

Full Length Research Paper

Expression of polyhedrin-hEGF fusion protein in cultured cells and larvae of *Bombyx mori*

Wei Yu, Jian Chen, Xiuling Zhao, Zhengbing Lv, Zuoming Nie, Yaozhou Zhang*.

Institute of Biochemistry, Zhejiang Sci-Tech University, Hangzhou 310018, China.

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For mass production of human epidermal growth factor (hEGF), silkworm baculovirus expression vector system (BEVS) was adopted in this study. hEGF gene was in-frame fused with polyhedrin (Ph) gene under the control of Ph promoter and was used to co-transfect BmN cell with the modified *Bombyx mori* baculovirus DNA to obtain recombinant virus. The ELISA showed a maximum expression on day 4 in larvae and pupae. Both cellular extracts and haemolymph of silkworm larvae infected with rBacPh-EGF could all support the proliferation of Balb/c 3T3 cell. The corresponding materials from BmN cell and silkworm larvae infected with wild virus also indicated a weak effect of up-regulation on 3T3 cells proliferation. The animal study showed that both pupae infected with rBacPh-EGF virus and wild virus protected the gastric mucosa against ethanol-induced damage in rats, although the protection from pupae infected with wild virus was slightly weak. The mechanism is under investigating.

Key words: Human epidermal growth factor, fusion protein, baculovirus expression vector system.

INTRODUCTION

Human epidermal growth factor (hEGF), a 6.2kD polypeptide of 53 amino acids, exists in whole human body fluid, secretion and most of the tissues. hEGF could induce epidermis proliferation and deratosis and its sequence was reported by Cohen and Carpenter in 1975. It is known that EGF plays an important role in stimulating the proliferation and differentiation of skin tissue and corneal (Kurten et al., 2005; Tripathi et al., 1990).

Because of the importance of hEGF in basic research and clinical medicine, it has aroused extensive interest to develop new method to produce hEGF in large scale. The traditional way to get hEGF is purification from animal urine fluid. However, hEGF concentration in urine fluid is very low (1 mg/1000 L). The purity and production

rate cannot reach the request of industrialization production because of the complexity of hEGF molecular structure in the disulphide and other functional groups. Another resource is from genetic engineering technology. Up to now, the hEGF gene has been expressed in *Escherichia coli* (Gan et al., 1992; Ptitsyn and Altman, 1999), *Bacillus brevis* (Ebisu et al., 1996), and *Saccharomyces cerevisiae* (Topczewska et al., 1993). However, compared to eukaryotic expression systems the biological activity and glycosylated modification of expressed products are lower in *E. coli* and *B. brevis*. Despite the high level of *S. cerevisiae* expression system for the production of hEGF, the expression level and cost are not satisfied with the therapeutic applications in humans.

The development of recombinant protein production in the silkworm baculovirus expression vector system (BEVS) may overcome these limitations and thus may facilitate the use of genetically modified edible proteins. There is the potential for not only low-cost but also high-capacity production. Using the *Bombyx mori* nuclear polyhedrosis virus (BmNPV) of the baculovirus

*Corresponding authors E-mail: yaozhou@zist.edu.cn Tel: +86-571-86843198. Fax: +86-571-86843198.

expression system also eliminates concerns regarding pathogens that could potentially be transmitted to humans. The baculovirus is non-infectious to vertebral animals, and the system itself is safe (Carbonell and Miller, 1987; Herrington et al., 1992). These features make the silkworm system an ideal expression and delivery package for producing oral medical proteins.

Previous studies showed that secretion of hEGF from gastrointestinal tract can avoid damage to mucosa by Neostigmine and Aspirin, and boost up the resistance of gastric mucosa by stimulating the secretion of mucus glycoprotein (Kelly et al., 1990; Konturek et al., 1991). Study of the protection of hEGF in gastric lumen might provide a new approach to treat ulceration in clinic. Therefore, in this study, we intensively explored the highly effective ways to express hEGF with silkworm BEVS as well as its pharmaceutical function.

