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# Chrysalises as natural production units for recombinant subunit vaccines☆

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ABSTRACT

The baculovirus vector expression system (BEVS) combines cultured insect cells and genetically modified *Autographa californica* nuclear polyhedrosis virus (*Ac*MNPV)-derived baculovirus vectors. This expression system has been widely used for the expression of hundred of proteins for more than 30 years, existing commercial products manufactured at large scale by this methodology, mainly subunit vaccines. At an industrial scale, insect cells, as any other cultured cells, require artificial media and a strict control of environmental sterile conditions in the complex and expensive bioreactors. Here we describe an efficient alternative to produce recombinant biologics using the versatile and productive baculovirus vectors. It consists in natural biocapsules (pupae from *Trichoplusia ni* (Hübner) Lepidoptera), containing millions of insect cells in perfect physiological conditions, ready to be programmed by a genetically modified *Ac*MNPV-derived baculovirus vector to produce large quantities of any recombinant protein. This technology, denominated CrisBio, has been tested to produce dozens of proteins, reaching productivities on the range of milligrams per infected pupa, that can be translated into dozens of vaccine doses, for example. The biologics production by CrisBio was industrialized with the design of both insect rearing and pupae storage single-use plastic devices, compatible with machines specifically designed for the automation of pupae manipulation and inoculation. These devices and machines reduce manual operations, increase batches consistency and facilitate the scaled production of any recombinant protein. As a mode of examples, the productivity in CrisBio technology platform of two virus-like particle (VLP) vaccine antigens is described in this work.

## 1. Introduction

Insects are the most diverse and numerous class of animals that populates the Earth and we have used them for thousands of years to obtain everyday products, such as honey, silk and, more recently, as a source of proteins for the animal food industry. They have also been key in the advancement of some scientific disciplines like *Drosophila melanogaster* to study genetic, physiology, microbial pathogenesis or evolution (Holden-Dye and Walker, 2018). Insects are amazing organisms in terms of population expansion. As a mode of example, the Lepidoptera *Trichoplusia ni* (cabbage looper; *T. ni*), extensively used to produce recombinant proteins (Guijarro-Pardo et al., 2017; Alonso-Padilla et al., 2010; Escribano-Romero et al., 2013; Gomez-Casado et al., 2011; Gomez-Sebastian et al., 2012; Perez-Filgueira et al., 2006, 2007a; Perez-Martin et al., 2010), may produce in theory, starting with a couple of moths, around 250 million individuals in three life cycles (12 weeks). Additionally, insects have a metabolic machinery unique in the animal kingdom. *T.ni* larvae growth at a daily rate of 20 % of its weight, growing 5000 times in around 10 days (from larva stage 1–5). Some insect cells have also the record for protein productivities. For example, the remarkable productivity of silk gland cells from *Bombyx mori*, where approximately 1000 cells produce up to 300 mg of silk proteins, even more protein than the most productive mammalian cell (Jha et al., 1990).

Since the late 80′, it has been demonstrated in numerous publications the efficiency of insects producing recombinant proteins (Guijarro-Pardo et al., 2017; Alonso-Padilla et al., 2010; Escribano-Romero et al., 2013; Gomez-Casado et al., 2011; Gomez-Sebastian et al., 2012; Perez-Filgueira et al., 2006, 2007a; Perez-Martin et al., 2010; Jha et al., 1990; Medin et al., 1990; Wurm, 2003; Miyajima et al., 1987; Maeda and Furusawa, 1987; Higashihashi et al., 1991; Shi et al., 1996; Utomo DIS et al., 2019). In most cases, scientists used *Bombyx mori* and *T. ni* insects for this purpose. The programming of these insects to produce the desired recombinant protein was performed by manual inoculation of larvae with the baculoviruses Bombyx mori nucleopolyhedrovirus (BmNPV) or *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), respectively. Moreover, a product for animal health based on an interferon, Virbagen Omega (Virbac; http://www.med-vet.fr/medicament-virbagen-omega-10-mu-p1266), was approved by the EMA in 2004 for its commercialization in Europe. Despite the productivity of this

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methodology to produce recombinant biologics, the use of insects as living biofactories was never fully implemented at industrial scale as it happened with the production in tobacco plants (https://www.medicago.com/en/). The improvement of synchronized insect rearing at high concentrations, the reduction or elimination of manual procedures in rearing and inoculation processes and the optimization and definition of the production batches are essential requirements for establishing a robust industrial process based on the use of insects for production of recombinant proteins.

