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Expression of recombinant herpes simplex virus type 2 glycoprotein D by high-density cell culture of *Spodoptera frugiperda*

Tao LIU[1], Ji-Feng LIU[2], Hui-Jun DONG[3], Wei ZHENG[1], Zhi-Cheng HUANG[1] & Shui-Fen ZHU[1]

[1] *Microbiology Laboratory, Hangzhou Center for Disease Control and Prevention, Hangzhou 310021, People's Republic of China; e-mail: liuniu73@yahoo.com.cn*
[2] *Department of Dermatology, The Third Hospital of Hangzhou, Hangzhou 310009, People's Republic of China*
[3] *Lunan Pharmaceutical Group Corporation, Linyi 273400, People's Republic of China*

Abstract: Herpes simplex virus type 2 (HSV-2) is the major cause of genital herpes in humans. The glycoprotein D of HSV-2 (gD2) is a promising subunit vaccine candidate for the treatment of genital herpes. The aim of the present study was to express a biologically active recombinant gD2 in eukaryotic baculovirus system in quantities sufficient for further studies. Human cDNA encoding a gD2 protein with 393 amino acids was subcloned into the pFastBac HTb vector and the recombinant protein was expressed in *Spodoptera frugiperda* (Sf9) cells by high-density cell culture. In a stirred bioreactor, the key limiting factors including glucose concentration, glutamine concentration and dissolved oxygen (DO) were optimized for high-density cell growth. The Sf9 cell density could reach 9.6×10^6 cells/mL and the yield of recombinant gD2 protein was up to 192 mg/L in cell culture under the optimal conditions of 15 mM glucose, 0.4 g/L glutamine and 40% DO. Production of significant amounts of pure, full-length gD2 opened up the possibility to investigate novel functions of gD2. Moreover, the purified recombinant gD2 protein revealed a partial prophylactic immune function in genital herpes of guinea pigs infected with HSV-2.

Key words: high-density cell culture; herpes simplex virus type 2; glycoprotein D; Sf9 cells; guinea pig.

Abbreviations: DO, dissolved oxygen; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; gD2, glycoprotein D of herpes simplex virus type 2; HSV-2, herpes simplex virus type 2; MEM, minimum essential medium; MOI, multiplicity of infection; PBS, phosphate buffered saline; PCR, polymerase chain reaction; Sf9, *Spodoptera frugiperda*.

Introduction

Herpes simplex virus type 2 (HSV-2) is the primary cause of human genital herpes that is a common sexually transmitted disease with increasing incidence in China. The disease affects both normal and immunosuppressed adults, and is associated with increased susceptibility to the human immunodeficiency virus (Perez et al. 1998; Chen et al. 2000). Chemotherapeutic agents, such as valacyclovir or acyclovir, have helped control recurrences and reduce but not completely eliminate transmission. There is still a need for development of additional therapies or vaccines to reduce transmission of HSV-2 (Sacks et al. 2004).

An ideal prophylactic vaccine can prevent infection, although partially effective prophylactic vaccines may still be useful if they shift the threshold of infection, or if they prevent or ameliorate disease. Subunit vaccines contain only the immunogenic components of a pathogen and, therefore, they are potential alternatives to the live-attenuated virus vaccines, replication-defective virus vaccines or DNA vaccines due to their higher degree of safety.

The most promising HSV-2 vaccine targets have been the viral envelope glycoproteins, especially the 393 amino acid residues long gD protein (Eisenberg et al. 1985; Welling et al. 1991). It has been proven that the glycoprotein D of HSV-2 (gD2) is a component of the virion envelope that plays an essential role in HSV-2 entry into susceptible mammalian cells, and so it is a primary vaccine candidate for genital herpes treatment in humans (Pertel et al. 2001). It has also been proven that HSV-2 gD2 vaccine alone or with various adjuvants, such as alum and water/oil emulsions, 3-O-deacylated monophosphoryl lipid A and GPI-0100, etc., can prevent or reduce the severity of primary genital herpes, and thereby reducing or eliminating recurrent genital herpes in various degrees (Lee et al. 2002; Stanberry et al. 2002; Quenelle et al. 2006; Fotouhi et al. 2008; Bernstein et al. 2010).

To date, although prophylactic vaccines to prevent HSV-2 acquisition have been tested with limited success, the gD2 has still been in the focus of efforts to develop subunit vaccine; its medical application has therefore been highly anticipated (Garnett et al. 2004; Stanberry 2004). As large amounts of gD2 are required for such applications, an efficient and economical way of producing gD2 must be established.