MATERIALS AND METHODS

Reagents, cell line, *B. mori* larvae and rats

E. coli Strain TG1, BmN cell line, plasmid pBacPAK8, *B. mori* linearized baculovirus and rat Balb/c 3T3 cell line were preserved in our laboratory. BmN cell and Balb/c 3T3 cell medium TC-100, DMEM and FBS were purchased from Gibco-BRL (Gaithersburg, USA). Plasmid pBS-EGF (with His-tag at the end) and pET22 were provided by Professor Xiangfu Wu (Institute of Biochemistry, Shanghai, China). Restriction endonucleases, T4 DNA ligase, Taq, DOSPER, protease K, X-gal and related reagents were purchased from Roche Diagnostics (Germany). Standard rhEGF was purchased from Promega (Madison, USA). Mouse anti-hEGF and goat anti-mouse IgG (HRP) antibodies were provided by Sino-American Biotechnology Company (SABC, Shanghai, China). Fifth-instar silkworm *B. mori* larvae (Jingsongx-Haoyue, Showa) were fed fresh mulberry leaves and reared under a photoperiod schedule of 12 h light and 12 h darkness at 25±1 °C. Rats were provided by Zhejiang Academy of Medical Sciences (ZJAMS, Hangzhou, China) with average weight of 200-250 g, and having equal males and females. Gastric ulcer medicine (Ranitidine) was purchased from Zhejiang University Hospital (ZJUH, Hangzhou, China).

Expansion of hEGF gene and Ph gene

In order to obtain target hEGF fragment, primers for PCR amplification from plasmid pET22-EGF were designed as follows: forward primer 5' TGGATCCAACCTCCGACTCTGAA (*Bam*HI) and reverse primer 5' AGCCGGATCTCAGTGGTG.. The condition for the PCR were 3 min at 94 °C, cycling parameters were 45 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C (35 cycles), followed by hold at 72 °C for 10 min.

In order to obtain Ph (polyhedrin) gene from AcMNPV, the primers for PCR amplification from AcMNPV were designed as follows: forwards primer 5' GGATATCATGGAGATAATTTAAAT (*Eco*RV) and reverse primer 5' GGGATCCACGACCTTCGATGAGA ACTTGATGAC (*Bam*H I). The condition for the PCR were 3 min at 94 °C, cycling parameters were 45 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C (35 cycles), and hold at 72 °C for 10 min.

Construction of the recombinant transfer vector and recombinant baculovirus

To improve the production level of hEGF, 348 bp of Ph gene and 92

bp of polyhedrin promoter of AcMNPV were introduced to the 5' end of the hEGF gene. hEGF gene was then fused in-frame to the carboxy-terminal of Ph gene; the fusion gene Ph-EGF was then cut out with *Eco*RI and *Eco*RV, and was cloned between *Eco*RI and *Eco*RV sites of pBacPAK8. The result plasmid was named pBacPAKPh-EGF and the Ph-EGF fusion gene was right under the control of polyhedrin promoter.

Co-transfection was performed as described in our previous work (Gong et al., 2005). Briefly, the purified recombinant transfer vector pBacPAKPh-EGF and linearized viral DNA of modified baculovirus Bm-BacPAK6 digested with *Acl*I were used for co-infection in cultured BmN cells. After purification by three rounds of plaque isolation, the recombinant virus was selected and then identified further by PCR amplification and genomic DNA hybridization. The fragment of the full-length Ph-EGF gene was used as a probe, the recombinant plasmid as a positive control. Finally, the recombinant virus stock was prepared and the dilution of the virus was calculated using Reed-Muench method.

Expression of rPh-EGF in BmN cells, larvae and pupae

BmN cells (5×10⁶ cell/flask) were infected with the recombinant virus at a medium dose (MOI=10), using wild-type virus infection as control. The infected cells were cultured at 27 °C, and the supernatant and cultured cells were collected at 1~6 days post-infection. After centrifugation to remove the cell fragment, the supernatant was collected. The cells were resuspended in 0.2 ml PBS and then the cells were lysed by repeated freezing and thawing or gentle sonication. Then the samples were stored at -20 °C for further analysis after trituration and centrifugation.

The fifth instar *B. mori* larvae (4th) were needle inoculated with the recombinant viral solution into the body cavity, using wild-type virus injection as control. The infected larvae haemolymph was then collected at 1-6 days post-inoculation and centrifuged to remove the insoluble impurities. Then the haemolymph samples were stored at -20 °C. After pupation 2-3 days, pupae were infected with the recombinant virus rBmBacPh-EGF, using wild-type virus injection as control. Thereafter, the pupae were harvested at 4 days post-infection and stored at -20 °C.

