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Purification of recombinant rotavirus VP7 glycoprotein for the study of *in vitro* rotavirus-like particles assembly

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ABSTRACT

Rotavirus VP7 is a glycoprotein that forms the viral capsid outerlayer and is essential to the correct assembly of triple-layered rotavirus-like particles (RLPs). In this work, a novel purification strategy was designed to allow obtaining highly pure monomeric VP7 required for the RLPs *in vitro* assembly. VP7 production kinetics in baculovirus–insect cells at cell concentration at infection (CCI) of 1×10^6 cells mL^{-1} was compared in terms of VP7/glycoprotein 64 (gp64) ratio at different multiplicity of infection (MOI). The best productivity was achieved at MOI of 0.1 plaque forming unit (pfu) $cell^{-1}$ and time of harvest of 80 h post-infection. After preliminary clarification steps, the proteins eluted from Concanavalin A were concentrated and loaded onto size exclusion chromatography. The polishing step was anion exchange chromatography with Mono Q. The high resolution of this column resulted in separation of monomers from dimers of VP7. Overall, the purification protocol yielded high level of purity (>90%). Purified VP7 was characterized by MALDI-TOF mass spectrometry and SDS-capillary gel electrophoresis. The M_W and apparent M_W were determined as 31.6 and 39 kDa, respectively, confirming the efficacy of the proposed purification strategy that now enables RLPs assembly studies.

1. Introduction

Rotavirus, the most common cause of severe dehydrating diarrhoea in children worldwide [1], is a non-enveloped icosahedral virus composed of three layers. The core contains viral protein 1 (VP1), VP2, VP3 and viral RNA; the second layer is formed by VP6, and the third layer is formed by VP7 and spikes of VP4 (for a review, see Ref. [2]).

VP7 is a highly immunogenic glycoprotein grouped into 260 trimers per virus. The recognition that this neutralization antigen is a structural glycoprotein has stimulated numerous studies of the structure, biosynthesis, and functions of VP7. Biochemical analyses determined that it contains only *N*-linked high-mannose oligosaccharide residues, which are added co-translationally as this protein is inserted into the membrane of the endoplasmic reticulum [3]. From the amino acid sequence, its molecular weight (M_W) is 37.4 kDa whereas the apparent molecular weight ($M_{W_{app}}$) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is 34 kDa due to signal peptide cleavage [2,3]. The presence of Ca^{2+} binding site explains the VP7 dependence on this ion to form trimers [4]. Moreover, it is known that chelating agents, such as EDTA, promote VP7 detachment from the capsid, which leads to the loss of viral infectivity [5–7]. However decapsidation may be overcome by the *in vitro* recoating of native rotavirus double-layered particles (DLP) with recombinant VP7 and/or VP4 with an increase of infectivity by a factor of 10^7 [8].

Rotavirus-like particle 2/6/7 (RLP 2/6/7), a candidate vaccine against rotavirus infection, is composed by the main rotavirus structural proteins (VP2, VP6 and VP7) and can be produced using the insect cell–baculovirus expression vector system (IC–BEVS) [9,10]. The main drawback in the RLP production process is the assembly of the three VPs into stoichiometrically correct intact RLPs. In fact, less than 20% of the VPs produced assemble correctly into RLP due to either incorrect stoichiometric ratios or to inadequate thermodynamic aggregation conditions [9,11–14]. A recent approach to overcome incorrect assembly and heterogeneous virus-like particles (VLPs) formation is to disassemble and reassemble VLPs *in vitro* [15], which requires the availability of purified VLPs and/or each one of their structural recombinant VPs. Accordingly, it is crucial to

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develope efficient methods to produce and purify VP7 in order to study and later produce the *in vitro* assembly of RLP 2/6/7.

Rotavirus VP7 has been produced in several recombinant systems: *Escherichia coli* [16], insect cells [4,9,17] and other eukaryotic systems such as *Dictyostelium discoideum* [18] and stably transfected COS 7 cells [19]. However, to our knowledge, there are only two reports that address VP7 purification, both of which describing a method not suitable to be used for the *in vitro* assembly of triple-layered RLP 2/6/7 and also for future upscaling. In the first case, the authors expressed VP7 as a fusion protein with maltose binding protein (MBP) and then purified it by affinity chromatography with amylose resin [16]. In the second report, the authors accomplished pure VP7 by performing two chromatographic steps: immunoaffinity followed by Concanavalin A [4]. The main drawback in this purification scheme relies on the requirement of high amount of antibodies to be immobilized into chromatographic beads. Moreover, with immunoaffinity, all forms of VP7 (monomeric, dimeric and trimeric) are recovered.

