***Review Article***

**Transgenic Silkworms for Recombinant Protein Production: A Comprehensive Review of Expression Systems and Biotechnological Advances**

**ABSTRACT**

Recombinant proteins serve as essential components in medicine, diagnostics, and industrial biotechnology. While microbial and mammalian systems have traditionally dominated recombinant protein production, limitations in scalability, cost, and post-translational modifications have prompted exploration of alternative hosts. The domesticated silkworm (*Bombyx mori*) offers a unique and highly efficient platform due to its robust protein synthesis capacity, well-characterized silk gland biology, and availability of advanced genetic tools. This review explores the evolution of silkworm-based expression systems, including baculovirus-mediated and transgenic platforms, with a focus on tissue-specific expression within the middle and posterior silk glands. Key molecular engineering strategies such as the use of synthetic promoters, enhancers, and site-specific genome editing tools like TALENs and CRISPR/Cas9 are discussed for improving yield and expression fidelity. Applications in therapeutic protein production, including growth factors, antibodies, and vaccine candidates, are presented alongside considerations of biopharmaceutical quality. Challenges in downstream processing, scalability, and regulatory compliance are addressed, and future directions involving synthetic biology, AI-driven bioprocess optimization, and research into non-mulberry silkworm species are highlighted. Collectively, these advances position transgenic silkworms as a next-generation platform for sustainable, scalable, and high-quality recombinant protein production.

*Keywords:Transgenic silkworms; recombinant protein production; silk gland bioreactor; piggyBac; baculovirus; TALEN; CRISPR/Cas9; biopharmaceuticals; gene expression*

**1. INTRODUCTION**

Recombinant proteins are indispensable tools across biotechnology, medicine, and industry, serving as therapeutic agents, enzymes, diagnostics, and research reagents (Dimitrov, 2012; Puetz & Wurm, 2019). The growing global demand for these bioactive molecules necessitates efficient, scalable, and cost-effective production platforms. While traditional expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae*, and mammalian cell lines have dominated the field, they are often constrained by issues related to poor post-translational modifications, biosafety concerns, high production costs, and limited scalability (Walsh, 2014; Huang *et al.,* 2012).

In response, the domesticated silkworm (*Bombyx mori*) has emerged as a promising and innovative bioreactor for recombinant protein production. Domesticated for over 5,000 years for silk production, *B. mori* possesses a specialized silk gland capable of synthesizing vast amounts of protein in a short period, particularly during the final larval instar. On average, a silkworm can convert 20 g of mulberry leaves into approximately 0.5 g of silk protein, showcasing its efficiency and potential as a biomanufacturing system (Ma *et al.,* 2014).

Silkworm silk proteins are primarily composed of fibroin and sericin, which are synthesized in distinct regions of the silk gland. Fibroin, produced in the posterior silk gland (PSG), constitutes 70–80% of the total silk proteins and is composed of heavy chain (FibH), light chain (FibL), and fibrohexamerin (Fhx) in a 6:6:1 molar ratio (Adachi *et al.,* 2010; Inoue *et al.,* 2000). Sericin, accounting for 20–30% of silk proteins, is synthesized in the middle silk gland (MSG) and includes Ser1, Ser2, and Ser3, with Ser1 being the most abundantly expressed (Garel *et al.,* 1997; Michaille*et al.,* 1990; Takasu *et al.,* 2007). These proteins are secreted into the anterior silk gland (ASG) and spun into cocoons, with sericin serving as a hydrophilic glue coating the insoluble fibroin core (Xu *et al.,* 2014; Dong *et al.,* 2016).

The feasibility of using silkworms for heterologous protein expression was first demonstrated by Maeda *et al.* (1985), who expressed human α-interferon using a recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV). This pioneering work laid the foundation for baculovirus-based expression systems in lepidopterans. Since then, a wide range of proteins including cytokines, hormones, growth factors, and enzymes have been produced in silkworms using either viral vectors or transgenic approaches (Wurm, 2003).

Notably, transgenic systems based on the piggyBac transposon have significantly improved the reliability and yield of recombinant proteins produced in the silk gland (Tamura *et al.,* 2000). These systems utilize tissue-specific promoters, such as Ser1, to target protein expression to the MSG, facilitating easier extraction due to the water-solubility of sericin (Tomita *et al.,* 2007; Iizuka *et al.,* 2008; Wang *et al.,* 2013). Further optimization using enhancer elements and untranslated region (UTR) modifications has boosted transcriptional and translational efficiency. Moreover, mutants with impaired fibroin secretion can redirect the gland’s biosynthetic capacity to express foreign proteins, enhancing yields even further (Inoue *et al.,* 2005).