ELISA quantification of rPh-EGF and Western blotting

The recombinant Ph-EGF protein levels in BmN cells, silkworm larvae haemolymph and pupae were determined by quantitative ELISA assay. A 96-well microtiter plate was loaded with serial dilutions of the cell-lysed supernatant, haemolymph or pupae homogenate supernatant in bicarbonate buffer, pH 9.6 (15 mmol/l NaCO₃, 35 mmol/l NaHCO₃) and incubated overnight at 4 °C. The plate was washed three times in PBST (containing 0.05% Tween-20). The plate was blocked by incubation in 1% bovine serum albumin (BSA) in PBS for 2 h at 37 °C, followed by three washes with PBST. The plate was incubated in a 1:3000 dilution of rabbit anti-hEGF primary antibody (SABC, China) (100 µl/well) at 37 °C, for 2 h, followed by three washes with PBST. The plate was then incubated with a 1:1000 dilution of anti-rabbit IgG conjugated with horseradish peroxidase (SABC, China) (100 µl/well) at 37 °C for 2 h and washed three times with PBST. Finally, the chromogenic substrate O-phenylenediamine (ADRC, China) (100 µl/well) was added to the wells, and the plate was incubated for 20 min at 37 °C, to develop color, followed by the addition of 2 M H₂SO₄ (50 µl/well) to stop the reaction. The plate was cooled to room temperature, and the absorbance at 492 nm was measured in a Labsystems Multiskan MS ELISA plate reader (Labsystems, Finland).

For detection of the presence of recombinant Ph-EGF, the haemolymph samples were loaded in a 15% SDS-PAGE. The cell-