A wide range of therapeutic proteins and vaccines have been produced in *Spodoptera frugiperda* (Sf9) cells

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infected with recombinant baculoviruses, but most interesting proteins are expressed at unsatisfactory level (Ikonomou et al. 2003). Their application is hampered by low-density cells and expensive culture media. In order to improve volumetric productivity in a cost-effective manner, recombinant proteins are often produced in high-density cells during fermentation. Recombinant DNA technology and fed-batch cultivation are frequently used for the production of various recombinant proteins with high productivities (Krause et al. 2010).

In the present study, in order to improve the expression level of recombinant gD2 protein, we established a eukaryotic baculovirus system for expressing recombinant gD2 protein with high productivity in high-density cell culture of Sf9 cells, which made it possible to produce a large amount of purified gD2 protein for its functional investigations. Moreover, a model of guinea pig vaginal infection with HSV-2 with similarity of human genital herpes was established to evaluate potential vaccines and antiviral functions. Our *in vivo* studies confirmed that the purified recombinant gD2 protein could provide partial immunization protection against genital herpes of guinea pigs with HSV-2 challenge.

Material and methods

Cells and virus

The Bac-to-Bac expression system was purchased from Invitrogen (USA). Sf9 cells were kindly provided by Dr. Chen Yin (Zhejiang Provincial Center for Disease Control and Prevention, China) and Vero cells were conserved in our laboratory. Sf9 cells were cultured in SF-900 II SFM at 27℃, while Vero cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA) at 37℃ with 5% CO_2. All media contained 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 g/mL amphotericin. HSV-2 was isolated and confirmed in our laboratory previously.

Construction of recombinant gD2 baculovirus

Virus HSV-2 was amplified by infecting Vero cells and the viral DNA was extracted according to standard protocols (Ausubel et al. 1993; Sambrook et al. 1989). Sequences encoding full-length gD2 were amplified by PCR using following primers – forward: 5'-GGTGAATTCATGGGGCGTTT GACCTCC-3', and reverse: 5'-GTTAAGCTTGCGGGGA AACTCCTCTAGTA-3'; *Eco*R I and *Hin*d III sites were underlined, respectively. The PCR product was subcloned into the pFastBac HTb donor plasmid and identified with restriction enzyme analysis. The recombinant plasmid was transformed into the *Escherichia coli* DH10Bac competent cells for site-specific transposition of the gD2 DNA from the transposing vector to a bacmid DNA through lacZ gene disruption. Successful transposition was verified by PCR analysis using either M13/pUC or gD2-specific primers. Positive recombinant bacmids were used to transfect Sf9 insect cells for viral particle formation. All procedures for the production of viral particles were performed according to the manufacturer's manual (Bac-to-Bac, Invitrogen).

Recombinant gD2 baculovirus was harvested from supernatant centrifuged at 1,000 rpm for 5 min to remove cell debris and subjected to two additional cycles of plaque purification, then subsequently propagated two cycles to generate 1,000 mL of high titer virus (10^8 pfu/mL as determined by plaque assay) for gD2 expression. All virus stocks were stored at 4℃ and protected from light to ensure the maintenance of the titer.

Fed-batch culture of Sf9 cells

A 5 L stirred bioreactor (New Brusnwick Scientific, USA) was applied to culture Sf9 cells at high density for fed-batch fermentation. The bioreactor was inoculated with cells at a density of 2×10^5 cells/mL and an initial working volume of 2.5 L. For all cell culture, temperature was controlled at 27℃. Agitation rate was initially set to 50 rpm and progressively increased to 150 rpm. Dissolved oxygen (DO) was maintained at the proper range by regulating the flow of compressed and highly purified nitrogen, carbon dioxide, oxygen and air. The pH was controlled at 6.2 by adding 0.25 M HCl or 0.2 M NaOH. Nutrient solutions were composed from 15% (w/v) glucose solution, 2% (w/v) glutamine solution and amino acid solution (containing 50 mM cysteine, 50 mM lysine and 80 mM methionine). All were filtered with a 0.22 µm sterile filter. Samples were collected every 24 h for cell counting and metabolic analysis. Cell density was measured by microscopic counting with a hemacytometer.

Quantitative assay and purification of recombinant gD2

The Sf9 cells infected with recombinant baculovirus were harvested at 48–72 h post-infection and sonicated for 10 s at 50% ultrasonic power with 5 repeats and a 3–min period of cooling down on ice. The supernatant cleared by centrifugation was stored at −70℃ until protein purification.

