Adenovirus dodecahedron, a VLP, can be purified by size exclusion chromatography instead of time-consuming sucrose density gradient centrifugation

List of abbreviations: Ad, adenovirus; Ad3, adenovirus serotype 3; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; Dd, dodecahedron; EM, electron microscopy; FBS, fetal bovine serum; FT, flow-through; HRP, horseradish peroxidase; IEC, ion exchange chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; MW, molecular weight; Pb, penton base protein; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine, VLP, virus-like particle; WB, Western blot.

Abstract

 Adenoviral dodecahedron (Dd) is a virus-like particle (VLP) composed of twelve pentameric penton base (Pb) proteins, responsible for adenovirus cell penetration. It is generated spontaneously in the baculovirus system upon expression of the Pb gene of adenovirus serotype 3. This particle shows remarkable cell penetration ability with 200 000 to 300 000 Dd internalized into one cell in culture, conceivably delivering several millions of foreign cargo molecules to the target cell. We have used it in the past for delivery of small drugs as well as a vaccination platform, in which Dd serves as a particulate vaccine delivery system. Since development of new biomedicals depends strongly on the cost of their expression and purification, we attempted, albeit unsuccessfully, to obtain Dd expression in bacteria. We therefore retained its expression in the baculovirus/insect cells system but introduced significant improvements in the protocols for Dd expression and purification, leading to considerable savings in time and improved yield.

Introduction

Adenoviral dodecahedron (Dd) is a virus-like particle (VLP), composed of twelve copies of the pentameric penton base (Pb) protein that is responsible for adenovirus endocytosis [***1***]. It is generated spontaneously in the baculovirus system upon expression of the Pb gene of adenovirus serotype 3 (Ad3) [***2***]. This particle shows remarkable cell penetration ability with 200 000 to 300 000 Dd internalized into one cell in culture [***3, 4***]; it is thus able to carry multiple copies of large proteins such as immunoglobulins. In contrast to other VLPs that are held together by calcium ions or disulfide bonds, this smaller-than-the-virus of origin 28-Å symmetrical particle is stabilized by the N-terminal strand swapping between neighboring penton bases, which creates a sort of a steadying net [***5***]. Dd, a multivalent and biocompatible vector, can be engineered to deliver several millions of foreign cargo molecules to a single target cell. We used it for delivery of small drugs [***6, 7***] as well as a vaccination platform for delivery of a particulate vaccine [***8***]. Since development of new biomedicals depends strongly on the costs of their expression and purification, we undertook some improvements in the protocols of Dd expression and purification. In particular, we succeeded in elimination of the time-consuming, costly, poorly resolving and difficult to scale up sucrose density gradient ultracentrifugation step. This resulted in shortening the overall protocol and improving the VLP yield.

Materials and Methods

 Expression in *E. coli*. The gene encoding the Ad3 penton base protein was initially inserted into the pETDuet-1 vector between the EcoRI and HindIII sites under control of T7 promoter. In a second attempt, in order to obtain an untagged protein the gene encoding Ad3 Pb was cloned into the pET28 vector between the NcoI and HindIII sites under control of T7 promoter. The recombinant plasmids bearing either the His-tagged Pb gene (first attempt) or the untagged Pb gene were obtained after transformation of *E. coli* DH5α. The proper DNA sequence was confirmed by sequencing using the following primers:

vector specific primer

pET-Up – 5'- ATG CGT CCG GCG TAG AGG ATC-3'

T7-term – 5'- GCT AGT TAT TGC TCA GCG G -3'

and insert specific primer

pForbase – 5'- ACA TGT TCA GCA ACA AGT T-3'

For expression of the his-tagged protein, *E. coli* RosettaTM DE3 (Novagen) cells were transformed with pETDuet-1 containing the Pb gene insert and then selected on LB-agar plates containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Protein production was induced in LB medium by adding IPTG (0.4 or 1 mM) when the optical density (OD600) of the culture reached 0.5 (~3 h). The culture growth was continued overnight at 37oC. 100 μl portions of cultures were collected before induction and at different times thereafter. Bacteria were collected by centrifugation at 14 000 rpm for 10 min at 4oC and stored at -20oC until further processing. Expression of untagged Pb protein was carried out in Rosetta™ 2(DE3)pLysS and Rosetta-gami™ 2(DE3)pLysS strains (Novagen) under conditions described above, except that kanamycin (50 µg/ml) was used for selection of positive transformants.