In the present publication we describe the use of *T. ni* insect pupae as natural biocapsules to produce subunit vaccines. We have developed a robust and scalable process which includes single-use plastic devices for insect rearing and pupae stockpiling and transportation, as well as robotic solutions for silk elimination from the pupae and their manipulation and inoculation.

Here we describe, as a mode of example, the production of two virus-like particle (VLP) vaccines against the *Porcine circovirus* type 2 (PCV2) and the calicivirus producing the *Rabbit hemorrhagic disease virus* (RHDV) using CrisBio platform. PCV2 affects domestic pigs worldwide (Segales et al., 2005) and its viral particle is composed by a single structural protein (Cap), encoded by ORF2 (Nawagitgul et al., 2000). Rabbit hemorrhagic disease (RHD) is a highly contagious and lethal infection that affects both wild and domestic rabbits (*Oryctolagus cuniculus*) (Abrantes et al., 2012). Its etiological agent, the rabbit hemorrhagic disease virus (RHDV), is included in the Caliciviridae family (Ohlinger et al., 1990; Parra and Prieto, 1990). The genome of RHDV encodes for the coat protein (VP60), with a molecular weight of 60 kDa, which is able to self-assembly forming VLPs (Barcena et al., 2004).

## 2. Materials and methods

### 2.1. Insect rearing and pupae production

The implementation of controlled rearing conditions leads to the production of pupae batches at the same physiologic stage which confers high levels of productivity and high consistency batch to batch.

Disposable insect rearing boxes were seeded with a known amount of *T. ni* eggs deposited in a filter paper. The rearing boxes were then incubated for 8 days at 21−27 °C until pupation in 50–70 % of humidity conditions. The eggs hatched and evolved in these conditions from larval stage 1–5 and then enter in pupation in around 10 days. Insect diet was replaced along these days twice and the waste produced by insect larvae was cleaned periodically.

Once the pupation was completed, the silk was removed by a mild chemical alkaline treatment for three minutes in a specifically designed machine. After this process, pupae were rinsed with abundant water to remove chemical traces. Then, the pupae were dried and allocated automatically in disposable plastic trays for pupae storage and transportation.

These plastic trays containing 160 pupae were RFID labeled with the information about the expiry of the pupa and potentially with information about the virus and dose to be used in the inoculation process. Pupae in trays were stored up to 1 month refrigerated, keeping all production capacities.

### 2.2. Baculovirus generation

Two recombinant baculoviruses expressing the Cap antigen from the PCV2 and the VP60 from RHDV were developed in the present work to model CrisBio technology's productivity. Both antigens were shown to form VLPs when expressed in insect cells.

The most predominant sequence for the CapD protein published in the NCBI database was selected (GenBank ascension number: ABV21950.1 and 639 more entries). The resulting sequence defined was:

MTYPRRRFRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFN-TRLSRTIGYTVKKTTVRTPSWNVDMMRFNINDFLPPGGGSNPLTVPFEY-YRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKANALTYDPYVNYS-SRHTITQPFSYHSRYFTPKPVLDRTIDYFQPNNKRNQLWLRLQTTGNVD-HVGLGTAFENSIYDQDYNIRITMYVQFREFNLKDPPLNPK

The VP1 (also named VP60) protein from the RHDV genotype a (Perez-Filgueira et al., 2007b) was the following:

MEGKARTAPQGEAAGTATTASVPGTTTDGMDPGVVATTSVVTAEN-SSASIATAGIGGPPQQVDQQETWRTNFYYNDVFTWSVADAPGSILYTVQ-HSPQNNPFTAVLSQMYAGWAGGMQFRFIVAGSGVFGGRLVAAVIPPGIE-IGPGLEVRQFPHVVIDARSLEPVTITMPDLRPNMYHPTGDPGLVPTLVLSV-YNNLINPFGGSTSAIQVTVETRPSEDFEFVMIRAPSSKTVDSISPAGLLTTP-VLTGVGNDNRWNGQIVGLQPVPGGFSTCNRHWNLNGSTYGWSSPRFA-DIDHRRGSASYPGSNATNVLQFWYANAGSAIDNPISQVAPDGFPDMSFV-PFNGPGIPAAGWVGFGAIWNSNSGAPNVTTVQAYELGFATGAPGNLQP-TTNTSGSQTVAKSIYAVVTGTAQNPAGLFVMASGVISTPSANAITYTPQP-DRIVTTPGTPAAAPVGKNTPIMFASVVRRTGDVNATAGSANGTQYGTG-SQPLPVTIGLSLNNYSSALMPGQFFVWQLTFASGFMEIGLSVDGYFYAGT-GASTTLIDLTELIDVRPVGPRPSKSTLVFNLGGTANGFSYV