The integration of advanced genome-editing technologies has revolutionized the field. Site-specific nucleases including ZFNs, TALENs, and CRISPR/Cas9 enable precise genome modifications via double-stranded breaks followed by homologous recombination (Maeder *et al.,* 2008; Edgell, 2009; Boch, 2011; Mulepati*et al.,* 2014; Zhang *et al.,* 2014; Ma *et al.,* 2024; Tsubota *et al.,* 2025). Among these, TALENs have been successfully applied in *B. mori* to generate targeted insertions within the Ser1 locus, establishing in situ expression systems capable of producing high levels of functional recombinant proteins such as enhanced green fluorescent protein (EGFP) and human epidermal growth factor (hEGF) in both MSGs and cocoons (Wang *et al.,* 2013; Xu *et al.,* 2018; Zhang *et al.,* 2018)

Given its low-cost maintenance, high protein output, and ability to perform post-translational modifications similar to those of higher eukaryotes, the silkworm presents a robust and sustainable solution for recombinant protein production. It has also found applications in the textile, cosmetic, biomedical, and pharmaceutical industries (Alam *et al.,* 2022). Insect biotechnology, particularly using *B. mori*, is therefore rapidly advancing as one of the most promising areas for scalable and efficient molecular farming (Chen *et al.,* 2018). Among these, the use of transgenic silkworms as silk gland bioreactors especially through targeted genetic engineering has emerged as a central and transformative strategy for producing complex recombinant proteins at high levels and low cost.

This review aims to comprehensively summarize the recent advancements in transgenic silkworm biotechnology, with a focus on expression systems, molecular strategies, case studies, and future prospects in recombinant protein production.

**2. SILKWORM EXPRESSION SYSTEMS**

The silk gland of *Bombyx mori* is uniquely adapted for the synthesis and secretion of large quantities of proteins, making it an excellent candidate for use as a biological platform for recombinant protein expression.

**2.1 Baculovirus-Silkworm Expression System**

The baculovirus-silkworm system employs *Bombyx mori*nucleopolyhedrovirus (BmNPV) as a vector to introduce foreign genes into host cells most commonly within the silk gland where they are transiently expressed. This platform allows for high-yield protein production on a short timeline without the need to generate stable transgenic lines (Maeda *et al.,* 1985). Foreign genes are typically driven by strong viral promoters, such as the polyhedrin promoter, within recombinant baculoviruses that infect silkworm larvae or pupae. This system has been widely used to express biologically active proteins, including cytokines, enzymes, antibodies, and vaccines (Motohashi *et al.,* 2005). Nonetheless, there are several limitations such as complex vector construction, viral instability, batch variation, risks of viral contamination, and inconsistent post-translational modifications, particularly glycosylation (Kato *et al.,* 2010; Mori *et al.,* 1992). Despite these challenges, the system remains suitable for high-yield, short-term production and proof-of-concept studies.

**2.1.1 Expression of Recombinant Proteins in Silkworm Larvae and Pupae**

The first successful expression of a recombinant protein in *Bombyx mori* was the production of human interferon-alpha (IFN-α) using BmNPV with a polyhedrin promoter (Maeda *et al.,* 1985). However, the expressed proteins were often degraded in the hemolymph due to the activity of a viral cysteine protease. To counteract this, vectors lacking the protease gene were developed (Kato *et al.,* 2010). Silkworm larvae generally achieve higher expression levels than insect or mammalian cell cultures. For instance, mouse interleukin-3 activity in silkworm hemolymph was 20-fold and 10,000-fold higher than in BmN and COS7 cells, respectively (Miyajima *et al.,* 1987), and human butyrylcholinesterase activity was 23- and 280-fold higher than in BmN and CHO cells, respectively (Wei *et al.,* 2000;Kadono-Okuda *et al.,* 1995; Qiu *et al.,* 1994).

Expression systems using BmNPV mutants lacking both the cysteine protease and chitinase genes (BmNPV-*CP*− and BmNPV-*CP*−-*Chi*−) have been developed to prevent protein degradation and larval liquefaction (Suzuki *et al.,* 1997; Nagaya *et al.,* 2004; Lee *et al.,* 2006). Although BmNPV titers are generally lower in *B. mori* than in *Spodopterafrugiperda* (Sf-9) cells, hybrid viruses combining AcMNPV and BmNPV have been engineered to broaden host specificity and improve titers (Mori *et al.,* 1992; Maeda *et al.,* 1993; Kamita & Maeda, 1997). Using such hybrid viruses, significant yields of proteins like bovine interleukin-21 have been achieved (Muneta *et al.,* 2004). Silkworm pupae offer additional advantages as bioreactors they can be stored at 4°C and do not require feeding. Proteins such as human GM-CSF have been expressed in pupae, and post-translational modifications differ notably between pupae and larvae (Chen *et al.,* 2006) (Table 1).

**Table 1. Recombinant Proteins Expressed in Silkworms Using Baculovirus or Bacmid Systems**

|  |  |  |
| --- | --- | --- |
| **Proteins** | **Used Viruses or Bacmids** | **References** |
| Firefly luciferase | BmNPV | Palhan*et al.* (1995) |
| Human interferon-α | BmNPV | Maeda *et al.* (1985) |
| Human macrophage colony-stimulating factor | BmNPV | Qiu *et al.* (1994) |
| Human growth factor | BmNPV | Kadono-Okuda *et al.* (1995) |
| Rat interleukin-5 | Cysteine protease depleted BmNPV | Ishihara *et al.* (1999) |
| Human butyrylcholinesterase | BmNPV | Wei *et al.* (2000) |
| Bovine interleukin-21 | HyNPV | Muneta *et al.* (2004) |
| Bovine interferon-t | Cysteine protease depleted BmNPV | Nagaya *et al.* (2004) |
| Porcine lactoferrin | HyNPV | Wang *et al.* (2005) |
| Human granulocyte macrophage colony-stimulating factor | BmNPV | Chen *et al.* (2006) |
| EGFP-spider dragline silk fusion protein | BmNPV bacmid | Zhang *et al.* (2008) |
| Cholera toxin B | BmNPV | Gong *et al.* (2005) |
| Human stem cell factor | BmNPV | Han *et al.* (2004) |
| anti-BSA scFv | Cysteine protease and chitinase depleted BmNPV | Ishikiriyama*et al.* (2009) |
| Human anti-BSA IgG1 | Cysteine protease and chitinase depleted BmNPV | Park *et al.* (2009) |
| Human α2,6-sialyltransferase | Cysteine protease and chitinase depleted BmNPV | Ogata *et al.* (2009) |
| Human (pro)renin receptor | Cysteine protease depleted BmNPV | Du *et al.* (2008) |
| Human prorenin-(pro)renin receptor complex | Cysteine protease depleted BmNPV | Du *et al.* (2009) |