Therefore, in this work, we propose a new purification method for recombinant rotavirus VP7 produced in insect cells infected with recombinant baculovirus. After three chromatographic steps we were able to achieve monomeric VP7 with enhanced purity. The most challenging issue in the purification was the separation of VP7 from the baculovirus glycoproteins gp64 and vp39 due to their structural and biochemical resemblance. The analysis of purified VP7 by sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) allowed the final product characterization and M_W determination.

2. Experimental

2.1. Cells and baculovirus

VP7 was produced in *Spodoptera frugiperda* Sf-9 cell line obtained from the European Collection of Cell Cultures (ECACC no. 89070101, Salisbury, UK) using BEVS (Baculovirus Expression Vector System). The baculovirus, containing bovine RF rotaviral *vp7* gene, was kindly provided by Dr. Didier Poncet from Centre National de la Recherche Scientifique (Institut National Recherche Agronomique (INRA), Gif-sur-Yvette, France). The recombinant baculovirus was obtained by co-transfection of Sf-9 cells with recombinant plasmid pVL941 (containing *vp7* gene) and wild-type baculovirus DNA [17]. The cells were routinely cultured in 500-mL shake flasks (Corning Inc., Lowell, MA, USA) using a working volume of 50 mL in Sf-900 II SFM™ medium (Invitrogen, Paisley, UK). The baculovirus was amplified in 250-mL spinner flasks (Wheaton, Millville, NJ, USA) and titrated using a viability-based method [20].

2.2. Production of recombinant rotavirus VP7

Studies on VP7/gp64 production kinetics were performed in 500-mL shake flasks (Corning Inc.). All subsequent VP7 production for purification studies was carried out in 2-L fully controlled stirred tank bioreactor (B. Braun, Melsungen, Germany). The bioreactor was equipped with two Rushton turbines (standard geometry); *p*O2 was kept at 30% by agitation rate (70–250 rpm) and sparged aeration rate 0.03 vvm, conditions that were previously optimized in our group for the growth of Sf-9 cells [11].

Sf-9 cells were infected with a multiplicity of infection (MOI) of 5 pfu cell^{-1} at a cell concentration at infection (CCI) of 1.0×10^6 cells mL^{-1}, unless otherwise stated. Approximately every 12–24 h an aliquot was collected from the shake flask to assess cell concentration, viability and VP7 and gp64 production by Western blot.

Cell concentration and viability were assessed by haemocytometer counting (Brand, Wertheim, Germany) with cell viability evaluated by 0.4% trypan blue exclusion dye (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS).

2.3. Purification of recombinant rotavirus VP7

The purification was initiated by cell culture bulk centrifugation ($2000 \times g$, 15 min, 4 °C). The supernatant was filtered through a 0.1-μm depth filter (Sartorius, Göttingen, Germany) and loaded into a XK 16/20 column (1.6 cm × 9 cm) packed with 18 mL of Concanavalin A-Sepharose (GE Healthcare, Piscataway, NJ, USA) connected to an ÄKTA Explorer System (GE Healthcare) at 1 mL min^{-1}. The column was equilibrated and washed with 0.5 M NaCl in 20 mM Tris-HCl pH 7.4 whereas elution was carried out with 0.5 M methyl-α-D-mannopyranoside (Sigma, Saint Louis, MO, USA) in equilibration buffer. Eluted fractions were concentrated with a 10-kDa molecular weight cut-off (MWCO) ultrafiltration membrane in Vivaspin 20 (Sartorius) and then either desalted with Sephadex G-25 in HiPrep 26/10 column (GE Healthcare) or loaded into Superdex 200 HiLoad 26/60 (GE Healthcare). Eluted samples from each column were loaded into a 1-mL Mono Q 5/50 column (GE Healthcare) at 1 mL min^{-1} previously equilibrated with 20 mM Tris-HCl pH 7.4. Gradient elution was performed with 1 M NaCl in equilibration buffer with 90-column volumes (CV) until 0.5 M NaCl. The purity of VP7 in each chromatographic step was evaluated by SDS-PAGE and its band confirmed by Western blot.