The recombinant gD2 protein was determined by enzyme linked immunosorbent assay (ELISA) according to protocol by Ausubel et al. (1993). The ELISA standard curve was established by gD2 standard VTI540 derived from *Pichia pastoris* (Meridian life science, USA) with various concentrations from 1.6 to 100 ng/mL according to previous manual (Wang & Fan 2000). The primary antibody used was mouse anti-gD2 monoclonal antibody C65019M (1:700, Meridian life science, USA) and the secondary antibody used was goat anti-mouse IgG conjugated to horseradish peroxidase (1:2000, Roche, USA). The amounts of recombinant gD2 were calculated from OD_{490nm} values with an ELX_{800} Universal Microplate Reader (Bio-Tek Instruments, Inc., USA) that were in the descending portion of the ELISA standard curve for the gD2 standard.

The Bac-to-Bac expression system was added a hexahistidine tag to ensure effective one-step purification of recombinant gD2 on NTA-Ni^{2+} resin. The supernatant was incubated with pre-equilibrated (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole) NTA-Ni^{2+} resin (Qiagen) packed in a column. The gD2 protein was eluted with elution buffer supplemented with 300 mM imidazole. The recombinant gD2 protein was analyzed by 12% SDS-PAGE and Western blotting.

Measurement of glucose and glutamine

The residual glucose and glutamine concentrations were measured by Nova Bioprofile Biochemistry Analyzer (Bioprofile 100, USA).

Immunogenic assay in vivo
Female guinea pigs (~300 g) were purchased from Animal Research Center of Shanghai Medical College. The guinea pigs were randomly divided into three groups for 10 guinea pigs in each group, and provided free access to food and water. All animal studies were performed according to the approved protocols by Institutional Animal Care and Use Committee.

Three groups of guinea pigs were immunized with 0.2 mL of PBS, 0.2 mL of PBS containing 25 μg purified recombinant gD2 and 0.2 mL of PBS containing 25 μg commercially gD2 VTI540 by subcutaneous injection every other week for a total of three injections, respectively. In both models, the animals were challenged 14 days after the last immunization.

All guinea pigs were anesthetized with ketamine at the time point of 2 weeks following the final immunization and 2–3 mL of whole blood samples were collected. Blood was allowed to clot at room temperature and serum was collected and frozen for neutralizing antibody assay.

For genital herpes HSV-2 infection, viral inoculation was performed intravaginally. The herpes model of guinea pigs was constructed according to the modified method as previous description (Pronovost et al. 1982). After removing vaginal secretions, a Dacron swab soaked with 150 μL of 10^6 pfu/mL HSV-2 solution was inserted intravaginally and rotated several times. External genital lesions in guinea pigs were graded daily for 14 days post viral inoculation using an established scoring system: 1, redness or swelling; 2, a few small vesicles; 3, several large vesicles; 4, several large ulcers and maceration (Kern et al. 1978). The mean lesion score for all animals was calculated in a blinded manner.

Neutralizing antibody assays
The neutralization assay was performed with some modification using methods described previously (Maul 1991; Masayuki et al. 2005). Briefly, the serum samples were inactivated by heating at 56℃ for 30 min and diluted with MEM-3% FBS from 1:10 to 1:320. A total of 100 μL of test sample was mixed with 100 μL of 100 pfu of HSV-2. The mixtures were incubated at 37℃ for 1 h for neutralization and then inoculated in duplicate onto Vero cells in 96–well plates. Virus was absorbed for 1 h at 37℃ and then 100 μL of MEM-3% FBS was added to each well and incubated at 37℃ for 3 days. Virus and medium controls were included in each assay. Titers of sera were defined by logarithmic values of the final serum dilutions that produced more than 50% reduction in the number of viral plaques, when compared with the virus control.

Statistical analysis
All statistical analyses were undertaken by *t*-test. The significant difference was considered at the *P*-value less than 0.05.

Results

Construction of recombinant baculovirus
The full-length sequence of HSV-2 gD gene was firstly amplified by PCR using the designed specific primers. The gD2 open reading frame encoding a putative 393-amino acid protein revealed 99% similarity with known gD2 amino acid sequence by BLAST analysis (data not shown), which suggested the recombinant gD2 had similar properties with its original gD2. The gD2 gene was then subcloned into the *Eco*R I and *Hin*d III restriction sites of pFastBac HTb donor plasmid to construct pFastBac HTb-gD2. Recombinant pFastBac HTb-gD2 plasmid was transformed into DH10Bac competent cells. The positive white colonies containing recombinant bacmids were selected. All the results were as expected (data not shown).