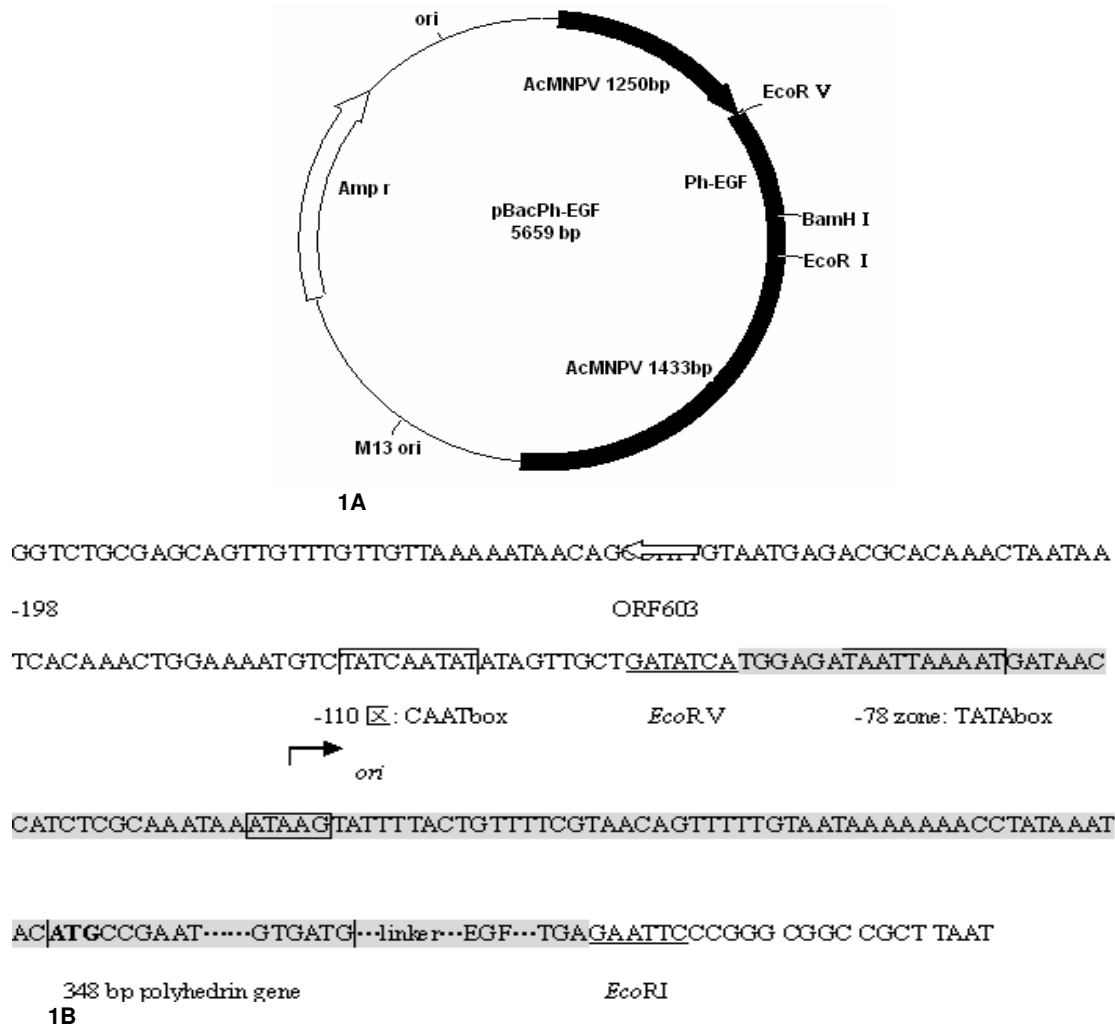


Figure 1. Construction of the recombinant transfer vector pBacPh-EGF.

lysed supernatant, haemolymph of fifth instar larvae or pupae were collected at 4 days post-infection, 10 times diluted and then loaded on 15% SDS-PAGE, using wild virus infected haemolymph and normal haemolymph as negative controls. After electrophoresis, the separated proteins were transferred to Hybond-P membrane (Amersham Biosciences), followed by hybridization with rabbit anti-hEGF primary antibody (SABC, China) and second antibody, anti-rabbit IgG conjugated with horseradish peroxidase (SABC, China).

Effect of rPh-hEGF on Balb/c3T3 cells

BmN cells (5×10^6 cell/flask) or silkworm larvae were infected with the recombinant virus rBmBacPh-EGF. The wild virus infected BmN cells and blank were used as negative controls. The cell-lysed supernatant was collected at 72 h post-infection. The haemolymph were collected at 4 days post-infection. The wild virus infected haemolymph and blank were used as negative controls. Balb/c3T3 cells were seeded in a 96-well microtiter plate ($5-7 \times 10^3$ cell/well), then loaded with serial dilutions of the cell-lysed supernatant or haemolymph, incubated for 48 h at 37°C. rhEGF (Promega) was

used as a standard sample with an initial concentration of 20 ng/ml. Balb/c3T3 cell viability was estimated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma) assay. Briefly, 1 mg/ml MTT (final concentration) was added to the well and incubated for 2 h at 37°C. The medium was removed and cells were lysed with 2-isopropanol containing 0.04 mol/L HCl. The absorbance measured at 570 nm was used to calculate the relative cell viability ratio.

Induction of gastric ulcer and treatment with rPh-hEGF

32 SD rats (ZJAMS, China) were divided into four groups randomly for different treatment, 8 in each group. All pupae were collected at 4 days post-infection. Group A: pupae fed infected with recombinant virus BmBacPh-EGF; Group B: pupae fed infected with wild-type virus; Group C: ranitidine fed; and Group D: physiological saline fed. Rats were gavaged with rPh-hEGF three times a day at a dose of 1 mg/kg body weight for three days continuously. After that the rats were fed with only water without food. The stomach was emptied after 48 h and fed ethanol (1 ml/250 g body weight). Then the rats were killed 1 h later.