 Protein analysis by Western blot. Cell pellets (from 100 μl of culture) were suspended in 20 μl of 3x Laemmli sample buffer and heated for 10 min at 100oC. 10 μl of each sample was loaded on a polyacrylamide gel and subjected to electrophoresis. Proteins were subsequently electrotransferred onto Immobilon P membrane, treated with rabbit anti-Dd (made in laboratory, and used at 1:40 000), followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Sigma, 1:100 000). Pierce ECL Western Blotting Substrate was used for detection of HRP on the immunoblots, which were then exposed to X-ray film and developed. The membranes were stained with Coomassie Brilliant Blue (CBB). Molecular weight markers of Fermentas (cat. no. 0671) were used throughout this work.

Ni-NTA column purification. The pellet from 50 ml of bacterial culture expressing the his-tagged Pb protein was suspended in 10 ml of 50 mM NaH2PO4 buffer, pH 8, containing 500 mM NaCl and 10 mg lysozyme. The mixture was incubated on ice for 30 min and then sonicated on ice with 10 s burst at high intensity with 10 s cooling period between each burst, using a sonicator equipped with a microtip. The lysate was centrifuged at 14 000 rpm for 15 min at 4oC. To the Ni-NTA agarose (1.5 ml) equilibrated with 2 x 6 ml lysis buffer, 10 ml lysate was added and incubated for 90 min at 4oC with shaking. Flow-through (FT) fraction was discarded by centrifugation at 800 g for 1 min at 4oC. The resin was subsequently washed five times with 10 ml portions of 50 mM NaH2PO4 at pH 8, containing 20 mM imidazole and 500 mM NaCl, and the protein was eluted with three 1 ml fractions of 250 mM imidazole in the same buffer. 10-μl samples were collected throughout the procedure for SDS-PAGE analysis.

Analysis on native agarose gels. Agarose gels (0.8%) were prepared according to Gallegos et al. [***9***]. Fractions of 1 ml obtained by elution from the Ni-NTA column were concentrated to final volume of 100 μl using Microcon centrifugal filter with molecular weight cut-off of 30 kDa (Millipore). Aliquots of 30 μl were mixed with 10 μl of loading buffer without SDS. Dd expressed in baculovirus expression system was used as a control. After electrophoresis the proteins were transferred onto Immobilon P membrane and analyzed by Western blotting.

Expression in the baculovirus system. For Dd production in eukaryotic cells two commercial Baculovirus Expression Systems Bac-to-Bac (Life Technologies) and FlashBacGold (Oxford Expression Technologies) were used, following the manufacturer’s protocols. A full-length Pb gene was cloned under the control of the polyhedrin promoter, between NspV and HindIII restriction sites in pFastBacDual and between HindIII and BamHI sites in pOET1 transfer vectors compatible with Bac-to-Bac and FlashBacGold expression systems, respectively.

In the Bac-to-Bac system, the pFastBacDual plasmid carrying the Pb gene was used for production of recombinant baculovirus upon specific transposition in DH10Bac™ *E. coli* cells. Baculovirus DNA was then used for transfection into Spodoptera frugiperda (Sf21) cells. In the FlashBACGold system, the recombinant baculovirus was obtained directly in Sf 21 insect cells by co-transfection with the pOET1 plasmid containing the Pb gene and FlashBacGold virus DNA. Virus stocks for expression of Dd in both systems were prepared by infection of Sf21 monolayers with supernatants obtained after transfection of insect cells. For protein expression *Trichoplusia ni* (High-Five, HF) cells (2x106 cells/ml) grown in suspension were infected with the recombinant baculoviruses at multiplicity of infection (MOI) 4 and cultured for 48 h at 27oC.