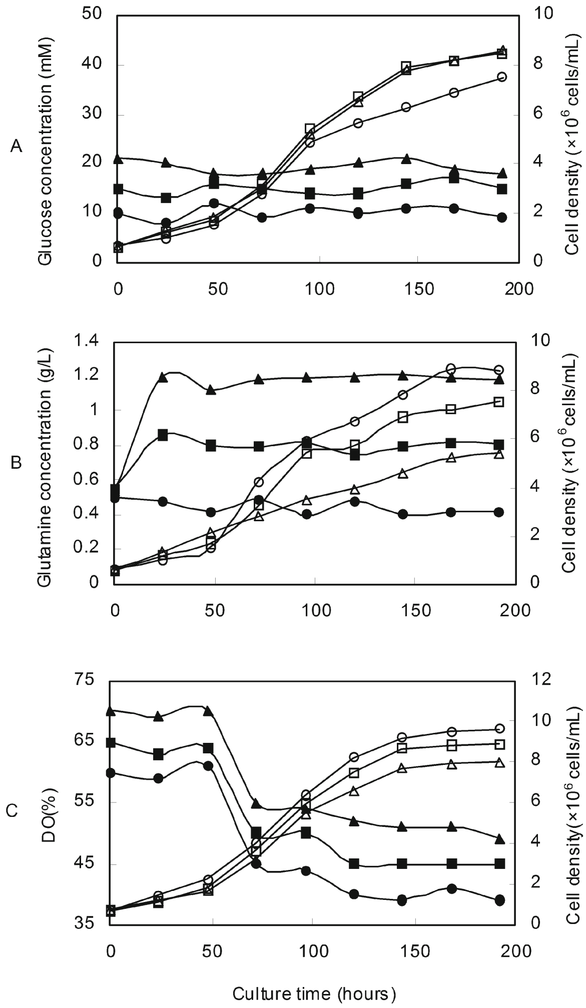

Fig. 1. The effects of various nutritions feeding and DO controlling on the cell growth. In all graphs, the hollow symbols represented the cell density at the corresponding conditions. (A) The effects of the glucose feeding on the cell growth. Glucose was fed as the pulse mode and determined at an interval of 12 h. (●), residual glucose concentration about ~10 mM; (■), residual glucose concentration about ~15 mM; (▲), residual glucose concentration about ~20 mM. (B) The effects of the glutamine feeding on the cell growth. Glutamine was fed as the pulse mode and determined at an interval of 12 h. (●), residual glutamine concentration about ~0.4 g/L; (■), residual glutamine concentration about ~0.8 g/L; (▲), residual glutamine concentration about ~1.2 g/L. (C) The effects of DO controlling on the cell growth. (●), ~40% of DO; (■), ~45% of DO; (▲), ~50% of DO.

Optimization of high-density cell culture of Sf9 and the gD2 protein expression
Fed-batch mode for insect cell or mammalian cell culture is probably the most attractive choice for the production of recombinant protein. Fed-batch operation could easily achieve the high cell density. In order to ob-