The encoding sequences for these proteins were synthesized by the company GenScript. The codon usage of the CapD encoding gene was optimized for its expression in insect cells (OptimumGene™-Codon Optimization algorithm). Both sequences contained the adequate flanking regions to facilitate their cloning in the donor plasmid TB3.2 (López-Vidal et al., 2015), containing an expression cassette which boost recombinant protein productivities in insect cells (Gómez-Sebastián et al., 2014). Once the TB3.2-modified donor plasmids with the CapD or VP1 genes were obtained, the Bacmids for the generation of the different baculoviruses were prepared in *E. coli DH10Bac* bacterial cells containing the mini Tn7-replicon. Then, the transfection of the bacmids in the regulatory Sf9-RVN cells (Glycobac, USA) was performed and a viral clone selection was made by two rounds of plaque cloning in order to obtain the passage I of the recombinant baculoviruses. Later, the virus seeds were passaged to obtain the working virus stocks. The baculovirus genome regions containing the expression cassette and the two foreign genes were sequenced to determine the integrity of the Cap and VP1 genes in the respective recombinant baculovirus. The resulting baculovirus were named ALG TB3.2 204 and ALG TB3.2. 131 for CapD and VP60 respectively.

### 2.3. Pupae inoculation

Plastic trays containing the pupae were allocated in the inoculation robot. This machine has an arm with a needle which moves along the plastic trays. The needle inoculated the pupae through small holes in the top of the trays, just above every pupa. The robot dispensed a maximum of 5 μl containing the adequate virus quantities previously determined experimentally to produce the maximum productivity of recombinant protein in a specific pupae incubation time. The inoculation speed was around 3000 pupae/h.

### 2.4. Recombinant protein expression and purification

After pupae infection, the plastic trays were allocated in chambers controlling temperature and humidity and incubated for 3–7 days depending of the protein an virus dose. After this period, the pupae were collected and stored under vacuum in plastic bags and frozen, before downstream processing. Recombinant protein contained in the frozen pupae was stable for at least 2 years in these conditions.

### 2.5. Protein extraction and VLP analysis

*T. ni* pupae containing the recombinant proteins were homogenized in extraction buffer containing phosphate-buffered saline (PBS), a reductant agent, a protease inhibitors cocktail, salts and detergents with the most

appropriate pH for each protein. Then, subsequent steps of clarification, diafiltration, purification and ultrafiltration were carried out in order to obtain purified VLPs.

Protein concentration, yield and level of purity were determined by SDS-PAGE analysis using 4–20 % or 12 % Mini-Protean TGX precast gels from Bio-Rad. Gels were stained with QC Colloidal stain (3 ng of sensitivity) in the case of concentration and yield evaluation and with SYPRO Ruby (1 ng of sensitivity) in the case of level purity analysis, both from Bio-Rad. Recombinant Cap and VP60 proteins produced in pupae were measured by band densitometry with the ChemiDoc™ XRS Gel Imaging System using Image Lab™ software (Bio-RadTM). A BSA standard curve was used for quantification.

Electron microscopy analyses were performed by conventional means. Briefly, purified VLPs (approximately 5 μ l) were applied to glow-discharged carbon-coated grids for 2 min. Samples were negatively stained with 2% (w/v) aqueous uranyl acetate. Micrographs were recorded with an EM 2000 Ex microscope (JEOL, Japan).

## 3. Results

### 3.1. CrisBio automated process for recombinant protein production

Fig. 1 shows a schematic representation of the protein production process by CrisBio technology using recombinant *Ac*MNPV baculoviruses and *T. ni* pupae. One of the limitations of insect rearing is the possibility of growing them in large concentrations and synchronizing their evolution from hatched egg to pupa stages. It can be only achieved providing to the insects a continuous access to the artificial diet to avoid niche competition. At larval stage, *T. ni* has a daily weight gaining of around 20 %. Differences in feeding and rearing temperatures may affect the evolution times required to reach the pupa stage, which is the production unit. In addition, it is important to reduce as much as possible the manual procedures related to artificial insect diet replacement and the cleaning of the waste generated by the larvae that could produce bacterial or fungi contaminations of the insect population. For that purpose, it has been developed a disposable insect rearing box with capacity to support the growing of up to 300 insects (Fig. 1, step 1). the number of insects per box was optimized to the continuous access of the insects to the diet ensuring a homogeneous insect size and evolution.