Dd stability assay.Dd samples of 10 l, obtained either only by fractionation on a sucrose density gradient or by fractionation followed by anion exchange chromatography, were dialyzed separately against 50 mM Tris or 20 mM Hepes, both of pH 7.5, with 2 mM EDTA and 150 mM NaCl, with or without 5% glycerol. Subsequently, the samples were stored at 4oC and controlled weekly for protein stability by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue.

Dd purification.Expressing cells lysate obtained by 3 cycles of thawing-freezing was dialyzed against 50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, clarified by centrifugation for 30 min at 30 000 rpm at 4oC and applied onto size exclusion chromatography (SEC) column XK 26 (GE Healthcare) packed with WorkBeads™40/10000 (Bio-Works), equilibrated and developed with the dialysis buffer. The column bed volume was 130 ml and the exclusion limit was 10 000 000 Da. 15 μl aliquots of 1 ml fractions were subjected to SDS-PAGE analysis. Fractions containing Dd were pooled and applied onto 5 ml anion exchange column (Bio-ScaleTM Mini Macro-Prep® High Q Centridge of Bio-Rad) previously equilibrated with the dialysis buffer containing 150 mM NaCl. Proteins were eluted with a linear NaCl gradient (0.15 - 1 M). During whole purification procedure buffers without glycerol were used, as glycerol did not influence Dd stability. Fractions of 900 l were collected and 15 μl aliquots were analyzed by SDS-PAGE, whereas 30 μl aliquots were analyzed by native agarose gel electrophoresis [***9***]. Dd sample of known concentration was used as a control. Protein bands were stained with CBB.

Protein and DNA content.Protein concentration was determined with Bradford assay. Alternatively, protein content within gel bands was estimated by comparison with bands containing various amounts of bovine serum albumin (BSA). DNA concentration was measured using NanoDrop 3300 Fluorospectrometer (Thermo Scientific). The DNA content in Dd samples was visualized by staining the gels with ethidium bromide prior to their staining with CBB.

Electron microscopy.Dd samples were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% sodium silicotungstate, pH 7.0. Micrographs were taken under low-dose conditions with a Jeol 1200 EX II microscope (Tokyo, Japan) at 100 kV and a nominal magnification of 49 000.

Dd cell entry (In-Cell ELISA). HeLa cells (2x104/well) were grown overnight to approximately 80% confluency in 96-multiwell plates (Grainer). After 3 washes with PBS various amounts of Dd preparations, ranging from 1 to 8 µg per well, were applied onto cells in EMEM medium without FBS. After incubation for 2 h at 37**ºC** the cells were rinsed three times with cold PBS and permeabilized with methanol at -20**ºC** for 10 min. Cells were further incubated with **blocking buffer (PBS with 5% FBS and 1% Tween 20)** overnight **at** 4oC. Subsequently p**rimary rabbit polyclonal antibody recognizing Dd was added at 1:40 000 and incubated for 1 h at RT.** After 3 washes with PBS containing 1% Tween 20, anti-rabbit HRP-conjugated antibody (Sigma) was added at 1:20 000 for 1 h at RT. Three PBS/1% Tween 20 washes were then performed and the peroxidase activity was revealed with TMB (Thermo Scientific). The reaction was stopped with 100 µl 2 M sulfuric acid and the absorbance was measured at 450 nm in Synergy HTi plate reader (Biotek). The mean of triplicate controls (cells in the absence of Dd) was subtracted from the mean of the triplicate readings for each point tested.

**Results**

 **Expression in bacteria.** We attempted to produce Dd in *E.coli* RosettaTM DE3 cells. A 65 kDa band corresponding to the combined molecular masses of 6xHis and Pb as well as several bands of lower molecular weight, were revealed by Western blot with polyclonal antiserum against Dd, which suggests that the Pb produced in this system might be unstable (Fig. 1A, lower panel). Lowering growth temperature from 37 to 20oC resulted in diminished Pb production and significantly increased the amount of shorter, anti-Dd responding bands (results not shown). The recombinant protein was mostly soluble and could be purified on a nickel column albeit in small amounts (Fig. 1A); the 65 kDa band was eluted in fraction E1, together with some additional proteins staining with CBB (Fig. 1A, lanes E1).

