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PRODUCTION OF RECOMBINANT PROTEINS IN INSECT CELLS

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ABSTRACT

Among the wide range of methods and expression hosts available for the heterologous production of recombinant proteins, insect cells are ideal for the production of complex proteins requiring extensive post-translational modification. This review article provides an overview of the available insect-cell expression systems and their properties, focusing on the widely-used Baculovirus Expression Vector System (BEVS). We discuss the different strategies used to generate recombinant baculovirus vectors and show how advanced techniques for virus titer determination can accelerate the production of recombinant proteins. The stable transfection of insect cells is an alternative to BEVS which has recently been augmented with recombinase-mediated cassette exchange for site-specific gene integration. We consider the advantages and limitations of these techniques for the production of recombinant proteins in insect cells and compare them to other expression platforms.

Keywords: Recombinant Protein Production, Insect Expression System, BEVS, Virus Titer Assay

1. INTRODUCTION

Many different expression systems are available for the production of recombinant proteins, each with numerous options. The production of a recombinant protein is usually motivated by an ambition to determine the protein structure, investigate its activity or search for interaction partners in order to unravel its mode of action. The simplest or most accessible system that meets minimum requirements is often chosen for initial expression studies and if successful these efforts are then scaled up for downstream applications.

1.1. Chemical Peptide Synthesis

Short linear peptides can be produced by total chemical synthesis, which has become more affordable and attractive as an option over time as laborious laboratory work has been replaced by companies offering peptide synthesis as a service. It is easier to purify peptides after chemical synthesis than after heterologous expression, because the starting material is less complex. Chemical synthesis reaches its limits when the peptide exceeds approximately 70 amino acids in length, contains a high proportion of challenging amino acids (e.g., arginine, cysteine, methionine and tryptophan) or requires post-translational modification. Although peptides containing multiple disulfide bonds can be synthesized (Reinwarth *et al*., 2012) success depends on chance and laborious procedures are required to verify the disulfide linkages.

1.2. Protein Production in Bacteria

Bacteria are usually the first type of system considered for the production of longer peptides or complete proteins, because high yields can be achieved in a short time and the cells can be propagated with relatively little effort. The production of bactericidal proteins can be challenging, although this can be achieved using specialized bacterial expression systems

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that maintain the expression vector as a low-copy-number replicon until just before the induction of gene expression so that minimal expression occurs prior to induction even when RNA polymerase is present in the cell (e.g., pETcoco™ vectors or CopyCutter™ cells). Alternatively, controlled induction can be achieved using a high-copy-number replicon combined with a promoter controlled by a non-endogenous RNA polymerase, e.g., a bacteriophage RNA polymerase that can be introduced by infection with the corresponding bacteriophage.The above strategies are only successful if the protein is mildly or moderately toxic. More potent toxins can be produced as fusion protein that abolishes toxicity, followed by the cleavage of the fusion partner after the initial purification step. This strategy can be very useful for production of small amounts of protein but becomes prohibitively expensive for large-scale production.

If the structure and/or function of the recombinant protein depend on disulfide bonds, proteolytic cleavage or any other post-translational processing, bacteria are less likely to be the ideal platform. The targeting of recombinant proteins into the periplasmic space can encourage the formation of disulfide bonds, but the yield tends to be much lower than that achieved by cytoplasmic expression. Under these circumstances, a eukaryotic expression system might be better, particularly if a signal peptide is present or an additional proteolytic cleavage step is expected without knowledge of the actual site, because these functions are often fulfilled by eukaryotic host cells.

1.3. Protein Production in Yeast

The production of recombinant proteins in yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris* combines the simple and inexpensive culture conditions of bacteria with the processing abilities of eukaryotic cells, thereby increasing the likelihood of proper folding and posttranslational modification. However, proteins with antifungal activity are difficult to produce in yeast cells and the glycosylation machinery in yeast differs significantly from human cells, resulting in hyperglycosylation which can mask the active sites of enzymes and reduce their activity, or create unusual epitopes which render the proteins immunogenic (Jayaraj and Smooker, 2009).

1.4. Protein Production in Mammalian Cells

Mammalian cells are well suited for demanding proteins, e.g., where authentic glycan structures are critical and Chinese hamster ovary cells in particular are widely used for the production of recombinant human therapeutic proteins (Kim *et al*., 2012). The fastest way to produce recombinant proteins in mammalian cells is transient transfection, although this is suitable for analytical experiments only because the most efficient transfection reagents are prohibitively expensive for large-scale applications. These limitations can be addressed by stable transfection, which also allows the selection of individual high-yielding cells to increase overall productivity. Viruses can be used as an alternative to transfection with naked DNA. This allows more efficient DNA transfer and thus higher yields, but the generation and amplification of the virus stocks requires additional resources and often increases the biosafety level of some parts of the experiment. The advantages of more sophisticated protein processing in mammalian cells are offset by the need for expensive media and equipment and the increased risk of contamination with human pathogens, making mammalian cells suitable for recombinant therapeutic proteins with a high cost of goods.

1.5. Protein Production in Insect Cells

Insect expression systems (Becker-Pauly and Stöcker, 2011) represent an adequate compromise between bacterial and mammalian systems. In insect cells, signal peptides are cleaved as in mammalian cells, disulfide bonds are formed in the endoplasmic reticulum and proprotein-converting enzymes are available for proteolytic processing. Established insect cell lines used for the production of recombinant proteins grow to higher densities than mammalian cells, thus smaller culture volumes are sufficient. Although insect cell cultures are less demanding than mammalian cells under standard laboratory conditions because shake or spinner flasks can be used and there is no need for a CO_2 atmosphere, the maintenance of sterility is equally important. In contrast to mammalian cell cultures, there is no increase in biosafety level if the heterologous gene is introduced by baculovirus infection. If the recombinant protein is derived from insects, an expression system based on insect cells is ideal unless the protein does not require post-translational modifications, in which case bacterial expression may still be the preferable option.