With this rearing box, the first step in CrisBio technology consisted in placing a known number of *T. ni* eggs in the rearing boxes, which hatched and evolved to the pupa stage in the same space. This rearing box was designed with aeration surfaces to reduce the humidity inside the box and internal walls facilitating the access of larvae to the diet. Insect larvae were self- distributed to occupy its own niche without competition. Upper and lower lids contain the inset diet and the frass respectively. Both lids are removable without manipulating the larvae.

Once the insects reached the pupae stage, the rearing boxes were introduced in an especially designed machine that automatically removed the silk from the cocoon by a mild alkaline chemical treatment (Fig. 1, step 2). Then, pupae were rinsed in the same machine with abundant sprayed water and then dried. The third step of CrisBio process consisted in the allocation of the de-silked pupae into disposable plastic trays for their storage and transportation (Fig. 1, step 4). For this purpose, single-use plastic trays were designed. The trays are composed by 160 alveoli to accommodate the pupae and a perforated top lid to facilitate the aeration and the inoculation of the pupae with the baculovirus. The manipulation of the pupae was carried out by another newly designed machine containing a Delta robot (Omron corporation), and an intelligent visual recognition system allowing the selection of the pupae by shape and size criteria (Fig. 1, step 3). The robot disperses the pupae, analyses their quality by physical criteria and discards those which not comply minimum requirements (Fig. 4). The robot arm picks and places the selected healthy pupae by a vacuum system at high speed in the alveoli of the plastic trays, previously RFID tagged with the information needed for the pupae traceability and inoculation instructions. The disposable trays, containing the living pupae, were then stored for weeks at 4 °C, keeping all the production capabilities of the pupae intact.

Finally, the fourth step of CrisBio technology consisted in the inoculation of the pupae allocated in the disposable pupae plastic trays. These trays, compatible with a specially designed baculovirus inoculation robot (Fig. 1, step 6), were allocated in the machine and injected with the previously established dose of the recombinant baculovirus vector by a needle connected to a precision pump which dispenses microliter volumes. After inoculation, the infected pupae were incubated for several days at a previously determined temperature (Fig. 1, step 7). Infection dose and incubation conditions were determined protein by protein in experiments analyzing the kinetics of specific protein synthesis under different conditions. Then, pupae were collected and stored frozen in plastic bags under vacuum until downstream processing (Fig. 1, steps 8–

