Appl Microbiol Biotechnol (2002) 59:45–50
DOI 10.1007/s00253-002-0976-x

ORIGINAL PAPER

L. Maranga · P. Rueda · A.F.G. Antonis · C. Vela
J.P.M. Langeveld · J.I. Casal · M.J.T. Carrondo

Large scale production and downstream processing of a recombinant porcine parvovirus vaccine

Received: 14 November 2001 / Revised: 12 February 2002 / Accepted: 15 February 2002 / Published online: 16 April 2002

Abstract Porcine parvovirus (PPV) virus-like particles (VLPs) constitute a potential vaccine for prevention of parvovirus-induced reproductive failure in gilts. Here we report the development of a large scale (25 l) production process for PPV-VLPs with baculovirus-infected insect cells. A low multiplicity of infection (MOI) strategy was efficiently applied avoiding the use of an extra baculovirus expansion step. The optimal harvest time was defined at 120 h post-infection at the MOI used, with the cell concentration at infection being 1.5×10^6 cells/ml. An efficient purification scheme using centrifugation, precipitation and ultrafiltration/diafiltration as stepwise unit operations was developed. The global yield of the downstream process was 68%. Baculovirus inactivation with Triton X-100 was successfully integrated into the purification scheme without an increase in the number of process stages. Immunogenicity of the PPV-VLPs tested in guinea pigs was similar to highly purified reference material produced from cells cultured in the presence of serum-containing medium. These results indicate the feasibility of industrial scale production of PPV-VLPs in the baculovirus system, safety of the product, and the potency of the product for vaccine application.

L. Maranga · M.J.T. Carrondo (✉)
Instituto de Biologia Experimental
e Tecnológica/Instituto de Tecnologia Química
e Biológica IBET/ITQB, Apartado 12, 2781-901 Oeiras, Portugal
e-mail: mjtc@itqb.unl.pt
Tel.: +351-21-4427787, Fax: +351-21-4421161

P. Rueda · C. Vela · J.I. Casal
Inmunología y Genética Apl. S.A. (INGENASA),
Hnos. García Noblejas 41, 40, 28037 Madrid, Spain

A.F.G. Antonis · J.P.M. Langeveld
Institute for Animal Science and Health (ID-Lelystad),
P.O. Box 65, 8200 AB Lelystad, The Netherlands

M.J.T. Carrondo
Laboratório de Engenharia Bioquímica,
Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa,
2825-114 Monte da Caparica, Portugal

Introduction

Porcine parvovirus (PPV) is a major cause of reproductive failure in swine. PPV is a ubiquitous virus present worldwide that causes increasing economic losses due to the industrialisation of pig farming (Martinez et al. 1992). PPV is a small, non-enveloped, icosahedral virus. The viral capsid contains 50–60 molecules of the major structural protein VP2 (M_r 64 kDa) (Casal 1996).

The baculovirus expression vector system, due to its high productivity and ability to achieve rapid implementation, has been widely used for the production of proteins for structural and other studies (Maranga et al. 2002). Expression of PPV capsid protein VP2 in insect (*Spodoptera frugiperda*) cells infected with a recombinant baculovirus leads to the production of large amounts of VP2 that self-assembles into empty PPV virus-like particles (VLPs); PPV-VLPs have been shown to be highly immunogenic (Martinez et al. 1992).

VLPs offer several advantages as antigens or immunogens since they present conformational epitopes more similar to native particles, leading to more relevant antibody reactivity or immune system response. Also, since the productivity of PPV in tissue culture is much lower than VLP production, the capsids could provide a cost-effective vaccine against the natural virus.

