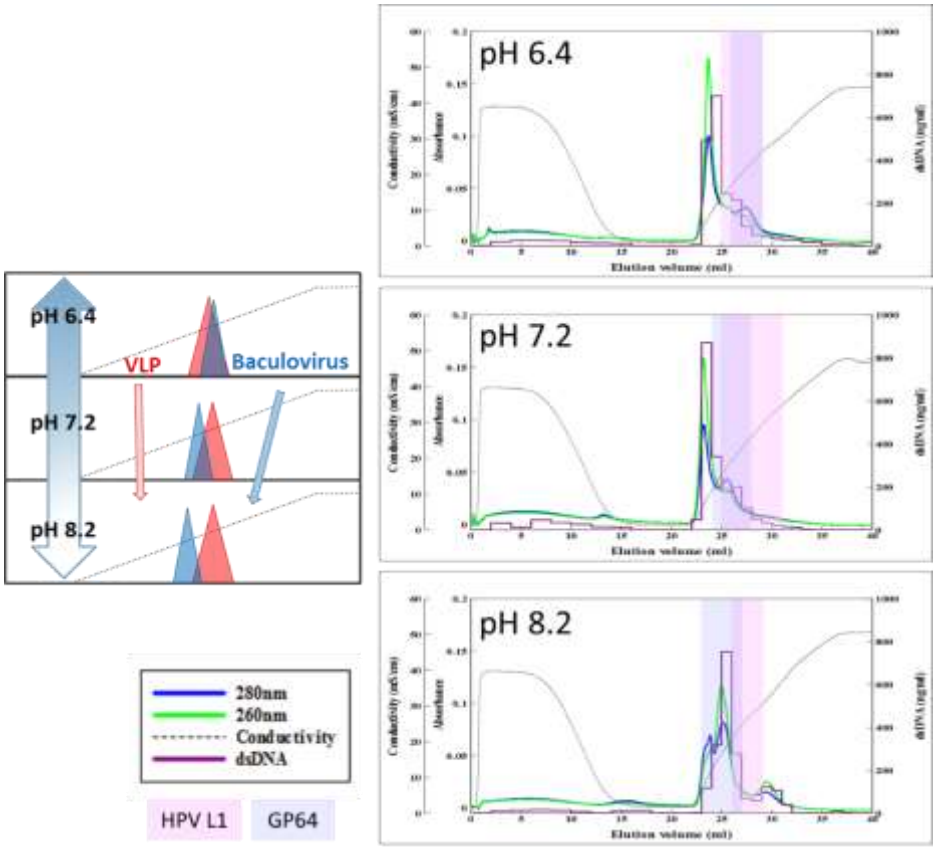
Blue lines: absorbance at 280 nm; Green lines: absorbance at 260 nm; Dotted lines: conductivity (mS/cm); Purple lines: DNA concentration (ng/ml); Pink areas: elution position of HPV L1 confirmed by western blotting. Flow through and elution fractions are 1–12 and 13–41, respectively. Whereupon the flow through fractions consist of 15 ml and the elution fractions of 7.5 ml.



Supplement Figure S4 GigaCap Q-650M Column Elution Check 1 (ϕ 4.6 x 35 mm). A different batch HPV6b L1 expressed in silkworm fat body was carried out five times. DNA and protein show in each case similar elution behavior. Sample was diluted in saline and loaded 2 times (9.6 ml sample) (1); was diluted in PBS and loaded once (9 ml sample) (2); was diluted in PBS and loaded 2 times (9 ml sample) (3); was diluted in saline and loaded once (4.9 ml sample) (4); was diluted in saline and loaded 2 times (4.9 ml sample) (5). Flow rate was 1 ml/min and the linear gradient was done within 20 ml up to 1.5 mol/l NaCl in10 mM sodium phosphate buffer (pH 7.2). Lines and pink areas are the same as those of Supplement Figure 2.