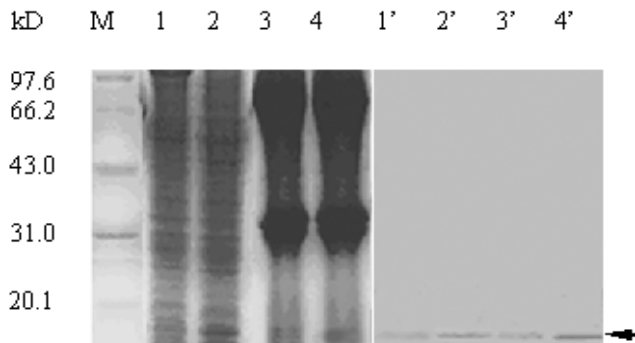


Figure 2. Western blotting analysis of BmN cells and larvae haemolymph infected with recombinant (rBmBacPh-EGF). Lane 1: BmN cells infected with wild-type virus; lane 2: BmN cells infected with recombinant virus; lane 3: haemolymph of larvae infected with wild-type virus; lane 4: haemolymph of larvae infected with recombinant virus. Arrow specifies Ph-EGF protein.

Analysis of gastric ulcer rats

For the evaluation of the prevention effect of silkworm-synthesized Ph-EGF fusion protein on gastric ulcer in rats that had been fed silkworm pupae synthesizing Ph-EGF fusion protein or pupae lacking Ph-EGF fusion protein, ethanol was used as a gastric ulcer inducer. Rats were killed 1 h later after feeding with ethanol; stomachs with different treatment were harvested and fixed in 1% formalin and embedded in paraffin, stained with haematoxylin and eosin. Ulcer degree standard was calculated by damage index (sum length of each focus in entire stomach). The damage index of physiological saline group was taken as 100%, and the inhibition percent of other groups were calculated. The degree of ulcer was evaluated using Image Analyzer (Leica Qwin).

Statistical analysis

All data were presented as mean \pm SEM. With all statistical analyses by t-test, differences were considered statistically significant for $P < 0.05$.

RESULTS

Construction of recombinant virus and expression of Ph-EGF in *B. mori* cells and larvae

Recombinant plasmid pBacPh-EGF was identified by PCR and DNA sequencing, indicating that Ph-EGF fusion gene was inserted into transfer vector pBacPAK8 correctly under the control of polyhedrin promoter (Figures 1A and B). The Ph-EGF gene in recombinant baculovirus (named BmBacPh-EGF) was confirmed by direct PCR of viral genomic DNA and further analyzed by genomic DNA dot hybridization. As expected, the PCR amplification of recombinant viral genomic DNA showed a specific band of 180 bp. The dot blot results revealed the presence of a fusion gene identical to the positive control, while the wild-type baculovirus did not show the

gene-specific dot (data not show). These evidences indicated that the Ph-EGF gene was inserted into the baculovirus genome under the control of polyhedrin promoter. According to the Reed-Muench formula, the dilution of the recombinant virus was 3.3×10^8 puf/ml.

After infection by this recombinant virus, BmN cells and silkworm larvae presented typical symptoms of BmNPV infection, simultaneously, Ph-EGF fusion protein was produced inside the cells and silkworm body cavity, respectively.

Immune reactivity detection of recombinant Ph-EGF expression in BmN cells, silkworm larvae and pupae