Production of VLPs has been reported at different multiplicities of infection (MOI) ranging from high (Cruz et al. 1998) to low (Zheng et al. 1999). The use of high rather than low MOI has been preferred for two main reasons: easier harvest time definition and elimination of the so-called ‘passage effect’ from the baculovirus inoculum. The passage effect results in a decrease with time (passage number) of the fraction of virus that carries the recombinant gene (Casal 1996). This phenomenon is similar to that observed in wild-type baculovirus AcMNPV, where highly passaged virus generates few polyhedra phenotypes (Krell 1996). Although the use of a high MOI will result in easier optimal harvest time definition, the use of low MOI at large scale is preferential since an intermediate step of virus inoculum pro-

duction is avoided. Also, industrial processes will necessarily use virus inoculum from tested and certified master banks, meaning that the use of a low MOI strategy will also extend the life span of these banks due to the 100–1,000-fold lower consumption of virus inoculum per batch.

Large scale production of PPV-VLPs will require the use of serum-free culture media to facilitate downstream processing and decrease the overall process cost. Veterinary products derived from biotechnology should be purified with a minimal number of steps. The unit operations employed should be simple and expensive chromatographic steps should be avoided due to their weight in process cost. Therefore, a process based on precipitation/centrifugation steps was envisaged and its applicability was demonstrated. The presence of baculovirus might conceivably be a problem for the registration and safety requirements of the recombinant vaccine, so a baculovirus inactivation strategy was tested and confirmed. Three different baculovirus inactivation procedures – pasteurisation, treatment with detergents and alkylation with binary ethyleneimine (BEI) – have already been tested with PPV-VLPs and the integrity and immunogenicity of the VLPs after inactivation has been studied (Rueda et al. 2001). Pasteurisation is the simplest method because it does not require specific equipment or reagents; however, a significant fraction of VLPs was disrupted after the treatment. BEI is the preferred inactivating agent for veterinary vaccines (Bahnemann 1990); however, it presents serious disadvantages given the prolonged inactivation time requirement (48 h) and its carcinogenicity. Viral inactivation by treatment with Triton X-100 is a simple, rapid and effective alternative, and might represent an attractive method to avoid the use of carcinogenic substances. Global process integration should then specify VLP purification along with baculovirus inactivation in a minimal number of steps.

Until now, PPV-VLPs have been produced using Sf9 cells cultured in the presence of serum protein. For safety reasons however, the current standard is to obviate the use of animal protein in vaccines (Hulst et al. 1993; Hulst and Moormann 1996). Therefore, serum-free production of PPV-VLPs in Sf21 cells has been undertaken in this study.

Materials and methods

Cells and virus

A clonal isolate (Sf21) of *S. frugiperda* IPBL-SF21-AE adapted to grow in serum-free medium (Hulst and Moormann 1996) was used. Cells were maintained in 500 ml (100 ml working volume) Erlenmeyer flasks operated as a repeated batch culture at 27°C and 90 rpm. Inoculum of 2–4×10^5 cells/cm^3 was used. When the cells reached 3–4×10^6 cells/ml, the culture was diluted 10 times with fresh SF900II medium (Gibco, Glasgow, UK).

Recombinant baculovirus AcAs3-PPV (Casal 1996) was adapted to grow in Sf21 cells, and was used for viral infections.

Cell growth and infection experiments

Cell growth and infection were performed in 2 l and 25 l fully controlled stirred bioreactors (Braun, Melsungen, Germany), respectively, with two Rushton turbines (standard geometry). The 2 l bioreactor was inoculated at 3–5×10^5 cells/ml with cells from the Erlenmeyer flasks. The 25 l bioreactor was inoculated at 2–4×10^5 cells/ml with cells from the 2 l bioreactor. The PO_2 was controlled at 30% by sequentially varying the agitation rate, the aeration rate and the oxygen partial pressure in the gas inlet.

Analytical methods

Cell concentration was determined by haemacytometer cell counts (Brandt, Wertheim/Main, Germany) and cell viability was evaluated by trypan blue dye exclusion (Merck, Darmstadt, Germany) in 0.4% PBS.

PPV-VLP titre was determined by ELISA using a quantitative protocol with the commercial kit INGEZIM PPV DAS (Ingenasa, Madrid, Spain).