Although protein glycosylation takes place in all eukaryotic organisms, the glycosylation patterns differ among species. These differences are more prominent between lower and higher eukaryotes, but also present between different mammalian cell lines and can influence protein solubility, half-life, activity and interactions with other molecules (Hossler *et al*., 2009). Glycosylation in insect cells is similar but not identical to that in mammalian cells (Katoh and Tiemeyer, 2013; Altmann *et al*., 1999). The expression system should resemble the glycan patterns of the

source of the recombinant protein as far as possible, if glycosylation is a critical property of the protein that will affect its behavior. For example, the Insect Metalloproteinase Inhibitor (IMPI) representing the only known peptide capable of specifically inhibiting virulence associated microbial metalloproteinases such as aureolysin, bacillolysin, pseudolysin and vibriolysin is currently developed as a template for the rational design of new drugs (Vilcinskas, 2011). The IMPI has been reported to be glycosylated at N48 with GlcNAc2Man3, showing fucosylation to different extents (Wedde *et al*., 2007). Consequently, recombinant production of properly glycosylated peptides for functional studies required an appropriate expression system such as *Drosophila* Schneider cells (Clermont *et al*., 2004).

Signal peptides from diverse origins can direct proteins into the endoplasmic reticulum of insect cells and are properly cleaved. There are also signal peptide sequences available on expression or transfer vectors that are known to promote the efficient secretion of heterologous proteins.

Furin-type proprotein-converting enzymes have been identified in *Spodoptera frugiperda* (Cieplik *et al*., 1998), *Trichoplusia ni* (Wang *et al*., 2006) and *Drosophila melanogaster* (Roebroek *et al*., 1991; 1992) and many reports confirm the efficient cleavage of recombinant proteins in insect cells (Clermont *et al*., 2004; Metz *et al*., 2011; Smolenaars *et al*., 2005). Similarly, we have reported furin-mediated cleavage of the IMPI resulting in two peptides with distinct activities against metalloproteinases (Clermont *et al*., 2004; Wedde *et al*., 2007). Here, we demonstrate the production and cleavage of a gloverin-like antimicrobial peptide (AMP) proprotein from *Galleria mellonella* (**Fig. 1**). The full-length coding sequence of the AMP, including the endogenous signal peptide and propeptide with a proprotein convertase cleavage site, was used for expression in the *Drosophila* Expression System. While the precursor molecule was detected in the cell lysate, the correctly-processed mature AMP was secreted into the cell culture supernatant. The latter proved to be of the same size as the AMP where the coding sequences of the endogenous signal peptide and propeptide were replaced by the signal sequence of the *D. melanogaster* BiP protein. Likewise, correct processing of the gloverin-like AMP was observed in the Baculovirus Expression Vector System, when a recombinant baculovirus was generated with the Bac-to-Bac™ expression system allowing the full-length AMP coding sequence to be delivered (**Fig. 2**).

If the yields of a recombinant protein are high, the expression system may fail to process every polypeptide. Such capacity constraints can be overcome by the expression of human furins (Bruinzeel *et al*., 2002; Laprise *et al*., 1998) or lepidopteran furins (Cieplik *et al*., 1998). If the proteolytic cleavage sites of a recombinant protein are known, it is advisable to use a well-established signal peptide sequence that is processed efficiently in the host cell, fused directly to the coding sequence of the mature target protein.

2. INSECT CELL EXPRESSION SYSTEMS

If insect cells are chosen as an expression platform, it is necessary to choose between stable transfection and infection with a baculovirus vector. As discussed above for mammalian cells, transient transfection in insect cells is only suitable for the production of analytical amounts of recombinant protein.

The most widely used insect cell lines for the production of recombinant proteins following the stable integration of exogenous DNA are Schneider 2 (S2) cells from the late embryonic stages of *Drosophila melanogaster* (Schneider, 1972) and Sf-9 cells from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* (Vaughn *et al*., 1977).

2.1. Stably-Transfected *Drosophila* Cells

In the commercially available *Drosophila* Expression System (DES) (Life Technologies), which can be traced back to the work of Johansen *et al*. (1989), the exogenous gene is placed downstream of the constitutive *D. melanogaster* actin promoter (Chung and Keller, 1990) or the inducible *D. melanogaster* metallothionein promoter (Bunch *et al*., 1988; Maroni *et al*., 1986). The expression vector is then introduced into S2 cells by calcium phosphate precipitation, together with a selection plasmid containing an antibiotic resistance gene. The ratio of these two plasmids favors the expression vector to ensure that resistant cells also contain the primary transgene and to maximize the copy number of the primary transgene to achieve higher yields (Johansen *et al*., 1989). The cells are cultivated under antibiotic selection for at least three weeks to isolate stable transformants, with optional single-cell cloning and screening for high-producer subclones if there is sufficient time. If the primary transgene is controlled by the metallothionein promoter, gene expression is induced by the addition of sub-millimolar concentrations of copper ions, which do not restrict cell viability. The choice between intracellular expression and secretion depends on the purification strategy. For the secretion of proteins lacking a native signal peptide, the *Drosophila* Expression System provides the signal sequence of the *D. melanogaster* BiP protein, an immunoglobulin-binding chaperone (Kirkpatrick *et al*., 1995).

