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Quantification of rotavirus-like particles by gel permeation chromatography

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Abstract

There is a lack of accurate and practical methods that require only small amounts of sample for quantifying virus-like particles (VLP). In this work, gel permeation (GP) HPLC was used to quantify double-layered rotavirus-like particles (dlRLP) produced in insect cells. The proposed methodology utilized two columns in series (pore sizes of 200 and 50 nm) and had a high precision (relative standard deviation below 5%). GP-HPLC not only allowed the routine quantification of dlRLP, but also of assembly intermediaries and other viral structures present in the samples. For the first time, kinetics of dlRLP accumulation could be followed. This methodology is valuable for designing new production processes and for optimizing dlRLP monitoring.

Keywords: Rotavirus-like particles; HPLC; Gel permeation

1. Introduction

Virus-like particles (VLP) are of interest to various fields, including vaccines, gene therapy, and nanotechnology [1–3]. The production of VLP is complex, as it often requires the simultaneous expression of several proteins that must assemble into multi-layered structures [4]. A productive process for VLP production should consist of recombinant proteins in a high concentration and at a ratio between them that maximizes their assembly into complete VLP. However, the design of such a process is difficult as the quantification of VLP is not a trivial task. An efficient method for quantifying VLP must differentiate between monomeric and assembled proteins, as well as between incomplete assembled intermediaries and complete VLP. Moreover, it should require a small sample volume and a short analysis time to be routinely used for following VLP accumulation kinetics. Such a methodology would not only be valuable for process monitoring and design, but would also provide information on the factors that limit VLP assembly. Possible effects of assembly conditions (temperature, ionic strength, pH, protein concentration, or the concentration of different monomers and the stoichiometry between them) could be easily and quantitatively evaluated.

Several techniques employed to follow VLP production kinetics are based on immunoassays, where the antibody utilized recognizes epitopes present when various proteins or monomers of the same protein interact with each other to form multimeric structures [5–7]. However, the sole presence of the epitope does not guarantee that it forms part of an assembled and complete VLP. It is possible that such an epitope is present in incomplete assembled intermediaries, or in groups of a recombinant protein that may lack other proteins required for assembly of the VLP of interest. An alternative approach for monitoring VLP is their purification by ultracentrifugation and quantification of the recovered material [8]. Nonetheless, this methodology has other drawbacks. Namely, the efficiency of the purification process may be unknown, and may result in quantification errors. Also, the presence of other structures conformed by various proteins, such as other viruses or assembly intermediaries, may pellet during ultracentrifugation and cause an overestimation of the

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amount of complete VLP. The problems of VLP quantification are also partially present in virus production processes, where infective particles are traditionally quantified by infectivity assays, but the actual number of viral particles remains unknown [9]. In this field, electron microscopy (EM) and ion exchange HPLC have been used [10]. However, several problems remain. EM is not a quantitative technique, and several factors, such as sample heterogeneity, may yield incorrect results [10]. In addition, it is time consuming and requires equipment not always available in a production facility. Ion exchange HPLC is a suitable technique [10], but its implementation must be performed in a case-to-case basis, as it relies on the charge of the viral particle as well as that of the contaminants. A new technique successfully applied is quantitative real-time PCR, which quantifies the amount of viral genetic material present, but not the amount of assembled, possibly empty, capsids [11,12]. An alternative is gel permeation (GP) HPLC, which relies solely on the size of the particle to be separated.

An interesting model for studying VLP assembly is the rotavirus. Rotaviruses are a cause of acute infantile gastroenteritis with approximately 600,000 annual deaths worldwide [13]. The rotavirus capsid is composed of four major proteins that assemble into three concentric layers. The core (50 nm in diameter) is formed by 60 dimers of VP2 (molecular mass of 102,500 per monomer) and the proteins VP1 and VP3 (less than 2% of the total protein content). The second layer (70 nm in diameter) is formed by 260 trimers of VP6 (molecular mass 44,000), and the third contains 780 molecules of VP7 and 60 spikes formed by dimers of VP4 [13–15]. When VP2 is expressed alone or with the other structural proteins, single, double, or triple layered rotavirus-like particles (RLP) can be obtained [16,17]. Interestingly, VP6 has a structural polymorphism and, in the absence of the other rotaviral proteins, can assemble into tubes, spheres, or trimers [18]. Double-layered RLP (dlRLP), containing VP2 and VP6, are useful as vaccines for human or veterinary use as they elicit immune responses and boost immunity after immunization with live virus [19,20]. In addition, they provide the advantages of other VLP. Namely, the absence of viral genetic material makes them non-infectious, and it is possible to engineer VP6 to display epitopes from other sources. Other applications of dlRLP include virus tracing, biosensors, and basic research [2,21]. When VP2 is expressed in the insect cell-baculovirus expression vector system (IC-BEVS), single-layered particles remain either intracellular, or in the insoluble fraction if cells are ruptured [22]. Upon expression of both VP2 and VP6, dlRLP accumulate in the cytoplasm of insect cells and are released to the culture supernatant when cells lyse [22]. The use of the IC-BEVS has advantages over other expression systems, as it is versatile and highly productive [2]. Nonetheless, the presence of baculovirus can be a drawback as it can be difficult to differentiate them from RLP. In this work, dlRLP were produced in the IC-BEVS, and the conditions for their quantification by GP-HPLC were determined. Utilizing GP-HPLC, the production kinetics of dlRLP in small-scale cultures could be followed. This technique is a valuable tool for efficient process development and monitoring.

2. Experimental

2.1. High performance liquid chromatography

Analyses were performed in a chromatographic system from Waters (Milford, MA, USA), which included a 600S controller, 626 pumps, and a 996 photodiode array detector and a 2475 fluorescence detector connected in series. Absorbance was measured at 280 nm and fluorescence was measured at excitation and emission wavelengths of 484 and 510 nm, respectively, with a gain of 10 and an attenuation of 64. The gel permeation columns used were Ultrahydrogel 500 and Ultrahydrogel 2000, both with a diameter of 7.8 and 300 mm of length (Waters, Milford, MA, USA). Such columns have a pore size of 50 and 200 nm, respectively. A 10 mM Tris–HCl, 0.1 mM EDTA buffer pH 8.0 was used as mobile phase, except where otherwise indicated (reagents from Sigma–Aldrich, St. Louis, MO, USA). Such a buffer is optimal for enhanced green fluorescent protein (EGFP) fluorescence [23]. Where indicated, ionic strength of the mobile phase was increased by the addition of sodium chloride. An isocratic flow of 0.9 mL/min was used in all runs. The following standards were used to calibrate the columns: fluorescent nanospheres of 40 nm in diameter (Invitrogen, Carlsbad, CA, USA), detected by excitation and emission wavelengths of 505 and 515 nm, respectively; EGFP (BD Biosciences Clontech, Palo Alto, CA, USA); bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA); and a rabbit IgG (Rockland Immunochemicals Inc., Gilbertsville, PA, USA).