Viral Infection

Baculovirus AcAs3-PPV encoding the VP2 gene of PPV under the control of the very late p10 promoter and the *Lac*Z gene under the control of the *Drosophila hsp*70 promoter (Casal 1996) were used for viral infections, and titre was determined by a modified plaque assay (Dee and Shuler 1997). Cells were infected at an MOI =0.05 pfu/cell.

PPV-VLP purification

Cells were harvested at different times after infection and centrifuged at 200 *g* for 15 min, resuspended in 25 mM Na_2HCO_3, pH 8.3, at a density of 2×10^7 cells/ml and lysis was allowed to occur for 20 min. Afterwards, cell debris was removed by a 10,000 *g* centrifugation step for 15 min.

For large-scale PPV-VLP purification, the bioreactor was harvested at 120 hpi (hours post infection) and processed as described above. PPV-VLPs were further precipitated by using a saturated solution of ammonium sulphate (pH 7.0), up to a final concentration of 20%. The pellet was recovered by centrifugation at 10,000 *g* for 15 min and resuspended in PBS.

Baculovirus inactivation and ultrafiltration

Baculovirus was inactivated by addition of Triton X-100 and tributyl phosphate (TBP) (Merck, Darmstadt, Germany) to concentrations of 1% and 0.3%, respectively, and leaving for 30 min at room temperature (25°C) (Rueda et al. 2001). PPV-VLPs were then diafiltered by tangential flow ultrafiltration at constant volume with 10 volumes of PBS with a 300 kDa polysulfone ultrafiltration membrane (Sartorius, Goettingen, Germany) at a constant pressure of 50 kPa (0.5 bar). Briefly, the pressure was built up with a circulation pump (model 502S; Watson-Marlow, Falmouth, England) at a cross-flow speed of 20 cm s^{-1} and the retentate containing the VLPs was totally recirculated. The temperature of the feed tank was controlled at 15°C. Finally, the product was dead-end filtered using a Millipore Durapore filter (Millipore, Bedford, Mass.) at a pressure of 100 kPa (1 bar).

SDS-PAGE analysis

Samples were mixed with an aliquot of sample buffer (0.0625 M Tris-HCl, pH 6.8) with 3% SDS, 10% glycerol, 5% dithiothreitol and 0.002% bromophenol blue and heated at 100 C for 3 min.

SDS-PAGE was carried out on 9% polyacrylamide gels with 4% stacking gels (Laemmli 1970) for 1 h at 150 V. The gel was then washed for 10 min with water and the protein bands were visualised by incubation with GelCode Blue Stain Reagent (Pierce, Rockford, Ill.) in accordance with the instructions from the supplier. A HMW-SDS Marker Kit (Amersham Pharmacia, Uppsala, Sweden) was used as protein marker.

Electron microscopy

Grids were placed on top of a drop of PPV-VLP suspension for 10 min. Negative staining was achieved by transfer to a drop of phosphotungstic acid for 3 min. The grids were blotted from the edge and air dried before examination by transmission electron microscopy.

Animals and potency testing

As a reference animal for potency testing, 400–600 g homebred female guinea pigs were used. Animals were free of PPV antibodies at the start of the experiment. The guinea pigs had access to water, hay and concentrates ad libitum. Animals were housed in groups of five guinea pigs in open boxes of about 1 m^2 on sawdust. Immunisations were based on previous work. A critical dose of 73 ng PPV-VLPs in 0.5 ml was administered intramuscularly in a single injection (Rueda et al. 2001). This vaccine was formulated using Carbopol (Carbopol 934 PH, 3×10^6 Da; Goodrich, Cleveland, Ohio) at a concentration of 4 mg/ml. Animals were bled by orbital puncture at days 0 and 14, and by cardiac puncture at day 28. As a reference for immunogenicity, PPV-VLP antigen produced and purified from baculovirus-infected Sf9 cells (Rueda et al. 2001) was used.