Source: Kato *et al.,* 2010

**2.1.2 Development of BmNPV Bacmid System**

Traditional recombinant BmNPV generation is labor-intensive, requiring co-transfection and multiple virus amplification steps, which may take 3–6 months (Xiang *et al.,* 2010; Kato *et al.,* 2010). To streamline this process, a baculovirus shuttle vector (bacmid) replicable in *E. coli* was developed (Luckow *et al.,* 1993). The Bac-to-Bac system facilitates rapid and simultaneous generation of multiple recombinant viruses.Motohashi *et al.* (2005) adapted this system for *B. mori* by creating a BmNPV bacmid. Recombinant protein expression was achieved simply by injecting the bacmid DNA into silkworm larvae or pupae, bypassing the need for baculovirus stock preparation. This system significantly accelerates recombinant protein production (Fig. 1).



**Fig. 1.Comparative workflow of Conventional BmNPV Expression System and BmNPV Bacmid System for recombinant protein production in silkworm larvae. (A) In the conventional BmNPV system, the target gene is co-transfected with wild-type BmNPV into B. mori cells, followed by virus purification, amplification, and injection into larvae; total process time ranges from 12–25 weeks. (B) In the bacmid system, recombinant BmNPV bacmid DNA is constructed in E. coli and directly injected into larvae, allowing protein recovery within 2–3 weeks, significantly reducing production time.**

Source: Kato *et al.,* 2010

**2.2 Transgenic Silkworm Expression System**

A major advancement in *B. mori* genetic engineering was the development of a stable germline transformation system based on the piggyBac transposon (Tamura *et al.,* 2000). This system enables stable insertion of foreign genes into the silkworm genome, allowing for heritable and tissue-specific protein expression, especially in the silk gland.

Genes are typically placed under silk gland-specific promoters, such as *Ser1* (for MSG) and *FibH* (for PSG), ensuring localized synthesis. One of the key advantages of this system is the ability to harvest recombinant proteins directly from cocoons, particularly when expressed in sericin, which is water-soluble and readily extractable. These transgenic lines are heritable, enabling consistent protein production without repeated transfection or infection (Tomita *et al.,* 2003). Moreover, the absence of viral elements improves biosafety.Enhancements have been made using regulatory elements like viral enhancers (hr3), optimized 3′-UTRs (*Ser1PA*), and signal peptides that enhance translation and secretion (Iizuka *et al.,* 2008; Wang *et al.,* 2013). Mutant silkworms lacking fibroin secretion have been generated to redirect glandular resources toward recombinant protein synthesis (Inoue *et al.,* 2005).Genome editing technologies like TALEN and CRISPR/Cas9 have also enabled site-specific integration of transgenes, improving reproducibility and expression efficiency (Wang *et al.,* 2013; Xu *et al.,* 2018). These advances have facilitated high-yield production of therapeutic proteins like human epidermal growth factor (hEGF) and EGFP.

**2.2.1 Germline Transformation Using piggyBac Transposon**

The piggyBac transposon, first isolated from *Trichoplusiani*, functions via a cut-and-paste mechanism, allowing efficient integration of large DNA fragments into AT-rich genomic regions (Li *et al.,* 2013; Yusa, 2015; Zhao et al., 2016). The key elements include inverted terminal repeats (ITRs) and a transposase enzyme. In the system developed by Tamura *et al.* (2000), the green fluorescent protein (GFP) gene, driven by the BmA3 promoter, was flanked by ITRs and co-injected with a helper plasmid expressing transposase. Approximately 2% of G1 offspring were successfully transformed. This method has since been used to express a wide range of medically relevant proteins in silkworms.

**2.3 Silk Gland-Specific Expression Systems in *Bombyx mori***

The silk gland of *B. mori* serves as the central organ for producing recombinant proteins, leveraging high levels of endogenous fibroin and sericin gene expression. Selection of an appropriate silk gland expression system is critical and depends on the desired characteristics of the target protein (Xu, 2014) (Fig. 2).

**2.3.1 Fibroin Light Chain (FibL) Expression System**

The FibL-based system was the first to express foreign proteins in silkworms. Recombinant proteins are secreted into the PSG lumen as free macromolecules. The system typically includes a 5′-flanking sequence, 3′-flanking sequence, and partial FibL cDNA (*Xu, 2014*). Tomita *et al*. (2003) and Xue *et al.* (2012) successfully used this system to express human collagen and human granulocyte-macrophage colony stimulating factor (GM-CSF), respectively. However, the native FibL competes with the recombinant FibL for disulfide bonding with FibH, leading to reduced expression (Tatematsu, 2012). To overcome this, Inoue *et al.* (2005) developed FibL-deficient silkworm strains for enhanced protein yield.