2.2. Production of dlRLP

dlRLP were produced by coinfection of High Five® insect cell cultures with two recombinant baculoviruses, one containing the gene of the fusion protein GFPVP2 (strain RF) and a second containing the gene of VP6 (strain SA11) [22]. Both baculoviruses contain the recombinant gene under the *polh* promoter. The baculovirus containing the gene of the fusion protein GFPVP2 was kindly provided by Dr. Jean Cohen (INRA, France), and has been described in detail by Charpilienne et al. [24]. The baculovirus containing the gene of VP6 was kindly donated by Dr. Susana López (IBT-UNAM). Insect cells were cultured in Sf900II medium (Invitrogen, Carlsbad, CA, USA) in 250 mL shaker flasks (60 mL working volume) agitated at 115 rpm and maintained at 27 °C. Cultures were infected at a cell concentration of 0.5×10^6 cell/mL with five plaque forming units/cell of each virus. Viability and total cell concentrations were determined with a hemacytometer and a Coulter Counter (Beckman Coulter, Fullerton, CA, USA), respectively. Viral titers were determined as described by Mena et al. [25].

To follow the kinetics of dlRLP production, 1 mL samples were taken daily and centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was concentrated to 100 μL with a Nanosep Omega centrifugal device (Pall Sciences, East Hills, NY, USA) with a 300,000 molecular weight cut-off (MWCO). The retentate was filtered with a 0.22 μm GHP Acrodisc (Pall Sciences, East Hills, NY, USA), and injected to the HPLC system. The cellular pellet was resuspended in 10 mM Tris 0.1 mM EDTA buffer, sonicated for 2 min in a VibraCell sonicator (Sonics and Materials, Newtown, CT, USA), and clarified by centrifugation at 14,000 × *g* for 10 min at 4 °C. One hundred and fifty microlitres of the supernatant were treated with 20 μL of a solution of DNAse I and RNAse I (2600 U/mL of each enzyme) (Sigma–Aldrich, St. Louis, MO, USA) in reaction buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 150 mM NaCl). Nucleic acids were digested for 1 h at 37 °C. Tubes were centrifuged and 100 μL of the supernatant were injected into the HPLC system after filtration with 0.22 μm GHP Acrodiscs. The concentration of dlRLP was determined by comparison with the fluorescence of an EGFP standard. When needed, EGFP fluorescence was also measured with a spectrofluorimeter Perkin-Elmer LS55 (Wellesley, MA, USA), with slit widths of 2.5 and 20 nm in the excitation and emission pathways, respectively.

2.3. Purification of dlRLP by isopycnic centrifugation in cesium chloride gradients

Cultures were harvested at 48 h post infection (hpi) and treated as described for the samples. Culture supernatants were ultracentifuged over a 35% sucrose cushion at 112,700 × *g* for 30 min at 4 °C in a SW-28 rotor and an Optima L-90K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). The pellet obtained was resuspended in TNC buffer (Tris–HCl 10 mM, EDTA 1 mM, NaCl 150 mM). Cesium chloride was added to yield a concentration of 0.42 g/mL (refractive index of 1.3631 at 25 °C). The sample was centrifuged at 148,930 × *g* for 18 h at 4 °C in a SW 50 rotor (Beckman Coulter, Fullerton, CA, USA). Two bands were obtained and characterized by immunoblotting in gels and electron microscopy (data not shown). The upper band contained complete, not aggregated dlRLP, and was recovered for further analysis.

The supernatant obtained from the cellular pellets after sonication was also subjected to a cesium chloride gradient as described before, but without a sucrose gradient. The band containing dlRLP was desalted by ultrafiltration through a Nanosep Omega centrifugal device (Pall Sciences, East Hills, NY, USA) with a 300,000 MWCO. dlRLP were resuspended in TNC buffer, and their identity was confirmed by Western blot.

2.4. Electrophoresis and Western blot

The samples were run in denaturing sodium dodecyl sulfate-polyacrylamide gels (4% stacking gel, 8 or 12% separating gels) and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% low-fat dried milk in phosphate buffered solution (PBS) and incubated for 1 h with a mouse monoclonal anti-EGFP antibody (BD Biosciences Clontech, Palo Alto, CA, USA) in PBS with 0.1% low-fat milk, followed by incubation for 1 h with a goat anti-mouse antibody conjugated with peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Membranes were then incubated with a rabbit anti-rotavirus polyclonal serum (kindly provided by Dr. S. López, IBT-UNAM) for 1 h, followed by incubation for 1 h with a goat anti-rabbit antibody conjugated with peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Membranes were washed three times in PBS with 0.1% low-fat milk after incubation with each antibody. Peroxidase activity was detected by reaction with carbazole. Alternatively, in some cases, individual detection with either one of the primary antibodies was performed.

2.5. Electron microscopy

dlRLP were visualized by negative staining in an electron microscope. Twenty five microlitres of sample were fixed for 1 min in a 200 mesh grid coated with Formvar-carbon (Structure Probe Inc., West Chester, PA, USA). The grid was washed, stained with 2% uranile acetate (Structure Probe Inc., West Chester, PA, USA), rinsed, and left to dry. Samples were observed with an electron microscope Jeol 1200EXII (Jeol, Peabody, MA, USA).