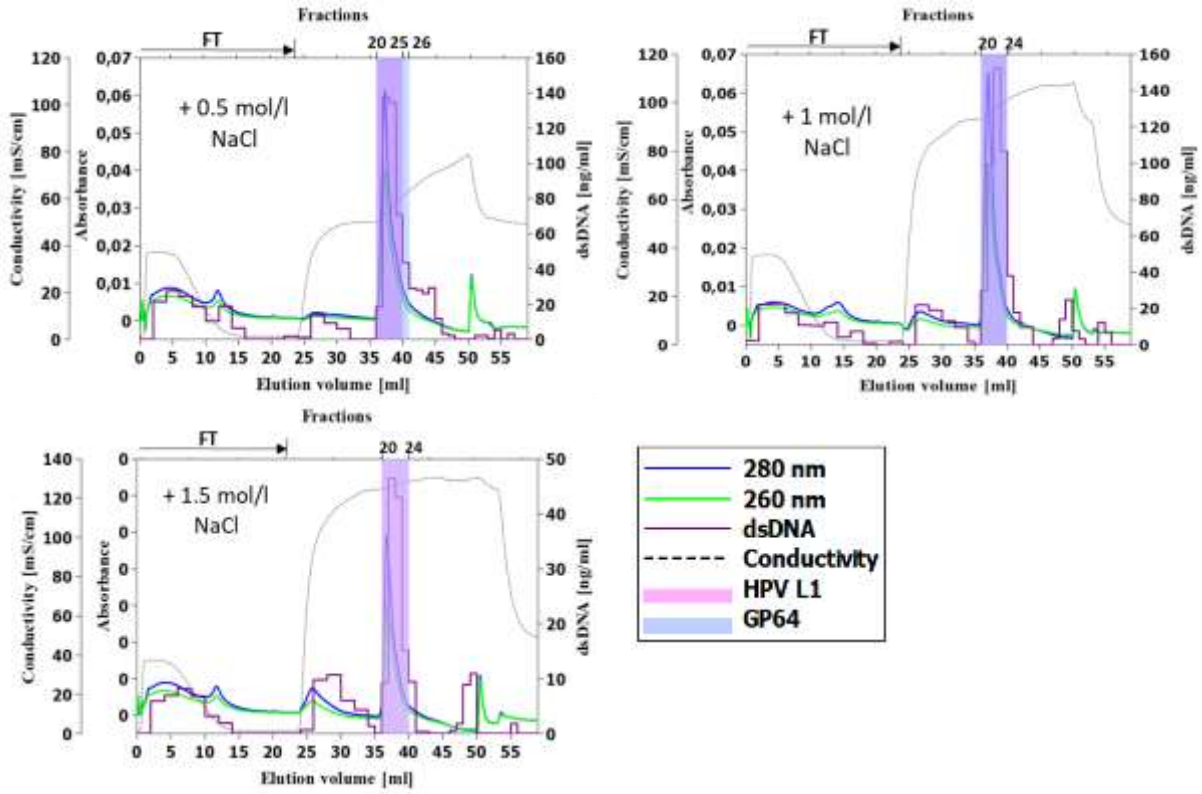


Supplement Figure S5 Confirmation of reproducibility of GigaCap Q-650M using large column (Ø 16 x 35 mm). The GigaCap Q-650M column chromatography was up scaled, and the experiment was 6 times repeated. Flow rate was 10 ml/min and the linear gradient was done within 20 ml. For No. (4) 46 ml was loaded, for the other experiments 60 ml. The linear gradient was done within 240 ml up to 1.5 mol/l NaCl in10 mM sodium phosphate buffer (pH 7.2).The chromatograms 1 to 6 show the same elution behavior. Lines and pink areas are the same as those of Supplement Figure 2.

