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Insect Larvae: A New Platform to Produce Commercial Recombinant Proteins

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Abstract: In Biotechnology, the expression of recombinant proteins is a constantly growing field and different hosts are used for this purpose. Some valuable proteins cannot be produced using traditional systems. Insects from the order Lepidoptera infected with recombinant baculovirus have appeared as a good choice to express high levels of proteins, especially those with post-translational modifications. Lepidopteran insects, which are extensively distributed in the world, can be used as small protein factories, the new biofactories. Species like *Bombyx mori* (silkworm) have been analyzed in Asian countries to produce a great number of recombinant proteins for use in basic and applied science and industry. Many proteins expressed in this larva have been commercialized. Several recombinant proteins produced in silkworms have already been commercialized. On the other hand, species like *Spodoptera frugiperda, Heliothis virescens, Rachiplusia nu, Helicoverpa zea* and *Trichoplusia ni* are widely distributed in both the occidental world and Europe. The expression of recombinant proteins in larvae has the advantage of its low cost in comparison with insect cell cultures. A wide variety of recombinant proteins, including enzymes, hormones and vaccines, have been efficiently expressed with intact biological activity. The expression of pharmaceutically proteins, using insect larvae or cocoons, has become very attractive. This review describes the use of insect larvae as an alternative to produce commercial recombinant proteins.

Keywords: Insect larvae, biofactories, recombinant proteins, baculovirus.

INTRODUCTION

There is a fast growing demand for low cost processes to produce biologically active biomolecules, such as eukaryotic proteins, glycoproteins, peptides and lectins. In Biotechnology, there are several systems currently available to express recombinant proteins. Bacteria, yeast and mammalian cells, followed by insect cell cultures, are commonly used as hosts to produce recombinant proteins in a short time [1, 2]. Insect cell systems, including the baculovirus expression system are broadly applied to produce proteins with biotechnological or pharmaceutical purposes. The system provides a post-translational modification pattern similar to that of mammalian cells when an eukaryotic environment is required [3]. The proteins expressed by this system are correctly folded and are usually biologically active [4]. In most cases, the insect cell lines Sf9, Sf21 or HiFive have been used as hosts. Nine products developed in the baculovirus-insect cell system have already been approved for human and veterinary use [5]. However, the most important drawback of recombinant protein production in insect cell culture is its high cost at industrial scale because of the tissue-culture specialized facilities and reactors needed [6]. Besides, at industrial scale, the risk of contamination is rather high. According to Vermasvuori *et al*. HIV-1 Nef production using insect cell-based strategy was four times more expensive than *in Escherichia coli* [7]. Using directly live insect larvae as "biofactories" is a low-cost alternative to scale up the production of recombinant proteins. This approach is very attractive and the manufacturing cost could be reduced up to four hundred times in contrast with insect cell cultures [8-11]. In this review, we describe the use of baculovirus expression system and insect larvae as biofactories to scale up recombinant protein expression.

THE BACULOVIRUS EXPRESSION SYSTEM: GENERALITIES AND CONSTRUCTION

Baculoviruses are rod-shaped DNA viruses with double-stranded circular genomes of 80-180 kbp in size. The Baculoviridae family infects only arthropod populations, i.e., these types of viruses do not replicate in vertebrates, plants and microorganisms [12]. This makes the baculovirus ex-

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pression system safe for the development of recombinant proteins of human and veterinary application.

The baculovirus expression system has become a recognized platform for the production of gene therapy vectors and vaccines in insect cell lines but also insect larvae can be used [3]. In biotechnology, two species are broadly applied as vectors to express recombinant proteins in insect larvae: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which is the most widely used baculovirus expression vector**,** especially in American and European countries and *Bombyx mori* nucleopolyhedrovirus (BmNPV), mainly adopted in China, India, Japan and other Asian countries. During the natural infection process, baculoviruses produce two viral phenotypes with specific biological properties: occlusion-derived virus (ODV) and budded virus (BV). ODVs enable the horizontal virus transmission from insect to insect through large (1-5 μm) structures called occlusion bodies (OB) or polyhedra, within which the viruses are embedded. Polyhedra, that contaminate larval food, are ingested and ODVs are released in the alkaline environment, infecting the midgut columnar epithelial cells, the only susceptible cell type. After the first round of infection, the BVs produced by the ODV-infected midgut disseminate the infection from cell to cell within the host because all cell types are susceptible to this viral form. Based on this, insect larvae can be infected either by intrahemocelical injection of BV or orally with OB contained in the diet [13].