3. Results and discussion

3.1. Analysis of dlRLP purified by cesium chloride gradients

Charpilienne et al. [24] demonstrated that the fusion protein GFPVP2 assembles into RLP similar to those formed by native VP2. The use of GFPVP2 allows the visualization and identification of VP2 and RLP by fluorescence at excitation and emission wavelengths of 484 and 510 nm, respectively [22,24]. Such observations were performed in cells producing GFPVP2 or GFPVP2 and VP6 utilizing a confocal microscope. This advantage was utilized in this work to facilitate the development of the quantification methodology. An Ultrahydrogel 500 column was used as a first approach for evaluating the utility of GP-HPLC for the quantification of dlRLP. For setting up the GP-HPLC system, two standards were injected. First, a standard of fluorescent-labeled spheres with a diameter of 40 nm resulted in a single peak that eluted at 5.52 min (Fig. 1A). As the diameter of the spheres is close to the pore size of the column, it is likely that such an elution time corresponded to the void volume of the column. The standard of pure EGFP (molecular mass 27,000) eluted at 8.98 min (Fig. 1A). dlRLP recovered from cesium chloride gradients were used to characterize the sys-

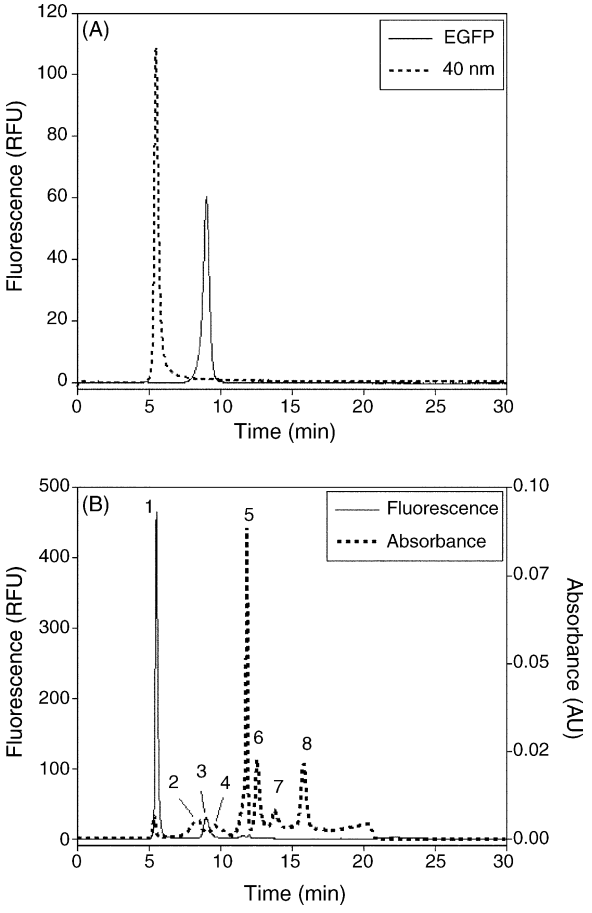

Fig. 1. Characterization of the GP-HPLC system with the Ultrahydrogel 500 column. (A) Pure EGFP and 40 nm fluorescent spheres. (B) dlRLP recovered from cesium chloride isopycnic gradients.

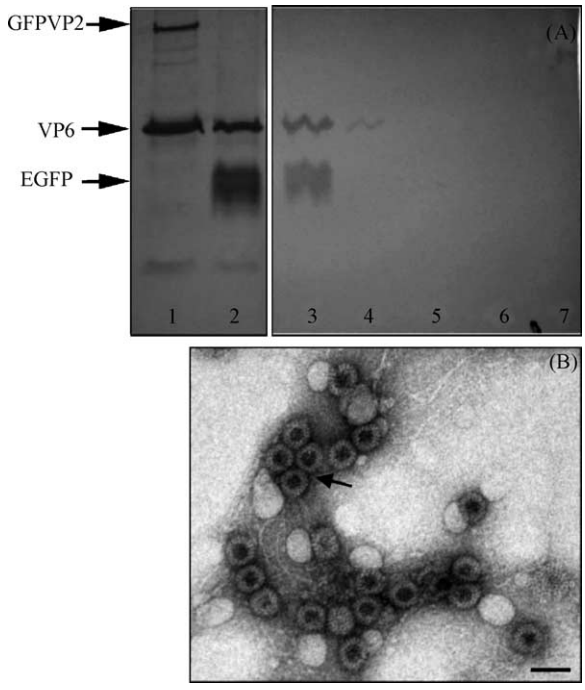

Fig. 2. Identification of the peaks obtained in chromatograms shown in Fig. 1. (A) Western blot. Both membranes were blotted with both anti-rotavirus and anti-EGFP primary antibodies. Lane 1, peak 1; lane 2, peak 3; lane 3, peak 2; lane 4, peak 5; lane 5, peak 6; lane 6, peak 7; lane 7, peak 8. (B) Electron microscopy of peak 1. Arrow indicates dlRLP. Scale bar 100 nm.

tem. The chromatograms obtained from both fluorescence and absorbance are shown in Fig. 1B. Seven peaks were detected by absorbance; of these only the first was also detected by fluorescence. Such a peak eluted at 5.51 min, a retention time similar to that of the 40 nm spheres. This suggests that peak 1 contained dlRLP, which have a diameter of 70 nm and should not enter the column pores. Western blot and electron microscopy confirmed the identity of peak 1 as dlRLP (Fig. 2). Namely, peak 1 was collected (2.3 μg) and loaded into a gel for Western blotting. The Western blot showed that it contained both GFPVP2 and VP6 (Fig. 2A). Observations by electron microscopy of peak 1 showed double-shelled spherical structures with a diameter of 69.5 ± 2.5 nm ($n = 10$) (Fig. 2B). The morphology of the observed spherical structures was identical to that previously reported for dlRLP [16]. The area of peak 1 (Fig. 1B) detected by fluorescence was 64.5 times higher than its area detected by absorbance. The fusion with EGFP clearly increased the sensitivity of detection. Nonetheless, dlRLP could also be detected by absorbance (Fig. 1B). The other peaks detected by absorbance, except peak 4 that could not be efficiently collected for its analysis, were also analyzed by Western blot (Fig. 2A). VP6 and EGFP were detected in peak 2, while VP6 was barely detectable in peak 5. The other peaks contained proteins that could not be detected by the anti-rotavirus serum or a monoclonal antibody to EGFP.

Purification of VLP by cesium chloride gradients is a method that did not yield highly pure material, as can be concluded from the existence of six additional peaks detected by absorbance in the RLP sample (Fig. 1B). The presence of impurities in viral particles purified by cesium chloride gradients has been previously reported [26,27]. Also, incubation of viral particles with cesium chloride at the concentrations typically used in gradients destabilizes the particles, most probably due to high ionic strength [26,28]. Despite of these drawbacks, cesium chloride gradients were used to generate material for characterizing the system, as this is the traditional methodology most commonly used. The concentration of dlRLP in relation with unassembled proteins was of 2.3%, as determined by the relation of peak areas measured by absorbance (Fig. 1B). It is possible that the unassembled proteins in the material recovered from cesium chloride gradients were present in the original sample, or that they resulted from particle disassembly during isopycnic centrifugation or during desalting. The presence of unassembled proteins in material recovered from ultracentrifugation underlines the errors that can occur in the quantification of VLP by this traditional method.