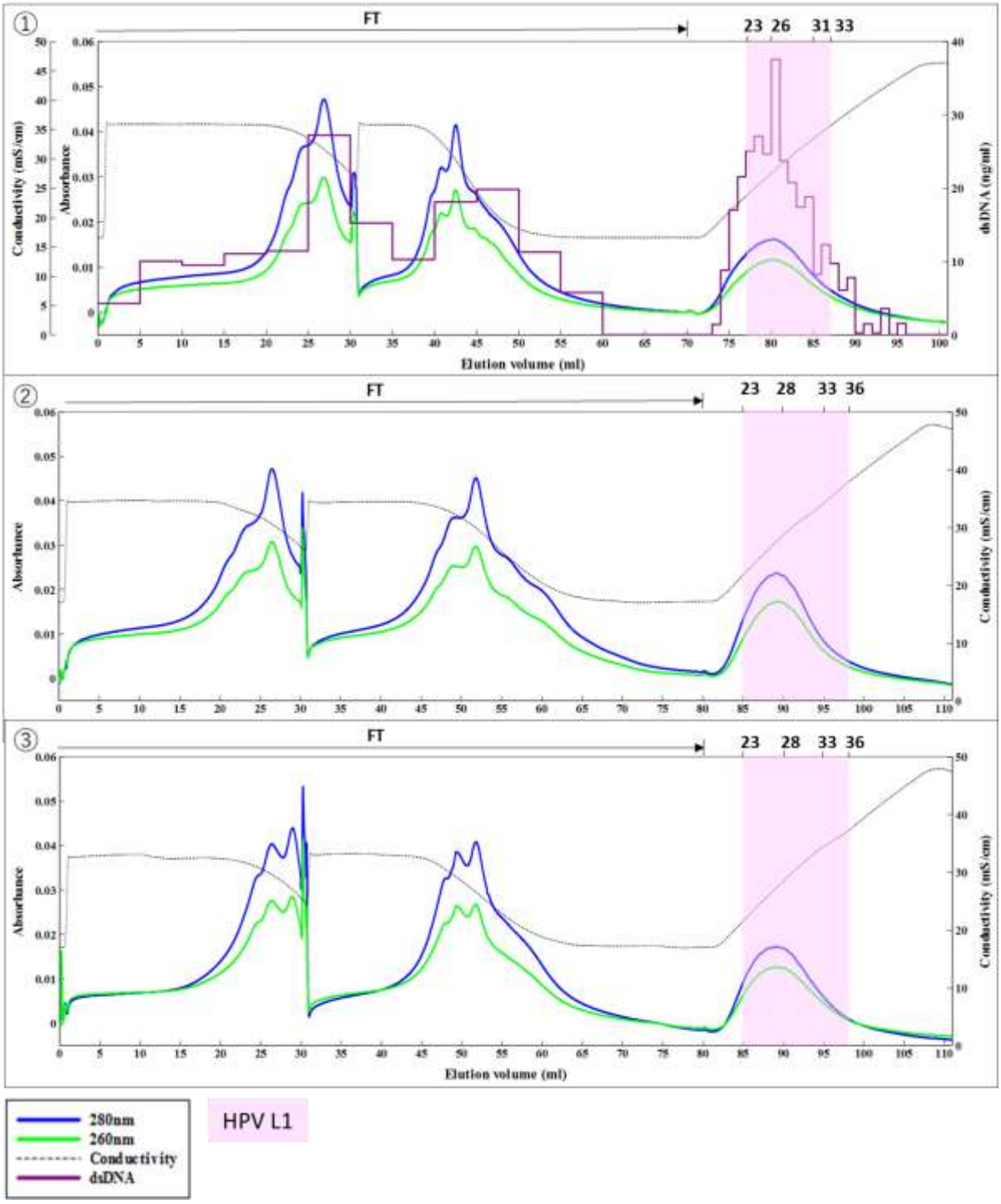


Supplement Figure S6 Effect on pH on the elution behavior of VLP and baculovirus in the CHT column chromatography.