2.4. Gel electrophoresis and Western blot

Samples for SDS-PAGE and Western blot were diluted (1:1) in NuPAGE LDS™ sample buffer (Invitrogen) and incubated at 70 °C for 10 min. Electrophoretic separation was carried out on precast NuPAGE® Tris-Glycine gels (Invitrogen) in NuPAGE® MES SDS running buffer (Invitrogen) according to the manufacturer's instructions. The molecular weight marker was SeeBluePlus 2™ pre-stained standard proteins (Invitrogen). The gels were either stained with Coomassie SimplyBlue™ SafeStain (Invitrogen) or transferred onto nitrocellulose membranes. Densitometry analysis (ImageQuant® version 5.0, Molecular Devices, Sunnyvale, CA, USA) was performed in the gels and membranes to estimate VP7 purity and concentration, respectively.

Immunochemical staining for detection of VP7 was carried out with a goat polyclonal anti-rotavirus antibody (Abcam, Cambridge, UK). Blots were developed after incubation with an alkaline phosphatase conjugated anti-goat IgG antibody (Sigma) using 1-stepTM NBT/BCIP blotting detection reagent (Pierce, Rockford, IL, USA). In order to confirm the contamination by gp64 and to calculate VP7/gp64 ratio, the membranes were also incubated with a monoclonal anti-gp64 antibody (eBioscience, San Diego, CA, USA) followed by an alkaline phosphatase conjugated anti-mouse IgG antibody (Sigma).

For native electrophoresis analysis samples were mixed with NativePAGE™ sample buffer (Invitrogen) and directly loaded into NativePAGE Novex® 3–12% gels (Invitrogen). Apart from the running buffer that was special for native electrophoresis (Novex® Tris-Glycine Native Running Buffer, Invitrogen) the procedure followed and material was the same as described above for the SDS-PAGE.

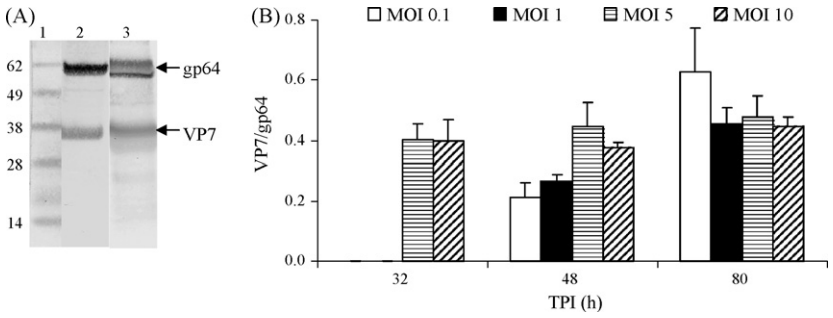

Fig. 1. Analysis of preliminary purification results by Western blot for assessment of contaminants: (1) molecular weight marker; (2) cell culture supernatant; (3) eluate from Concanavalin A. Samples were simultaneously incubated with goat anti-rotavirus and mouse anti-gp64 antibodies (A). Effect of multiplicity of infection (MOI) in VP7/gp64 ratio at different time post-infection (TPI); 32, 48 and 80 h (B). MOI tested were 0.1 pfu cell^{-1} (white), 1 pfu cell^{-1} (black), 5 pfu cell^{-1} (horizontal lines) and 10 pfu cell^{-1} (diagonal lines).

2.5. Characterization of purified recombinant rotavirus VP7

Besides SDS-PAGE and Western blot, mentioned above, other analytical techniques were used for further characterization of purified VP7, namely MALDI-TOF-MS and SDS-CGE.

For MALDI-TOF-MS analysis, VP7 was mixed with an equal volume of 2% (v/v) trifluoroacetic acid (TFA)/water. Desalting and concentration of the samples prior to MALDI analysis were performed using custom-made chromatographic micro-columns containing a C8 resin (EMPORE C8 disks, 3M, St. Paul, MN, USA) equilibrated with 0.1% (v/v) TFA. The retained proteins were eluted directly onto the MALDI plate using 10 mg mL^{-1} of sinapinic acid matrix prepared in 70% (v/v) acetonitrile and 0.1% (v/v) TFA.

Spectra were acquired with a PerSeptive Biosystems MALDI-TOF-MS Voyager-DE STR (Framingham, MA, USA) equipped with delayed extraction, and a 337-nm N2 laser, in linear mode using optimized laser intensity. A mass range of 20–100 kDa was acquired. External mass calibration of the spectra was performed using a standard mixture of proteins, Protein calibration Mix3 (LaserBio Labs, Sophia-Antipolis, France). The individual spectra were processed by Data Explorer 4.2 software (Applied Biosystems, Foster City, CA, USA) used for baseline correction, noise removal and peak detection.

