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Diapausing Pupa of Wild Silkmoths as an Ideal Model of Bioreactor

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Abstract Baculovirus expression vector (BEV) system is frequently used in many aspects of biological science for producing large quantities of recombinant proteins, because of its advantageous characteristics, especially capability to perform higher eukaryotic posttranslational modifications, over the conventional microbial expression systems or newly developed cell-free systems.

Recently, we have developed a novel BEVS with a cell line (NISES-AnPe-428, AnPe), a nucleopolyhedrovirus (AnpeNPV), a transfer vector pApCH1 and diapausing pupae of the Chinese oak silkmoth *Antheraea pernyi*. When we expressed β-galactosidase gene (*lacZ*) under the control of the polyhedrin promoter in *A. pernyi* pupae, β-galactosidase activity higher than any other BEV systems such as AcNPV/Sf9 or High5 cell system and BmNPV/*Bombyx mori* larva system was detected. In addition, using diapausing pupae of another wild silkmoth, *Samia cynthia pryeri*, as an alternative host, much higher activity was detected. We have also succeeded to avoid proteolytic degradation and liquefaction in the virus-infected pupae by removing the cathepsin gene from the AnpeNPV genome.

All of our results indicate that the diapausing pupa of wild silkmoth is an excellent natural bioreactor, or an insect factory, for the recombinant protein production. Further studies on this BEV system will reveal molecular mechanisms underlying efficient production in the diapausing pupa and enable us to design a novel insect cell culture system mimicking the pupal cellular conditions.

Key words: diapausing pupa, *Antheraea pernyi, Samia cynthia pryeri*, baculovirus, insect cell, bioreactor

Introduction

There are many kinds of gene expression system; prokaryotic systems including *E. coli* and other bacteria, a variety of eukaryotic systems from microorganisms like yeasts, fungi to multicellular higher eukaryotes like plants, insects and mammals, and recently developed cell free systems using extracts of *E. coli* and wheat germ. For users interested in expression of their target genes, insect expression systems, such as baculovirus vector and *Drosophila* S2 cell are just options to choose or not.

Among four major expression systems, *E. coli*, yeast, insect and mammalian systems, insect systems enable relatively high level production of eukaryotic gene products which are structurally and functionally similar to their native counterparts by its posttranslational modification capabilities closer to mammalian system. For users demanding gene products with such properties at lower costs, insect systems are suitable options to try.

The baculovirus expression vector (BEV) system is a predominantly used insect expression system and has several advantageous characteristics for the recombinant protein production (O'Reilly *et al.*, 1992). Its strong very late

promoters of the polyhedrin and p10 genes enable high-level expression of heterologous genes. Recombinant eukaryotic proteins produced in insect cells are usually correctly folded and modified as higher eukaryotic fashions so that possess structures and biological activities similar to the native counterparts. Because of limited narrow host range of baculovirus, recombinant viruses are very safe. During the recent progress in use of baculovirus as a gene delivery vector for human gene therapy, no replication and viral gene expression in human cells are demonstrated. Thus the BEVS system is now frequently used, probably next to *E. coli* system, in many fields of both basic and applied biology as a valuable tool.

Even with these advantages, several limits of the BEV system, such as lytic/batch fashion of production which make normal cellular protein processing machinery unfunctional at later stage of infection and make it impossible task to produce recombinant proteins continuously, cost of cell culture media and laborious task of rearing insect larvae, which become important problems when considering scale up, insect specific posttranslational modification properties, which is especially problematic when considering use of recombinant glycoproteins for pharmaceuticals because slight difference in structure of oligosaccharide chain cause allergenic responses or quick clearance in applied human bodies as described later, and patented methodology, hamper the practical large scale production of recombinant proteins.

To overcome the problems, several improvements have been made by genetic engineering of virus genome and, more recent, modifying host cell metabolism, by development of alternative virus/host systems employing new cell lines and insect species, which have never been used previously, and by optimizing conditions and facilities helpful for scale up, such as developing low cost serum-free media and artificial diets and designing full automatic bioreactor and insect factory (Goosen *et al.*, 1993: Richardson, 1995; Jarvis, 1997).

Newly established baculovirus expression vector system using diapausing pupae of wild silkmoths

Recently, we have developed a novel BEV system, *Antheraea pernyi* nucleopolyhedrovirus (AnpeNPV)/diapausing pupa system (Wang *et al.*, 2000, Kobayashi *et al.*, 2001), expecting higher production levels in giant *A. pernyi* pupa than the existing systems such as *Autographa californica* NPV (AcNPV)/Sf9 and High5 cell system and *Bombyx mori* NPV (BmNPV)/*B. mori* larva system. We also hope for better protein processing properties in the new system.