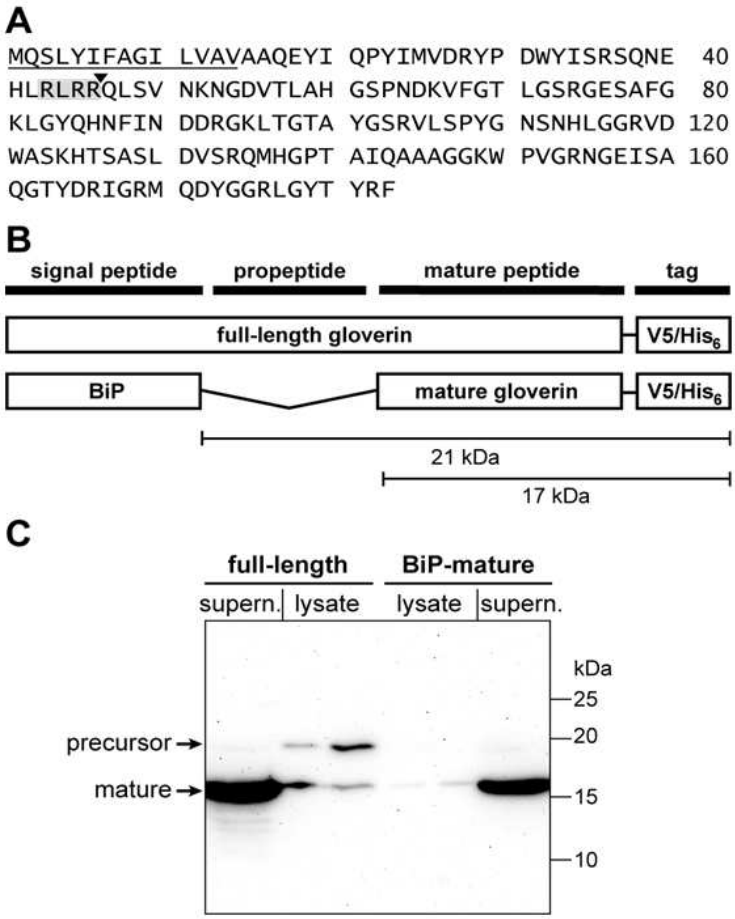

Fig. 1. Expression of G. mellonella gloverin-like AMP in the *Drosophila* Expression System. (A) Amino acid sequence of *G. mellonella* gloverin-like AMP with signal peptide (underlined), proprotein convertase recognition sequence (gray background) and cleavage site (triangle). (B) Cloning strategy: Either the full-length Coding Sequence (CDS) of the gloverin-like AMP or the CDS of the BiP signal peptide and mature gloverin-like AMP, in each case with a C-terminal V5/His_6 sequence, was inserted into the expression vector. (C) Expression of either full-length gloverin-like AMP or the mature peptide fused to BiP signal sequence was induced in stably-transfected S2 cells by addition of 500 µM $CuSO_4$. After 24 hours the cell culture supernatant was collected and the cells were lysed. After separation by SDS-PAGE, proteins were transferred to a PVDF membrane and immunostained with an anti-His_6 antibody

More detailed information and protocols can be found in the DES® manual, in Schetz and Shankar (2004) and Moraes *et al*. (2012). Those references also contain lists of proteins successfully produced in S2 cells, stating yields in the range of 0.1-20 mg per liter of cell culture.

2.2. Stably-transfected Lepidopteran Cell Lines

Although Sf-9 and High Five™ cells are typically associated with the baculovirus expression vector system, they are also used for stable transfection. High-level constitutive expression is typically achieved by using the Immediate Early (IE) promoters from Multicapsid Nuclear Polyhedrosis Viruses (MNPV). These promoters facilitate high-level expression in lepidopteran and dipteran cell lines (Hegedus *et al*., 1998; Lin and Jarvis, 2013). In the commercially available InsectSelect™ System (Life Technologies), the gene of interest is placed under control of the IE-2 promoter from *Orgyia pseudotsugata* MNPV (Theilmann and Stewart, 1992). Similarly, in the InsectDirect™ System (Novagen), the gene of interest is placed under control of the IE-1 promoter from *Autographa californica* MNPV (Guarino and Summers, 1987) together with the hr5 enhancer element (Guarino and Summers, 1986).

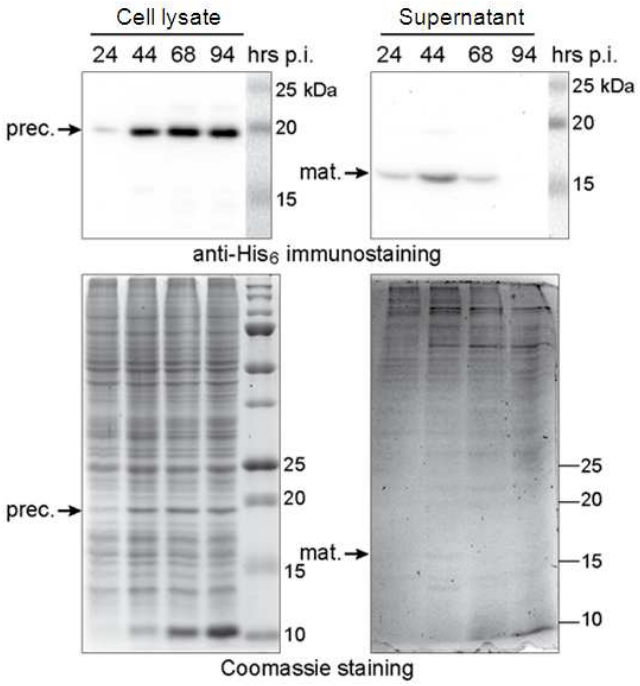

Fig. 2. Production of *G. mellonella* gloverin-like AMP with a C-terminal V5/His_6-tag in baculovirus-infected High Five™ cells. The gene was expressed from the polyhedrin promoter upon infection. At different times post infection (p.i.), the cell culture supernatant was collected and the cells were lysed. After separation by SDS-PAGE, the total protein was stained with Coomassie Brilliant Blue (lower panel) or transferred to a PVDF membrane and immunostained with an anti-His_6 antibody (upper panel). Bands representing precursor (prec.) or mature (mat.) gloverin-like AMP are indicated by arrows

Stably-transfected cells are selected using either a resistance gene cassette linked on the expression vector or unlinked on a separate plasmid. Transfection and selection results in the isolation of cell clones carrying multiple copies of the integrated transgene and there is a positive correlation between copy number and expression levels (Jarvis *et al*., 1990). The InsectSelect™ System can typically achieve yields in the range 0.2-50 mg L^{-1} (Life Technologies InsectSelect™ System manual; Gouveia *et al.*, 2010; Morais and Costa, 2003).