VP7 was analysed by SDS-CGE as previously described [21]. Briefly, samples were diluted (1:1) in sample buffer from CE-SDS kit (Bio-Rad, Hercules, CA, USA) containing 5% (v/v) mercaptoethanol (Sigma) and incubated at 90 °C for 10 min. The analyses were carried out in a Beckman P/ACE MDQ capillary electrophoresis system (Fullerton, CA, USA) with diode array detection. For $M_{W_{app}}$ determination, VP7 was further mixed with a standard solution of lysozyme (from hen egg white, Fluka, Seelze, Germany) and BSA (96%, Sigma) prior to injection.

Finally, purified VP7 concentration was determined by BCA assay kit (Pierce).

3. Results and discussion

3.1. VP7 production in Sf-9 cells

The aim of this work was to obtain highly pure monomeric recombinant rotavirus *vp7* that can be used for future studies of RLP 2/6/7 *in vitro* assembly and possible crystallographic investigation for VP7 structure elucidation.

The result of a preliminary purification scheme for VP7 collected from the 2-L bioreactor at MOI of 5 pfu cell^{-1} after centrifugation and supernatant loading into Concanavalin A chromatography is shown in Fig. 1A. The presence of a major contaminant before (lane 2) and after (lane 3) the affinity chromatography led to the conclusion that it consisted of a glycoprotein with mannose residues. After analysing the M_W, it was hypothesized that this

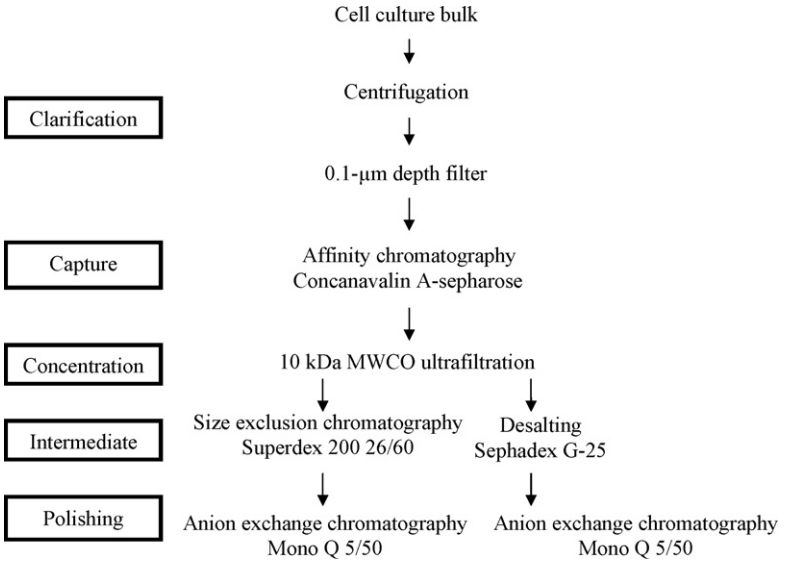

Fig. 2. Flow-chart of VP7 purification process showing two different intermediate steps: size exclusion chromatography using Superdex 200 26/60 and desalting using Sephadex G-25.

contaminant could be the baculovirus envelope glycoprotein gp64, which was then confirmed by Western blot. The identification of this contaminant led to a re-evaluation of the impact of the main upstream conditions (MOI and time of harvest, TOH) on the final VP7 purity. These conditions would not only increase relative VP7 productivity but also facilitate the purification process.

The production of recombinant proteins and VLPs with IC-BEVS has been deeply explored and reviewed [22,23], although few works discussed the impact of upstream conditions in downstream process and the final protein purity afterwards [24].

The effect of MOI of 0.1, 1, 5 and 10 pfu cell^{-1} in VP7/gp64 ratio was investigated in shake flasks (Fig. 1B). The use of a low MOI (0.1 pfu cell^{-1}) resulted in a higher VP7/gp64 ratio, which greatly contributed to simplify VP7 purification. In spite of higher TOH, previous reports preferred a low MOI strategy to increase volumetric productivity [13,24]. Apparently a low MOI, apart from increasing product yield, also prevents the production of free-gp64 trimers (unassembled baculovirus), which greatly contributes to VP7 purity.

A complex relationship exists amongst MOI, time of infection (TOI), TOH and product titre [13]. Although no extensive optimization was performed for VP7 production, the TOH should be approximately 80 hpi to reduce baculovirus and intracellular protein contaminants originated from cell lysis. From these results it was decided to use a MOI of 0.1 pfu cell^{-1} and TOH of 80 hpi to produce VP7 for the development of the purification scheme.