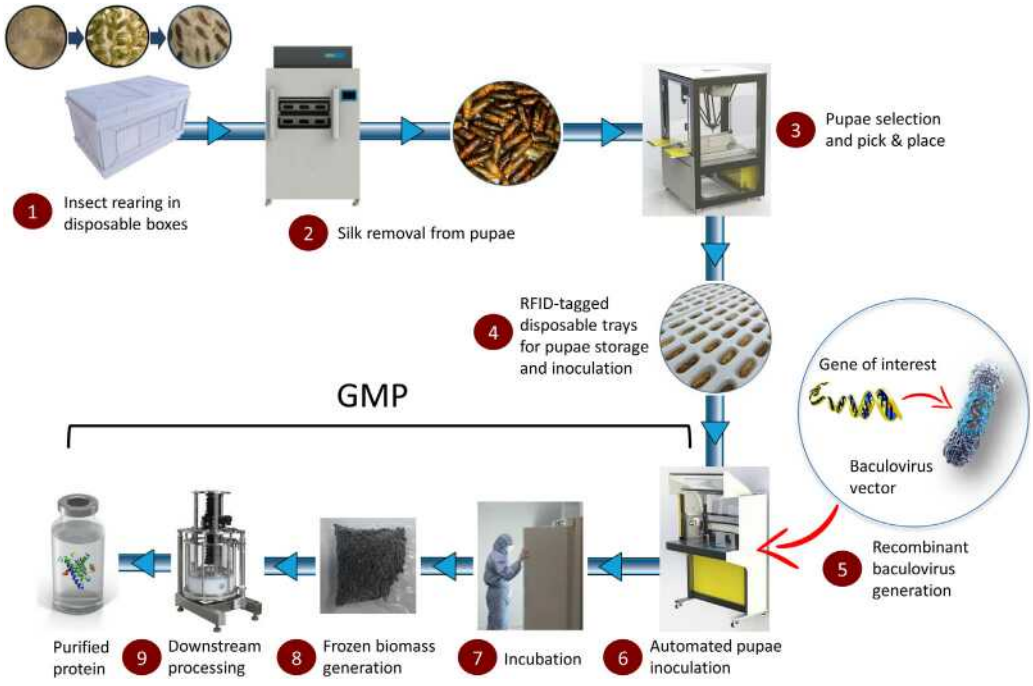


**Fig. 1.** Schematic CrisBio-based recombinant subunit vaccine production process described in 9 steps, from insect rearing and pupae production to recombinant protein extraction and purification. Step 1: Chrysalises production in disposable insect rearing boxes. Step 2: elimination of silk from chrysalises. Step 3 and 4: Pick & place of pupae into RFID-labeled plastic trays. Step 5: recombinant baculovirus generation. Step 6: automated pupae inoculation with the recombinant baculovirus. Step 7: Infected pupae incubation. Step 8: infected pupae harvesting and freezing. Step 9: recombinant protein purification.

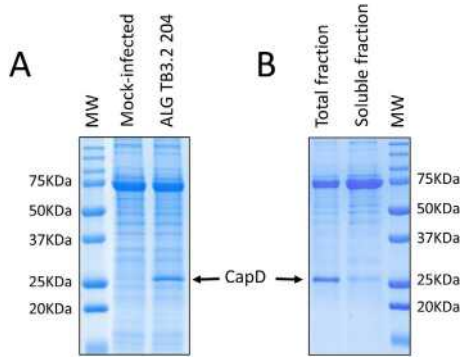


**Fig. 2.** CapD protein expression in CrisBio. A) The crude extracts (TF) from infected pupae were loaded in a SDS-PAGE gel. A standard curve was created with densitometry measurement of BSA standards and used to calculate estimates CapD expression by gram of infected biomass. B) Analysis of the CapD protein solubility in absence of non-ionic detergents.

9).

The expression of dozens of recombinant proteins have been tested in CrisBio technology. Here, we summarize the expression of two well-known vaccine antigens forming virus-like particles (VLP), describing their productivity in the insect pupae platform.

### 3.2. Production of Porcine circovirus type 2 VLP-derived vaccine in pupae

Different doses of the baculovirus ALG TB3.2 204 expressing the CapD protein were used to inoculate pupae batches. Infected pupae were incubated during different days at several temperatures, determining the optimal production conditions (not shown). The CapD protein was expressed as a major single band of 26KDa in extracts resolved in SDS-PAGE gels (Fig. 2A). At the optimal expression conditions, the productivity was around 5 mg ± 11 % of recombinant protein per gram of infected biomass measured in 5 independent production batches and calculated by densitometry of stained gels using a BSA curve. When the proteins were extracted from pupae with a buffer PBS pH7.2, the CapD protein remained mainly in the non-soluble fraction of the extracts (Fig. 2B).

Then, to purify the recombinant protein, hypothetically forming VLPs, we established a general protocol shown in Fig. 3A. CapD could be easily solubilized from the insoluble fraction using a buffer with a high salt concentration and in the presence of a low percentage of a non-ionic detergent, eliminating a large part of the contaminants. The rest of the downstream was improved introducing clarification, diafiltration and filtration steps, resulting in a simple and cost-efficient process, lacking chromatographic steps, with an overall yield of around 70 % and antigen purity between 70–80 %, more than sufficient for an animal vaccine (Fig. 3B).

In order to demonstrate that CapD is capable of forming VLPs when expressed in *T. ni* pupae we carried out an analysis of the purified CapD by transmission electron microscopy (negative staining). The three-dimensional structure adopted by the PCV2 VLPs corresponds to an icosahedron (20-sided polyhedron) with a diameter of 17−22 nm. Fig. 3C shows an image taken from a sample of purified VLPs. A large number of structures, corresponding in shape and size with the VLPs described for the porcine circovirus type 2, could be seen, confirming the viability of the CrisBio technology for the production of Cap-derived VLPs.