Protein detection by fluorescence resulted in an additional peak not detected by absorbance, peak 3 with an elution time

of 8.98 min (Fig. 1B). Such an elution time coincided with that observed for pure EGFP (Fig. 1A). Western blot analysis showed that the peak contained EGFP and VP6. It is possible that the presence of VP6 was due to the inclusion of material from peak 2 during the manual recollection of peak 3, as both peaks overlap. The area of peak 3 detected by fluorescence corresponded to 11% of the area of peak 1. The presence of EGFP can only be explained by a proteolytic degradation of GFPVP2, which could have occurred in the intact dlRLP, before dlRLP assembly, or after dlRLP destabilization. Proteases have a Stoke's radius between 2.9 and 1.5 nm [29], while the dlRLP contains channels with a diameter of approximately 1 nm in their inner part and 6.5 nm in the surface [30,31]. Thus, it is unlikely that a protease could enter the inner core, where the EGFP fragment of the fusion protein is located, and cleave GFPVP2 while it is assembled in dlRLP. Therefore, GFPVP2 degradation must occur when the protein is exposed to the extra RLP environment. Interestingly, determination of the relation between absorbance and fluorescence of peak 1 (Fig. 1B) remained fairly constant (65.47 ± 1.4) in injections of three different samples, indicating that the ratio between EGFP fluorescence and total protein in the dlRLP was constant regardless of the amount of cleaved GFPVP2 detected. Such evidence and that cleaved GFPVP2 was found in material recovered from cesium chloride gradients suggest that the cleavage of GFPVP2 occurred after dlRLP destabilization, and not before its assembly into dlRLP. No evidence of a dlRLP population lacking EGFP was obtained. In accordance with this observation, studies of the in vivo localization of GFPVP2 have demonstrated that GFPVP2 is not intracellularly cleaved [22]. Namely, localization of GFPVP2 as determined by EGFP fluorescence was identical to that determined by immunostaining of the VP2 part of the fusion protein.

It should be noted that the presence of macrostructures different to dlRLP is expected in samples from insect cell cultures, as infected cells produce baculoviruses. Both the baculovirus and dlRLP cannot enter the pores of the Ultrahydrogel 500 column, as both structures have a diameter larger than the pore size (the baculovirus has a diameter of 50 nm and a length between 200 and 300 nm [2]). To discriminate between baculoviruses and dlRLP, a second column with a pore size of 200 nm was added in series before the Ultrahydrogel 500 column. It should be noted that the use of only the Ultrahydrogel 2000 column did not result in an efficient separation of dlRLP (data not shown). All results presented in the following sections were obtained using both columns in series.

3.2. Optimum assay conditions

The initial conditions of the assays were based on optimum EGFP fluorescence. However, other conditions may result in better resolution of dlRLP without a decrease in fluorescence. Different ionic strengths, pH, and temperatures were tested. It has been reported that such factors affect the assembly and stability of viral capsids [18,32], so it was also important to assess dlRLP stability. A concentration of 1 μg/mL of dlRLP obtained from cesium chloride gradients was used in all experiments. All conditions were tested by duplicate injections after equilibration of the columns with at least 10 column volumes. In the case of temperature, injections were performed after 2 h at constant temperature. When the Ultrahydrogel 2000 and 500 were connected in series, the retention time of the peaks that corresponded to dlRLP and EGFP increased to 14.8 and 21 min, respectively (ionic strength of 5.8 mM, pH 8 and 37 °C, Fig. 3A). An increase in the area of the EGFP peak in relation to the dlRLP peak was observed.

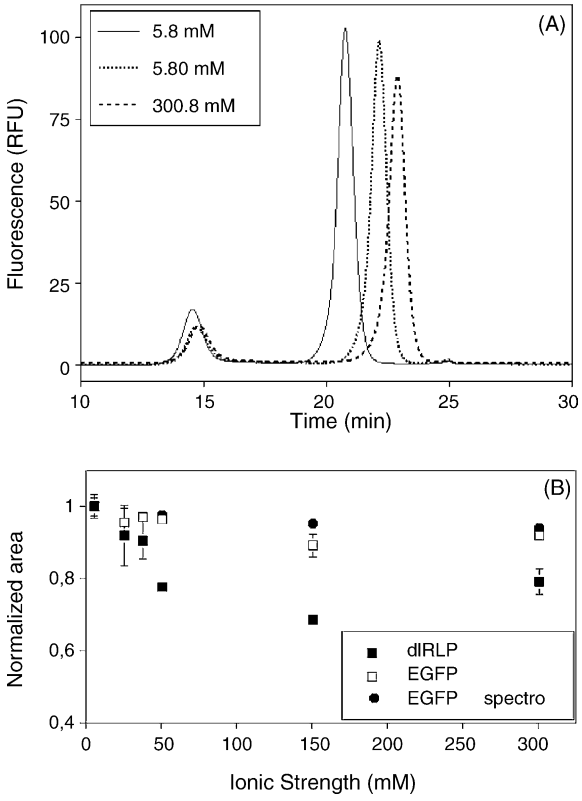

Fig. 3. Effect of ionic strength in the migration of the material recovered from cesium chloride gradients in the Ultrahydrogel 2000 and 500 columns in series. (A) Representative chromatograms at different ionic strengths. (B) Effect of ionic strength on peak area. The fluorescence of EGFP measured in a spectrofluorimeter at the different ionic strengths is shown for comparison (EGFP spectro). Media and difference between two measurements are shown. In some cases, error bars are smaller than symbols.

To evaluate the effect of ionic strength, NaCl was added to the mobile phase and pH and temperature were maintained at 8 and 37 °C, respectively. As the ionic strength increased from 5.8 to 300.8 mM, the retention time of both the dlRLP and EGFP peaks increased by 13.8 s and 2.13 min, respectively (Fig. 3A). It is possible that such changes in the retention time were caused by a modification of protein structure, as a decrease of Stoke's radius has been detected when proteins are subjected to high ionic strengths [33,34]. Such changes appear to be confined to the protein's tertiary structure [35]. The higher increase in the retention time of EGFP in com-