Serology

Anti-PPV activity was measured by haemagglutination inhibition (HI) test and indirect ELISA. Sera were stored at –20°C prior to testing. For the HI assay, sera were diluted in kaolin (1:4) and a 25% suspension of guinea-pig erythrocytes (1:2). Serial 2-fold dilutions, with a maximum of 1:16,384, of the pre-treated sera were made in U-shaped microtitre plates. A 50 µl suspension containing 4 haem-agglutinating units of PPV was added. Plates were incubated for 45 min at room temperature and 50 µl of a 1% suspension of guinea-pig erythrocytes was added to each well. After an incubation period of 1.5 h the test result was read. The HI-titre was expressed as the 10log of the reciprocal of the highest serum dilution that completely inhibited haemagglutination. Samples were considered negative if not inhibiting at dilutions of ≥1:8.

For testing of anti-PPV antibodies, a commercial indirect ELISA (INGEZIM PPV, INGENASA, Madrid, Spain) was used according to the manufacturer's instructions. Briefly, PPV-VLP-coated plates were incubated with serial dilutions of sera for 1 h at 37°C. After washing, plates were incubated for 1 h at room temperature with peroxidase-labelled protein A (Sigma) diluted 1:5,000. Bound enzyme was detected by adding 2,2′-azino-di[3-ethyl-benzthiazolinesulfonat(6)]diammoniumzout (Sigma) as substrate and stopped after 10 min with 2% SDS.

Neutralising antibodies in guinea pig sera were assayed in a micro virus neutralisation (VN) test using a dilution of 1:1,500 of the PPV10-p85 INGENASA PPV virus (Kamstrup et al. 1998). Sera were tested in a 2-fold dilution starting at 1:100. Serum-virus mixtures were incubated for 2 h prior to addition of a monolayer of ST cells. After an incubation of 5 days at 30°C, the presence of virus was revealed by immunoperoxidase staining with a PPV-positive rabbit serum after fixation of the monolayer with alcohol as previously described (Botner et al. 1994).

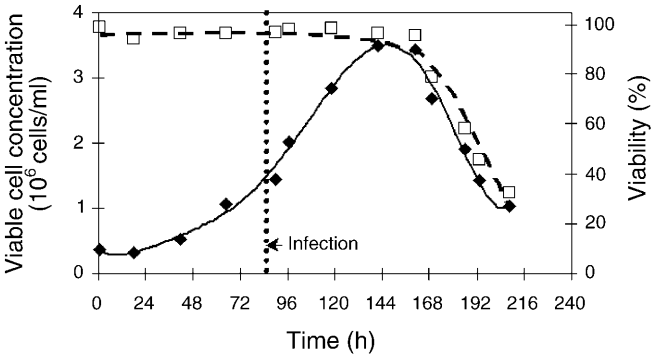

Fig. 1 Growth curve of infected insect cells in a 25 l bioreactor for porcine parvovirus virus-like particle (PPV-VLP) production. The cells were infected at 1.5×10^6 cells/ml with a multiplicity of infection (MOI) =0.05. *Black diamonds* Viable cell concentration, *white squares* viability

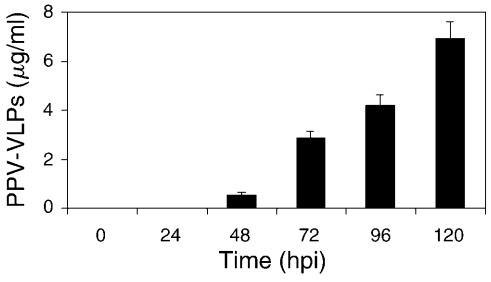

Fig. 2 Product expression profile at different times after infection in a 25 l bioreactor. The cells were infected at 1.5×10^6 cells/ml with an MOI =0.05. The bioreactor was harvested at 120 h post infection (hpi)

Results

Process development

The growth curve (Fig. 1) of the bioreactor run shows that the use of a low MOI is a feasible option for large scale (25 l) production. The cells were infected at a cell concentration at infection (CCI) of 1.5×10^6 cells/ml and grown to a maximum cell concentration of 3.6×10^6 cells/ml. This maximum cell concentration is still lower than the maximum cell concentration attainable in uninfected cultures (5×10^6 cells/ml, data not shown). The use of low MOI at this scale was reproducible; several bioreactor runs were conducted with similar growth curves.