2.3. Site-specific Gene Integration for Stable Expression

Transfection followed by the selection of stable transformants results in a heterogeneous pool of cells with a range of productivities depending on the transgene copy number and site of integration. Maximum yields can be achieved by single-cell cloning, although this is time consuming because it involves clonal expansion from one cell to a culture size suitable for analysis and subsequent screening for high-producer clones.

To remove this time-consuming step, cell lines have been developed which allow site-specific transgene integration at a site that has proven appropriate for high-yield protein production and transgene stability. For this purpose, a reporter gene and a resistance gene cassette flanked by recombinase recognition sites are randomly integrated into the genome using standard procedures. After single-cell cloning by limiting dilution, reporter gene expression is used to screen for high-producer clones. Any gene of interest can then be integrated into the genome of this master cell line at the same site by Recombinase-Mediated Cassette Exchange (RMCE) (Turan *et al*., 2011; 2013). The cells are super-transfected with a plasmid carrying the transgene flanked by compatible recombinase recognition sites and a plasmid providing the recombinase coding sequence, allowing the transgene to be exchanged for the integrated reporter gene. The productivity of cell lines generated using this method was shown to be similar to the master cell line (Turan *et al*., 2011; 2013).

Site-specific gene integration by RMCE was pioneered for the production of recombinant antibodies in Chinese

hamster ovary cells (Huang *et al*., 2007; Kito *et al*., 2002), but has also been used in *D. melanogaster* whole insects (Horn and Handler, 2005), cultured silkworm cells (Nakayama *et al*., 2006) and recently in Sf-9 cells (Fernandes *et al*., 2012).

2.4. Baculovirus Expression Vector System (BEVS)

The Baculovirus Expression Vector System is widely used for the production of recombinant proteins in insect cells and has extensively been reviewed (Jarvis, 2009; van Oers, 2011). It is well suited for coexpression of heterologous genes in order to produce multi-protein complexes or to provide specialized proteins for enhanced processing (e.g., chaperones) (Sokolenko *et al*., 2012). The BEVS gains in importance for the production of recombinant protein vaccines, since the first substances have been approved for human use by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA). These and other vaccines in clinical development have been extensively reviewed (Mena and Kamen, 2011; Cox, 2012). Further interesting fields of application of the BEVS in human therapy are the production of Virus-Like Particle (VLP) based vaccines and the use of baculovirus as a vector in gene therapy (Rychlowska *et al*., 2011).

Gene transfer in the BEVS is facilitated by highly-efficient baculovirus infection followed by episomal replication and expression, which removes the need to select integrated transgenes and high yields are insured by the availability of strong viral promoters, particularly the *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin promoter which is activated during the very late phase of virus infection (Smith *et al*., 1983). In the wild-type virus, the polyhedrin promoter is responsible for the production of the major occlusion-body matrix protein. Another strong very late-stage promoter controls expression of the viral p10 protein (Kuzio *et al*., 1984; Williams *et al*., 1989) forming cytoskeletal-like structures whose function is still not completely understood (Carpentier *et al*., 2008).

2.5. Generation of Recombinant Viruses

Before host cells can be infected, a recombinant baculovirus is generated by inserting the transgene into a transfer vector, which is then used for recombination with the virus genome. Several different systems are available using distinct recombination strategies, each with the goal of simplifying and expediting recombination and the subsequent selection process.

Originally gene transfer was achieved by homologous recombination in insect cells. The transgene was introduced into a transfer vector with a viral promoter (e.g., the polyhedrin promoter) flanked by viral DNA sequences matching the target locus. Following the cotransfection of insect cells with the transfer vector and viral genome, homologous recombination produced recombinant baculovirus DNA in which the target locus was replaced by the transgene. Because only a small portion of cells contained the recombinant virus, this strategy incorporated a step allowing the screening of recombinants. If the polyhedrin locus was targeted, the nuclei of cells infected with the recombinant virus would not contain polyhedral occlusion bodies (occ^- phenotype) and could therefore be distinguished from the occ^+ phenotype of cells infected with parental (non-recombinant) virus. This selection procedure can be made more straightforward by replacing the polyhedrin gene of the parental virus with the bacterial *lacZ* gene, so that cells containing parental virus form blue plaques in the presence of X-gal whereas successful recombination would remove the *lacZ* gene and the plaques appear colorless. A linearized parental virus lacking part of the essential gene downstream of the polyhedrin locus (ORF1629) can significantly increase the recovery of recombinant viruses. In this case, the transfer vector carries the missing sequence of that essential gene in addition to the transgene. Only double recombination with the transfer vector (i.e., at both recombination sites) produces a circular virus genome with the essential gene restored, allowing virus replication (Kitts and Possee, 1993). This strategy is used in the BacPAK™ Baculovirus Expression System (Clontech) and in the BaculoGold™ Baculovirus Expression System (BD Biosciences), which also provides the *lacZ* gene for blue/white screening.

The restoration of a defective essential gene by recombination makes it unnecessary to select recombinant viruses using the laborious plaque assay. However, partial deletion of the essential gene is achieved by restriction digestion, which inevitably leaves a small number of intact molecules that could contaminate the virus preparation with non-recombinant virus particles. A control reaction including the parental virus but no transfer plasmid indicates the magnitude of this problem on a case-by-case basis.

An enhanced version of this technology completely eliminating the need for plaque purification is commercially available as the *flash*BAC™ Baculovirus Protein Expression System by Oxford Expression Technologies (Hitchman *et al*., 2011; Possee *et al*., 2008; Zhao *et al*., 2003). The parental virus also features a deletion of the essential ORF1629 gene, but no

restriction digestion is necessary to achieve this deletion. Because partial deletion of the ORF1629 gene makes it impossible to amplify the virus genome in insect cells, a Bacterial Artificial Chromosome (BAC) was inserted into the polyhedrin locus to facilitate propagation in bacterial cells. This virus genome isolated from bacteria cannot be replicated in insect cells unless there is recombination with a transfer vector, which complements the ORF1629 sequence and inserts the transgene into the polyhedrin locus.

