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Design of Signal Peptide Bombyxin and its Effect on Secretory Expression Efficiency and Levels of *Helicobacter pylori* Urease Subunit B in Silkworm Cells and Larvae

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ABSTRACT

*This study employed a Bac-to-Bac/*Bombyx mori *bioreactor to mass-produce immunogenic urease subunit B (UreB) from* Helicobacter pylori. *The signal peptide* bombyxin *from* B. mori *was used to promote secretory expression to improve expression levels and was designed and integrated into the UreB gene to generate the Bacmid/BmNPV/(signal peptide)-UreB* baculovirus *expression system. To determine whether the* bombyxin *signal peptide resulted in secretory expression of recombinant UreB (rUreB) and to determine the secretory efficiency, we tested the secretory expression level of rUreB in Bm5 cells using ELISA. To further investigate whether secretory expression affected cell viability, cells were evaluated using 0.4% trypan blue staining, and Bacmid/BmNPV/UreB without the signal peptide served as a control. The above recombinant bacmid constructs were injected to silkworm larvae, and the secretory expression level of rUreB was detected using SDS-PAGE and semi-quantitative western blot analysis. The results indicated that the* bombyxin *signal peptide directed the secretory expression of rUreB and that this expression improved the viability of Bm5 cells. Moreover, the results showed that the expression level of rUreB was 1.5 times higher with the Bacmid/BmNPV constructs containing the* bombyxin *signal sequence than those without the signal sequence. These results demonstrate that secretory expression can enhance rUreB expression levels and is likely to aid in the large-scale expression and yield of rUreB in silkworm larvae.*

Key words: baculovirus expression system, *Bombyx mori*, signal peptide, *Helicobacter pylori* urease subunit B, secretory expression

INTRODUCTION

The baculovirus expression system (BES) has been widely used to express many types of eukaryotic and prokaryotic recombinant proteins at high levels. BES is highly effective in cultured insect cells because it is driven by the strong p10 and polyhedron promoters. *B. mori* nucleopolyhedrovirus (BmNPV) infects not only silkworm cell lines but also pupae and larvae, and protein expression levels using silkworm pupae or larvae are 10-100-fold higher than those using *B. mori* cell lines, indicating that pupae and larvae are better suited for the mass production of recombinant proteins. Indeed, it has been shown that silkworm pupae and larvae can be utilized in the BmNPV BES system instead of silkworm cell culture and that this is more conducive to large-scale recombinant protein production. Furthermore, scaled-up production of recombinant proteins is more easily performed in silkworm pupae or larvae than in silkworm cells (Muneta et

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al. 2003; Rabah et al. 2004; Wang et al. 2005). In addition, silkworms can be conveniently reared at much lower cost using mulberry leaves or an artificial diet throughout the year, making large-scale and successive production of heterologous proteins both possible and economical. BmNPV in BES also eliminates concerns regarding pathogens that could potentially be transmitted to humans, as the baculovirus is an invertebrate virus and is not infectious to vertebral animals due to its strict host specificity. The system itself is safe for the environment and humans (Herrington et al. 1992; Yamamoto et al. 1999). Proteins produced with silkworm bioreactors have also been demonstrated to retain their natural activity. Therefore, the use of silkworms as a production system for for large-scale and low-cost industrial mass production of recombinant proteins is very promising (Maeda 1994). In our previous reports, we successfully employed a practical BmNPV bacmid system to recombinantly express the urease subunit B (rUreB) protein in silkworm pupae using recombinant bacmid baculoviruses, resulting in prophylactic and therapeutic effects against *H. pylori* infection in mice (Zhang et al. 2011; Zhang et al. 2012). Indeed, it is important and of particular value to achieve efficient rUreB expression for medical and industrial applications. Theoretically, the amount of protein expressed is approximately 30% of the total protein generated by the strong polyhedrin and p10 promoters; nonetheless, exogenous proteins are generally not well expressed at high levels. Although the reason for this is unknown, it is possible that rUreB may be better expressed via secretory expression using signal peptides. Signal peptides, however, might be inefficiently recognized by the protein translocation machinery found in insect cells. Tessier et al (1991) reported that insect cells inoculated with a baculovirus harboring the gene encoding propapain fused to a honeybee melittin signal peptide sequence secreted more than fivefold the amount of the papain precursor protein fused to a plant signal peptide. In this paper, we explore the effects of a signal peptide derived from bombyxin, an insulin-like secretory peptide from the brain of the silkworm (Adachi et al. 1989), on the expression of rUreB in the BmNPV bacmid system. To investigate secretory efficiency, the effects on rUreB protein secretion were examined in Bm5 cells, and the ability of the signal peptide to direct rUreB protein secretion was further determined in silkworm larvae. The purpose of the present study was to improve Bac-to-Bac/BmNPV BES expression of recombinant UreB for medical and industrial applications and for potential use as an edible vaccine against *H. pylori* infection in humans.