This figures show the different elution behavior of VLP and baculovirus with changing pH. Flow rate was 1 ml/min and the linear gradient was done within 15 ml of sodium phosphate gradient (10 mmol/l to 600 mmol/l) at pH 6.4, 7.2 or 8.2. Samples were 10 ml from the AIEX eluate. Blue line: absorbance at 280 nm; Green lines: absorbance at 260 nm; Dotted lines: conductivity (mS/cm); Purple lines: DNA concentration (ng/ml); Pink areas: elution position of HPV L1 confirmed by western blotting; Purple areas: elution position of BmGP64 confirmed by western blotting. **Left side figure:** The shift of VLP and baculovirus corresponded by pH of the elution buffer in CHT column chromatography.

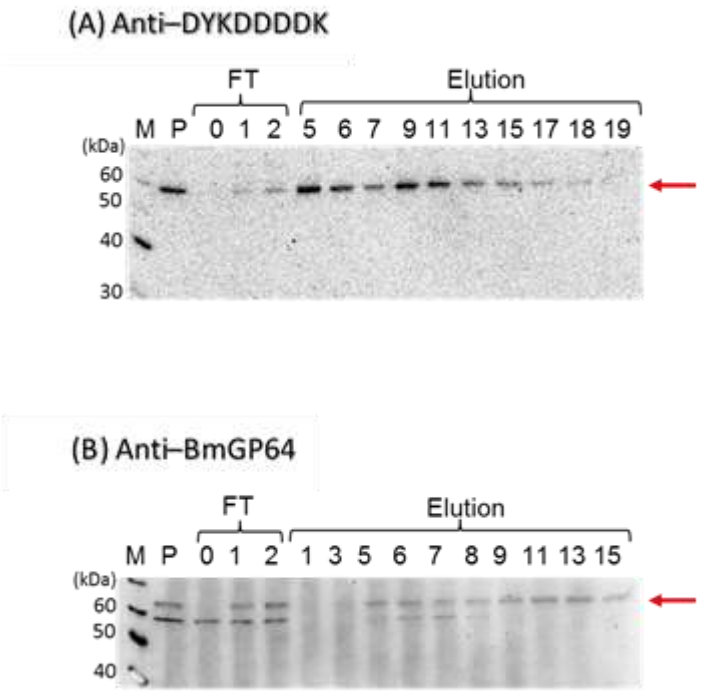


Supplement Figure S7 CHT column chromatography (Ø 4.6 x 35 mm) using different NaCl concentrations in the elution buffer. This figure shows the different behavior of the proteins during the purification when the NaCl concentration in the elution buffer was changed. Flow rate was 1 ml/min and the linear gradient was done within 15 ml (10 mmol/l to 600 mmol/l sodium phosphate). Samples were 10 ml from the AIEX eluate. Blue lines: Absorbance at 280 nm; Green lines: absorbance at 260 nm; Dotted lines: conductivity (mS/cm); Purple lines: DNA amount (ng/ml); Pink areas and blue areas indicate elution positions of HPV L1 and BmGP64 confirmed by western blotting, respectively.