An older technology also avoiding plaque purification makes use of a baculovirus shuttle vector (bacmid), i.e., a virus genome that can be propagated in *E. coli* (Luckow *et al*., 1993). This is known as the Bac-to-Bac™ Baculovirus Expression System (Life Technologies). The competent virus can be generated and selected in bacterial cells. In the first step, the transgene is inserted into a donor vector downstream of the polyhedrin or p10 promoter next to a gentamicin resistance gene. This expression cassette is flanked by the right and left arm of the Tn7 transposon, thereby forming a mini-Tn7 element. The bacmid carries a mini-*att*Tn7 target site into which the mini-Tn7 from the donor vector is inserted by site-directed transposition (Barry, 1988). The bacterial cells containing the bacmid also provide a helper plasmid encoding the Tn7 transposase, which accomplishes transposition following transformation of the cells with the donor plasmid. Bacterial colonies carrying the recombinant bacmid can be identified by blue/white screening. By inserting the expression cassette from the donor vector into the bacmid, a DNA sequence encoding the LacZα peptide is destroyed. Therefore the LacZα coding sequence can no longer complement a *lacZ* deletion on the bacterial chromosome, so the bacteria are unable to form blue colonies in the presence of a chromogenic substrate such as X-gal. After purification of the recombinant bacmid DNA from selected clones, insect cells can be transfected for the production of recombinant viruses.

In the BaculoDirect™ Baculovirus Expression System (Life Technologies) the transgene is inserted into the virus genome by lambda recombination, a property of bacteriophage λ (Nash, 1981). A mixture of recombination enzymes is used to move the transgene from the transfer vector (a Gateway® entry vector in this case) to the virus genome, inserting it between specific attachment (*att*) sites flanking the relevant DNA elements. The crude reaction mixture is then used for the transfection of insect cells, leading to the production of recombinant and parental viruses. The parental virus genome also contains the Herpes Simplex Virus type 1 Thymidine Kinase (HSV1 TK) gene within the recombination target site, allowing the negative selection of cells containing the recombinant virus (Godeau *et al*., 1992). TK expressed after the transfection of insect cells phosphorylates the nucleoside analog ganciclovir, which is added to the culture as a selection reagent. Phosphorylation in turn allows ganciclovir to be incorporated into DNA thus inhibiting DNA replication. By recombination with the transfer vector, the HSV1 *tk* gene is lost from the virus genome and the recombinant virus can be replicated in the presence of ganciclovir.

2.6. Virus Amplification and Protein Production

Although technologies for the generation of recombinant viruses differ considerably, the subsequent steps of virus amplification and protein production follow a common procedure but it is first necessary to determine the virus titer, i.e., the concentration of infectious virus particles. Although it is possible to produce recombinant proteins without this information, it would be necessary to establish standardized experimental conditions or perform yield-orientated optimization. Several different techniques are discussed below. Whichever titer assay is selected, the cell line used for titer evaluation should be the same as intended for production. Furthermore, it is advisable to use the same virus titer assay once selected, because the results from different methods are not necessarily comparable.

Because the number of virus particles in the recombination experiment is too low to infect a production culture, virus amplification is achieved by infecting a log-phase insect cell culture at a low Multiplicity Of Infection (MOI), which is the ratio of infectious virus particles to cells. The number of consecutive rounds of amplification depends on the initial amount of virus and the amplification efficiency. Virus mutation may also reduce infectivity and no more than three consecutive passages should be used because serial passaging results in the accumulation of defective virus particles with extensive mutations, requiring co-infection with wild-type virus for replication and thereby interfering with wild-type virus replication (Kool *et al*., 1991). An early virus generation should therefore be used for amplification or a new transfection experiment should be carried out. High MOI values should also be avoided for virus amplification, because the number of cells infected with both wild-type and defective viruses would increase (Zwart *et al*., 2008).

Cell culture infection for protein production usually involves a high MOI (~5) to ensure that all cells are infected simultaneously and therefore show similar and reproducible expression kinetics, but significantly lower

MOI values are beneficial for certain proteins or processes (Liebman *et al*., 1999; Steed *et al*., 1998; Wong *et al*., 1996). An MOI of 1 is not sufficient to infect all cells at once, since the distribution of viruses and subsequent infection follow statistical principles. The MOI that achieves the highest yield is dependent on the cell line, cultivation conditions and the method of virus titer determination and should therefore be determined experimentally to ensure maximum yields.

The optimal harvest time must also be evaluated experimentally, because it is strongly dependent on culture and infection conditions, the promoter and the nature of the target protein. In the example shown in **Fig. 2**, the concentration of the recombinant protein in the cell culture supernatant peaked two days post infection, but the precursor was still present at high levels in the cells two days later. This may reflect the decline in ability of the cells to process and secrete the target protein because of the virus infection and the increased proteolytic activity of the medium reflecting accumulation of enzymes released from lysing cells.

2.7. Virus Titer Determination by Plaque Assay

It is helpful to determine the titer of the available virus stock before infecting insect cells because without knowledge of this crucial parameter, it is not possible to optimize the expression conditions and achieve maximum yields or to generate comparable results under standardized conditions with different lots of virus preparations. Likewise, the virus titer is a valuable piece of information for virus amplification and the successful generation of high-titer virus stocks.