Native agarose gels permit visualization of dodecahedric VLPs formed of twelve pentameric Pbs [***6***]. The His-Pb expressed in bacteria and eluted from the nickel column was analyzed on 0.8% agarose gel under native conditions. Western blot analysis revealed that the recombinant protein migrated considerably slower than the control Dd expressed in the baculovirus expression system (Fig. 1B, right panel, compare lanes C (control Dd) and E1). The recombinant protein mobility is characteristic of free penton bases [***6***], which implied that the Pb produced in Rosetta and organized into free pentameric penton bases is not able to assemble into complete, symmetric dodecahedric virus-like particles.

 Our experiments with the N-terminal mutants of Dd expressed in the baculovirus system revealed that the Dd assembly process does not support elongation of Pb N-ends since it resulted in production of only free pentameric Pbs (unpublished data). As the N-terminally His-tagged protein had an extended N-terminus, this might have led to lack of VLP formation. Therefore, the Pb gene was recloned into the pET28 vector and expression of untagged Pb protein was undertaken in Rosetta strains that were designed to enhance the expression of eukaryotic proteins with rare codons. A 62 kDa band corresponding to the molecular mass of Ad3 Pb protein was barely detected on Western blots (Fig. 1C, right panel). Predominantly low molecular weight material reacting with anti-Pb antibody was observed and the kinetics of Pb expression suggested protein instability during expression under these conditions. Of note, even the production of these shorter Pb fragments was decreasing during expression longer than 4 h.

 **Expression in the baculovirus system with Bac-to-Bac or FlashBacGold systems.** These two baculovirus expression systems are both based on infection of insect cells with the recombinant baculovirus carrying the gene of a heterologous protein, which triggers gene expression. However, while in Bac-to-Bac system the formation of a recombinant baculovirus is carried out in bacterial cells, the FlashBacGold system allows recombinant baculovirus formation by recombination directly in insect cells, which shortens the procedure. Importantly, the FlashBacGold system offers plasmids with two baculovirus genes of enzymes protease (cathepsin) and chitinase removed [***10***], which should enhance production of the recombinant protein and improve its stability.

 It appears that the yield of Dd expression was slightly higher for the FlashBacGold system (Fig. 2A). In addition, Dd obtained in the FlashBacGold system and purified on sucrose density gradient followed by ion-exchange chromatography (IEC) was proteolytically somewhat more stable than that obtained in the Bac-to-Bac system (Fig. 2B, note the absence of shorter proteins in FlashBacGold fractions). Furthermore, the analysis on native agarose gels suggested that Dd obtained in the Bac-to-Bac system was less uniform (Fig. 2C). Therefore, we have chosen the FlashBacGold system for further expression of Dd. Moreover, it seems that the presence of glycerol is dispensable as Dd was stable also in the absence of 5% glycerol. Interestingly, Dd expressed in the FlashBacGold system and purified was not proteolyzed for at least 7 weeks upon storage at 4oC; only samples fractionated by sucrose density gradient (Gr) showed proteolysis with time of storage (Fig. 2D).

**Dd purification.** Dd was routinely purified in our laboratory by a simple two-step procedure [***6***]. However, we wished to eliminate the sucrose density gradient step, which provides rather low-resolution, and is tedious and difficult to implement for large Dd batches. With this aim we used the CIMmultusTM QA monolithic column (BiaSeparations, Slovenia), suitable for separation of large molecules [***11***]. Indeed, this column separated Dd from the pentameric Pb and the rest of the cell extract components well. However, we were unable to regenerate the column despite using two such columns purchased at the interval of several months. Therefore, we were obliged to forego the use of monolithic column. Instead, we implemented a new two-step purification protocol with the first step being size exclusion chromatography (SEC), whereby Dd together with large cell extract components were recovered in the first elution peak (Fig. 3A). SEC proved to be very effective as the yield of Dd obtained by gel filtration purification was significantly higher (93%) than that obtained by sucrose density gradient centrifugation (~55%, Fig. 3D). In the second step, fractions containing Dd were pooled and resolved on an anion exchange column. The results of gel analysis confirmed that samples purified in this way are homogenous (Fig. 3B) and free of contamination by nucleic acids. Importantly, in comparison to the old protocol, the time required for the entire 2-step procedure was reduced from almost 3 days to 4-5 hours. When a 3-fold larger amount of Dd expressing cells was used, a similar yield of Dd per cell was obtained, which showed that this procedure is suitable for larger scale production of VLPs.