**2.3.2 Fibroin Heavy Chain (FibH) Expression System**

The FibH expression system is widely adopted, particularly for generating high-performance silk materials for biomedical use. The FibH promoter demonstrates stronger transcriptional activity than the FibL promoter (Tatematsu, 2012). Three structural variants, R1, R2, and R3 have been described, with the R3 configuration (including the N- and C-terminal domains of FibH) being the most efficient (Xu, 2014). Although FibH is primarily expressed in the PSG, target proteins expressed here can be more readily extracted from the MSG (Xu, 2014). Teulé*et al.* (2012) engineered silkworms with spider silk gene sequences under FibH control, resulting in silk fibers with exceptional strength, exceeding even natural spider silk.

**2.3.3 Sericin-1 (Ser1) Expression System**

Sericin expression involves three main genes: Ser1, Ser2, and Ser3. Ser1 and Ser3 contribute to cocoon sericin, while Ser2 is more associated with larval silk (Kunz *et al.,* 2016). Promoter activity studies by Tatematsu*et al.* (2010), using a GAL4/UAS-driven EGFP system, revealed strong activity from the *ser1* promoter in both MSG and PSG, while *ser3* showed moderate activity in MSG and *ser2* showed little to none. To enhance expression levels, the system can be supplemented with the baculovirus-derived enhancer hr3 and the transactivator IE1 (Tomita *et al.,* 2007). These modifications have enabled more robust and targeted production of recombinant proteins within sericin layers.



**Fig. 2.Schematic Representation of Promoter Constructs Used for Silk Gland-Specific Expression in Transgenic Silkworms. a)TheFibH-expression system, b) FibL-expression system, c) Fhx-expression system, d) Ser1-expression system, and e) Ser3-expression system. Each construct is shown with key regulatory elements (enhancers, promoters, introns, polyA/terminator), gene of interest (GOI), and relative sequence lengths.**

Source: Xu, 2014

**3. MOLECULAR ENGINEERING STRATEGIES**

**3.1 Promoters and Enhancers**

The selection of appropriate promoters is a critical factor in determining both the yield and tissue specificity of recombinant protein production in *Bombyx mori*. Among the most frequently employed promoters is the sericin-1 (Ser1) promoter, which facilitates targeted expression in the middle silk gland (MSG)—a tissue specialized in the synthesis and secretion of sericin proteins. Incorporation of regulatory elements such as the hr3 enhancer from BmNPV and optimized 3′ untranslated regions (Ser1PA) significantly boosts the transcriptional efficiency of the Ser1 promoter (Tomita *et al.,* 2007; Iizuka *et al.,* 2008).

For posterior silk gland (PSG)-specific expression, fibroin heavy chain (FibH) and fibroin light chain (FibL) promoters are widely used due to their high transcriptional activity. However, one of the main limitations of using FibH/L promoters is that recombinant proteins often become incorporated into the insoluble fibroin matrix of the cocoon, making protein recovery more challenging (Xu, 2014; Tatematsu, 2012). Therefore, promoter selection must consider not only the expression level but also downstream processing requirements based on the solubility and localization of the target protein.In the development of transgenic silkworm platforms, the FibL promoter with its 5′-flanking regulatory regions has been used to achieve targeted expression of recombinant proteins in the PSG. Using this configuration, proteins have been secreted into the silk gland lumen alongside endogenous fibroin proteins, reaching up to 0.84% of total cocoon shell weight (Tomita *et al.,* 2003). Subsequently, an improved system employing the FibH promoter increased recombinant protein accumulation to approximately 15% (w/w) of cocoon shell weight (Tomita *et al.,* 2007; Zhao *et al.,* 2010).

Parallel advancements in the transposon-based sericin expression system have allowed ectopic expression of exogenous proteins under the control of the Ser1 promoter. This system has been further optimized by modifying both promoter elements and 3′ UTR sequences, enhancing transcriptional and translational efficiency (Tomita *et al.,* 2007; Iizuka *et al.,* 2008; Wang *et al.,* 2013). In particular, silkworm lines deficient in fibroin secretion have demonstrated increased recombinant protein yields, as the secretory machinery is redirected toward the production of foreign proteins (Inoue *et al.,* 2005).Given that sericin is hydrophilic and generally non-allergenic, it has found applications in biomedical materials, such as wound healing agents (Aramwit et al., 2012). Building on this potential, a sericin in-fusion expression system was used to express human epidermal growth factor (hEGF), a peptide involved in epidermal wound healing and keratinocyte stem cell proliferation—in transgenic silkworm cocoons (Nanba *et al.,* 2013). The hEGF gene (183 bp, ~7.2 kDa) was integrated using TALEN-mediated genome editing. Out of 640 wild-type eggs injected with TALEN mRNA and donor plasmid, four fluorescent-positive G1 broods were obtained from 65 total broods, yielding a transformation efficiency of 6.2%.