MATERIAL AND METHODS

Signal peptide design

The bombyxin signal sequence from silkworm was based on the report of Adachi et al (Adachi et al. 1989) and modified with the aid of SignalP 3.0 (http://www.cbs.dtu.dk/services/ signalp/), which is based on an artificial neutral network and hidden markov models (HMM) (Bendtsen et al. 2004).

Construction of recombinant Bacmid/BmNPV signal peptide)-UreB

The recombinant bacmid system Bacmid/BmNPV/*UreB* was previously established in our laboratory (Zhang et al. 2011). The *UreB* gene of *Helicobacter pylori* (GenBank AY714224), pFastBacDual and DH10Bac/BmNPV *E. coli* were preserved in our laboratory. The *H. pylori UreB* gene fused to the bombyxin signal sequence was amplified by polymerase chain reaction (PCR) using primers synthesized at Sangon Biotech Co., Ltd. (Shanghai, China) (Table 1). To facilitate cloning, *Xho*I and *Kpn*I recognition sites were introduced into the 5′-terminal end of the forward primer (FP, underlined) and reverse primer (RP, underlined), respectively. The PCR product containing the bombyxin signal sequence was cloned into the Gateway cloning vector pENTR/D-TOPO (Invitrogen) according to the manufacturer-provided protocol. The isolated plasmid, pENTR/D/signal peptide-*UreB*, was recombined with the TaKaRa pMD19-TA cloning vector and sequenced using an ABI 3730 DNA Analyzer. The construction of Bacmid/ BmNPV/ (signal peptide)-*UreB* baculoviruses was performed as described in our previous report (Zhang et al. 2011).

Table 1 - PCR primers.

Name	Sequence (5'-3')
Bombyxin-F	CACCCTCGAGATGAAGATACTCCTTGCTA TTGCATTAATGTTGTCA
	ACAGTAATGTGGGTGTCAACACAACCGCG GGGTTCTCATCATC
UreB-R	TTGGTACCCTAGAAAATGCTAAAGAGTTG

Secretory expression of rUreB in Bm5 cells

Silkworm BmN5 cells were cultured with Sf-900 II Serum-Free Medium (SFM) (Invitrogen) supplemented with 1% antibiotic. Transfections of Bm5 cells with Bacmid/BmNPV/(signal peptide)-*UreB* and Bacmid/BmNPV/*UreB* (lacking the signal peptide) were performed as described in our previous report (Zhang et al. 2011). BmN5 cells, at a density of approximately 1.0×106 cells/mL, were infected with the viral transfection solution. The P1 viral stock was obtained at 120 h post-transfection from the cell medium. We amplified the viral stock through further infection of BmN5 cells using the P1 stock until the P3 stock was obtained (Zhang et al. 2011), and the P3 virus (106 viruses/10 µL) was used to infect BmN5 cells in six-well culture plates. The culture medium was collected and centrifuged every 12 from 72 to 120 h, and the culture supernatants and cell lysates were analyzed. The amount of secreted rUreB was determined using ELISA. The detailed procedure was described in our previous paper (Zhang et al. 2011). Secretory efficiency (%) = rUreB in culture supernatants/(rUreB in culture supernatants + rUreB in cells)×100.

Evaluation of BmN5 cell viability

The cells collected as described above were treated with culture medium, and a single-cell suspension was generated by repeated pipetting. The cell suspension was mixed with trypan blue according to the protocol included in the Trypan Blue Staining Cell Viability Assay Kit (Beyotime, Nantong, China). In this assay, live cells appear colorless and dead cells appear blue when observed under a microscope. We counted the number of dead cells and viable cells within a timeframe of three min: cell death rate (%) = the number of dead cells/(the number of viable cells + the number of dead cells)×100. The data represent the average viability of three separate experiments, and error bars indicate the standard deviation.