3.2. VP7 purification

The protocol developed herein for monomeric VP7 purification aimed at maximizing purity and therefore a purification based on extracellular VP7 was chosen in order to minimize host-cell protein contaminants (Fig. 2). Initially, when VP7 supernatant was loaded directly into Concanavalin-A column, the eluate analysis showed that VP7 was contaminated with the baculovirus glycoproteins gp64 (Fig. 1A) and vp39 (data not shown). For this reason, an additional clarification step with 0.1-μm microfiltration was added after the centrifugation in order to partially retain the baculoviruses [25]. The chromatographic capture step with Concanavalin A, like any affinity chromatography, is advantageous in terms of sample concentration and purification factor [26]. Nevertheless, all glycoproteins with mannose residues, such as gp64 and vp39, adsorbed to the affinity resin together with VP7 (Fig. 3A, lane 3) and consequently these contaminants were concentrated as well, leading to a low purity (<30%).

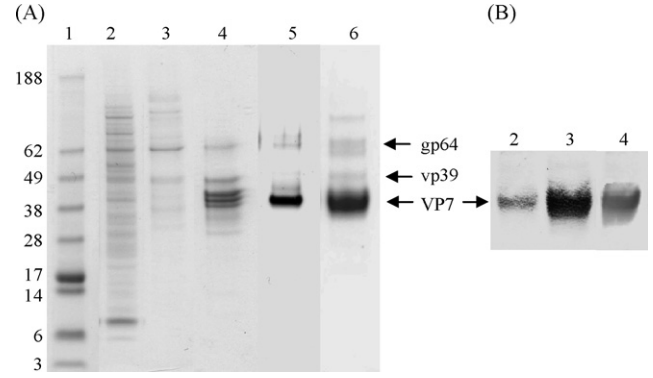

Fig. 3. SDS-PAGE (A), and Western blot (B) of samples from different steps of VP7 purification: (1) molecular weight marker; (2) sample loaded into Concanavalin A; (3) eluted sample from Concanavalin A; (4): peak 4 from Superdex 200 26/60; (5) VP7 from Mono Q (after Superdex 200); lane (6) VP7 monomer from Mono Q (after desalting).

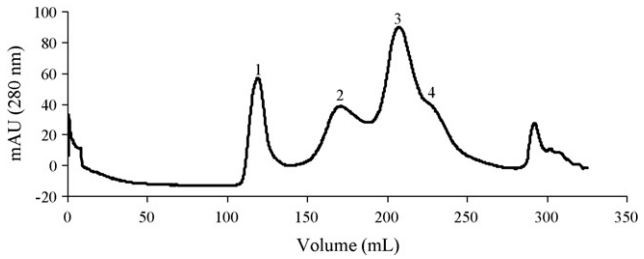

Fig. 4. Chromatogram of intermediate step of VP7 purification with Superdex 200 26/60: sample loaded was the concentrated eluate from Concanavalin A. Numbered peaks correspond to gp64 trimers (1), vp39 dimers and gp64 monomers (2), vp39 monomers and VP7 dimers (3) and VP7 monomers (4).

VP7 and gp64 have similar pIs: 3.89 (from amino acid sequence) and 3.15 [27], respectively, and tend to form dimers [18] and trimers [4,18,28]. Such similar structural and biochemical characteristics make it difficult to separate them. The high-resolution anion exchange Mono Q column, packed with monodispersed porous beads of polystyrene/divinyl benzene, was especially designed for the polishing step [29,30], although it has been used for one-step purification [31].

After elution from the affinity chromatography, and before loading into Mono Q, a buffer exchange had to be performed to eliminate the methyl-α-D-mannopyranoside and equilibrate the sample with the Mono Q buffer. At this stage, two intermediate steps were compared: size exclusion chromatography using