To compare production efficiency, a transposon-based Ser1-driven line (Ser–T–hEGF) was generated as a control alongside a Ser–2A–hEGF line, which used a 2A peptide strategy for in-fusion expression. In the Ser–2A–EGFP animals, high expression levels of EGFP were observed in both MSG and cocoon shells, attributed to the use of the entire Ser1 promoter region, which may enhance transcriptional efficiency.The hr3 enhancer, coupled with the native Ser1 signal peptide, has also been utilized to achieve spatially and temporally controlled expression of other medically relevant genes such as platelet-derived growth factor-B (PDGF-B) in transgenic silkworms (Chen *et al.,* 2018). For selection purposes, 3xp3-EGFP, which confers specific EGFP expression in the ocelli and compound eyes, was used as a visual genetic marker (Chen *et al.,* 2018).

Among PSG-targeted systems, FibH promoter-driven constructs remain the most widely used due to their strong transcriptional activity, with three major configurations: R1, R2, and R3, of which R3incorporating the FibH promoter and both N- and C-terminal regions of FibHhas proven the most effective (Tatematsu, 2012; Xu, 2014).Tatematsu*et al.* (2010) evaluated the activities of Ser1, Ser2, and Ser3 promoters using a binary GAL4/UAS expression system to drive EGFP expression. Strong promoter activity was observed for Ser1 in both the PSG and MSG, while Ser3 showed moderate activity in the anterior MSG. Ser2, however, demonstrated negligible activity in any silk gland region. Enhancer and trans-regulator elements such as hr3 and IE1 have been implemented to overcome these limitations and boost promoter performance (Tomita *et al.,* 2007).

Overall, two major classes of transgenic expression systems have been established in silkworms for recombinant protein production, PSG-targeted systems using FibH/FibL promoters (Tomita *et al.,* 2003; Royer *et al.,* 2005; Kurihara *et al.,* 2007; Ogawa *et al.,* 2007; Zhao *et al.,* 2010) and MSG-targeted systems using the Ser1 promoter, which includes a 528 bp 5′-flanking region, 54 bp 5′ UTR, and an 87 bp sericin-1 signal peptide sequence (Garel *et al.,* 1997; Xia *et al.,* 2004; Liu *et al.,* 2006; Tatematsu*et al.,* 2010). Modified vectors such as phSRSV and phSRSE, incorporating hr3 and Ser1PA, have also been designed for enhanced transgene expression (Liu *et al.,* 2006). Despite attempts to utilize Ser2 and Ser3 promoters, only the Ser1-based system has demonstrated consistent effectiveness for high-level recombinant protein expression in the MSG (Tatematsu*et al.,* 2010).

**3.2 Site-Specific Integration**

One of the key challenges in generating transgenic silkworms is the random integration of transgenes, which often results in position effect variegation or transcriptional silencing, leading to inconsistent expression. To address this, site-specific genome editing technologies, including TALENs and CRISPR/Cas9, have been introduced to allow targeted insertion of transgenes at well-characterized genomic loci (Wang *et al.,* 2013; Xu *et al.,* 2018; Ma *et al.,* 2024). Targeting the Ser1 locus within the MSG has proven to be particularly effective for achieving both high-level and consistent expression of recombinant proteins. These genome-editing tools generate double-strand breaks (DSBs) at specific target sites, promoting homology-directed repair (HDR) and enabling precise integration of exogenous DNA.

This site-directed strategy enhances the genetic stability, reduces line-to-line variability, and minimizes off-target effects, thereby improving the reproducibility of protein production. Successful applications include the stable expression of human epidermal growth factor (hEGF) and enhanced green fluorescent protein (EGFP) directly in silkworm cocoons (Zhang *et al.,* 2018; Xu *et al.,* 2018). Collectively, these molecular engineering strategies ranging from promoter/enhancer optimization to genome editing for site-specific integrationhave significantly improved the transcriptional and translational efficiency of recombinant protein production in silkworms, positioning them as a scalable and reliable biomanufacturing platform.

**4. APPLICATIONS AND CASE STUDIES**

The transgenic silkworm platform has emerged as a versatile and efficient system for the production of therapeutic proteins, primarily through targeted expression within the silk glands. By harnessing tissue-specific promoters, proteins can be accumulated either within the soluble sericin matrix or embedded in the fibroin fibers, facilitating downstream recovery and application in biomedical and pharmaceutical contexts.

**4.1 Recombinant Human Proteins**

A range of clinically relevant human proteins have been successfully expressed in Bombyx mori, affirming the feasibility of using silkworms as bioreactors (Table 2):