Evaluation of the AnpeNPV vector system by comparing the activity of recombinant enzyme, β-galactosidase, with other BEV systems indicated that productivity of diapausing *A. pernyi* pupa was the highest among BEV systems compared (Huang *et al.*, 2001). However, the significant proteolytic degradation was observed at 15 days post-infection when β-galactosidase activity in pupae reached the maximum level and at the same time liquefaction of pupal bodies became obvious. The cathepsin, virus encoding cystein protease, is usually considered as the main factor causing such proteolytic degradation as well as liquefaction of infected insect bodies by working together with the virus encoding chitinase. Thus, we tried to delete cathepsin gene and/or chitinase gene from AnpeNPV genome to avoid proteolytic degradation and liquefaction.

To identify the cathepsin gene and chitinase gene, we first made a genome map of AnpeNPV consisting of 54 *Pst*I fragments (Huang *et al.*, 2002). Utilizing the genome-wide high homology to the *Orgyia pseudotsugata* multicapsid NPV (OpMNPV), the AnpeNPV cathepsin and chitinase genes were found in the genome map exactly at the corresponding positions to the OpMNPV genome. We have constructed two recombinant AnpeNPVs lacking functional cathepsin gene and lacking both cathepsin and chitinase genes by replacing with the green fluorescent protein gene and evaluate the effects. Analysis of protein components demonstrated that both recombinants effectively avoided proteolytic degrada-

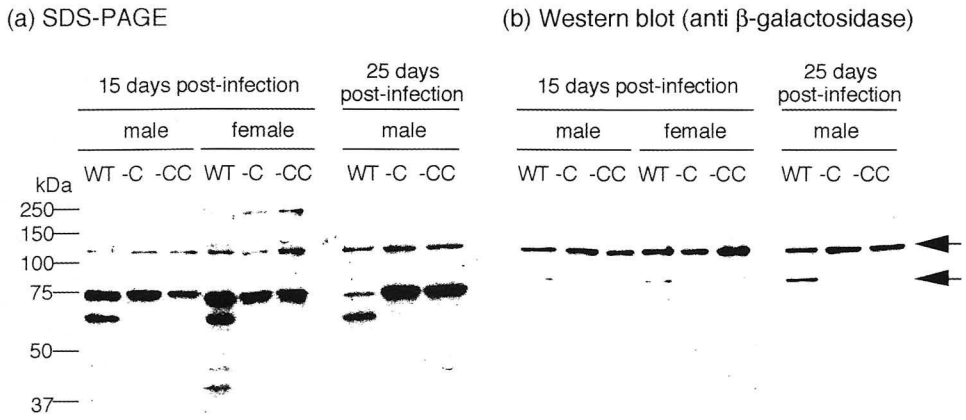

Fig. 1. SDS-PAGE (a) and Western blot (b) analyses of recombinant β-galactosidase produced in diapausing pupae of *Antheraea pernyi* (males and females at 15 days post-infection and males at 25 days post-infection) infected with one of three recombinant AnpeNPVs expressing β-galactosidase gene (WT: both cathepsin and chitinase genes intact virus, -C: cathepsin gene deficient virus, -cc: both cathepsin and chitinase genes deficient virus). Molecular masses of marker proteins are indicated on the left side. Arrows on the right side indicated positions of the intact (upper) and major degraded (lower) β-galactosidase gene products.

tion of β-galactosidase as well as arylphorin and vitellogenin even at 25 days post-infection (Fig.1). This means cathepsin gene deletion without chitinase gene deletion is enough for avoiding proteolytic degradation and liquefaction in virus-infected diapausing pupae.

We also evaluated productivities of other satruniid moth's pupae, such as *Samia cynthia ricini* and *S. c. pryeri*. The former does not diapause at pupal stage while the latter does. Both of them were infected with a recombinant AnpeNPV expressing β-galactosidase gene. At 15 days post-infection, *S. c. ricini* pupa liquefied completely but *S. c. pryeri* did not show any infected symptoms. Until 15 days post-infection, their β-galactosidase activities were almost the same levels as maximal level of *A. pernyi*. For *S. c. pryeri*, the activity continued to increase until 25 days post-infection and reached the maximum level 5 times higher than that of *A. pernyi* and *S. c. ricini* (Fig. 2), indicating the diapausing pupa of *S. c. pryeri* is the best protein productien factory.