Baculoviruses have two strong very late promoters: polyhedrin and p10. Both are commonly used to express foreign genes, because their products are non-essential for BV production and virus propagation in the cell culture. It must be note that most recombinant baculoviruses are constructed traditionally by replacing the polyhedrin gene (polh) with that of interest. This kind of recombinant baculovirus is identified as polyhedrin-minus genotype (polh-) because of its incapacity to generate polyhedra and thus, its low effectiveness to infect larvae by oral inoculation. Therefore, the application of polh- recombinant baculoviruses is restricted to the intrahemocelical infection of BV [14, 15]. The construction of a polh- recombinant baculovirus is not achieved by direct cloning of the foreign gene because the baculovirus genome is very large to manipulate. So, the classical way of cloning is by recombination: homologous recombination or transposition. The homologous recombination event is carried out inside the insect cells by cotransfection with the transfer vector that containing the foreing gene and the viral genome (Baculogold®, Pharmingen). On the other hand, the transposition event is carried out inside a bacterium by transposition between the transfer vector and a bacmid that containing the complete baculovirus genome and then the insect cells are transfected with a bacmid purified from the bacterial culture (Bac-to-Bac® system, Invitrogen). These two commercial systems for AcMNPV have been extensively reviewed [3, 5, 12]. The Bac-to-Bac®system was also developed for BmNPV by Motohashi *et al.* [16]. Once recombinant baculovirus has been established (3 weeks), it is used to infect larvae to produce recombinant proteins.

Since oral infection using polyhedra is a simpler method than intrahemocelical infection when a large number of insect larvae have to be infected, mainly at large-scale production of proteins. Alternatives to construct recombinant baculovirus with ability to produce polyhedra (polyhedrin-plus genotype or polh+) have been explored. Je *et al*. generated the bacmids pBmGOZA and pAcGOZA that allow obtaining recombinant BmNPV and AcMNPVpolh+ viruses, respectively. In this case, they introduced the gene of interest into the polyhedrin locus and polyhedrin gene was under the control of the *p10* promoter [17, 18]. Romero *et al*. obtained the polyhedra-plus genotype (polh+) recombinant baculovirus using a bacmid for insect larvae infection. Then, they spread the polyhedral suspensions on the larval free-phenol diet and fed the larvae with contaminated diet [19]. On the other hand, Lopez *et al*. developed a new insect stable cell lines that express polyhedrin and able to occlude recombinant baculovirus by trans- complementation to achieve an oral inoculum for insect larvae [20].

INSECT LARVAE SPECIES AND THEIR SUSCEPTIBILITY TO BACULOVIRUS INFECTION

The order Lepidoptera (butterflies and moths), the second largest order in the class Insecta, is a group of insects including more than 100,000 described species. A lot of them are considered destructive plagues during their larval stage and affect important crops of economic interest. *Spodoptera frugiperda, Spodoptera littoralis, Trichoplusia ni, Helicoverpa zea, Heliothis virescens* and *Rachiplusia nu* are some of the lepidopteran species extensively distributed all around the world. All are permissive hosts to AcMNPV infection. Particularly, there is some industrial interest in the larvae of *T. ni*, the cabbage looper moth, because it is an excellent host for AcMNPV and has been extensively used to produce several recombinant proteins in biotechnology [21, 22]. Nowadays, industrial-scale protein production in *T. ni* larvae is carried out by several companies (Chesapeake PERL, Savage, MD, Sysmex, Japan, and ALGENEX, Spain). On the other hand, *B. mori* larvae have economic importance in silk production and for this reason this species has been domesticated for thousands of years, usually cultivated on leaves of the mulberry *(Morus alba)*. Also, it has become an eukaryotic model system for scientific research. Although both the AcMNPV and BmNPV systems offer high level expression of genes of interest using larval hosts, the silkworm larva infected with recombinant BmNPV offers several additional advantages, i.e., it is easy to rear, it is large (120 mm long in the last stage) and easy to manipulate, it has a short life cycle (about 7 weeks), and it has its genetics and its biology well documented [23]. Silkworm pupae have also been used as bioreactors. Human granulocyte macrophage colony-stimulating factor and artificial influenza virus-like particles have been produced in pupae [24, 25]. In contrast, other species like *S. frugiperda, H. zea*, *H. virescens* and *T. ni* are also relatively easy to rear and produce similar or even more quantities per insect than *B. mori*. Additionally, the hemolymph that content high lipid is the principal source to collect the recombinant protein in *B. mori*. It makes more complex the production process in comparison to this other species in which the whole larva is processed for protein recovery.