* **Human Interferon Alpha (IFN-α):** Maeda *et al.* (1985) demonstrated the first successful application of a recombinant protein expression system in silkworms using the BmNPV vector. The **polyhedrin promoter**, known for its strong expression during the late phase of viral infection, facilitated high-level production of IFN-α marking a milestone in using silkworms for therapeutic protein synthesis.
* **Human Collagens:** Tomita *et al.* (2003) directed the expression of a fragment of human type III collagen in the posterior silk gland (PSG) under the control of the FibL promoter. Initial challenges in biological activity were attributed to inadequate levels of prolyl 4-hydroxylase α-subunits (P4Ha). Subsequent co-expression with B. mori P4Ha partially restored collagen functionality (Adachi *et al.,* 2006). Later, Adachi *et al.* (2010) successfully expressed the α1(I) collagen chain in the middle silk gland (MSG), establishing the feasibility of generating high-quality gelatin analogs for biomedical use.
* **Human Serum Albumin (HSA):** Ogawa *et al.* (2006) generated germline transgenic silkworms expressing recombinant HSA (rHSA) under the control of the Ser1 promoter, enhanced by the hr3 enhancer and IE1 trans-activator. The recombinant protein was secreted into the MSG lumen and deposited in the sericin layer, from which 2.8 mg of more than 99% pure rHSA was recovered from 2 g of cocoons. Structural and functional assays confirmed its equivalence to plasma-derived HSA.
* **Human Insulin Receptor (hIR):**Matsumoto *et al.* (2014) demonstrated the production of functional hIR in transgenic silkworms, which effectively reduced hemolymph sugar levels. This model presents a valuable system for pharmacokinetic and therapeutic investigations.
* **Adiponectin:** As an adipocyte-secreted hormone involved in glucose and lipid metabolism, adiponectin holds potential for treating metabolic disorders. The gene was expressed using an internal ribosome entry site (IRES)-mediated bicistronic system in transgenic silkworms, resulting in the detection of a 30 kDa functional monomeric form (Shin *et al.,* 2014). The biologically active recombinant protein presents prospects for managing insulin resistance and inflammation (Zhang *et al.,* 2018).
* **Human Cytokines:** Various cytokines have been expressed in silkworm silk glands, including acidic FGF (Wu & Cao, 2004), basic FGF (Hino *et al.,* 2006), IGF-I (Zhao *et al.,* 2009; Li *et al.,* 2011; Song *et al.,* 2014), and GM-CSF (Xue *et al.,* 2012). Functional bioassays confirmed their bioactivity in human or mouse models.
* **Human Acidic FGF (haFGF):** Wang *et al.* (2015) achieved a 5.6-fold increase in haFGF yield via targeted insertion into transcriptionally active sericin loci. The protein retained full mitogenic activity in NIH-3T3 cells.
* **Chimeric Anti-CD20 mAbs:** Tada *et al.* (2015) engineered transgenic silkworms to produce rituximab-analog chimeric mAbs for targeted cancer therapies. These antibodies displayed distinct N-glycosylation patterns, yielding improved antibody-dependent cellular cytotoxicity (ADCC) but reduced complement-dependent cytotoxicity (CDC) compared to those produced in CHO cells (Aoyama *et al.,* 2018).
* **Platelet-Derived Growth Factor-BB (PDGF-BB):** Recognized for its role in wound healing and angiogenesis, PDGF-BB was expressed in MSG under the Ser1 promoter by Chen *et al.* (2018). The recombinant protein, recovered at 0.33 mg/g cocoon with 82% purity, significantly promoted fibroblast proliferation and migration in vitro.
* **Vascular Endothelial Growth Factor (VEGF165):** VEGFs play a central role in promoting angiogenesis, a pivotal process in tumor progression from benign to malignant states. These growth factors not only stimulate neovascularization but also help protect tumor cells from apoptosis (Shibuya, 2011). To exploit this potential, Zhang *et al.* (2019) constructed transgenic silkworms expressing recombinant VEGF165 in the middle silk gland (MSG), where the protein was secreted into the cocoon. The successful expression and recovery of VEGF165 underscore the feasibility of using silkworms as a cost-effective platform for producing angiogenesis-related therapeutic proteins.
* **Recombinant PreS2-S Protein:** Hepatitis B virus (HBV) is a major cause of liver diseases, including cirrhosis and hepatocellular carcinoma. To develop a new and effective vaccine candidate against HBV, Abdurakhmanov *et al.* (2019) utilized the BmNPV-based expression system in Bombyx mori to produce the PreS2-S protein, a component of the HBV surface antigen family. The protein was successfully expressed in silkworm larvae, highlighting the cost- and time-efficient nature of this method. This recombinant PreS2-S protein is a promising candidate for HBV vaccine development.
* **Human Epidermal Growth Factor (hEGF):** Li *et al.* (2022) employed the transcription activator-like effector nuclease (TALEN)-mediated insertion into the sericin locus, achieving a more than 15-fold increase in hEGF production compared to standard piggyBac-based systems.

**4.2 Recombinant Animal Proteins**

* **Mouse Monoclonal Antibodies (mAbs):** Iizuka *et al.* (2009) demonstrated the expression of functional mouse mAbs in cocoon sericin layers, with simplified extraction and purification. The use of mAbs is a promising therapeutic method to prevent the infectious disease (Jahanshahlu& Rezaei, 2020).
* **Major Royal Jelly Protein-1 (MRJP1):** You *et al.* (2017) used the FibL promoter to express glycosylated MRJP1, highlighting the silkworm’s ability to perform complex post-translational modifications.
* **Thyroid Hormone Receptor (TRβ1):** Nakaya *et al.* (2020) utilized Bombyx mori to express mouse thyroid hormone receptor TRβ1, a nuclear receptor crucial for regulating metabolism and development. These receptors, TRα1 and TRβ1, are ligand-dependent transcription factors that modulate gene expression in response to thyroid hormones (Anyetei-Anum *et al.,* 2018). The transgene was introduced using a piggyBac vector under the control of the fibroin heavy chain (FibH) promoter, fused with an E. coli-derived glutathione S-transferase (GST) tag to facilitate purification. Expression was regulated using the GAL4/UAS binary system, and transformation efficiency was verified by EGFP fluorescence. Additionally, a separate transgenic construct was engineered to ensure posterior silk gland (PSG)-specific expression, enabling precise spatial targeting within the silk gland (Nakaya *et al.,* 2020).