The plaque assay is often regarded as the "gold standard" for virus titer determination and instructions can be found in the literature (O'Reilly *et al*., 1994) or in the manuals that accompany baculovirus expression systems. The plaque assay is the oldest and most widely used, providing accurate results without the need for expensive and specialized equipment. However, one disadvantage is the need to cover the cell monolayer (usually in 6-well plates) with a soft agar overlay after infection, which prevents the spread of newly-released virus particles and instead keeps them at the site of formation, where progressive cell lysis will eventually result in a plaque. This is a labor-intensive procedure and care must be taken not to disrupt the cell monolayer if medium is added to prevent drying or when the cells are stained for plaque assessment. Each well needs to be evaluated manually under a microscope at the assay endpoint. This makes it difficult to adapt the plaque assay for high-throughput screening. Furthermore, removing the viral inoculum from the culture 1 h after infection may cause the number of infectious particles to be underestimated (Dee and Shuler, 1997).

2.8. End-point Dilution Assay

The end-point dilution assay is similar to the plaque assay. A cell monolayer in 96-well plates is infected with several different dilutions of the virus stock. No agar overlay is used, so the virus spreading from infected cells can move freely throughout the well and infect all the cells. After a suitable incubation time, the number of wells with infected cells among several replicates is determined for every dilution step. From these counts, the dilution factor at which 50% of the wells show signs of infection is determined, yielding a 50% tissue culture infectious dose ($TCID_{50}$) value which is proportionate but not identical to the plaque forming units (pfu) value obtained using the plaque assay. Depending on the calculation method, the pfu/$TCID_{50}$ ratio is 0.69 (Bryan, 1957; Reed and Muench, 1938) or 0.56 (Wulff *et al*., 2012). Either method is reliable, but reproducibility depends on the consistent application of one method.

The end-point dilution assay removes difficulties associated with the agar overlay and it is not necessary to count plaques, only to judge whether a well show signs of infection or not. Although the readout procedure is less time consuming and needs less experience than the plaque assay, the manual inspection of all wells near the $TCID_{50}$ dilution is required.

2.9. Virus Counter®, Transmission Electron Microscopy, qPCR

The most significant disadvantage of the methods discussed above is the long duration of the assays (5-10 days before plaques or infection can be detected reliably). Because the virus titer must be determined before cells are infected, the use of either assay will extend the duration of the experiment considerably. In order to achieve the highest possible virus count, it is also advisable to determine the virus titer during virus amplification, thereby delaying protein production even further. Frequent virus titer determination is desirable to monitor process performance and reproducibility.

The Virus Counter® is a tool for virus titer determination that achieves measurement within 1 h. The method has been commercialized but was developed by an academic institution (Stoffel *et al*., 2005; Stoffel and Rowlen, 2005). Two different dyes are added to the virus suspension, which are non-specific stains for nucleic acids and proteins, respectively. The counter itself is a specialized flow cytometer, which counts all particles that are stained simultaneously by both dyes,

representing intact virus particles (Ferris *et al*., 2011). Because this method does not discriminate between infectious and non-infectious virus particles, the resulting values (virus particles per mL) are approximately tenfold higher than the pfu values determined by plaque assays from identical samples (Ferris *et al*., 2011). The results from both methods thereby show a linear relationship. The Virus Counter® is ideal for the frequent monitoring of virus titers in the baculovirus expression system as long as it is used consistently and the virus particle counts are not mixed up with values from other methods. Nevertheless, the quality of virus preparations varies from lot to lot, introducing the possibility of varying ratios of infectious and non-infectious virus particles.

If a transmission electron microscope is available, this can also be used to determine virus counts rapidly (Malenovska, 2013). Similar to the Virus Counter® method, intact virus particles are counted regardless of their ability to infect cells. Quantitative PCR is another rapid method for virus analysis (George *et al*., 2012; Lo and Chao, 2004), but it only measures the number of viral genomes, which may not be equivalent to the number of infectious virus particles.

2.10. Flow Cytometry Based Virus Titer Assay

A virus titer assay based on flow cytometry was described by Mulvania *et al*. (2004). This can be completed in less than two days and determines the number of infectious virus particles instead of total virus counts, making it superior to the other methods even though an expensive flow cytometer is needed. Depending on the capabilities of the flow cytometer, the final measurement can be recorded automatically and it may also be possible to adapt cell infection and immunostaining to a (semi)automated procedure.

The number of infected cells is determined by measuring the cell surface expression of the viral envelope protein gp64 (Whitford *et al*., 1989). This glycoprotein is necessary for virus take up by endocytosis (Blissard and Wenz, 1992; Volkman and Goldsmith, 1985) and is expressed on the surface of infected cells within a few hours after infection (Jarvis and Garcia, 1994). Besides its short timescale, this assay is appealing because the cell culture conditions can be adapted to the conditions used for protein production. Furthermore, because infected cells are counted instead of virus particles, the resulting titer value will be as meaningful as possible in the context of protein production.

A similar baculovirus titer assay measures the expression of Green Fluorescent Protein (GFP) as a marker of virus-infected cells (Malde and Hunt, 2004). The fluorescent protein allows direct analysis without a prior staining procedure. The *gfp* gene is controlled by the baculoviral p10 promoter, which extends the incubation phase after virus infection to 48 h and raises concerns about discrimination between primary and secondary infection events. Furthermore, *gfp* gene expression during the very late infection phase could compete with the expression of the target recombinant protein.

Ultimately, all the available virus titer assays provide sufficient information for monitoring and optimizing baculovirus-based protein expression experiments as long as the limitations are acknowledged and each method is applied consistently.

2.11. Cell Lines for Baculoviral Infection

The baculovirus expression vector system is often used with cell lines Sf-9 and Sf-21, both originating from cell line IPLB-SF-21 isolated from *Spodoptera frugiperda* pupal ovarian tissue (Vaughn *et al*., 1977), as well as BTI-TN-5B1-4, established from ovarian cells of the cabbage looper *Trichoplusia ni* (Granados *et al*., 1994) and better known under the brand name High Five™. Sf-9 cells were established as a denser and faster-growing subclone of Sf-21. When comparing different insect cell lines for baculovirus-based protein production, High Five cells achieved the highest yield (Hashimoto *et al*., 2010; Keith *et al*., 1999; Taticek *et al*., 2001), whereas the productivity of Sf-9 and Sf-21 cells was less sensitive to cell density (Wickham *et al*., 1992).