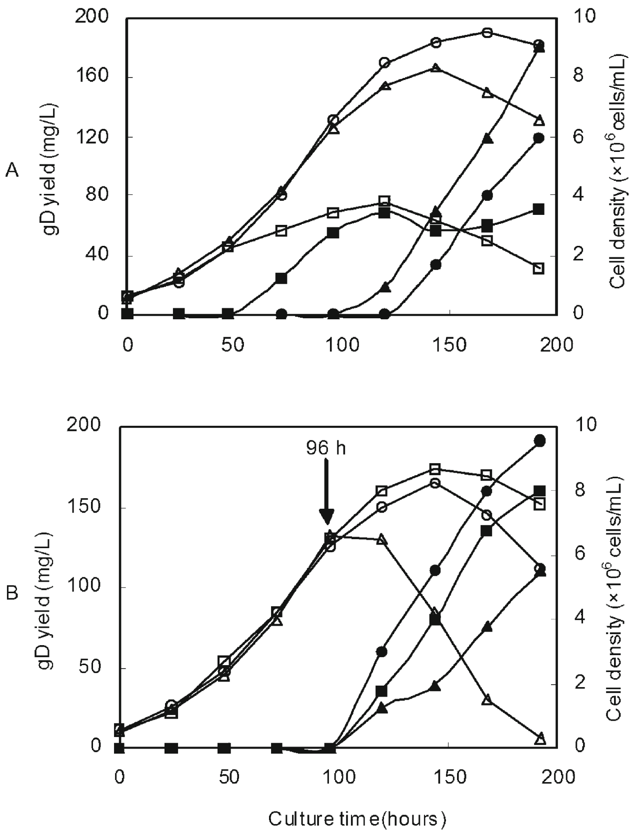

Fig. 2. Determination of the infection time and MOI in fed-batch culture. (A) The effects of infection time on the cell density and the gD2 protien yield. (■/□), protein yield/cell density when infected at the 48th; (▲/△), protein yield/cell density when infected at the 96th; (●/○), protein yield/cell density when infected at the 120th. (B) The effects of MOI on the cell density and the gD2 protein yield at the optimal infection time of 96th. (■/□), protein yield/cell density when MOI is 1; (●/○), protein yield/cell density when MOI is 5; (▲/△), protein yield/cell density when MOI is 10.

tain the high cell density, three critical factors including glucose level, glutamine level and DO were explored.

In the nutrition-limited fed-batch culture, the glucose feeding was started at 30 h when the initial glucose was consumed. After that the glucose concentration was controlled at the different levels as shown in Figure 1. The glutamine was controlled at the different initial concentration. The results showed that the cell density reached the maximum at the controlled concentration 15 mM of glucose and 0.4 g/L glutamine, respectively. Under the optimal nutrition fed-batch conditions, the cell density rose to 8.8×10^6 cells/mL. Following the optimization of nutrition feeding, the effects of DO on the cell growth were investigated. The results revealed that the final cell density decreased with the increase of DO level, which revealed that the higher oxygen concentration inhibited the cell growth. The cell density could reach 9.6×10^6 cells/mL under the appropriate DO level of 40% (Fig. 1).

In fed-batch culture baculovirus infection was done at the relatively higher cell density to ensure quantities of protein expression. In the present study, the insect cells were infected at different growth phases for evaluating the gD2 expression. The results revealed that the maximum yield of gD2 protein was achieved in Sf9 cells infected by recombinant baculovirus after 96 h with a cell density of 6.5×10^6 cells/mL (Fig. 2). The effect of multiplicity of infection (MOI) on recombinant gD2 expression was also evaluated. Fed-batch culture was infected after 4 days when the cell density reached up to 6×10^6 cells/mL. The highest yield of recombinant gD2 was obtained at MOI of 5 with 192 mg/L (Fig. 2).

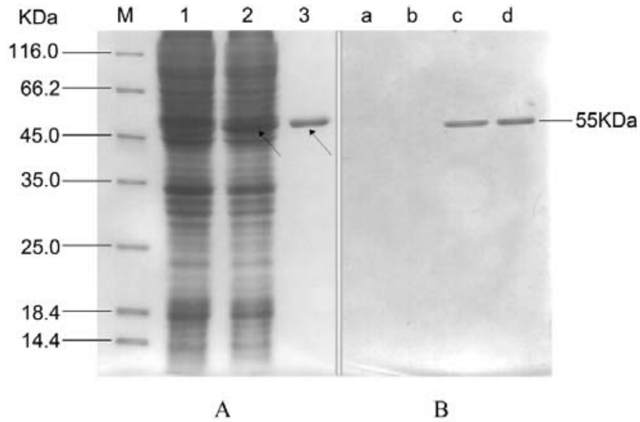

Fig. 3. Expression and identification of recombinant gD2 from baculovirus-infected Sf9 cells. (A) Expression of recombinant gD2 in baculovirus-infected Sf9 cells. The cell culture was fractionated by 12% SDS-PAGE and subsequently visualized by coomassie blue staining. M, protein marker; lane 1, uninfected Sf9 cell culture; lane 2, baculovirus-infected Sf9 cell culture; lane 3, purified recombinant gD2. (B) Western blotting analysis of recombinant gD2. Lane c, baculovirus-infected Sf9 cell culture; lane d, purified recombinant gD2. No western blot signal was observed from negative control (lanes a and b). Arrow represents the recombinant gD2 protein.

Table 1. Neutralizing antibody titers in guinea pigs prior to the challenge with HSV-2.