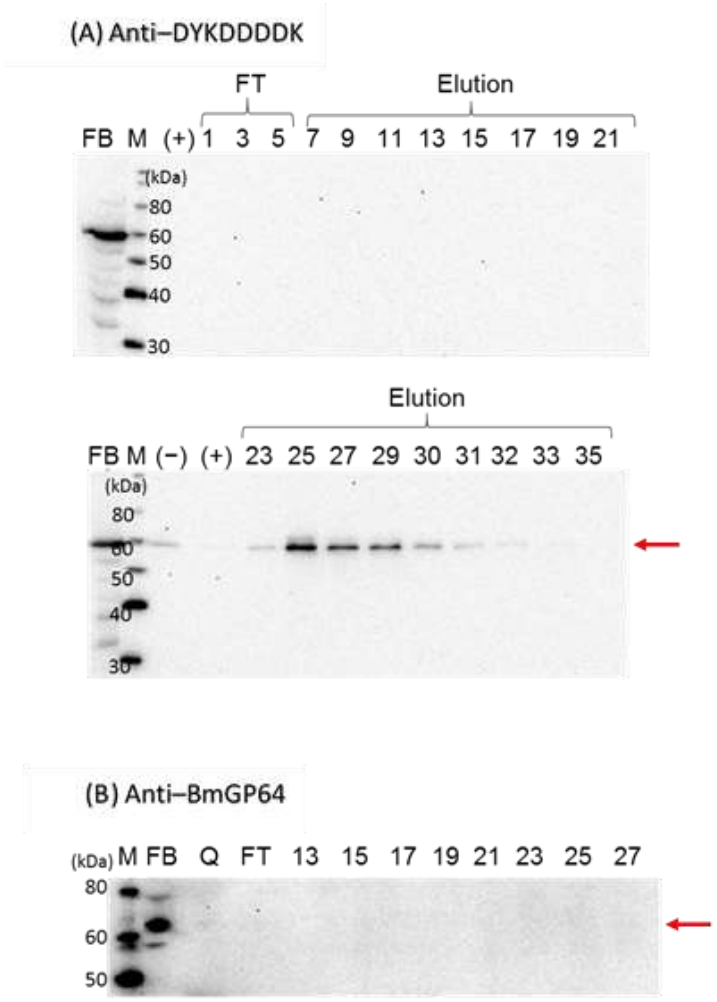


Supplement Figure S8 Reproducibility of CHT column chromatography using standard column (Ø 4.6 x 35 mm). Elution gradient was 150 to 600 mmol/l sodium phosphate (pH 7.2)and the experiment was 3 times repeated with loading amounts of 5 (42 ml), 6 (53 ml), and 5.8 (51 ml) mg/column. Flow rate was 1 ml/min and the linear gradient was done within 26 ml.

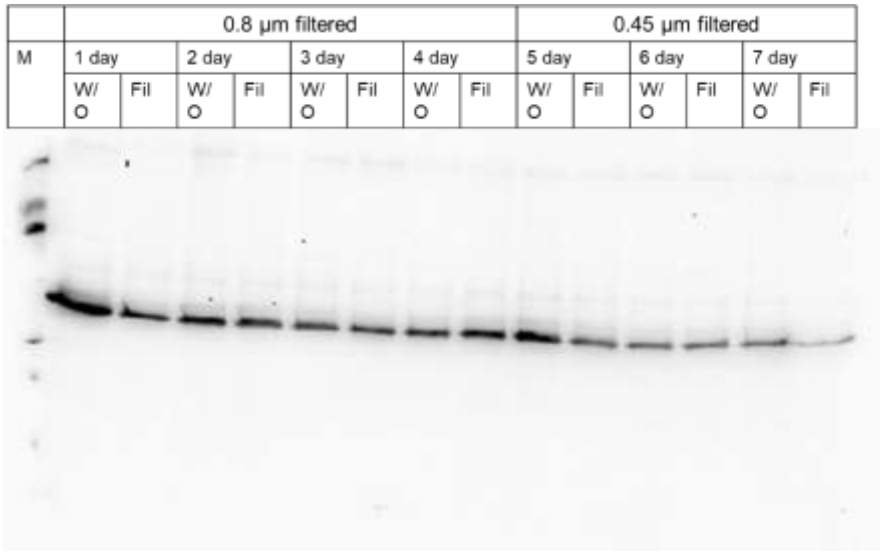
Blue lines: absorbance at 280 nm; Green lines: absorbance at 260 nm; Dotted lines: conductivity (mS/cm); Purple lines: DNA concentration (ng/ml); Pink areas: elution position of HPV L1 confirmed by western blotting. Flow through and elution fractions are 1–15 or 16 and 16 or 17–45 or 47, respectively. Whereupon the flow through fractions consist of 5 ml and the elution fractions of 1 ml.