Based on the productivities of the CapD protein reached with CrisBio technology and the purification yields in the described downstream process, we may conclude that the productivity of a pupae tray is around 112 mg of CapD protein. It represents a productivity of about 28 g of CapD per every 475 L capacity incubator, every 5 days. This amount of recombinant protein may be used to formulate 1,120,000 vaccine doses, calculated considering 25 μg of VLPs per dose. With a single incubator, working for a year in a continuous process, it would be possible to produce

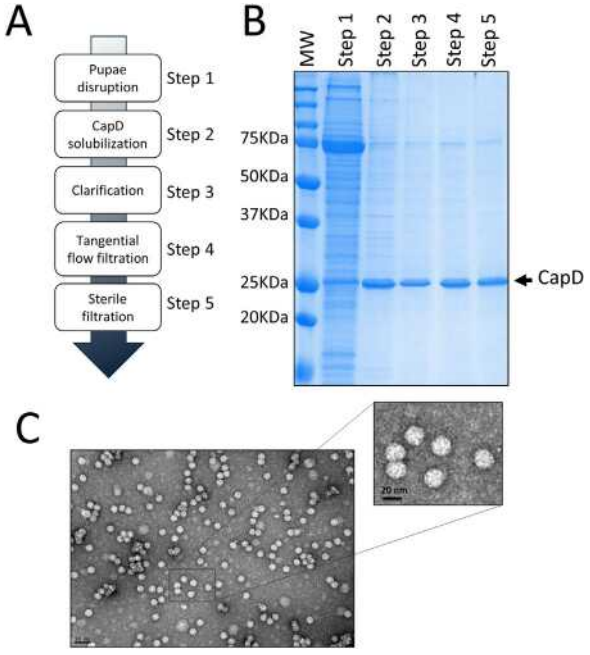


**Fig. 3.** Purification of CrisBio-derived CapD VLPs. A) General scheme of Cap D purification starting from infected pupae. B) Analysis of the resulting purity of CapD protein at the different downstream process steps. C. Electron micrograph of the final CapD vaccine product showing the resulting VLPs.

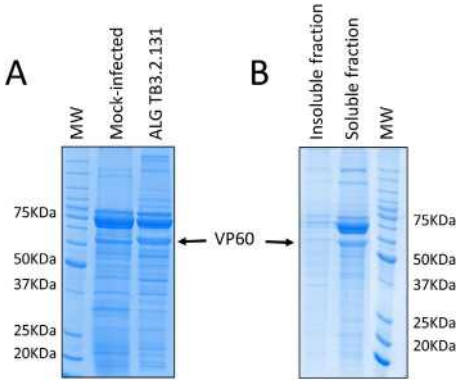


**Fig. 4.** VP60 protein expression in CrisBio. A) The crude extracts (TF) from infected pupae were loaded in a SDS-PAGE gel. A standard curve was created with densitometry measurement of BSA standards and used to calculate estimates VP60 expression by gram of infected biomass. B) Analysis of the VP60 protein solubility in absence of non-ionic detergents.

58.2 million doses of PCV2 vaccine.

### 3.3. Production of a Rabbit hemorrhagic disease virus (RHDVa) VLP-derived vaccine

As carried out with the CapD antigen, different doses of the baculovirus ALG TB3.2. 131 expressing the VP60 protein were used to infect pupae batches. Infected pupae were incubated during different days at several temperatures, determining the optimal production conditions (not shown). The VP60 protein was expressed as a major single band of 60KDa in extracts resolved in SDS-PAGE gels (Fig. 4A). At the optimal expression conditions, the productivity was in average of around 6.2 mg ± 7% of recombinant protein per gram of infected biomass, measured in 5 independent production batches and calculated by densitometry of stained gels using a BSA curve. When the proteins were extracted from pupae with a buffer PBS pH7.2 > 90 % of the VP60 protein remained in the soluble fraction of the extracts (Fig. 4B).

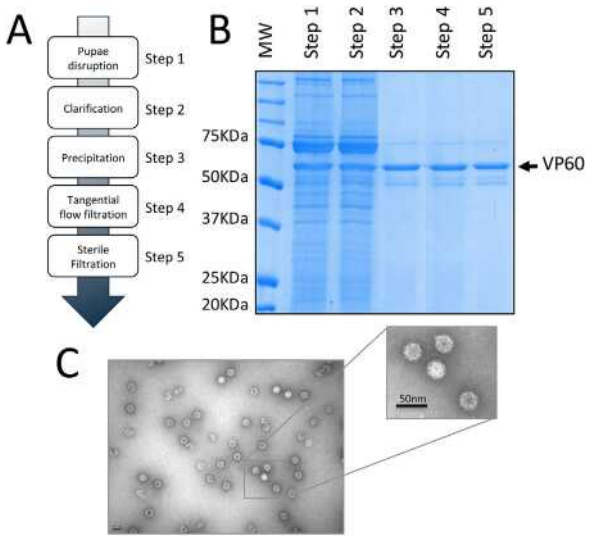


**Fig. 5.** Purification of CrisBio-derived VP60 VLPs. A) General scheme of VP60 purification starting from infected pupae. B) Analysis of the resulting purity of VP60 protein at the different downstream process steps. C) Electron micrograph of the final VP60 vaccine product showing the resulting VLPs.