Secretory expression of rUreB in silkworm larvae

Fifth-instar silkworm larvae were reared with artificial feed at 27°C and were injected with 5 µL P3 Bacmid/BmNPV/(signal peptide)-*UreB* or Bacmid/BmNPV/*UreB* viral stock per larva. The hemolymph of 10 silkworm larvae was collected at 108 h after inoculation of the recombinant bacmids. To prevent melanization, 5 mM phenyl-thiourea was immediately added to the collected hemolymph. The body cells were processed after hemolymph collection and suspended in a solution consisting of 5 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid sodium salt and 0.5% Triton X-100 and then sufficiently homogenized with a homogenizer and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was analyzed for rUreB expression using ELISA. The detailed procedure was described in our previous paper (Zhang et al. 2011). Secretory efficiency (%) = rUreB in hemolymph/(rUreB in hemolymph+ rUreB in body cells)×100.

SDS-PAGE and semi-quantitative western blot analysis of rUreB in silkworm larvae

To assess the expression level of rUreB in silkworm larvae, a semi-quantitative western blot analysis was performed. Fifth-instar silkworm larvae were inoculated with Bacmid/BmNPV/(signal peptide)-*UreB* or Bacmid/BmNPV/*UreB*. After 108 h, total protein in both the hemolymph and lysed body cells was quantified using the BCA protein assay kit (Sangon Biotech Co., Ltd, Shanghai, China) and separated by 12% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. Western blotting was carried out according to our previous paper (Zhang et al. 2011). The rUreB protein was detected using the EasyBlot ECL kit (Sangon Biotech Co., Ltd, Shanghai, China). The ECL substrate was utilized for generating chemiluminescent bands with X-ray film, and the protein bands were semiquantitatively measured via densitometric scanning using Image-Pro Plus 6.0.

RESULTS AND DISCUSSION

Characteristics of the bombyxin signal peptide

In this study, the bombyxin signal peptide, M K L I L L A I A L M L S T V M T V S T Q P R G S, was designed using SignalP 3.0 (Verdile et al. 2004). The probability of this sequence being a signal peptide is 1.000, and the maximum cleavage site probability is 0.995. The predicted cleavage site is Thr-Gln (indicated by the gray box).

Construction and proliferation of Bacmid/ BmNPV/signal-*UreB*

The target signal peptide-*UreB* gene was inserted into the MCS of the donor plasmid pFastBacDual at the *Xho*I and *Kpn*I sites under the control of the P10 promoter to generate a recombinant bacmid

baculovirus containing the target gene. PCR resulted in a ~4300 bp product (Fig. 1), which was consistent with the predicted size of the PCR product (2560 bp plus 75 bp for the signal sequences and 1704 bp for the *UreB* gene), thus indicating the successful generation of Bacmid/BmNPV-(signal peptide)-*UreB*. The transfected cells displayed the typical increase in nuclear size and cell diameter, stopped growing, and exhibited mass lysis after transfection for 120 h. After three transfection cycles, a high-titer P3 viral stock was obtained and protected from light at 4°C.

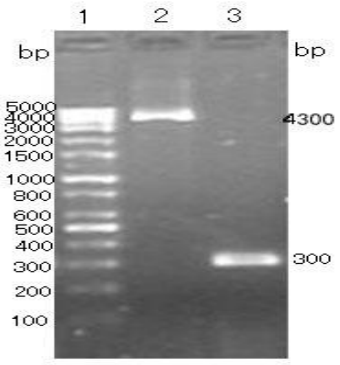

Figure 1 - Identification of Bacmid-signal-UreB 1. DNA marker, 2. PCR product using Bacmid-signal-UreB as the DNA template, 3. PCR product using Bacmid as the DNA template.