Product expression is only detected at 48 hpi (Fig. 2) and product accumulates intracellularly until 120 hpi. Laboratory tests have shown that the recovery starts to decrease after 120 hpi, probably due to PPV-VLPs disassembling at acidic pH after cell lysis. It was therefore decided to harvest the bioreactor at 120 hpi.

A sharp decrease in cell viability was observed after 72 hpi, most probably due to some toxicity effect of the intracellular accumulation of PPV-VLPs, although a productivity increase was still achieved at the late culture period. This highlights the fact that cell death, here measured as trypan blue uptake, could mask the real cell

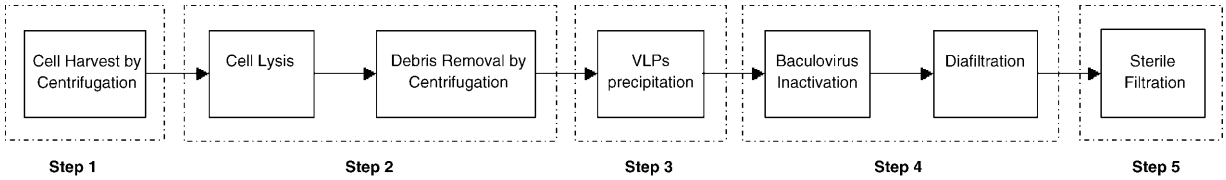

Fig. 3 Diagram of PPV-VLP downstream process developed and tested in this work

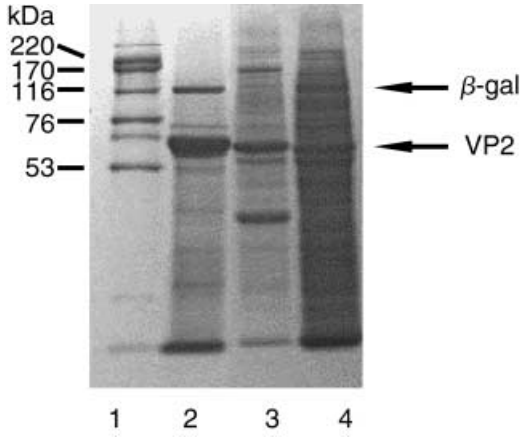

Fig. 4 SDS-PAGE gel of product purification. Lanes: *1* Molecular weight markers, *2* product after step 4, *3* product after step 3, *4* product after step 2. Process steps are defined in Fig. 3

physiological state after baculovirus infection due to membrane integrity being compromised after infection as the cell volume increases.

PPV-VLP purification

In accordance with the constraints defined in the introduction, a process scheme was designed and verified for low cost PPV-VLP purification (Fig. 3). The methodology followed was to first concentrate the product from the culture broth of the 25 l fermenters 5-fold (step 1). A volume reduction of 83% (from 25 to 4.2 l) was accomplished, decreasing the scale of equipment required in subsequent stages.

The cells were then lysed and the PPV-VLPs were stabilised at a basic pH. Large debris as contaminants were removed by a first centrifugation (step 2) and the product was further concentrated by salting-out with ammonium sulphate (step 3). The subsequent inactivation was integrated with diafiltration (step 4) in order to increase process performance by minimising the number of steps required. Good recoveries were obtained at all stages, with yields ranging from 81 to 95% (Table 1). The diafiltration step showed a high yield (88%) and no product was detected in the filtrate. Product losses were due to binding to the ultrafiltration membranes, which can be ascribed to the 10-volume renovation at diafiltration.

Baculovirus inactivation and capsid integrity

After downstream processing according to Fig. 3, PPV-VLPs were plaqued to confirm baculovirus inactivation. No virus was detected in any dilution (from 10^{-1} to 10^{-5}). However, cells died in the first dilutions (10^{-1} and 10^{-2}) due to the detergent present in the sample, even after diafiltration. SDS-PAGE analysis (Fig. 4) showed that the material was highly enriched with VP2 capsid protein.