Recently, BTI-Tnao38 cells from *Ascalapha odorata* were introduced as the cell line Ao38 (Hashimoto *et al*., 2010). These offer properties suitable for virus amplification and protein production, but later turned out to be a contamination of *Trichoplusia ni* origin, most likely a clonal derivative of the High Five cell line (Hashimoto *et al*., 2012).

Instead of cell lines also whole silkworm larvae and pupae are used as hosts for efficient baculovirus-based protein production (Kato *et al*., 2010; Usami *et al*., 2010).

2.12. Stable Transfection or Baculoviral Infection?

The choice between baculovirus expression vector systems and stably-transfected cells comes down to the effort required and demands in terms of product quality and yield.

One important difference between the two systems is the condition of the cell culture at the time of harvest. In the baculovirus expression system, the transgene is

usually controlled by a very late promoter so that protein production is maximized just before the cells are eventually lysed. A significant quantity of host cell proteins is therefore released into the culture supernatant and these will need to be removed during purification because they may compromise the stability of the target protein in the supernatant. The advantage of stably-transfected cells is that they remain healthy, allowing the continuous expression of target proteins without the need to expand new cultures for each production batch.

The baculovirus expression system produces the recombinant proteins more quickly than stably transfected cells because there is no lengthy selection process and in systems without plaque purification the first large-scale batch can be generated within 3-4 weeks (**Fig. 3**). In contrast, the selection of stably transfected cells takes 2-3 weeks (depending on the antibiotic), plus an additional 2-3 weeks to expand the cell culture and even more time if the cells are adapted to serum-free media. If serum-free expression conditions are required, it is worth testing the cells without serum during selection. Although the cells could die in the absence of serum even if they have integrated the resistance gene, this avoids the need for adaptation after selection. Further time is required for single-cell cloning. Even so, despite the short interval from vector construction to protein production using the baculovirus expression vector system, the virus maintenance, amplification and titration steps make the baculovirus system much more labor-intensive during the production phase than stably-transfected cells.

In terms of yield, the baculovirus expression system is generally superior because the strong very late promoters achieve high levels of expression. This does not apply to every protein, e.g., immediate early promoters in stably-transfected cells may be more efficient for the expression of membrane-bound or secreted proteins, because processing may already be compromised during the late stages of baculovirus infection (Jarvis *et al*., 1990; 1996).

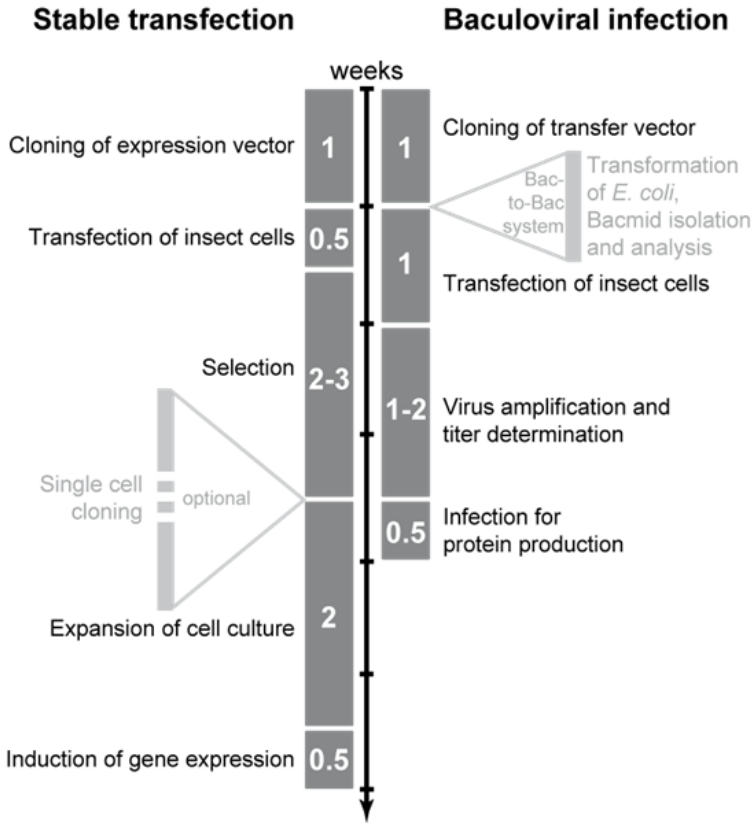

Fig. 3. Timeline comparison for protein production using stably-transfected cells or the baculovirus expression vector system. The BEVS timeline is only valid for systems that do not require plaque purification.

3. PROTEIN PURIFICATION CONSIDERATIONS

Although we do not discuss protein purification in detail in this article, we consider some ways to avoid known pitfalls and thus provide a starting point for further studies.

3.1. Affinity Tags and Fusion Proteins

Ideally, recombinant protein should be produced without modifying or augmenting the original polypeptide sequence and this is feasible if the native protein can be detected and isolated using existing tools, e.g., antibodies for purification or mass spectrometry for identification. Even without these tools, the purification of an unmodified protein could be achieved if the yield were sufficient for identification by SDS-PAGE followed by non-specific staining. In most cases however, an affinity tag or fusion partner makes the processes of detection and isolation more straightforward because standard procedures can be used e.g., for affinity chromatography (Arnau *et al*., 2006). The additional polypeptide sequence can also improve the yield for the protein of interest, e.g., by improving solubility or stability, or by reducing toxicity.