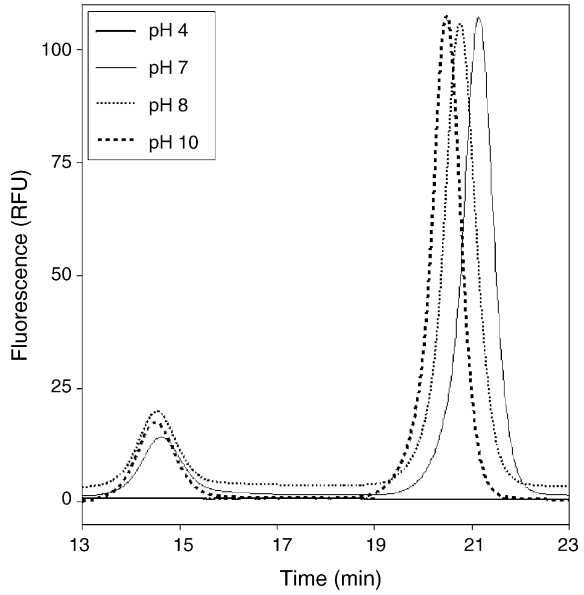

Fig. 4. Effect of pH on the migration of the material recovered from cesium chloride gradients in the Ultrahydrogel 2000 and 500 columns in series.

parison with dlRLP may be a result of the compact structure of dlRLP, which can limit protein mobility. As ionic strength was increased, the area of both peaks decreased (Fig. 3B). At 300.8 mM, the area of the dlRLP peak decreased 21%, while the area of the EGFP peak decreased 8%, in comparison with runs at 5.8 mM. The effect of ionic strength in fluorescence intensity was determined by measuring EGFP fluorescence directly in a spectrofluorimeter (Fig. 3B). The decrease of fluorescence observed in the spectrofluorimeter was equivalent to that observed in the EGFP peak. However, a higher fluorescence decrease was observed for dlRLP. It is possible that, as ionic strength increased, quenching of EGFP fluorescence was higher in the compact interior of the dlRLP core, in comparison with EGFP in solution. Further experimentation is needed to explain such a phenomenon. Even when the separation between the dlRLP and EGFP peaks was better at higher ionic strengths, it has the drawback that the area of the former peak decreased.

The effect of pH was evaluated at 37 °C and ionic strengths of 5.8, 25.8, 38.3, and 50.8 mM, and was the same regardless of the ionic strength tested. Results at 5.8 mM are shown in Fig. 4. No fluorescence was detected at pH 4, in accordance with previous reports regarding the stability of EGFP [23]. Increasing pH from 7 to 8 and 10 decreased the retention time of EGFP and slightly that of dlRLP (Fig. 4). No difference in the peak areas at pH of 7 or higher was detected.

The effect of temperature was evaluated at ionic strengths of 5.8 mM, and pH 8 and 10 (Fig. 5). No changes in retention times or in peak areas were observed at temperatures between 30 and 55 °C at pH 8 (Fig. 5A), or between 30 and 45 °C at pH 10 (Fig. 5B). However, at pH 10 and 55 °C, the area of the peak corresponding to dlRLP (peak 1) decreased and a new peak (peak 3) with a retention time of 16.4 min

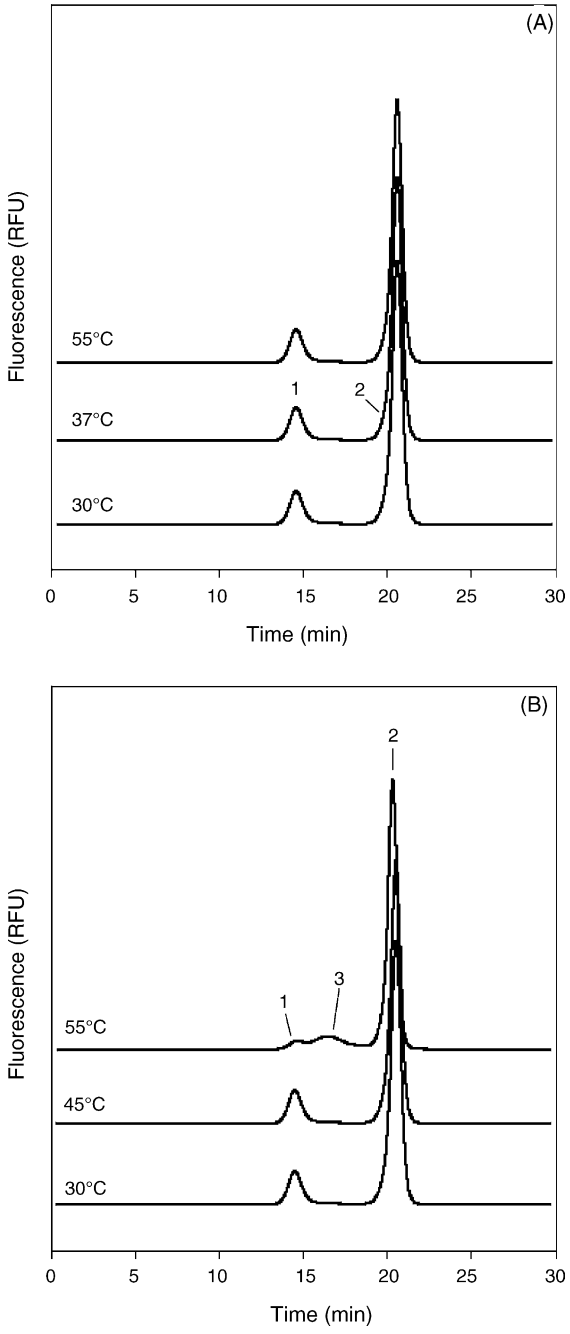

Fig. 5. Effect of temperature on the migration of the material recovered from cesium chloride gradients in the Ultrahydrogel 2000 and 500 columns in series. (A) At pH 8; (B) at pH 10. Only some representative chromatograms are shown.

appeared (Fig. 5B). The peaks obtained at 37 and 55 °C were collected and analyzed by Western blot (Fig. 6). Such an assay confirmed that peak 1 at 37 °C contained dlRLP, while peak 2 contained EGFP and VP6. Peak 1 at 55 °C was not detected in the Western blot, possibly because of the low protein concentration present, while GFPVP2 was detected in peak 2 at 55 °C, in addition to EGFP and VP6. Peak 3 contained GFPVP2. These results show that the conditions of 55 °C and pH 10 destabilized the dlRLP. The assembly state