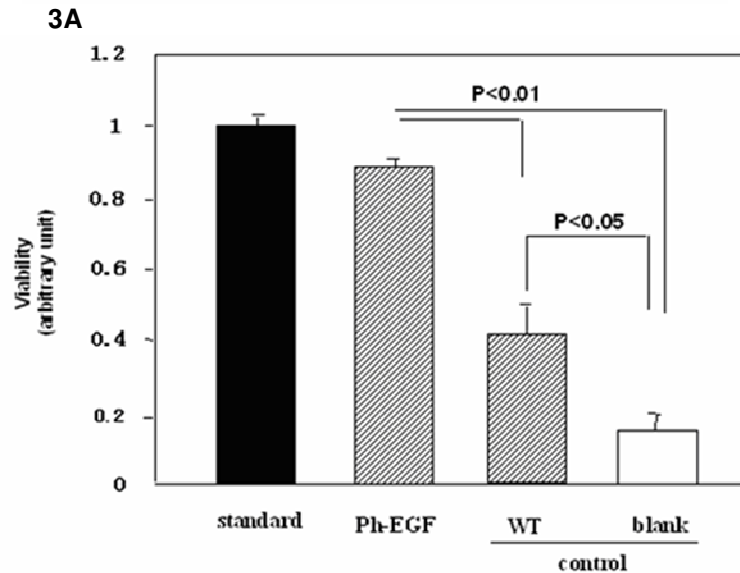
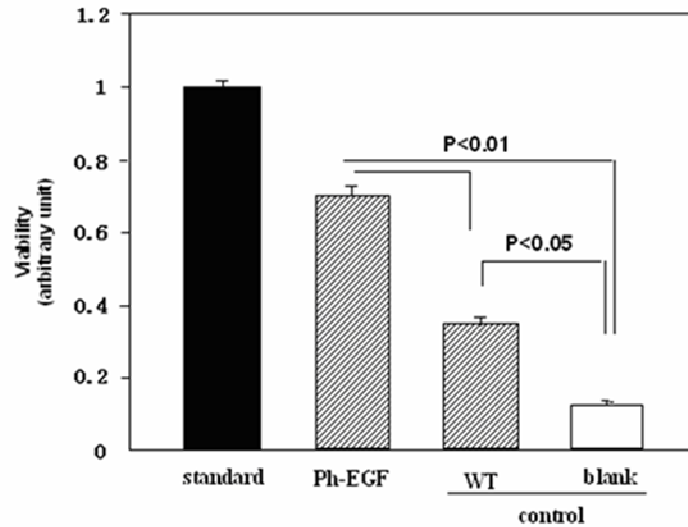
The ELISA results showed that the expression of Ph-EGF was mainly in cells extract. The haemolymph of fifth instar *B. mori* larvae and pupae inoculated with recombinant virus solution were collected everyday post-infection, the ELISA assay showed a maximum expression on day 4. The highest detectable level of Ph-EGF protein was $6 \sim 7 \mu\text{g}/2 \times 10^6$ cells in cell culture, 59.1 mg/L in silkworm haemolymph and 65 mg/L in pupae, respectively at the fourth day post-infection. Interestingly, either wild type virus infected cell-lysed supernatant or silkworm larvae haemolymph presented weak positive reaction. This meant that EGF-like protein maybe existed in normal BmN cell (silkworm) or silkworm BEVS itself and resulted in wild type virus infected cells and haemolymph reacting with hEGF antibody. Western blotting results (Figure 2) showed that silkworm-expressed Ph-EGF appeared as a 19 kDa specific band.

Proliferation effect of hEGF expressed in *B. mori* cell culture and silkworm larvae on Balb/c3T3 cells

Biological activity detection results revealed that both cellular extracts and haemolymph of silkworm larvae infected with recombinant virus could all support the proliferation of Balb/c3T3 cell ($P < 0.01$). Similar to ELISA results the corresponding cellular extracts and haemolymph infected wild type virus also indicated a weak effect of up-regulation of Balb/c3T3 cells proliferation ($P < 0.05$) (Figure 3A, 3B). It implied that there might be some EGF-like growth factor in BmN cells and haemolymph or in BEVS itself, which could enhance the proliferation of the cell line.

Oral administration of Ph-EGF fusion protein prevent gastric ulcer

The pathological study revealed that rat stomach presented obvious gastric mucosa damage in all groups after ethanol treatment, such as hyperemia, swelling and festers. When compared to the group treated with saline,



3B

Figure 3. Effect of cell extract (A) and haemolymph of larvae (B) on proliferation of Balb/c 3T3 cells. The cell-lysed supernatant were collected at 72 h post-infection, using wild-type virus infected BmN cells and blank as controls. The haemolymph were collected at 4 days post-infection, using wild-type virus infected silkworm haemolymph and blank as controls. Balb/c3T3 cells viability were evaluated 48 h after cell-lysed supernatant or haemolymph treatment by means of MTT assay (n=8), respectively. A, $P < 0.01$ recombinant virus infected cell lysed vs. wild virus infected cell lysed and blank; $P < 0.05$ wild virus infected cell lysed vs. blank. B, $P < 0.01$ recombinant virus infected haemolymph vs. wild virus infected haemolymph and blank. $P < 0.05$ wild virus infected haemolymph vs. blank. Values are expressed as mean \pm SEM. Similar results were obtained in three independent experiments.