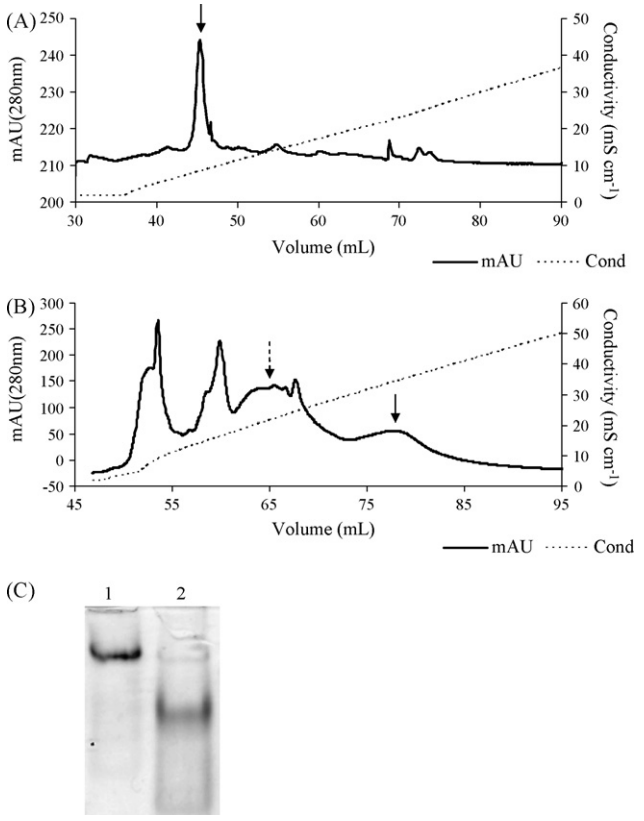

Fig. 5. Chromatogram of VP7 purification performed with a Mono Q 5/50. Loaded samples were eluate from Superdex 200 26/60 (A) and desalting using Sephadex G-25 (B). Solid and dashed arrows indicate the peaks corresponding to VP7 monomers and dimers, respectively. Native electrophoresis of VP7 monomer and dimer eluted from Mono Q (C); (1) dimer of VP7; (2) monomer of VP7.

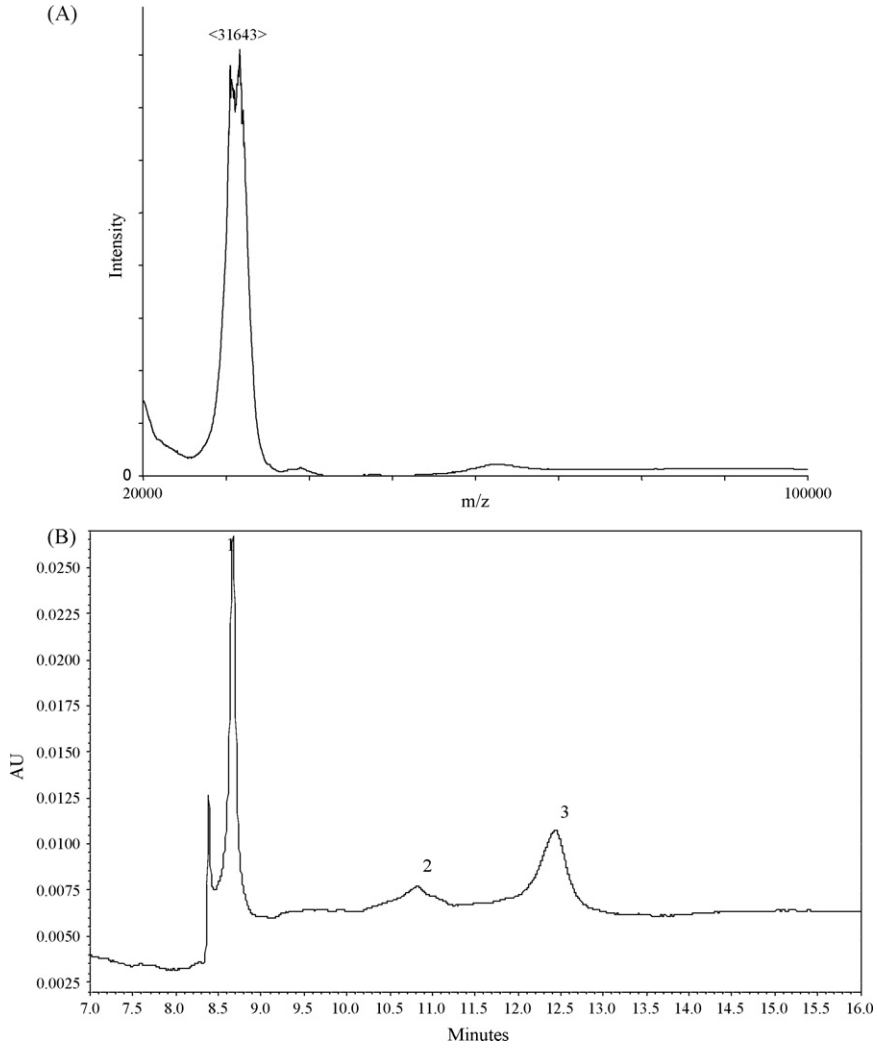

Fig. 6. Analysis of purified VP7 by MALDI-TOF-MS (A) and by SDS-CGE (B). Numbered peaks in (B) correspond to (1) lysozyme; (2) VP7; (3) BSA.