**Table 2. Recombinant Proteins Expressed Using Silk Gland Expression Systems**

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| --- | --- | --- | --- | --- |
| **Application fields** | **Target genes/fragments** | **Promoters**  | **Expressed regions** | **References** |
| **Fibroin Heavy Chain (FibL) Expression System** |
| Model proteins | EGFP  | FibH | PSG  | Kojima *et al.* (2007) |
| Animal-derived pharmaceutical proteins  | Feline interferon | FibH | PSG  | Kurihara *et al.* (2007) |
| Model proteins | EGFP  | FibH | PSG  | Zhao *et al.* (2010) |
| Silk-based biomaterials | Partial spider silk fibroin sequence  | FibH | PSG  | Zhu *et al.* (2010) |
| Silk-based biomaterials | Ca binding sequence  | FibH | PSG  | Nagano *et al.* (2011) |
| Silk-based biomaterials | Partial spider silk fibroin sequence  | FibH | PSG  | Teule´ *et al.* (2012) |
| Silk-based biomaterials | EGFP  | FibH PSG Iizuka et al. (2013) | PSG  | Iizuka *et al.* (2013) |
| Silk-based biomaterials | DsRed | FibH | PSG | Iizuka *et al.* (2013) |
| Silk-based biomaterials | Monomeric Kusabira orange  | FibH | PSG | Iizuka *et al.* (2013) |
| Animal-derived pharmaceutical proteins | Mouse thyroid hormone receptor TRβ1 | FibH | PSG | Nakaya *et al.* (2020) |
| **Fibroin Light Chain (FibL) Expression System** |
| Human-derived pharmaceutical proteins | Human type III procollagen  | FibL | PSG | Tomita *et al.* (2003); Adachi *et al.* (2006) |
| Human-derived pharmaceutical proteins | Human basic fibroblast growth factor  | FibL | PSG  | Wu &Cao (2004); Hino *et al.* (2006) |
| Model proteins | EGFP  | FibL | PSG  | Inoue *et al.* (2005) |
| Silk-based biomaterials | Partial collagen sequence  | FibL | PSG | Yanagisawa *et al.* (2007); Nakazawa *et al.* (2011) |
| Silk-based biomaterials | Partial fibronectin sequence  | FibL | PSG | Yanagisawa *et al.* (2007); Nakazawa *et al.* (2011) |
| Human-derived pharmaceutical proteins | Human l-opioid receptor  | FibL | PSG  | Tateno *et al.* (2009) |
| Human-derived pharmaceutical proteins | Human granucyto-macrophage colonystimulating factor  | FibL | PSG  | Xue *et al.* (2012) |
| Silk-based biomaterials | Single-chain variable fragment  | FibL | PSG  | Sato *et al.* (2012, 2014) |
| Animal-derived pharmaceutical proteins | Royal jelly protein (MRJP1) | FibL | PSG | You *et al.*(2017) |
| **Sericin-1 (Ser1) Expression System** |
| Silk-based biomaterials | Spider dragline silk (MaSp1)  | Ser1  | MSG | Wen *et al.* (2004) |
| Model proteins | EGFP  | Ser1  | MSG | Tomita *et al.* (2007) |
| Model proteins | DsRed | Ser1  | MSG | Tomita *et al.* (2007) |
| Human-derived pharmaceutical proteins | Human serum albumin  | Ser1  | MSG | Ogawa *et al.* (2007) |
| Model proteins | EGFP  | Ser1  | MSG | Iizuka *et al.* (2008) |
| Animal-derived pharmaceutical proteins | Mouse monoclonal antibody  | Ser1  | MSG | Iizuka *et al.* (2009) |
| Model proteins | EGFP  | Ser3  | MSG | Tatematsu*et al.* (2010) |
| Human-derived pharmaceutical proteins | Human type I collagen a1(I) chain  | Ser1  | MSG | Adachi *et al.* (2010) |
| Model proteins | DsRed | Ser1  | MSG | Wang *et al.* (2013) |
| Human-derived pharmaceutical proteins | Human adiponectin  | Ser1  | MSG | Shin *et al.* (2014) |
| Human-derived pharmaceutical proteins | Human acidic fibroblast growth factor (r-haFGF) | Ser 1 | MSG | Wang *et al.*(2015) |
| Human-derived pharmaceutical proteins | Human platelet derived growth factor (PDGF- B) | Ser 1 | MSG | Chen *et al.*(2018) |

Source: Xu, 2014; Wang *et al.,* 2015; Chen *et al.,* 2018

**4.3 Biopharmaceutical Quality**

For therapeutic applications, protein quality is of paramount importance, particularly regarding glycosylation, folding, and proteolytic stability. Transgenic silkworm expression systems exhibit enhanced fidelity in post-translational modifications (PTMs) relative to baculovirus-based approaches. Silk gland-derived expression benefits from eukaryotic machinery that more accurately replicates mammalian glycosylation and folding pathways. In contrast, baculovirus systems may suffer from inconsistent glycosylation, improper disulfide bonding, and proteolytic degradation due to viral cysteine proteases (Kadono-Okuda *et al.,* 1995; Kato *et al.,* 2010).