S. frugiperda, H. zea, *H. virescens* and *T. ni* have five or six larval stages depending on the temperature and food, reaching only 35-40 mm in length. All these species can be

reared under laboratory conditions, but while *B. mori* larvae in general are not susceptible to AcMNPV. *S. frugiperda, S. littoralis, T. ni, H. zea, H. virescens* and *R. nu* are not susceptible to BmNPV. Recently, Park *et al*. have reported that some silkworm strains are highly-permissive to AcMNPV [26]. Although, all larvae are susceptible to intrahemocelical infection, different host species have demonstrated some degree of developmental resistance when the virus is administered orally. Resistance to larvae infection increase with age, and this is decisive to choose the infection route [19, 27-29]. *H. zea* and *S. frugiperda* larvae are resistant to oral infection, however they are highly susceptible to infection with BVs injected into the hemocele. In contrast, *H. virescens* and *R. nu* have shown oral and intrahemocelical susceptibility [19, 20, 30, 31].

Insect larvae other than *B. mori* are not widely exploited, mainly due to the lack of knowledge in rearing and maintaining in laboratories [32]. *B. mori* is a very efficient host but it is mainly used in the silk industry. In contrast, *R. nu*, *S. frugiperda* and other lepidopteran species are plagues without any economic value. For this reason, it is interesting to explore these larvae as alternative hosts to produce recombinant proteins.

THE OPTIMIZATION OF THE PROTEIN PRODUCTION PROCESS IN LARVAE

The protein production process in larvae is a complex task, where one of the most important issues is the selection of the expression vector to be used. Another decision includes selecting larvae at the appropriate developmental stage. Larvae should be large enough to facilitate injection and yet not ready for pupae formation. Furthermore, insect protein synthesis capacity is optimal between the 4^{th} and the 5^{th}instars stages.

The time needed for recombinant protein expression should be monitored in each case because it may vary with each particular protein. To determine the expression level of the target protein, the larval extract is obtained by homogenization in a buffer that controls the melanization process [31]. Usually 3-5 days are required to reach the peak of protein expression. Once this optimization step is done and the protein quantified, the process can be linearly scaled up. The availability of automated rearing equipment and the fact that larvae are non-allergenic to human handlers make scale-up and mass production of recombinant proteins very attractive for commercial protein production. With an automated facility for mass rearing and controlled working conditions, it is possible to scale up to kilograms of protein-containing larvae per week. The yield of recombinant protein with post-translational modifications can reach values in the range of micrograms or milligrams per larva. Analyzing the different hosts and their parameters is of great importance when choosing the expression system. Romero, *et al*. evaluated larvae reared at 24°C as biological factories to produce horseradish peroxidase isoenzyme C (HRPC), a protein used in important biotechnological fields such as diagnostics, biocatalysts and biosensors. They compared their potential as expression hosts by infection with recombinant baculovirus, either by injection of BV or by oral administration of polyhedral. Of the different larvae studied, those of *S. frugiperda* presented the best in HRPC expression (137 µg per g of larvae) when intrahemocelically infected. Meanwhile, for oral infection, *R. nu* showed a high biotechnological yield of HRPC (110 µg per g of larvae) [14]. On the other hand, the same enzyme was expressed in yeast at a level of 0.1 mg l^{-1} [33] and 41.3 mg l^{-1} in Sf9 cells, but the cost of enzyme production was a hundred times more expensive than in the larval system [34]. Furthermore, the viral replication rate and the larval susceptibility are influenced by the temperature so this effect was also studied on the expression of HRPC [14, 35-37]. It has been observed that the HRPC expression in *S. frugiperda* and *R. nu* larvae increased 1.8 and 2.5-fold when the rearing temperature was increased from 24°C to 27°C [14]. On the other hand, when feline interferon alpha was expressed, the high temperature only accelerated the expression kinetics but had no influence on the yield [38]. Therefore, parameters should be evaluated according to the recombinant protein to be produced.

Changes in the expression baculovirus vector can be implemented to achieve higher levels of expression. For instance, Gong *et al*. reported an important increase in insect cell expression level of the cholera toxin subunit B-insulin by included the PPHS element in the coding sequence [39]. This element also allowed increasing HRPC yield up to 1.8-times in *R. nu* larvae due to the PPHS acted as a useful enhancer [14].