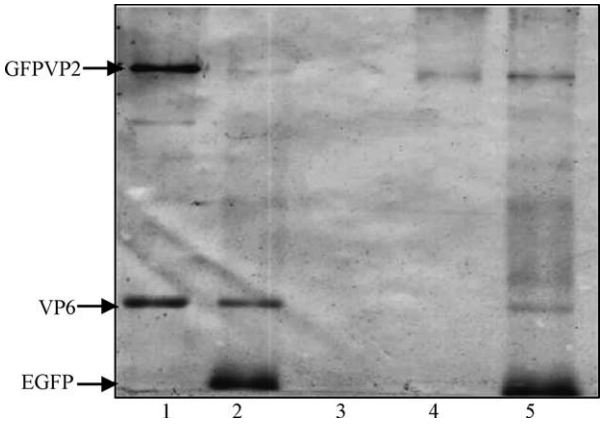

Fig. 6. Western blot of the peaks obtained from chromatograms shown in Fig. 5.The membrane was blotted with both anti-rotavirus and anti-EGFP primary antibodies. Lane 1, peak 1 at 37 °C and pH 8; lane 2, peak 2 at 37 °C and pH 8; lane 3, peak 1 at 55 °C and pH 10; lane 4, peak 3 at 55 °C and pH 10; lane 5, peak 2 at 55 °C and pH 10.

of GFPVP2 in peak 3 can be estimated from its molecular mass, by comparison with the migration time of purified protein standards. An IgG (molecular mass of 150,000) migrated at 20.078 min, bovine serum albumin (BSA, molecular mass of 67,000) migrated at 20.378 min, and EGFP migrated at 21.323 min. The migration time of all these proteins was higher than that of peak 3, indicating that GFPVP2 (molecular mass 120,300) in peak 3 was not in a monomeric form. It is possible that GFPVP2 was assembled in a multimeric structure, possibly single-layered RLP (formed only by GFPVP2) or another intermediary. Such results show the usefulness of the GP-HPLC method described here for identifying and quantifying assembly intermediaries, or to perform capsid stability assays, which is a fundamental issue for products based on VLP.

Based on the results obtained in this section, the conditions for quantification of dlRLP were set as ionic strength of 5.8 mM (buffer Tris 10 mM, EDTA 0.1 mM), pH 8.0 and 37 °C.

3.3. Quantification of dlRLP

The presence of GFP in the dlRLP allowed a higher sensitivity in the detection of dlRLP. An equimolar relation exists between VP2 and EGFP in the fusion protein; therefore, a dlRLP contains 120 molecules of EGFP. Charpilienne et al. [24] reported that the molar extinction coefficient of assembled GFPVP2 was similar to that of EGFP (53,000 M^{-1} cm^{-1} at 488 nm). Thus, a purified commercial EGFP can be used as a standard to quantify GFPVP2. The molecular masses of dlRLP and EGFP are 49.5×10^6 and 27×10^3, respectively. Therefore, 1 g of GFP is contained in 15.27 g of dlRLP. Utilizing such a relation, it was possible to quantify the concentration of dlRLP by measuring its fluorescence. A linear response of fluorescence versus concentration of EGFP was obtained between 5 and 100 ng/mL with a regression of $y = 39{,}661x - 28$ ($r^2 = 0.9991$), where y refers to peak area in relative fluorescence units (RFUs) and x to EGFP concentration in ng/mL. The mean and standard deviations of nine injections of the 5 ng/mL standard were 188,682 and 3246 RFUs, respectively. The relative standard deviation was 1.72%. From this data, the detection and quantification limits were calculated and resulted in 245 and 818 pg/mL, respectively. Extrapolating such values to concentrations of dlRLP, the limits of detection and quantification were 3.74 ng/mL (75 fM) and 12.49 ng/mL (252 fM), respectively. The precision of the method was evaluated by three sets of six injections of purified dlRLP performed in consecutive days. Table 1 shows the results obtained. The relative intra- and inter-assays standard deviations were below 5%. From dlRLP concentration, it is possible to calculate the number of particles present in a sample, by utilizing the Avogadro's number. A conversion factor of 1.216×10^7 particles/ng was obtained.

Table 1
Determination of the precision of the proposed assay

Injection	Area (RFUs)		
	Day 1	Day 2	Day 3
1	817907	810586	860589
2	863497	885320	850412
3	812200	830248	840265
4	817145	824315	843625
5	833469	815289	825014
6	811937	821318	830215
Intra day assay precision			
Mean	826025	831179	841686
S.D.	19969	27397	13029
R.S.D. (%)	2.41	3.29	1.54
Inter day assay precision			
Mean	832963		
S.D.	7981		
R.S.D. (%)	0.95		

dlRLP purified by cesium chloride gradients were repeatedly injected into the Ultrahydrogel 2000 and 500 columns in series at a ionic strength of 5.8 mM, pH 8 and 37 °C. RFU, relative fluorescence units; S.D., standard deviation; R.S.D., relative standard deviation.

To evaluate the utility of the proposed method for the quantification of unlabeled VLP, dlRLP concentration was also followed by absorbance, as shown in Fig. 7. A linear relation between peak area measured by absorbance and dlRLP concentration was obtained between 160 and 560 ng/mL, with a regression of $y = 0.0013x + 5.8998$ ($r^2 = 0.991$) (three different concentrations of dlRLP were injected in triplicate, see Fig. 7), where y is dlRLP concentration in ng/mL and x is peak area in absorbance per second. Such a result demonstrates the utility of the proposed method even in the absence of a reporter protein. However, to utilize absorbance to quantify dlRLP, the peak corresponding to dlRLP must have been fully identified and a dlRLP preparation with known concentration (obtained by utilizing EGFP, or another method) should be available.

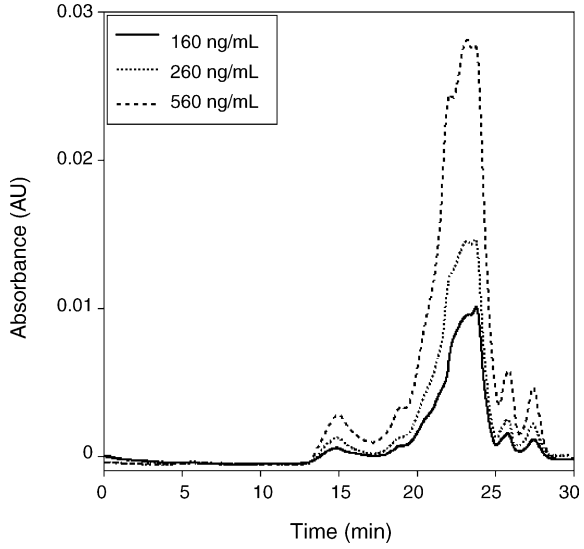

Fig. 7. The concentration of dlRLP can also be followed by absorbance. Three concentrations of dlRLP were injected to the HPLC and detected by absorbance at 280 nm. The regression obtained from the injection by triplicate of each concentration was $y = 0.0013x + 5.8998$ ($r^2 = 0.991$), where y is dlRLP concentration in ng/mL and x is peak area in absorbance units per second.