Electron micrographs of PPV-VLPs show the presence of ample amounts of capsids, in addition to debris thought to be residual material from the cellular production (Fig. 5). As reported by Rueda and co-authors

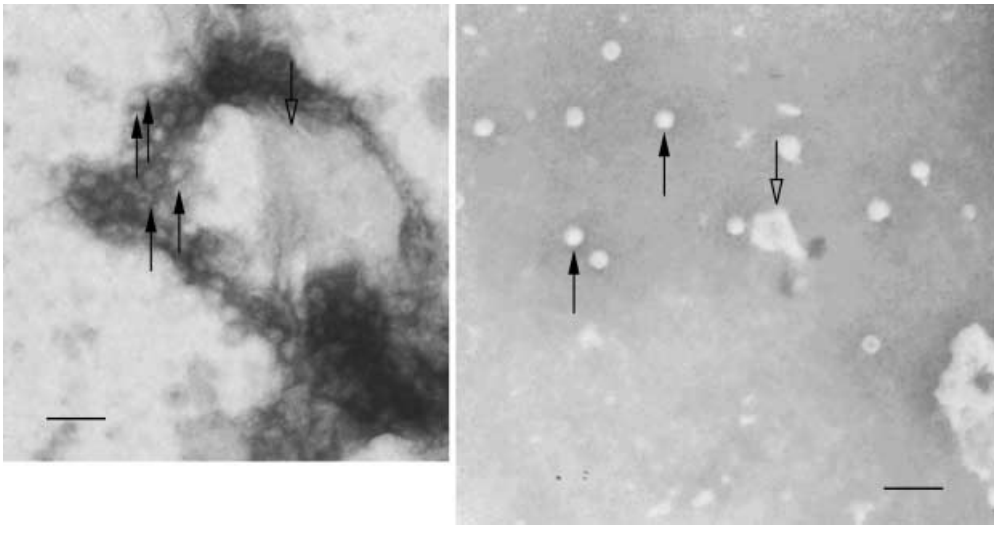

Fig. 5 PPV-VLPs from Sf21 cells. Ample intact PPV-VLP particles are present (*arrows*). *Open arrow* points to debris thought to be residual cellular material from the baculovirus-insect cell production system. Phosphotungstic acid stain. *Bar* 100 nm

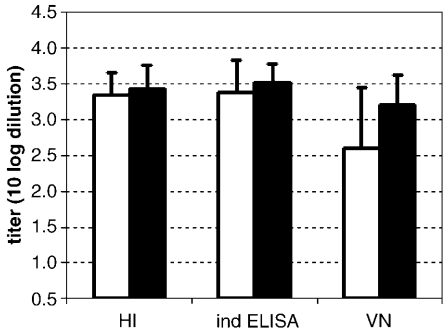

Fig. 6 Anti-PPV activity of large-scale PPV-VLPs in guinea pigs. Immunogenicity of large-scale PPV-VLPs from Sf21 cells was compared to a reference product processed and highly purified from Sf9 cells. Dose of PPV-VLPs administered was 23 ng. The materials were injected once into guinea pigs. Test results are expressed as average ±SD (n =5 animals per group). *HI* haemagglutination test, *ind ELISA* indirect ELISA, *VN* virus neutralisation test. *Open bars* Group of animals immunised with reference antigen, *closed bars* group with large-scale product. At day 0, antiviral activities were below detection level. Titres are expressed as 10log of the serum dilution factor

Table 1 Determination of the yield of each step in porcine parvovirus virus-like particle (PPV-VLP) purification. Process steps are defined according to Fig. 3

Step	1	2	3	4	5
Volume (cm^3)	4,200	4,200	410	5,032	4,780
VLPs ($\mu g/cm^3$)[a]	–	42	350	25	25
Yield (%)	–	–	81	88	95
Global yield (%)	–	–	–	–	68

[a] VLP concentration measured by ELISA

(Rueda et al. 2001) inactivation with Triton X-100 and TBP affects neither capsid stability nor immunogenicity.