To purify the recombinant VP60 protein, we established the protocol shown in Fig. 5A. VP60 could be easily precipitated from the soluble fraction using ammonium persulfate in order to reduce the insect-derived contaminants, mainly hexamerins (the most abundant proteins in pupae extracts). The rest of the downstream consisted in a tangential flow filtration and an additional filtration steps to reach an sterile vaccine preparation. This downstream process, free of chromatographic steps, was simple and cost-efficient, with an overall yield of around 70 % and antigen purity >80 %, more sufficient for an animal vaccine (Fig. 5B).

RHDV mature virions are spherical, non-enveloped, icosahedral particles of 32−40 nm in diameter, whose capsid consists of 90 dimers of capsid protein VP60. In order to demonstrate that the VP60 protein is capable of forming VLPs when expressed in *T. ni* pupae we carried out an analysis of the purified VP60 by transmission electron microscopy (negative staining). Fig. 5C shows an image taken from a sample of purified VLPs. A large number of structures could be seen that corresponding in shape and size with the VLPs described for the rabbit virus, confirming the viability of the CrisBio technology for the production of RHDV VLPs.

Based on the productivities of the VP60 protein reached with CrisBio technology and the purification yields with the described downstream process, we may conclude that the productivity of a pupae tray is around 138 mg of VP60 protein. It represents a productivity of about 34.5 g of VP60 per every 475 L capacity incubator every 5 days. This amount of recombinant protein may be used to formulate 6.9 million vaccine doses, considering 5 μg per dose. With a single incubator, working for a year in a continuous process, it would be possible to produce 358 million doses of RHDVa vaccine.

## 4. Discussion

The demand for biopharmaceutical products is set to see a significant increase over the next few years. As a consequence, the processes used to produce these products must be able to meet market requirements, including the efficient and economical production of human and animal vaccines. Bioreactors-based production technologies have several shortcuts, independently of the cell type cultured inside. The most relevant are the extreme complexity of the technical facilities and processes, the elevated fixed and operative costs, the limitations in scalability and flexibility and, last but not least, the technology robustness, which decreases with the sophistication of the bioreactors. The production in insect cells at large scale is not an exception and requires a high degree of technical expertise. This complexity inherent to the production in bioreactors, jointly with the elevated fixed investments required, make the production of recombinant biologics at industrial scale inaccessible to many mid-size companies or to the pharmaceutical industry in developing countries.

CrisBio technology is, in opposite to the standard operating procedures used with bioreactors, extremely simple and robust. In addition, the capital expenditures and operation costs in this technology platform are only a fraction in comparison to those required in bioreactor-based technologies. Also, the training of personnel to operate CrisBio to produce a sophisticated biotech product, can be completed in a short period of time without high technical skills requirements. This fact is extremely important in non-technically sophisticated companies or in developing countries.

Another advantage of CrisBio technology is its linear scalability, which only depends on the number of pupae infected in the pre-established conditions. The consistency of production in *T. ni* pupae is very high and comparable to the production in cultured insect cells by using the same recombinant baculovirus vector. CrisBio is not affected by media quality, relative gases concentration or cells contaminations as it happens with bioreactor-based technologies. Regularly, productivities of recombinant proteins in CrisBio, measured by recombinant protein quantities obtained per gram of fresh insect biomass, vary in average from 2 to 5 mg/g of final product, with record productivities in some cases of >10 mg/g. This can represent a productivity per pupae tray of 60–150 mg of product, enough protein to formulate thousands of vaccine doses, depending of protein quantity used per dose. An inoculation robot with a single syringe may infect 3000 pupae per hour (around 18 pupae trays). However, this robot may be designed to work with 4–8 needles, multiplying dramatically the inoculation speed. Additionally, the process is highly versatile, because the infected pupae biomass can be stored frozen at -20 °C for at least two years without observing significant recombinant protein degradations, as we demonstrated with the PCV2 vaccine antigen (data not shown). Then, it is possible to stockpile high amounts of frozen insect biomass containing the recombinant protein, generating a single batch for the downstream process. This versatility facilitates the formulation of a vaccine in an emergency situation, by stockpiling of biological material with a reduced production cost, and assuming that for most technologies, downstream purification is more expensive than upstream processes.