TRANSGENIC LARVAE TO MODIFY INSECT GLYCOSYLATION PATTERN

Insect larvae are a low cost alternative to produce proteins of pharmaceutical interest that often require mammalian-like post-translational modification [3]. Mammalian glycoproteins produced in insects are often biologically active. However, insect cells generate simpler N-glycans than mammals [5] .Native protein produced by mammalian cells is of complex-type, terminally galactosylated or sialylated. However, the structure of N-glycans in insect recombinant glycoproteins is paucimannose and the cells are unable to add terminal galactose and sialic acid residues [40]. Therefore, the recombinant glycoproteins produced in the baculovirus expression system are less stable in blood than native mammalian glycoproteins [41]. To solve this limitation, several strategies have been implemented, such as transgenic insect cell lines that stably express mammalian glycosylation enzymes or co-expressing these enzymes and the foreign gene [5]. These technologies would bring about the expression of recombinant protein with mammalian-type N-glycans in insect larvae. For instance, the method to generate transgenic silkworm is to inject the PiggyBac-Transposon with a target construct into eggs [4]. In transgenic silkworm, some pharmaceutical recombinant proteins have already been expressed successfully in the silk gland and produced in cocoons with a level of one to few hundred µg per mg of cocoon weight [42]. Human collagen [43], feline interferon [44] and mouse monoclonal antibody [45] have been produced using this system. Therefore, insect larvae expression system would be use for the production of mammaliam glycoproteins, if the enzyme glycosylation pathway is enhanced [3].

THE BACULOVIRUS SYSTEM IN INSECT LARVAE AS AN ALTERNATIVE PLATFORM FOR PHARMACEUTICAL PROTEIN EXPRESSION

Insect larvae as "Biofactoies" are used as a low-cost alternative for protein production. The use of larvae was pioneered [46]. Nowadays, two commercial recombinant proteins for veterinary use are produced in *B. mori* by Toray Ind. Inc. (Tokyo, Japan): Intercat, a drug composed of feline interferon, and Interdog, a drug composed of canine interferon gamma. Also, feline interferon is currently marketed in Europe under the name Virbagen Omega (Virbac).

The list of biopharmaceutical recombinant proteins produced in this system, especially in the last years, is endless. Many enzymes [19, 31, 47, 48], antibodies [49-53], vaccines [11, 54-60], diagnostic proteins [21, 61-66], hormones [67, 68], lectins [69], and cytokines [38, 70-74] have been efficiently expressed in insect larvae with good yield using recombinant baculoviruses as vectors. Some of the proteins of biomedical importance expressed in larvae infected with BmNPV or AcMNPV are well summarized in works [3, 4, 9]. Tables **1** and **2** provides a summary of relevant biopharmaceutical recombinant proteins expressed in insect larvae infected with recombinant AcMNPV and BmNPV respectively.

For example, mouse anti-botulinum antibody fragment (Fab) has been expressed in *T. ni* larvae with a total yield of 1.1 μg per g of larvae [52]. Similar results have been obtained with antigen protein-based virus-like particles (VLPs). Deo *et al*. described the expression of Rous sarcoma VLPs in silkworm larvae. The yield of the VLPs was approximately 8.2-fold higher than that obtained in stable cell lines [60]. High levels of recombinant wheat germ agglutinin have been obtained in *R. nu*, where yields reached 346.6 μg per g of larvae. Moreover, Urtasun *et al* developed a simpler purification process to purify this protein based on aqueous two-phase system coupled to affinity chromatography using chitosan mini-spheres [69]. Feline interferon alpha has been expressed in *R. nu* larvae with a yield of 116 μg g larvae or 3.7×10^6 U per ml and in *S. frugiperda* larvae 22 μg per g of larvae or 1.1×10^6 U per ml [38]. Feline interferon and canine interferon have also been expressed in *B. mori* larvae with a yield of 1.2 $x10^8$ U per ml of body fluid [70] and 528 μg per larvae [71] respectively. The high yield of Influenza A H1N1 neuraminidase obtained in *R. nu* larvae (1.2 mg per g of larvae) results in a very attractive cost-effective alternative to conventional cell culture-based methods for the expression of this important influenza antigen [59]. Dojima *et al.* have expressed antibodies in whole insects at a range of g of purified Fab/kg of larvae [73].

All these results indicate that insect larvae are an attractive platform for application in vaccine development.