Samples of Dd expressed in the FlashBacGold expression system, those purified by sucrose density gradient followed by anion exchange chromatography and those purified by SEC and anion exchange chromatography were analyzed by negative stain electron microscopy. The analysis confirmed similar structure of dodecahedric particles and additionally showed their stability at 4oC within two months after Dd purification (Fig. 3C). Finally, the In-Cell ELISA analysis showed no visible difference in cell entry of Dd purified with the old procedure and the new one based on SEC and IEC (Fig. 3E).

Discussion

We have already demonstrated successful application of adenovirus dodecahedron as a vector for delivery of small molecule anticancer agents and as a vaccination platform for foreign antigens [***6, 7, 12, 8***]. For further preclinical studies and possible future therapeutic uses, a large amount of homogeneous VLP material is needed. This prompted us to undertake optimization of VLP production and elaborate a scalable purification procedure.

Initially we tested the possibility of Dd production in a bacterial system since some VLPs can be formed upon heterologous expression in bacteria. For example, the filamentous nanoparticles derived from papaya mosaic virus capsid protein (PapMV CP) produced in bacteria were proposed as a vaccination platform [***13***]. Also polyoma VLPs can be obtained *in vitro* upon expression of the coat protein VP1 alone or together with minor capsid proteins [***14***]. Similarly, the capsid protein of the hepatitis E virus (p239) that was expressed in *E. coli* in the form of inclusion bodies could be renatured from urea, forming VLPs [***15***]. However, according to the authors the building blocks could not be associated into tight and orderly VLPs, thus making the p239 VLP unsuitable for high-resolution structural studies. Moreover, it seems that in numerous cases of prokaryotic expression, VLPs formation does not occur inside bacteria but rather during the purification/renaturation process, sometimes with changes in pH, ionic strength or sequence [***16, 17***]. In contrast, upon expression in eukaryotic systems, such as insect cells or yeast, polyoma VP1 is obtained only as an icosahedral VLP [***18***].

From our structural and biochemical studies we know that Dd does not contain post-translational modifications or disulfide bonds [***5***], so the known disadvantages of bacterial expressing systems were of no importance for eventual Dd expression. We expressed in bacteria two variants of Pb, the untagged one and one as a fusion protein with 6 histidines at the N-terminus. A full-length soluble Pb was obtained upon expression of His-tagged protein but with rather low yield. The majority of protein recognized by anti-Dd serum was in the form of low molecular weight bands, suggesting low protein stability. Importantly, His-Pb purified from bacteria was in the form of free pentameric penton bases that did not assemble into dodecahedra.

During the replication cycle of Ad serotype 3 native virus-like particles called dodecahedra-fibers are produced from penton base and fiber proteins in Ad3 infected mammalian cells [***19***]. Recombinant dodecahedra, with or without the fiber protein (dodecahedra-base), could be produced in baculovirus expression system/insect cells [***2***], which showed that no viral scaffolding proteins are required for penton base assembly into Dd. In such a scenario the lack of Dd production in *E. coli* cells could conceivably be explained by the fact that bacterial chaperones are functionally unable to replace the eukaryotic ones.

Since the bacterial system could not be used for Dd expression, we attempted to optimize VLP production in insect cells. For this purpose we compared two commercial baculovirus systems, Bac-to-Bac and FlashBacGold. The quality of Dd preparations generated in both systems was comparable. At the end, we have chosen the FlashBacGold system for further use because of its simplicity and faster generation of the recombinant baculovirus. Moreover, a 3-months storage in a refrigerator brought no visible Dd proteolysis, and negative stain electron microscopy showed a homogenous Dd preparation with only a few free pentameric penton bases.