All of our results clearly demonstrated that diapausing pupae are excellent natural bioreactors for the recombinant protein production. Users do not have to establish insect rearing facilities but just purchase the pupae and store in refrigerator until injecting recombinant viruses for the protein production. Virus infection and protein harvest are very easy and can be performed by automatic injectors and processors. Productivity by pupae is much

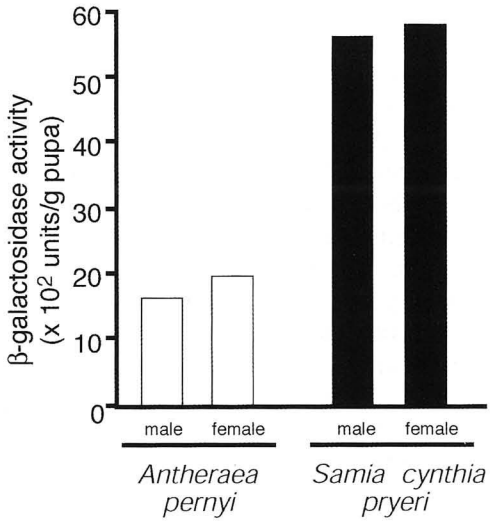

Fig. 2. Comparison of maximal activity of β-galactosidase produced by diapausing pupae (males and females) of *Antheraea pernyi* and *Samia cynthia pryeri*.

higher than those of insect cell lines and *B. mori* larvae. In addition, there is a report that posttranslational modification such as α-amidation, which never occurs in cell culture, occurred in larvae and more efficiently in pupae (Hellers and Steiner, 1992).

Altering protein processing properties of insect cells by metabolic engineering

The insect cells do possess enough potentials and possibility for pharmaceutical glycoprotein production. Because N-glycosylation pathway of insect is very similar to that of human, only we have to do is to solve these crucial problems, addition of strong allergenic core alpha1,3-fucose and inability or extreme inefficiency of complex type N-glycan formation (Altmann *et al.*, 1999).

We have evaluated N-glycosylation property of *A. pernyi* cells by analyzing N-linked oligosaccharide added on recombinant insect peptide hormone, prothoracicotropic hormone, PTTH, which has one N-glycosylaion site in each subunit of homodimer. We occasionally obtained results indicating mammalian-type complex N-glycans were formed on PTTH by *A. pernyi* cells (Kobayashi, 2001), although a recent study did not support (Nagaya *et al.*, 2002). Whether of not it is true, the efficiency of complex N-glycan formation in *A. pernyi* cells is very low and insufficient for practical production of pharmaceutical glycoproteins.

Therefore, it seemed to be much better to improve the N-glycosylation property as well as other posttranslational modifications of the lepidopteran cell line by metabolic engineering than by searching and evaluating other cell lines. Without abundant and complete genome informatics, it is impossible to accomplish the altering metabolic pathways of insect cells rationally and efficiently. The best and fastest way seemed to take advantages of abundant genome informatics of *Drosophila melanogaster*, whose genome sequence was completely determined (Adams *et al.*, 2000).

So we decided to use *Drosophila* S2 cell as a model system for metabolic engineering of insect N-glycosylation pathway. For S2 cells, foreign genes are easily introduced and expressed by transformation using cotransfection with a drug resistant gene plasmid or infection of recombinant baculovirus at very high titer (Culp *et al.*, 1991; Lee *et al.*, 2000). Gene silencing using RNA interference is technically well established (Hammond *et al.*, 2000). It may be possible to activate useful genes in the genome which are inactivated in the cell line but active certain tissues at specific developmental stage of *Drosophila* by artificial expression of limited number of master genes. And it also may be possible to generate insect stem cells.

We have already established transformed *Drosophila* S2 cells expressing PTTH under control of hsp70 promoter and found the similar N-glycosylation pattern to those of lepidopteran cells used in BEV systems (data not shown). Using this S2 cell system, we will establish methods for metabolic engineering of N-glycosylation pathway, and then transfer the techniques to lepidopteran cells. The same strategy can be also applicable for metabolic engineering of any other posttranslational modification pathways.

Conclusion; a dream

One of our goals is fusion of metabolic engineering and diapause. As demonstrated by AnpeNPV vector system, diapausing pupae of wild silkmoths are ideal bioreactors having long shelf lives more than one year and performing extremely high-level production of recombinant proteins without any nutritional inputs. If we can create kinds of insect stem cells which enter the diapausing states by artificial manipulations based on the genetic mechanisms involved in insect diapause, we will apply metabolic engineering to such diapausing cells and conserve them in the refrigerator until using for protein production, or eating as nutritious foods.

Developing such artificial diapausing pupae is almost a dream that will come true for me.

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