Secretory efficiency of rUreB expression in Bm5 cells and silkworm larvae

In BmN5 cells, rUreB protein expression was assessed every 12 from 72 h to 120 h post-infection with the P3 recombinant bacmid (Fig. 2). The rUreB protein was barely detectable in the culture medium after 72 h. The secretory expression level gradually increased from 84 h (10.3 ± 1.6%) to 108 h (32.5 ± 4.6%), but the secretory efficiency exhibited almost no change and gradually decreased from 108 h (32.5 ± 4.6%) to 120 h (31.7 ± 4.8%). In the control bacmid without the signal peptide sequence, the secretory expression level was low from 84 (1.1 ± 1.2%) to 108 h (8.6 ± 3.8%) ($p < 0.01$) but significantly increased from 108 to 120 h (23.2 ± 3.6%), which may be because cell lysis occurs at late stages of infection, with protein being released into the culture medium. In silkworm larvae, the secretory efficiency of the rUreB protein was detected at 108 h post-infection. As shown in Figure 2, approximately 32% (31.8 ± 4.7%) of the total rUreB protein expressed was secreted into the hemolymph; the secretory efficiency of the control without the signal peptide was only 9.4 ± 4.8%. These results suggested that the *UreB* gene fused to the bombyxin signal peptide can be expressed and secreted by both BmN5 cells and silkworm larvae.

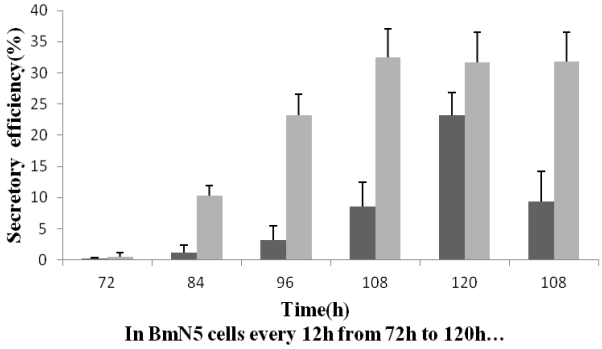

Figure 2 - Secretory efficiency of rUreB in BmN5 cells and in silkworm larvae. The *UreB* gene fused to the signal peptide sequence (gray bars) compared to the control without the signal peptide sequence (black bars). BmN5 cells: Secretory efficiency (%)=rUreB in culture supernatants/(rUreB in culture supernatants + rUreB in cells)×100. Silkworm larvae: Secretory efficiency (%)=rUreB in hemolymph/(rUreB in hemolymph+ rUreB in body cells)×100.

Cell viability

Secretory proteins undergo processing in the endoplasmic reticulum (ER): effective production and secretion of mature proteins requires correct folding in the ER. However, the accumulation of unfolded proteins in the ER (ER stress) leads to activation of the unfolded protein response (UPR) (Ron and Walter 2007), and cells tend to die if the protein load in the ER exceeds the folding capacity. As shown in Figure 3, trypan blue staining assays demonstrated that the number of dead cells carrying the *UreB* gene fused to the signal peptide decreased slightly compared with the control not containing the signal peptide sequence. This result showed that rUreB secretory expression using the signal peptide did not reduce cell viability.

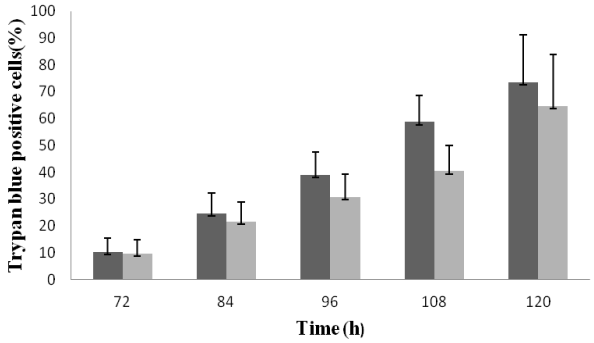

Figure 3 - Trypan blue staining assays. The number of dead cells expressing the *ureB* gene gene fused to the signal peptide sequence (gray bars) compared to the control without the signal peptide sequence (black bars).

SDS-PAGE and semi-quantitative western blot analysis

To examine the secretory expression levels of rUreB in silkworm larvae, SDS-PAGE and semi-quantitative western blots were performed using Coomassie brilliant blue staining and enhanced chemiluminescence, respectively (Fig. 4). The protein bands on western blots were quantified using semiquantitative densitometric scanning.