In conclusion, we improved Dd purification significantly. Although our earlier two-step purification protocol consisting of sucrose density gradient fractionation followed by anion exchange chromatography was quite simple, it required much time and was rather costly. With the new procedure of size exclusion chromatography as the initial step in VLP purification [***20***], it was possible to purify Dd from crude cell lysate in one instead of 3-4 days. We also showed that this protocol is suitable for scaling up, as a threefold larger amount of expressing cells yielded the usual amount of Dd per cell. Our analysis confirmed that replacing sucrose gradient fractionation with SEC did not affect Dd structure, stability or cell penetration capability.

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Figure legends:

Fig. 1. Pb expression in Rosetta. (A) His-Pb purification on Ni-NTA column. Samples of 10 μl were applied. Upper panel - membrane after protein transfer stained with CBB. Lower panel - Western blot analysis with anti-Dd Ab. MW – molecular weight markers, NI – non-induced, Lys – lysate, Sol – fraction soluble after sonication, Insol – pellet after sonication, FT - flow through, W - wash, E1, E2 – eluted fractions. (B) Native agarose gel analysis of His-Pb expressed in bacteria. Left panel – CBB-stained transfer membrane, right panel - Western blot. C – control Dd expressed in the baculovirus system; E1, E2 – fractions eluted from the Ni-NTA column with 250 mM imidazole and concentrated ~ 10 fold on Microcon, applied in 30 μl each. (C) Untagged Pb protein expression in Rosetta™ 2(DE3)pLysS or in Rosetta-gami™ 2(DE3)pLysS. Equivalent of 50 μl portions of culture collected at indicated periods were resolved by SDS-PAGE and analyzed by Western blot with anti-Dd serum. MW – molecular weight markers, Dd - control Dd expressed in the baculovirus system, NI - non-induced.

Fig. 2. Comparison of Dd expression in Bac-to-Bac and FlashBacGold baculovirus expression systems. (A) Sucrose density gradient purification. Samples of 15 μl from 1 ml sucrose density gradient fractions were resolved on SDS-PAGE and stained with CBB. (B) Fractions obtained after two-step purification (sucrose density gradient, dialysis and Q-Sepharose, 15 μl from 0.9 ml fractions) were run on SDS-PAGE and stained with CBB. (C) Native agarose analysis. 30 μl from the 0.9 ml fractions purified by two-step protocol were resolved on native agarose gels and stained with CBB. (D) Proteolysis of Dd expressed in FlashBacGold system during storage at 4oC. Dd samples after either one-step (Gr) or two-step purification (Q), were dialyzed against either 50 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl with or without 5% glycerol, or 20 mM Hepes pH 7.5, 2 mM EDTA, 150mM NaCl with or without 5% glycerol, 10 l aliquots were resolved on SDS-PAGE and stained with CBB. The stability of dialyzed Dd samples was evaluated one day after dialysis (upper panel) and seven weeks later (lower panel). MW - molecular weight markers.

Fig. 3. Novel two-step Dd purification procedure with gel filtration (SEC) followed by anion exchange chromatography. (A) SEC profile, with inset showing SDS-PAGE analysis of the E1 peak (samples of 15 μl from 1 ml gel filtration fractions). (B) Dd purified by SEC and anion exchange chromatography. Samples of 15 μl from 0.9 ml Q-column fractions were resolved on SDS-PAGE and stained with CBB (upper panel). SEC and Q-column purified Dd samples (30 μl from 0.9 ml fractions) were electrophoresed on native agarose gel and stained with CBB (lower panel). MW - molecular weight markers, CCL - crude cellular lysate (diluted tenfold) Dd – control VLP, SEC – fraction obtained after gel filtration. (C) Negative stain EM images of Dd expressed in the FlashBacGold system, purified by the old method (upper panel, 0.9 mg/ml) and the new method (lower panel, 0.15 mg/ml); scale bar corresponds to 100 nm. (D) Yield differences in the two methods of Dd purification. (E) Dd cell entry, analyzed using the In-Cell ELISA assay. Increasing amounts of Dd were applied on HeLa cells and allowed to penetrate for 2 h at 37**ºC**.

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