Furthermore, transgenic approaches enable spatially restricted expression in either MSG or PSG, promoting homogeneity and simplifying purification—especially when sericin-targeted expression is employed. These advantages collectively support the use of transgenic silkworms for producing high-quality, biologically active recombinant proteins suitable for therapeutic applications.

**5. CHALLENGES AND FUTURE PROSPECTS**

The utilization of transgenic silkworms, particularly *Bombyx mori*, as bioreactors for recombinant protein production has shown significant promise. However, several technical, regulatory, and scalability barriers must be addressed to transition this platform from experimental use to industrial-scale biopharmaceutical manufacturing.

* 1. **Technical and Operational Challenges**
* **Efficient purification from complex silk matrices:**One of the most persistent obstacles lies in the downstream processing of recombinant proteins expressed within the silk gland. While proteins expressed in the sericin-rich middle silk gland (MSG) are more easily extracted due to the solubility of sericin in aqueous buffers, those embedded within the posterior silk gland (PSG)-derived fibroin matrix pose a greater challenge. Fibroin is highly insoluble and forms strong intermolecular β-sheet structures that complicate protein recovery. Advanced purification strategies, including the use of engineered affinity tags, enzymatically cleavable linkers, or targeted expression into cocoons with reduced silk protein backgrounds such as fibroin-deficient silkworms, are being developed to overcome this bottleneck.
* **Scaling up and process standardization:**Although *B. mori* is well-suited for mass rearing and has been cultivated at scale for centuries, commercializing transgenic silkworm-based protein production introduces challenges. These include maintaining transgene stability across generations, ensuring uniform larval development, controlling environmental conditions, and preventing microbial contamination. Additionally, scaling from laboratory to industrial production lacks standardized bioprocess protocols specific to insect bioreactors, which can lead to batch-to-batch variability and reduced reproducibility.
* **Regulatory barriers for human therapeutic use:**To gain approval for clinical application, recombinant proteins must meet strict regulatory criteria regarding safety, efficacy, consistency, and purity. Challenges include validating that silkworm-derived proteins have appropriate post-translational modifications such as glycosylation, ruling out immunogenicity or toxicity, and ensuring that no viral or insect-derived contaminants are present. Developing regulatory frameworks and Good Manufacturing Practice (GMP) protocols specifically tailored to insect expression systems is essential to facilitate clinical translation.

**5.2 Strategic Innovations and Future Prospects**

* **Synthetic biology and expression optimization:**Advancements in synthetic biology provide powerful tools for enhancing expression control in the silkworm silk gland. Engineered promoters, enhancers (baculoviral hr3), untranslated regions (UTRs), and signal peptides can be customized for high-level and tissue-specific expression of foreign proteins. Additionally, the use of inducible or environmentally regulated systems may improve biosafety and reduce metabolic burden on the host organism. These tools allow for precise spatiotemporal control of gene expression and improved expression stability over multiple generations.
* **Expanding the host range to non-mulberry silkworms:**Although *Bombyx mori* is the most extensively studied and domesticated silkworm species, there is growing interest in exploring non-mulberry silkworms such as *Antheraeamylitta*, *Antheraeapernyi*, and *Samiacynthiaricini*. These wild species produce stronger or different silk types and may offer unique advantages in terms of silk composition, immune responses, and adaptability to alternate expression conditions. Some preliminary studies have demonstrated successful transgene integration and protein expression in non-mulberry silkworms, though these systems remain less genetically tractable compared to *B. mori*. Further development of genomic tools and transformation techniques could unlock their potential for specialized protein production.
* **AI-driven design and process optimization:**Integration of artificial intelligence and machine learning is poised to revolutionize recombinant protein design and silkworm-based bioprocess development. AI models can predict optimal codon usage, protein folding patterns, and secretion signals tailored to the silkworm's translation machinery. Moreover, digital twins and AI-powered control systems could monitor and optimize larval rearing conditions, infection timing (in baculovirus systems), and harvest cycles, thereby increasing yield consistency and reducing production costs.

**6. CONCLUSION**

Transgenic *Bombyx mori* represents a transformative and biologically efficient platform for recombinant protein production. Leveraging the high secretory capacity of the silk gland and precise genetic control mechanisms including promoter engineering, enhancer elements, and genome editing has enabled the synthesis of biologically active, therapeutically relevant proteins at industrially viable scales. The use of both middle and posterior silk glands offers spatial flexibility for expressing proteins with varying biochemical properties, while mutant silkworm lines deficient in silk protein secretion further boost yield efficiency. Despite these advances, challenges persist in protein purification, large-scale rearing, and satisfying regulatory standards for therapeutic use. Moreover, extending transgenic approaches to non-mulberry silkworms offers untapped potential for broader host compatibility and novel biomaterial applications. Future integration of synthetic biology, artificial intelligence, and systems biology will be crucial to optimizing gene constructs, codon usage, and rearing protocols. With these innovations, transgenic silkworms are poised to become a core component of next-generation biomanufacturing platforms for pharmaceuticals and beyond.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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