Supplement Figure S9 Western blot analysis of each fraction in the large-scale CHT column chromatography. (A) Detection of HPV L1 protein by western blot. The L1 protein was detected using an anti-DYKDDDDK tag monoclonal antibody as a primary antibody. (B) Detection of BmGP64 in each fraction by western blot. BmGP64 was detected using anti-BmGP64 antibody as the primary antibody. M: Magic Mark XP; P: Q sample after column purification pool sample. Red arrows in (A) and (B) indicate position of the HPV L1 and BmGP64, respectively.



Supplement Figure S10 Fraction analysis in heparin column chromatography. (A) Detection of HPV L1 protein by western blot. Anti-DYKDDDDK tag monoclonal antibody was used as the primary antibody. (B) Detection of BmGP64 by western blot. Anti-BmGP64 antibody was used as the primary antibody. M: Magic Mark XP; FB: HPV 6b L1 expressing fat body (× 10); Q: Large Q pool; (+): CHT Fr.4-17 pool + 0.45 μm filter; (-): CHT Fr.4-17 pool; FT: Flow through; 1 to 35: Fr. No. Red arrows in (A) and (B) indicate position of the HPV L1 and BmGP64, respectively.



Supplement Figure S11 Influence of freezing/thawing and filtration on the stability/amount of HPV L1 VLP shown with Western Blot. An original unprocessed fat body sample was several days in a row thawed, filtered and frozen again. Each time two samples were taken for analysis, one after thawing and one after filtration before freezing again. The first four days a 0.8 µm filter was used and for the last three days a 0.45 µm filter was used. Both filter show the reduction of the HPV L1 protein. The decreasing is with the 0.45 µm filter even stronger with the 0.8 µm filter. For the gel 1:10 dilution of the samples were mixed 1:1 with SDS-buffer and 15 µl was loaded on each lane. M: YesBlot Western Marker I; W/O: sample after thawing/without filtration; Fil: filtrated sample.

Supplement Table ST1 Influence of freezing/thawing and filtration on the stability/amount of HPV L1 VLP as shown in Supplement Fig. S11.

	0.8 µm filtered								**0.45 µm filtered**					
	1 day		2 day		3 day		4 day		5 day		6 day		7 day	
	W/O	Filtered	W/O	Filtered	W/O	Filtered	W/O	Filtered	W/O	Filtered	W/O	Filtered	W/O	Filtered
Average area	286.5	102	117.5	111.5	87.5	87	84.5	100	125	90.5	69	57	77	32.5
Average area [%]	20.07	7.15	8.23	7.81	6.13	6.09	5.92	7.01	8.76	6.34	4.83	3.99	5.39	2.28
Intensity Order of Area	1	5	3	4	8	9	10	6	2	7	12	13	11	14
Degradation [%] (1 is 100 %)	0.00	64.40	58.99	61.08	69.46	69.63	70.51	65.10	56.37	68.41	75.92	80.10	73.12	88.66
Degradation because of freezing [%]	0.00	///	-15.20	///	21.52	///	2.87	///	-25.00	///	23.76	///	-35.09	///
Degradation because of filtering [%]	///	64.40	///	5.11	///	0.57	///	-18.34	///	27.60	///	17.39	///	57.79

This table shows the degradation of HPV L1 VLPs. The data was obtained using densitometry. The program ImageJ was used to calculate the area of the HPV L1 bands obtained from the Western Blot. The densitometry was done twice with the same base membrane and the Table shows the average values. The trend of HPV L1 loss is clear, even if there is a high variability in the degradation through freezing. On the other hand, the degradation because of filtration is clearly shown. The intensity order shows the descending order of the calculated area size. W/O: sample after thawing/without filtration.