the gastric mucosa of pupae synthesizing Ph-EGF fusion protein group were better protected. Treatment of rats with gavaging rPh-EGF produced significant reduction of experimental gastric ulcer with a reduced total damage index. Representative gastric mucosa islet (Figure 4A1) and section (Figure 4B1) from a rat fed pupae infected with recombinant virus had a damage index of 65.61 ± 7.42 ($P < 0.01$) and the protection rate approximated 50% (Table 1). In addition, similar to biological activity detection results, the wild virus pupae

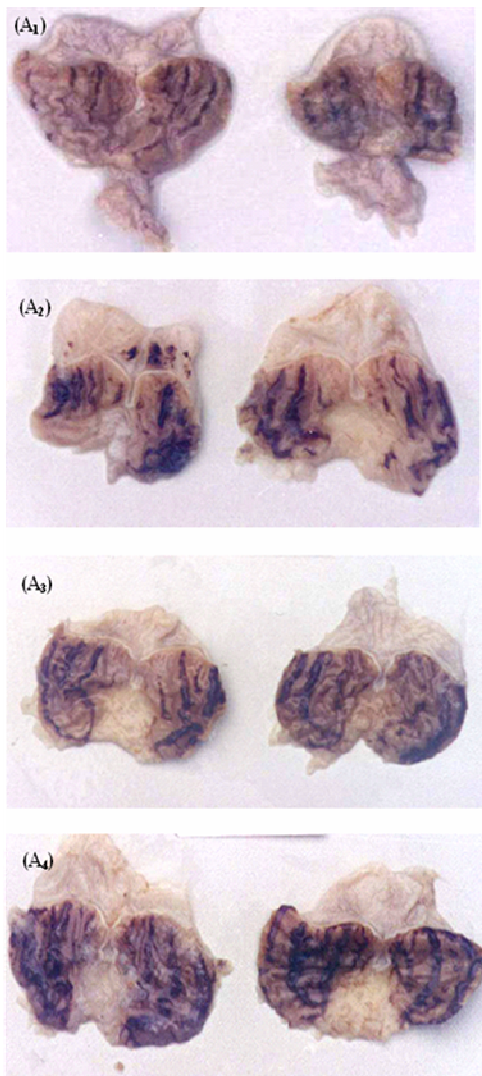
group also presented certain protective effect on gastric mucosa damage (Figures 4A2 and B2) ($P < 0.05$) (Table 1). Ranitidine group has no significant difference (Figures 4A3 and B3) (Table 1).

DISCUSSION

In recent years, increasing number of foreign genes have been expressed in BEVS, and impressive progress has

Table 1. Effect of different treatment on ethanol induced gastric mucosa damage in rats.

Group	Mouse number	Damage index (mm)	Protection (%)	P
physiological saline	8	130.83±16.05	0	
Ranitidine	8	115.75±7.18	12	>0.05
wild virus	8	83.26±8.57	37	<0.05
recombinant virus	8	65.61±7.42	50	<0.01

**Figure. 4A**

been made in modifying this expression system. According to previous studies, the coding region of the carboxy-terminal half of polyhedrin could promote the expression level of a foreign gene in *B. mori* (Marumoto et al., 1987). In this study, in order to enhance the expression level of hEGF, we constructed a fusion protein by ligating hEGF with a coding region (116 aa.) of the N-terminal polyhedrin. Our investigation demon-