3.4. Quantification of dlRLP in insect cell cultures

To evaluate the utility of the proposed method for the quantification of dlRLP from insect cell cultures, a culture was coinfected with two recombinant baculoviruses, one containing the gene of GFPVP2 and the other the gene of VP6, each with a multiplicity of infection (MOI) of 5 pfu/cell. When VP2 and VP6 are coexpressed, not only dlRLP can be obtained, as VP2 is able to assemble into cores [17,22]. The cores are not liberated to the culture supernatant, as they accumulate in the cytoplasm in very compact aggregates, as observed by electron microscopy of High Five® insect cells expressing VP2 [22], and their recovery from the pellet after cellular sonication requires the addition of tensoactives [17]. In this work, no tensoactives were added to the cellular pellet to avoid recovery of cores. Thus, the only macrostructures in the samples were dlRLP and baculoviruses. Fig. 8A shows the chromatogram obtained when a sample from the cell pellet collected at 48 hpi was analyzed as described in Section 2. A new peak (0) eluted at 12.3 min, before the peak containing dlRLP. Such a peak was analyzed by Western blot (Fig. 8B and C). No GFPVP2 was detected. A small amount of VP6, possibly due to a contamination by the dlRLP peak was obtained. The unknown peak was recollected and

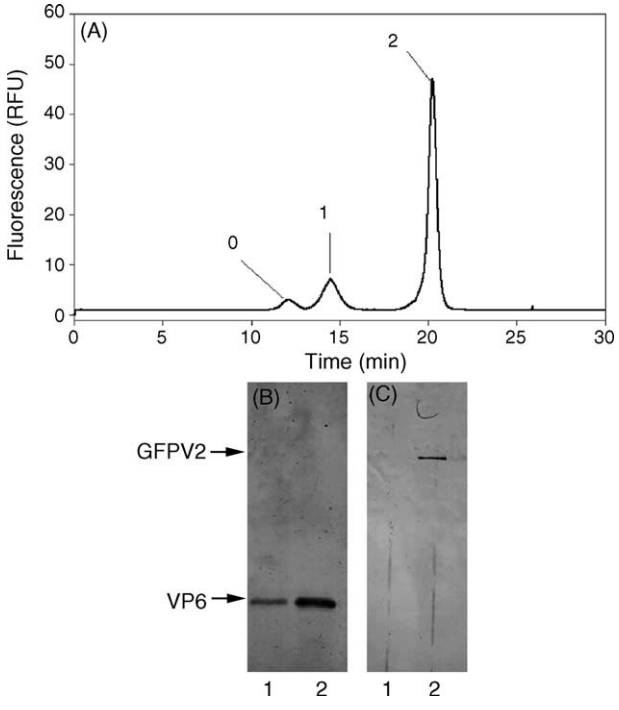

Fig. 8. (A) Typical chromatogram obtained from the intracellular material of insect cells in the Ultrahydrogel 2000 and 500 columns in series. (B) Western blot of the peaks obtained blotted with anti-rotavirus primary antibody. Lane 1, peak 0; lane 2, peak 1. (C) Western blot of the peaks obtained blotted with anti-EGFP primary antibody. Lane 1, peak 0; lane 2, peak 1.

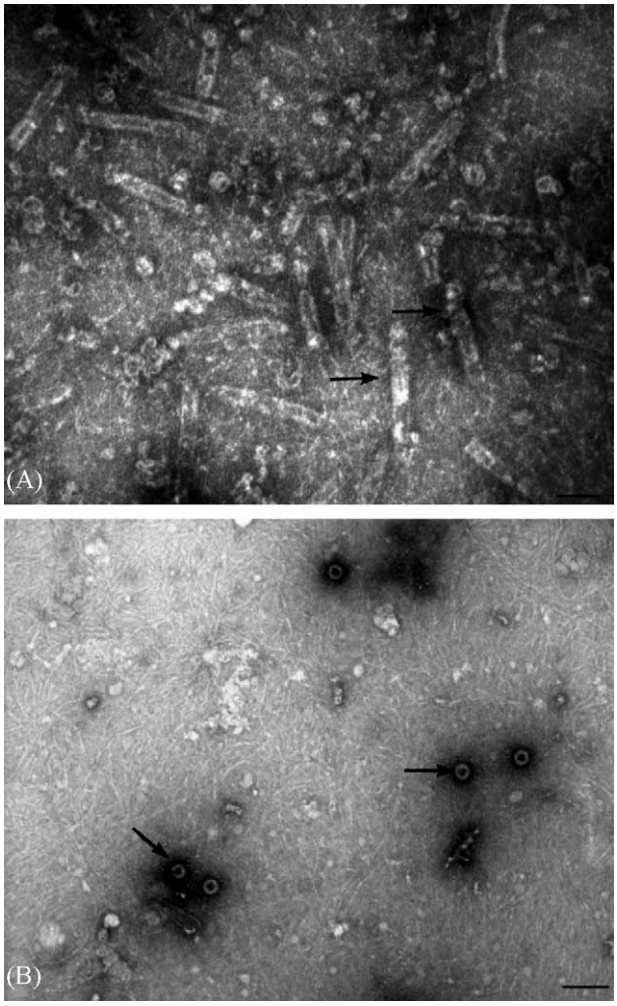

Fig. 9. Electron microscopy of the peaks recovered from the chromatogram shown in Fig. 7. (A) Peak 0, scale bar 100 nm. (B) Peak 1, scale bar 200 nm. Arrows indicate baculovirus and dlRLP in panels A and B, respectively.