EXTRACTION AND PURIFICATION OF RECOMBINANT PROTEINS

The downstream processing of recombinant proteins produced in insect larvae has not been deeply studied. The diversity of proteins which have been produced in insect larvae is so great that the development of standard methods becomes

Table 1. Biopharmaceutical proteins expressed in *R. nu*, *S. frugiperda* and *T. ni* larvae infected with recombinant AcMNPV.

Proteins	Host	Expression level	References
Horseradish peroxidase isoenzyme C	*R. nu*	480 μg per g larvae	[19]
Feline interferon-α	*R. nu*	116 μg per g larvae	[38]
Neuraminidase (strain H1N1)	*R. nu*	1.2 mg per g larvae	[59]
Germ agglutinin	*R. nu*	346.6 μg per g larvae	[69]
Horseradish peroxidase izoenzyme C	*S. frugiperda*	315 μg per g larvae	[19]
Feline interferon-α	*S. frugiperda*	22 μg per g larvae	[38]
Rabbit haemorrhagic disease virus capsid protein	*T. ni*	2 mg per larvae	[11]
Human adenosine deaminase	*T. ni*	8-9 mg/22 larvae	[47]
Human II phospholipase A2	*T. ni*	800 μg per ml haemolymph	[48]
Recombinant single chain antibody against MHC Class II DR molecule epitope	*T. ni*	2.8-3.2 mg per g of larvae	[50]
Glycoprotein G of viral haemorrhagic septicemia virus	*T. ni*	0.3 mg per larva	[53]
Human papillomavirus-like particles	*T. ni*	18-21 mg per g larvae	[56]
Hemagglutinin (strain H1N1)	*T. ni*	113 μg per larvae	[57]
Human epidermal growth factor	*T. ni*	9.1 mg per g larvae	[72]
Human fibroblast growth factor-2	*T. ni*	2.6 mg per g larvae	[72]
Human keratinocyte growth factor-1	*T. ni*	3 mg per g larvae	[72]

Table 2. Biopharmaceutical proteins expressed in *B. mori* pupae and larvae infected with recombinant BmNPV.

Protein	Expression Level	References
Human interferon-alpha	0.3 mg/15 larvae (after purification)	[23]
Granulocyte macrophage colony stimulating factor	100 µg per pupa	[24]
Virus-like particle from H5 hemagglutinin	2,000 µg per pupa	[25]
Human type III procollagen	70 µg per pupa	[43]
Feline interferon	1-5 mg per pupa	[44]
Mouse monoclonal antibody	139 -319 ng/0.1 mg cocoon	[45]
Mouse interleukin-3	0.5 mg per larvae	[46]
Human hepatitis B virus surface antigen	750 µg per larva and 690 µg per pupa	[55]
Hemagglutinin (strain H5N1)	500 µg/30 larvae	[58]
RSV-gag virus like particle	384 mg/10 larvae (after purification)	[60]
Human parathyroid hormone	70 mg per L of haemolymph	[67]
Human growth hormone	20-50 µg per larva	[68]
Feline interferon	$1.2x10^8$ U per ml of haemolymph	[70]
Canine interferon alpha	528 µg per larva	[71]
Bovine interferon-τ	4.55 mg/ 100 larvae	[74]