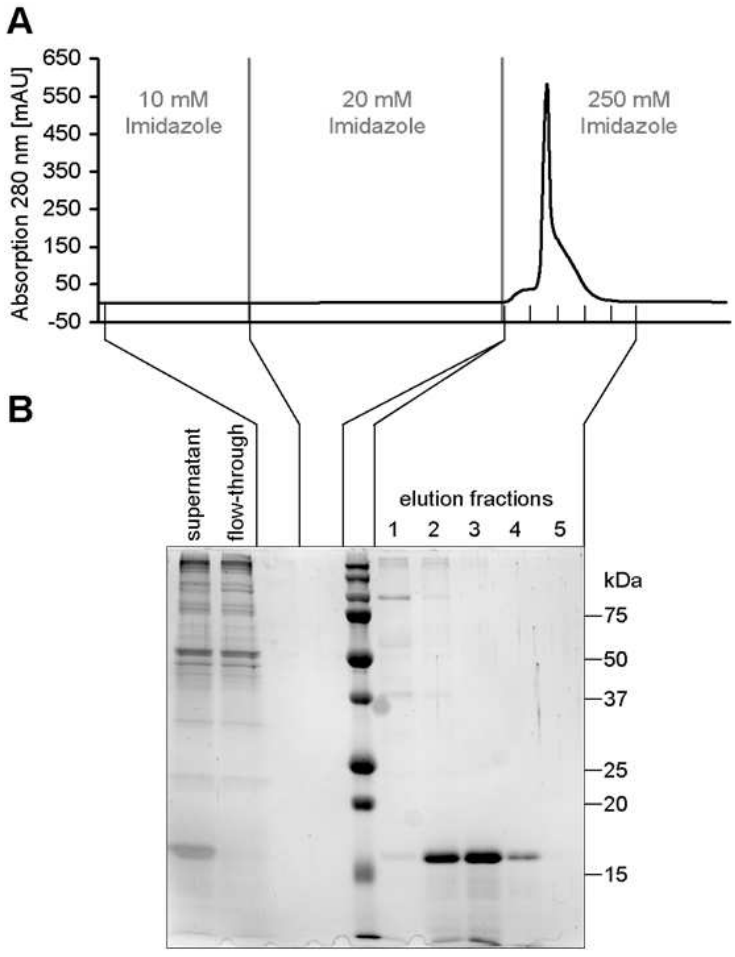

Fig. 4. Purification of *G. mellonella* gloverin-like AMP from culture supernatant of stably-transfected S2 cells by Immobilized Metal Affinity Chromatography (IMAC). (A) Chromatogram of column wash with two different imidazole concentrations followed by elution with 250 mM imidazole. (B) Samples of supernatant and elution fractions separated by SDS-PAGE and stained with Coomassie Brilliant Blue

Although protein tags facilitate production and purification, they can also alter protein function and should therefore be removed prior to downstream applications. Tag removal is usually achieved by cleavage with a specific protease at a recognition site introduced during vector construction. Suitable enzymes and recognition sequences are described by Waugh (2011). After cleavage, an additional purification step is usually necessary to remove the affinity tag and enzyme. If the intention is to obtain the native protein sequence, care must be taken to select an enzyme that cleaves without leaving residual amino acids surrounding the recognition site attached to the target protein. Only a few enzymes possess such attributes and this only applies to N-terminal fusions (e.g., enterokinase and factor Xa). Therefore native signal sequences and/or propeptides are incompatible with the intention to make use of an epitope tag or fusion partner which is to be cleaved off without leaving residual traces of the fusion partner.

3.2. Insect Cell Culture Media and IMAC

Six consecutive histidine residues (His_6) are often used as an affinity tag, allowing the purification of recombinant fusion proteins with an anti-polyhistidine antibody or by Immobilized Metal-Ion Affinity Chromatography (IMAC). In the example depicted in **Fig. 4**, *G. mellonella* gloverin-like AMP with a C-terminal V5/His_6 affinity tag was produced with the *Drosophila* Expression System. The molecule was purified from the cell culture supernatant by binding to Ni^{2+} ions immobilized on agarose beads. The chromatography column was washed with low concentrations of imidazole before the His-tagged protein was eluted by increasing the imidazole concentration to 250 mM.

It is important to note in this context that serum-free insect cell culture media are usually incompatible with IMAC because the immobilized Ni^{2+} and Co^{2+} ions are stripped off the resin when the medium is applied. The substance responsible for this effect is unknown, but histidine, triglycerides, sterols, phospholipids and non-ionic detergents may each play a role. Dialysis or diafiltration of the cell culture supernatant before IMAC can circumvent this issue but this adds a laborious additional step. Interestingly, we found that this step can be avoided by adding divalent metal ions directly to the cell culture supernatant like in the example presented in **Fig. 4**, but this is not always successful.

Furthermore, insect cell culture media tend to be acidic, particularly after use (pH 5-6), whereas the binding of His_6-tagged proteins to IMAC columns is most efficient under slightly alkaline conditions. Therefore the pH of the insect cell culture supernatants should be increased before direct application to an IMAC column, but not above pH 7 since this encourages the precipitation of media ingredients.

4. CONCLUSION

Although bioinformatics provides useful theoretical information about proteins, empirical experiments are still needed to confirm protein structures and functions and this means it is necessary to produce recombinant proteins in heterologous systems. Among the many available expression hosts, insect cells offer a combination of high yields and the ability to carry out complex post-translational modifications. Both the baculovirus expression vector system and stably-transfected insect cells can produce large amounts of high-quality recombinant protein. The BEVS offers rapid progress from sequence to protein and achieves the higher yield, but stably transfected cells are easier to handle once they are established. The BEVS has been improved by the development of recombination techniques that avoid plaque purification and novel approaches for virus titer determination, whereas stably transfected cells have been improved by the development of master cell lines that allow site-directed gene integration. Detailed protocols are provided by Murhammer (2007), O'Reilly *et al*. (1994) and in the manuals provided by the manufacturers of different expression systems. Although these sources describe the impressive yields that can be achieved with particular expression systems, these are likely to reflect ideal situations and each protein must be evaluated on a case-by-case basis.

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