Superdex 200 26/60 (Fig. 4) and desalting using Sephadex G-25, both preceded by an ultrafiltration step to concentrate the sample. Finally, eluted samples from both columns were loaded separately into an anion exchange column (Mono Q) (Fig. 5A and B, respectively). As expected, the first approach for the intermediate step (Superdex 200 26/60) gave rise to higher VP7 purity when compared to the second approach (>90% and 70%, respectively).

The great advantage of using Superdex 200 26/60 was to remove gp64 trimers, which were eluted in peak 1 (Fig. 4). In peak 3 the majority of vp39 contaminants were removed, together with some VP7 (probably dimers). However, most of VP7 (monomers) could be recovered in peak 4 (with purity of 72%), which was confirmed by SDS-PAGE and Western blot analysis (lane 4, Fig. 3A and B, respectively). The VP7 from this step (Fig. 4, peak 4) was then loaded into Mono Q (Fig. 5A) and eluted at 8 mS cm^{-1} (Fig. 5A, solid arrow), thus accomplishing the separation from the minor contaminants gp64 and vp39 monomers initially observed (lane 5, Fig. 3A).

Interestingly, in the second approach, VP7 eluted from Mono Q at 20 and 30 mS cm^{-1} (Fig. 5B, dashed and solid arrows, respectively). From native electrophoresis observations one could say that VP7 in those peaks are dimers and monomers, respectively (Fig. 5C). Galadari et al. have already observed that an enzyme from rat brain eluted as two peaks in Mono Q: the first, at lower conductivity, corresponded to heterotrimers and the second to heterodimers [32].

Another interesting result found was the different VP7 interactions with Mono Q beads. In the sample with more contaminants (second approach of intermediate step) VP7 adsorbed more strongly to Mono Q, eluting at a higher conductivity. Also, VP7 monomers were less pure than in the first approach (lane 6, Fig. 3A). Contrarily, in a purer sample (first approach of intermediate step) VP7 eluted earlier due to weaker interaction with Mono Q. It seems that, apart from interacting with the beads, VP7 monomers display inter- and intra-molecular interactions, with the contaminants vp39 and gp64 and with VP7 monomers themselves (dimers and trimers), respectively.

Purified VP7 obtained with the first approach yielded 0.2 mg of monomeric VP7 per litre of bioreactor bulk with a final purity of more than 90%. In a previous report the authors obtained somewhat higher values, 0.25–0.5 mg L^{-1} but for all forms of VP7 with 99% of purity using immunoaffinity chromatography specific for the RRV strain [4].

3.3. VP7 characterization

The preliminary characterization of purified VP7 showed that it is in monomeric form and its M_W is in accordance with the value

of 31.2 kDa from the amino acid sequence [3]. Also, the VP7 M_W obtained by MALDI-TOF-MS (31.6 kDa) (Fig. 6A) is similar to that previously reported for rotavirus RRV VP7 monomers (32.2 kDa) [4] and VP7 from RLP 2/6/7 (32.7 kDa) [21]. The $M_{W_{app}}$ found by SDS-CGE (39 kDa) (Fig. 6B) is higher than the value for VP7 from RLP 2/6/7 (34 kDa) [21] but lower than the 46 kDa obtained by gel filtration chromatography [4]. VP7 peak in both MALDI spectrum and electropherogram is rather broad probably as a result of heterogeneity due to different isoforms. The non-globular shape of VP7, apparent in electron cryomicroscopy-based reconstructions of the virion [33], probably accounts for the differences in expected M_W from amino acid sequence, M_W from MALDI and $M_{W_{app}}$ from SDS-CGE.

4. Conclusion

Overall, the final purity of VP7 achieved (>90%) is enough not only to perform *in vitro* assembly of triple-layered RLP 2/6/7 but also for crystallization studies, further biochemical and immunological characterization and development of a quantification technique like enzyme-linked immunosorbant assay (ELISA).