As it is shown in Fig. 1, the full automation of this technology includes the machine to dissolve the cocoons, the selection and pick & place of the pupae into disposable storage plastic trays by another machine device, and the automated pupae inoculation with the recombinant baculovirus using an inoculation robot. All these automations introduced in CrisBio avoided manual procedures increasing the consistency of the technology from batch to batch and also reinforcing its robustness. The capacities of the three robots designed for the pupae manipulation with CrisBio technology worked coordinately with respect to the number of individuals processed. Theoretically, infecting 18 pupae trays per hour, it is possible to produce the pupae biomass required to obtain around 2.3–2.6 g of an antigen to formulate a recombinant subunit vaccine. Meaning, in the case of RHDV, between 520,000 vaccine doses/h (5 μg/dose), and for PCV2, between 73,440 vaccine doses/h (25 μg/dose), in both cases, final product.

Because they are eukaryotes, insect cells also can process newly synthesized proteins in many different ways. Thus, recombinant proteins can be typically folded, chemically modified, trafficked, and assembled into highly authentic, soluble end products (reviewed in references (Jarvis (1997); Kost et al., 2005; Shi and Jarvis, 2007). With CrisBio production platform complex VLP structures requiring the self-assembling of one, two or three proteins have been produced, as well as other VLPs formed after protease processing of polyproteins (unpublished results). In all cases, the CrisBio-derived structures are indistinguishable in size and shape from that obtained in mammalian cells. A clear example of the competence of the pupae system to produce correctly formed VLPs

are those described in the present work and based on the self-assembling of Cap and VP60 proteins from porcine circovirus type 2 and rabbit hemorrhagic disease virus respectively.

In general, insect protein glycosylation pathways are far simpler than the corresponding pathways of higher eukaryotes (Shi and Jarvis, 2007; Jarvis, 2003). While native mammalian glycoproteins often have *complex* type *N*-glycans with terminal sialic acids, insect cell-derived recombinant glycoproteins usually have much simpler side chains, known as *paucimannosidic N*-glycans, at sites normally occupied by complex, terminally sialylated structures (Kost et al., 2005). By the analysis of the glycosylation of a human antibody expressed in *T. ni* insects we have demonstrated that it was the same that the achieved in the Sf9 cell line using the same baculovirus vectors (data not shown). The differences found in the glycosylation pattern with respect to that obtained in mammalian cells didn't affect the function of the recombinant antibody in terms of affinity or its function *in vitro*. We consider that the conclusions of the many studies realized in this particular posttranslational modification in insect cells could be applied to the production in CrisBio technology.

## 5. Conclusion

*T. ni* pupae represent an excellent alternative to cultured insect cells to produce recombinant biologics using baculovirus vectors. Significant process simplifications, costs reduction (CAPEX and OPEX), technical flexibility, practically unlimited linear scalability, speed of development and productivity are some of the advantages of CrisBio technology with respect to bioreactors-based production technologies. Last but not least, the use pupae instead of larvae for recombinant protein production has numerous advantages such as the possibility of automation of the insect manipulation and inoculation processes, the stockpiling of the pupae for several weeks before inoculating them with the baculovirus vectors (and two years after the insect biomass generation containing the recombinant protein) and the option of transporting the pupae to another location for inoculation and protein production, in a similar way that fertile eggs are used for vaccines production. All in all, CrisBio is an excellent novel platform to transform protein production to enable universal access to biotechnology products.

## CRediT authorship contribution statement

**José M. Escribano:** Project administration, Funding acquisition, Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Miguel Cid:** Methodology, Validation, Investigation, Writing - review & editing. **Edel Reytor:** Methodology, Formal analysis, Investigation. **Carmen Alvarado:** Methodology, Validation, Investigation. **María C. Nuñez:** Methodology, Investigation. **Susana Martínez-Pulgarín:** Methodology, Investigation. **Romy M. Dalton:** Methodology, Validation, Writing - review & editing.

## Declaration of Competing Interest

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors José M. Escribano, Miguel Cid, Edel Reytor, Carmen Alvarado and Romy M. Dalton are inventors in a PCT patent application filed for the use of pupae from *Trichoplusia ni* for recombinant proteins production.

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