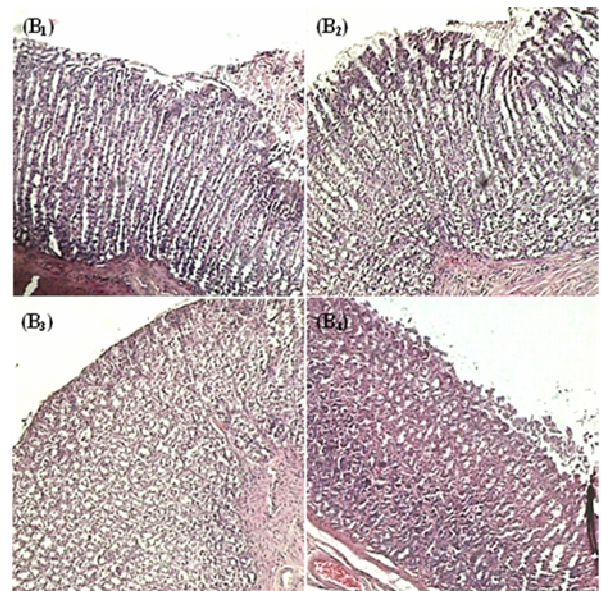


Figure 4. Degree of gastric mucosa damage (A) and pathology section of gastric mucosa (B) in rat after different treatment. (A₁) Degree of gastric mucosa damage from a rat fed pupae infected with recombinant virus (rBmBacPh-EGF). (A₂) Degree of gastric mucosa damage from a rat fed pupae infected with wild-type virus. (A₃) Degree of gastric mucosa damage from a rat fed Ranitidine Hydrochloride Capsule (control). (A₄) Degree of gastric mucosa damage from a rat fed physiological saline (control). (B₁) A representative gastric mucosa section from a rat fed pupae infected with recombinant virus. (B₂) A representative gastric mucosa section from a rat fed pupae infected with wild-type virus. (B₃) A representative gastric mucosa section from a rat fed Ranitidine Hydrochloride Capsules (control). (B₄) A representative gastric mucosa section from a rat fed physiological saline (control). (original magnification×100).

strates the use of polyhedron coding sequence can enhance the expression level of the Ph-EGF gene with BEVS. The expression level of the fusion protein Ph-EGF was 6~7 $\mu\text{g}/2 \times 10^6$ cells in cell culture and 59.1 mg/L in haemolymph, respectively. Compared with *E. coli* (25~30 mg/L) (Ptitsyn et al., 1999) and *Bacillus brevis* (1 mg/L) (Ebisu et al., 1996) expression system, the expression level of hEGF in BEVS is higher. Furthermore, these short sequences have no effect on the biological activity and immunogenicity of the fusion protein.

The potential use of silkworm as an expression system or “bioreactor” for production of useful protein for clinical strates use offers several advantages. It can be use for

(i) large-scale production of foreign proteins, (ii) as an edible medicine if expressed in an edible silkworm pupa or (iii) as a delivery system for oral protein drugs. In contrast to the conventional prokaryotic expression system, large-scale production of purified hEGF in bacteria involves the use of expensive fermentation techniques and stringent purification protocols (Lebens et al., 1993). As compare with other eukaryotic expression systems, the expression of heterologous proteins in the silkworm bioreactor is under the control of the strong polyhedron promoter, allowing levels of expression of up to 20% of total cell protein (Massotte, 2003). It is obvious that the protein

production capacity of silkworms predominates over that of any other industrial system in use today. In addition, since silkworms can be conveniently reared with mulberry leaves at much lower cost or with artificial diet throughout the year, large-scale and successive production of rhEGF is possible. The approach established here is probable one of the most economical and efficient ways of producing rhEGF. The cost of producing 1 kg of recombinant protein in silkworm is much lower than the cost of producing the same amount by *E. coli* or *S. cerevisiae* fermentation.

Evidence is mounting that EGF may have more important physiological effects rather than pathological effects on peptic ulcer healing (Calabro et al., 1995). EGF has been shown to enhance healing of gastric ulcers when given subcutaneously or orally in the drinking water (Itoh et al., 1990). Our studies showed that expressed hEGF presented certain protection effect on gastric ulcer, which was simultaneously proved by the pathological section. In addition, it is discovered that the normal silkworm pupae sample also presented weak protection effect on damage gastric membrane induced by ethanol. Therefore, is it possible that EGF family members exist in silkworm body itself? Further studies are required to investigate it in the future. Thus, it is difficult to identify the disturbance degree from EGF-like in silkworm itself to Ph-EGF fusion protein in promoting cell proliferation and preventing damage to gastric ulcer in this research. Further work will involve purifying this fusion protein from silkworm blood or cutting the EGF multi-peptides to determining the biological activity of the purification fusion protein or the hEGF again.

In summary, we have demonstrated the feasibility of using silkworm for the production of hEGF. To our knowledge, this is the first report on the production of functional hEGF using silkworm. The availability of large quantities of rhEGF that the silkworm provides should greatly facilitate the future research and testing of these proteins for potential application in medicine. It is hoped that silkworm will not only become a valuable commercial source of hEGF, but will also provide a convenient, feasible and effective means of treating clinical diseases, such as gastric mucosa damage, by the oral delivery of hEGF.

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