Potency testing in guinea pigs

Two groups of guinea pigs were immunised with PPV-VLP preparations, one with reference antigen, and the other with the large-scale product. In HI, indirect ELISA, and VN tests, the immunogenicity of the samples was comparable, leading to 10log titres between 2.6 and 3.5 (Fig. 6).

Discussion

Process design is an important step when developing feasible and economic veterinary vaccines. Constraints in the final price of the antigen impose the use of rational approaches in products derived from biotechnology. The goal of minimising the number of process steps is a prerequisite if industrial application is intended. In this work, the use of a low MOI with the baculovirus-insect cell system was efficiently applied to the production of PPV-VLPs. Batch fermentation processes traditionally employ intermediate to high MOI, necessitating two parallel scale-up processes – one for cells and one for virus (Wong et al. 1996). Using a low MOI, it is possible to infect large bioreactors directly from a frozen stock. In our case we used a virus inoculum volume of 15 ml for a bioreactor volume of 25 l. If an MOI of 5 was used a virus inoculum volume of 4–20% of total bioreactor volume would have been necessary, which could lead to an increase in process complexity (Maranga et al. 2002). A virus concentration step before infection could have been performed instead to avoid the addition of spent medium to the reactor vessel; this option has been reported for very high cell concentrations (Elias et al. 2000). However, another step would need to be included in the production scheme, leading to increased complexity and costs.

Veterinary vaccines do not impose the same final purity requisites as human vaccines, thus purification schemes should reflect that fact. Although inactivated natural virus vaccines are not very well characterised, it is commonly accepted that the safety and efficacy of biotechnology derived products is increasingly demanding. For this reason, a simple but reliable purification scheme for PPV-VLPs, including a baculovirus inactivation step, was tested and its applicability was demonstrated. Globally, the process follows a CiPP strategy (capture, intermediate purification, polishing). The process was designed taking only the molecular weight and density properties of the particles into account.

Product capture by centrifugation was a reliable method since other inexpensive techniques like sedimentation led to high product losses (data not shown). Stabilisation of PPV-VLPs was integrated with cell lysis in a hyposmotic buffer.

The use of ultrafiltration in downstream processing of VLPs has been described previously (Hu et al. 1999; Cruz et al. 2000). In our study similarly high recoveries were obtained (>85%).

After final purification (step 4) some impurities are still observed (Fig. 4). Although this could pose some problems for the registration and safety requirements of the vaccine, it is generally accepted that veterinary vaccines do not impose the same regulatory requirements as human vaccines. Highly pure PPV-VLPs can be obtained by gel-filtration chromatography (Rueda et al. 2001), but the inclusion of this expensive chromatographic technique would make a cost-effective baculovirus-derived veterinary vaccine extremely difficult to achieve.

Nevertheless, to register a vaccine it is necessary to fulfil several regulatory requirements, and it is particularly important to be sure that there is no replicative agent, such as live baculovirus. After VLP precipitation (step 3, Fig. 3), a reasonably high concentration of baculovirus was detected by plaque assay and PCR (data not shown); therefore, in order to avoid the spread of genetically-modified microorganisms, it has been necessary to introduce an inactivation step in the purification process. Different methods of baculovirus inactivation have been reported (Rueda et al. 2001). Since baculovirus is an

enveloped virus, Triton X-100 is a simple method able to disrupt the lipid viral envelope without affecting the protein-protein interactions that maintain the VP2 particle structure of the PPV-VLP. Indeed, this procedure was selected in the VLP downstream process because Triton X-100 is not very expensive and the use of carcinogenic agents such as BEI can thus be avoided. Nevertheless, BEI is one of the most commonly used inactivating chemical substances in veterinary vaccines.

Although the data concerning the yield of the existing inactivated PPV vaccine is not known, our process has a yield of ten doses/ml of culture medium, which can be defined as very cost-effective.

Several studies have pointed out that a long term vaccination program is a cost effective method for controlling PPV-induced reproductive failure in pigs (Parke and Burgess 1993; Gardner et al. 1996); the process described in this work could be applied to large-scale production of a PPV vaccine to supply such a program.