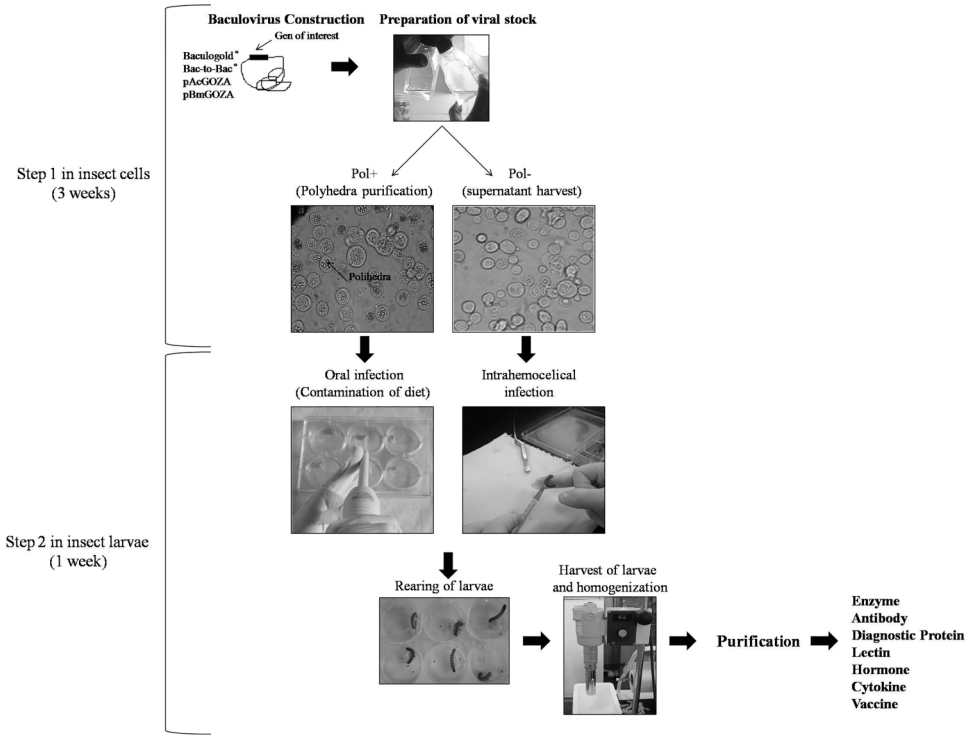

Fig. (1). The production process of recombinant protein in insect larvae. The production process of recombinant protein in insect larvae involves a first step in insect cells and a second step in larvae. Step 1 in insect cell: The gene of interest is incorporated into the baculovirus genome (Bac-to-Bac®, Baculogold®, pAcGOZA, pBmGOZA). The construction of the recombinant baculovirus and preparation the virus stock takes 3 weeks. If the virus is pol+ genotype, polyhedra is purified from insect cell; however, if the virus is pol- genotype, the supernatant is harvested to infect insect larvae. Step 2 in Insect larvae: After the viral stock is ready, the process to produce and purified recombinant protein from larvae takes only 1 week. Larvae are infected by oral infection (Virus pol+) or intrahemocelically (Virus pol-) with recombinant baculovirus. Larvae can be reared under laboratory conditions and fed on a diet at 23–25 ºC in a 70% humidified chamber, with a 16:8 photoperiod (L:D). After 3-5 days, larvae are harvested and homogenized. Finally, the recombinant protein (enzyme, antibody, diagnostic protein, lectin, hormone, cytokine, vaccine) is purified using standard techniques.

difficult. The choice of a specific downstream strategy is based on the scale of operation, localization of the target protein and the expression yield. The aim of the downstream step is to separate contaminant proteins present in the host, viral proteins, DNA and viral particles. One of main difficulties is the high activity of proteases that may degrade the recombinant protein during larval sacrifice by homogenization. The product quantity and quality is strongly influenced by the time of harvest. As the viral cycle progresses, cells lyses and a significant amount of intracellular proteases and glycosidases appear in the homogenate. The first step is to achieve a clarified homogenate of insect larvae. This step is generally achieved by centrifugation or filtration to separate tissues and remove lipids. The clarified solution should have a yellow-green color. Besides, it is important to inhibit the melanization process during disruption to avoid protein yield loss because this interferes with the purification process [75].

The procedure to purify recombinant proteins from insect larvae is similar to those commonly used to proteins from other organisms according to standard techniques [5]. Ion exchange and affinity chromatography have demonstrated to be efficient to purify different proteins from larval extracts [14, 38, 74]. In general, proteins are fused to histidine tags and then purified by ion-metal affinity chromatography. Biotechnology industry demands fast, efficient and inexpensive downstream processes for the recovery and purification of important proteins. However, insect larvae extracts brings about some drawbacks, as example, the regeneration of the chromatographic matrices is complicated and their half-life decreases considerably due to the complexity.

Some low-cost alternatives for the purification procedure, such as aqueous two-phase systems and chitosan minispheres, are being implemented [69, 76]. However, the purification system selected will depend on the physicochemical characteristics of the recombinant protein and its contaminants.

CONCLUSION

Biopharmaceuticals represent the fastest growing sector of the global pharmaceutical industry, driven by a rapid and successful manufacture of recombinant protein-based drugs. To fulfill the demand, it is crucial to increase the throughput of expression systems and purification processes.

Insect larvae infected with baculoviruses can serve as natural biofactories to synthesize proteins of interest *in vivo*. Fig. (**1**) summarizes the whole process to produce recombinant protein in insect larvae. The high yield of protein reported in homogenates harvested from 3-5 days post-infection at very low production costs makes biofactories a very attractive alternative to traditional hosts like yeast or mammalian cells. However, further efforts must be made to improve the downstream processing of recombinant proteins.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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