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References

[1] M.A. Franco, J. Angel, H.B. Greenberg, Vaccine 24 (2006) 2718.
[2] J.B. Pesavento, S.E. Crawford, M.K. Estes, B.V. Prasad, Curr. Top. Microbiol. Immunol. 309 (2006) 189.
[3] M.K. Estes, J. Cohen, Microbiol. Rev. 53 (1989) 410.
[4] P.R. Dormitzer, H.B. Greenberg, S.C. Harrison, Virology 277 (2000) 420.
[5] M.C. Ruiz, A. Charpilienne, F. Liprandi, R. Gajardo, F. Michelangeli, J. Cohen, J. Virol. 70 (1996) 4877.
[6] M.C. Ruiz, M.J. Abad, A. Charpilienne, J. Cohen, F. Michelangeli, J. Gen. Virol. 78 (1997) 2883.
[7] S. Martin, M. Lorrot, M.A. El Azher, M. Vasseur, J. Virol. 76 (2002) 552.
[8] S.D. Trask, P.R. Dormitzer, J. Virol. 80 (2006) 11293.
[9] H.L. Vieira, C. Estevao, A. Roldao, C.C. Peixoto, M.F. Sousa, P.E. Cruz, M.J. Carrondo, P.M. Alves, J. Biotechnol. 120 (2005) 72.
[10] S.E. Crawford, M. Labbe, J. Cohen, M.H. Burroughs, Y.J. Zhou, M.K. Estes, J. Virol. 68 (1994) 5945.
[11] P.E. Cruz, A. Cunha, C.C. Peixoto, J. Clemente, J.L. Moreira, M.J. Carrondo, Biotechnol. Bioeng. 60 (1998) 408.
[12] B. Jiang, M.K. Estes, C. Barone, V. Barniak, C.M. O'Neal, A. Ottaiano, H.P. Madore, M.E. Conner, Vaccine 17 (1999) 1005.
[13] L. Maranga, T.F. Brazao, M.J. Carrondo, Biotechnol. Bioeng. 84 (2003) 245.
[14] L.A. Palomares, S. Lopez, O.T. Ramirez, Biotechnol. Bioeng. 78 (2002) 635.
[15] L.K. Pattenden, A.P. Middelberg, M. Niebert, D.I. Lipin, Trends Biotechnol. 23 (2005) 523.
[16] A.H. Choi, M. Basu, M.M. McNeal, J.D. Clements, R.L. Ward, J. Virol. 73 (1999) 7574.
[17] M.A. Franco, I. Prieto, M. Labbe, D. Poncet, F. Borras-Cuesta, J. Cohen, J. Gen. Virol. 74 (1993) 2579.
[18] K.R. Emslie, J.M. Miller, M.B. Slade, P.R. Dormitzer, H.B. Greenberg, K.L. Williams, J. Virol. 69 (1995) 1747.
[19] M.S. Poruchynsky, C. Tyndall, G.W. Both, F. Sato, A.R. Bellamy, P.H. Atkinson, J. Cell. Biol. 101 (1985) 2199.
[20] J.A. Mena, O.T. Ramirez, L.A. Palomares, Biotechniques 34 (2003) 260.
[21] M.C. Mellado, C. Franco, A. Coelho, P.M. Alves, A.L. Simplicio, J. Chromatogr. 1192 (2008) 166.
[22] L. Maranga, P.E. Cruz, J.G. Aunins, M.J. Carrondo, Adv. Biochem. Eng. Biotechnol. 74 (2002) 183.
[23] L.A. Palomares, S. Estrada-Mondaca, O.T. Ramirez, in: S.S. Ozturk, S. Hu W (Eds.), Cell Culture Technology for Pharmaceutical and Cell-based Therapies, Taylor & Francis, New York, 2006, p. 627.
[24] L. Maranga, P. Rueda, A.F. Antonis, C. Vela, J.P. Langeveld, J.I. Casal, M.J. Carrondo, Appl. Microbiol. Biotechnol. 59 (2002) 45.
[25] M. Tellez, Membr. Technol. 2005 (2005) 4.
[26] S.R. Narayanan, J. Chromatogr. 658 (1994) 237.
[27] L.E. Volkman, P.A. Goldsmith, Virology 139 (1984) 295.
[28] A.G. Oomens, S.A. Monsma, G.W. Blissard, Virology 209 (1995) 592.
[29] M.M. Keshwani, D.B. Ross, T.J. Ragan, T.K. Harris, Protein Exp. Purif. 58 (2008) 32.
[30] R. Devos, Y. Guisez, S. Cornelis, A. Verhee, J. Van der Heyden, M. Manneberg, H.W. Lahm, W. Fiers, J. Tavernier, G. Plaetinck, J. Biol. Chem. 268 (1993) 6581.
[31] Y.J. de Kok, M.P. Geurds, E.A. Sistermans, M. Usmany, J.M. Vlak, B. Wieringa, Mol. Cell. Biochem. 143 (1995) 59.
[32] S. Galadari, K. Kishikawa, C. Kamibayashi, M.C. Mumby, Y.A. Hannun, Biochemistry 37 (1998) 11232.
[33] B.V. Prasad, W. Chiu, Curr. Top. Microbiol. Immunol. 185 (1994) 9.