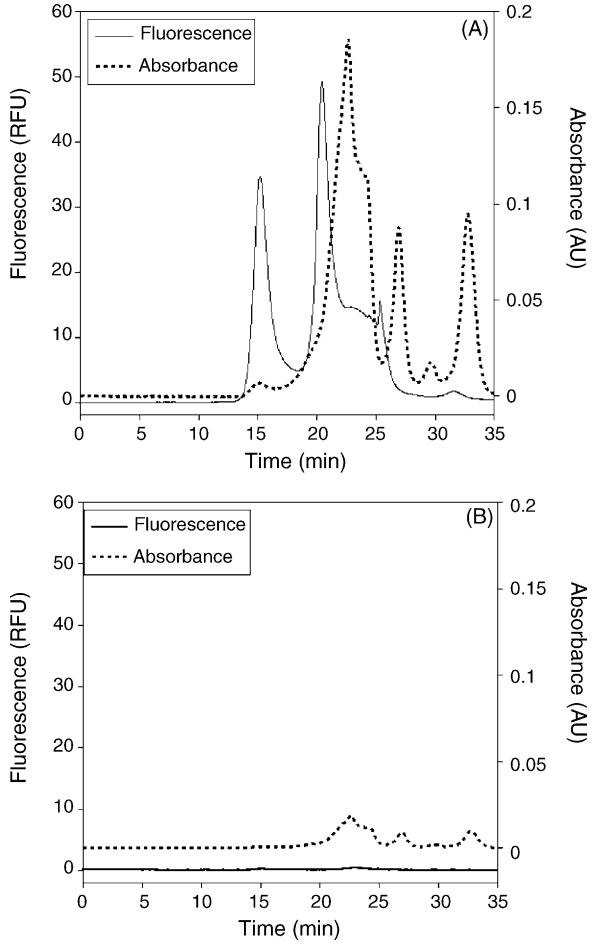

Fig. 10. (A) Typical chromatogram obtained from the injection of culture supernatants at 72 hpi. (B) Chromatogram obtained from the supernatant of an uninfected exponentially growing culture.

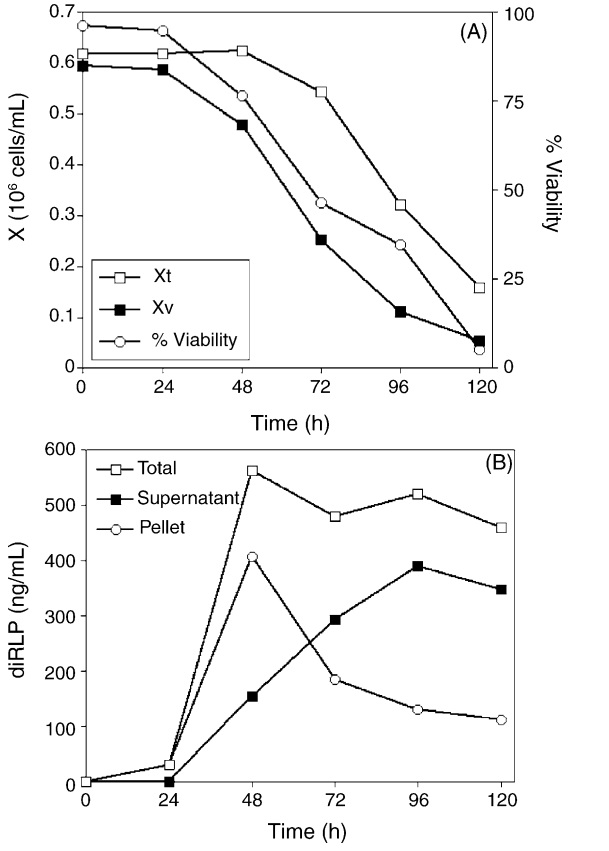

Fig. 11. Kinetics of the production of dlRLP in insect cell cultures followed by the GP-HPLC quantification method proposed here. (A) Cell concentration and viability kinetics. (B) Concentration of dlRLP.

observed by electron microscopy. Viral structures with a rod shape of 276 nm (±15.1) in length and approximately 50 nm in diameter were observed. Such structures were recognized as baculoviruses (Fig. 9A). The identity peak 1 was confirmed as dlRLP both by Western blotting and electron microscopy (Figs. 8B, C, and 9B). The utilization of the two columns in series allowed the separation of both viral structures, dlRLP and baculovirus, a task that could not be achieved when a single column was used.

The chromatograms obtained from injection of a sample of culture supernatant at 72 hpi is shown in Fig. 10 and compared with samples obtained from uninfected cultures processed similar to those of infected samples. The peaks corresponding to dlRLP and EGFP were detected by fluorescence in the infected culture supernatant. After the EGFP peak, broad peaks of unidentified material were observed. The dlRLP peak could also be detected by absorbance, and represented from 0.15 to 0.2% of the total protein detected at 280 nm. None of the fluorescent peaks could be detected in the supernatant of an uninfected exponentially growing culture (Fig. 10B). Furthermore, the dlRLP peak detected by absorbance in infected cultures was not present in uninfected cultures. Moreover, the total protein content detected by absorbance was much lower in the uninfected culture.

Utilizing the technique proposed in this work, kinetics of the accumulation of dlRLP in insect cell culture was followed (Fig. 11). Such results show, for the first time, the kinetics of intra- and extra-cellular dlRLP production. In contrast to other methods used for quantifying VLP, results shown in Fig. 11 leave no doubts on particle integrity and identity. As expected for a culture infected with a total MOI of 10 pfu/cell, cells did not grow after infection ($t = 0$ hpi). dlRLP could be detected intracellularly at 24 hpi. Viability decreased from 95% at 24 hpi to 76% at 48 hpi. The maximum total concentration of dlRLP (563 ng/mL) was obtained at 48 hpi. At this time, 90% of the dlRLP were intracellular. Such results are in accordance with previous reports showing that dlRLP assemble intracellularly, as determined by electron microscopy observations of insect cells coexpressing VP2 and VP6 [22]. As viability decreased, dlRLP were liberated to the cytoplasm until 96 hpi. After this time, no increase of the extracellular concentration of dlRLP was observed. At 120 hpi, 90% of the dlRLP were in the supernatant. These results demonstrate that routine quantification of dlRLP by the GP-HPLC system proposed here is possible.

4. Conclusions

The use of GP-HPLC with two columns in series allowed the routine quantification of dlRLP produced by insect cells, both intracellular and in culture supernatants. Such a methodology required small sample volumes and a simple sample treatment before injection into the HPLC. The use of a fusion protein with GFP allowed the detection of small amounts of dlRLP (1.5 fM dlRLP), but quantification utilizing absorbance at 280 nm was also possible, after the peak corresponding to dlRLP was identified and a preparation with known concentration was available. The best quantification conditions were obtained utilizing an ionic strength of 5.8 mM, pH of 8, and 37 °C. The proposed method had a high precision, with intra- and inter-assay standard deviations below 5%. The method was also capable of detecting and quantifying multiproteic assembly intermediaries, as well as other viral structures, such as baculovirus. The availability of this technique will allow the characterization of the production process of dlRLP, including production kinetics. This information is valuable for process design and monitoring. A very important feature of the method described here is its capability for quantifying complex viral structures with the confidence of considering only the structure of interest. To our knowledge, quantification of dlRLP during production was achieved for the first time. The GP-HPLC system proposed here should be easily implemented for the quantification of other VLP or viral particles.

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