Acknowledgements The authors acknowledge and appreciate the financial support received for the project from the European Commission (BIO4-CT98-0215) and a grant to L.M. from Fundação para a Ciência e Tecnologia – Portugal (PRAXIS XXI/BD/16136/98). We thank Frans Wagenaar for electron microscopy, Marieke van Es for serology, and Dr. Luuk Hilgers for assistance in vaccine preparation.

References

Bahnemann HG (1990) Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine. Vaccine 8:299–303

Botner A, Nielsen J, Bille-Hansen V (1994) Isolation of porcine reproductive and respiratory syndrome (PRRS) virus in a Danish swine herd and experimental infection of pregnant gilts with the virus. Vet Microbiol 40:351–360

Casal JI (1996) Parvovirus diagnostic and vaccine production in insect cells. Cytotechnology 20:261–270

Cruz PE, Cunha A, Peixoto CC, Clemente J, Moreira JL, Carrondo MJ (1998) Optimization of the production of virus-like particles in insect cells. Biotechnol Bioeng 60:408–418

Cruz PE, Peixoto CC, Devos K, Moreira JL, Saman E, Carrondo MJT (2000) Characterization and downstream processing of HIV-1 core and virus-like particles produced in serum free medium. Enzyme Microb Technol 26:61–70

Dee KU, Shuler ML (1997) Optimization of an assay for baculovirus titre and design of regimens for the synchronous infection of insect cells. Biotechnol Prog 13:14–24

Elias CB, Zeiser A, Bedard C, Kamen AA (2000) Enhanced growth of Sf-9 cells to a maximum density of 5.2×10(7) cells per ml and production of beta-galactosidase at high cell density by fed batch culture. Biotechnol Bioeng 68:381–388

Gardner IA, Carpenter TE, Leontides L, Parsons TD (1996) Financial evaluation of vaccination and testing alternatives for control of parvovirus-induced reproductive failure in swine. J Am Vet Med Assoc 208:863–869

Hu YC, Bentley WE, Edwards GH, Vakharia VN (1999) Chimeric infectious bursal disease virus-like particles expressed in insect cells and purified by immobilized metal affinity chromatography. Biotechnol Bioeng 63:721–729

Hulst MM, Moormann RJ (1996) Classical swine fever virus diagnostics and vaccine production in insect cells. Cytotechnology 20:271–277

Hulst MM, Westra DF, Wensvoort G, Moormann RJ (1993) Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. J Virol 67:5435–5442

Kamstrup S et al (1998) Mapping the antigenic structure of porcine parvovirus at the level of peptides. Virus Res 53:163–173

Krell PJ (1996) Passage effect of virus infection in insect cells. Cytotechnology 20:125–137

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Maranga L, Cruz PE, Aunins JG, Carrondo MJT (2002) Production of core and virus-like particles with baculovirus infected insect cells. Adv Biochem Eng Biotechnol 74:183–206

Martinez C, Dalsgaard K, Lopez de Turiso JA, Cortes E, Vela C, Casal JI (1992) Production of porcine parvovirus empty capsids with high immunogenic activity. Vaccine 10:684–690

Parke CR, Burgess GW (1993) An economic assessment of porcine parvovirus vaccination. Aust Vet J 70:177–180

Rueda P, Fominaya J, Langeveld JP, Bruschke C, Vela C, Casal JI (2001) Effect of different baculovirus inactivation procedures on the integrity and immunogenicity of porcine parvovirus-like particles. Vaccine 19:726–734

Wong KTK, Peter CH, Greenfield PF, Reid S, Nielsen LK (1996) Low multiplicity infection of insect cells with a recombinant baculovirus: the cell yield concept. Biotechnol Bioeng 49:659–666

Zheng YZ, Greenfield PF, Reid S (1999) Optimized production of recombinant bluetongue core-like particles produced by the baculovirus expression system. Biotechnol Bioeng 65:600–604