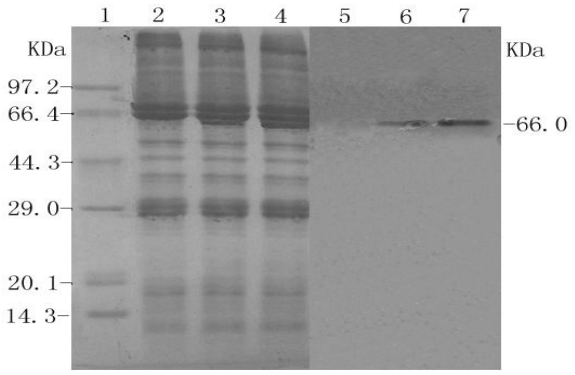

Figure 4 - Analysis of recombinant UreB using SDS-PAGE and western blotting 1. Protein marker, 2. Silkworm larvae at 120 h post infection with wild virus, 3. Silkworm larvae at 120 h postinfection with recombinant Bacmid/BmNPV/*UreB*, 4. Silkworm larvae at 120 h post-infection with recombinant Bacmid/BmNPV/signal-*UreB*, 5. Western blotting of control (post-infection with wild virus), 6. Western blotting of UreB (post-infection with recombinant Bacmid/BmNPV/*Ure*B), 7. Western blotting of UreB (post-infection with recombinant Bacmid/BmNPV/ signal-*UreB*).

The absorbance values of the rUreB band without the signal peptide were 120 ± 16, whereas the value for the rUreB band fused to the signal peptide was 183 ± 27; thus, the expression level in silkworm larvae was approximately 1.5-fold higher when the bombyxin signal peptide was included.

Various types of genetic manipulation techniques and alterative technologies have been implemented to improve expression levels for the mass production of proteins in insect cells, including site-directed and random mutagenesis (Verdile et al. 2004), promoter exchange and signal peptide insertion (Golden et al. 1998), and increased plasmid copy number (Deml et al. 1999). The secretory expression of recombinant proteins in host cells is also a highly effective strategy. In this process, the signal peptide and certain molecular chaperones play critical roles in enhancing the secretion of recombinant proteins (Chang et al. 1997; Tate et al. 1999; Higgins et al. 2003; Zhang et al. 2003; Kato et al. 2004). Although several studies have reported the successful secretion of recombinant proteins in insect cells (Chen et al. 2000; Alam et al. 2002), there are few reports to date on the secretion of exogenous proteins into the hemolymph of silkworm pupae or larvae. Signal peptides are indispensable for exogenous protein secretion into the hemolymph in silkworm pupae or larvae, and an ideal signal peptide must be properly cleaved by a signal peptidase.

As shown in Figure 4, rUreB proteins of the same molecular weight were observed using the bacmids with and without the signal peptide, demonstrating that the bombyxin signal peptide can be cleaved correctly by a signal peptidase in silkworm larvae and that the signal peptide's sequence was thus properly designed. Moreover, for optimal protein expression, it is important that a protein is correctly folded in the ER: if an expressed protein is correctly folded, no effect is observed on cell viability; however, poor protein folding will likely to lead to cell death due to ER stress (Ron and Walter 2007). In addition, cells maintain optimum biological activity under certain physiological environmental conditions. When exogenous proteins are highly expressed, there is a greater impact on cell growth because exogenous proteins are retained in the cells, affecting their activity, which is a reason that secretory expression is more conducive to the survival of the host cell. Trypan blue staining confirmed that the cells undergoing secretory expression had increased viability (Fig. 3). Viral infection has a greater impact on the host cell and can lead to

lysis: in the late stage of viral infection, host cell lysis occurs, and exogenous proteins are released into the cell culture medium or into the silkworm hemolymph. Although there was no significant difference in the secretory efficiency of rUreB expressed with the bombyxin signal peptide compared to that without the signal peptide (Fig. 2), rUreB was more highly expressed (1.5-fold higher) when fused to this signal peptide (Fig. 4). This is the first report demonstrating the use of a signal peptide to enhance the expression of rUreB in silkworms. The results indicate that use of this signal peptide is an effective way to improve rUreB protein expression in silkworms.

CONCLUSION

The results of this study confirmed that silkworm larvae are an optimal system for the mass production of *H. pylori* rUreB. The bombyxin signal peptide is an effective strategy for improving rUreB protein expression in silkworms and can be correctly cleaved by a signal peptidase and folded in the ER of silkworm larvae. The expression levels of rUreB were 1.5-fold higher using the bacmid containing the bombyxin signal sequence than with the bacmid lacking the signal sequence. This is the first report demonstrating the use of a signal peptide to enhance the expression of rUreB in silkworms.

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