Immunization group	Neutralizing antibody titer[a]
Recombinant gD2	3.42 ± 0.29
PBS	0.27 ± 0.00
Commercial gD2 VTI540	3.39 ± 0.32

[a]Data are presented as mean logarithmic values of (log10) neutralizing antibody titers.

Purification of recombinant gD2

Sf9 cells were cultured in bioreactors and infected with recombinant baculovirus. Cultured cells were harvested and the gD2 was purified as described in Material and Methods with the purity evaluation by SDS-PAGE. For further characterization, the separated proteins were probed by mouse anti-gD2 monoclonal antibody through Western blotting analysis. The recombinant gD2 had similar molecular weight as the original gD2 with approximately 55 kDa, while no specific band was observed in uninfected Sf9 cells culture (Fig. 3).

In vivo assays

The serum neutralization titers of the blood collected from immunized guinea pigs are listed in Table 1. The

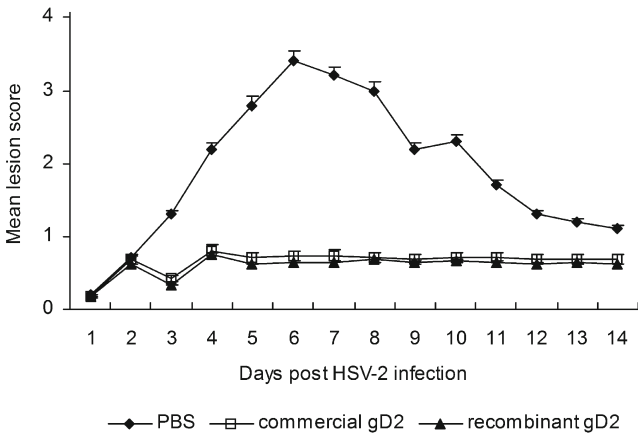

Fig. 4. Effect of recombinant gD2 immunization on lesion development in guinea pigs infected with HSV-2.

immunization of purified recombinant gD2 greatly increased neutralizing antibody levels when compared with animals administered with PBS alone ($P < 0.05$). No significant difference in immunization between the recombinant gD2-immunized group and the commercially available gD2-immunized group was observed.

The reduction in genital lesion scores is an important parameter for evaluating vaccine and antiviral therapy. Mean lesion scores for each day were calculated based on the sum of group lesion scores and the number of animals in each group. The group immunized with recombinant gD2 revealed a partial protection on guinea pigs against HSV-2 challenge when compared with the group immunized with PBS alone ($P < 0.05$). However, no significant difference in the reduction of lesion scores between the recombinant gD2-immunized group and the commercially gD2-immunized group was observed (Fig. 4).

Discussion

Infection with HSV-2 remains a prevalent and potentially serious health problem, especially in neonates and immunocompromised individuals. The gD2 is a primary vaccine candidate for the application in humans. Associated with both the virus envelope and the infected cell surface, gD2 has been shown to play an indispensable role in early events of virus infection and to serve as an important target of cross-reactive neutralizing antibody and cellular immune responses (Bernstein & Stanberry 1999).

The recombinant gD2 can be expressed in both recombinant prokaryotic and eukaryotic hosts. Since gD2 is easy to form inclusion bodies in *E. coli*, further studies for its expression in other systems have highly been required. The baculovirus expression system has a number of potential benefits, such as high expression level, correct folding and post-translational modification, and production of biologically active proteins for analysis (Liu et al. 2005). In addition, since one of our main reasons for producing the recombinant gD2 protein was for *in vivo* studies, we preferred to use the baculovirus system to avoid the testing and removal of endotoxin that may contaminate *E. coli* products.

Fed-batch cultivation has been used for production of various recombinant proteins with high productivities (Jeong & Lee 1999). High-cell density fermentation offers many advantages including increased productivity, reduced cost, labour-saving technique allowing the elimination of hundreds of flasks and reduced risk of contamination compared with other traditional methods. As for the insect cell/baculovirus expression system, high cell density can guarantee the highly effective production of protein. We initiated this study for optimizing the cultural conditions of Sf9 cells at high cell density to produce a large amount of recombinant gD2. Because glucose, glutamine and DO are the most essential factors affecting cell growth and viability of Sf9 cells, these factors have been investigated with the aim of increasing the final cell density. In this study, the concentrations of glucose, glutamine and DO were optimized in the feeding process. Higher cell density could be achieved at the conditions of 15 mM glucose and 0.4 g/L glutamine. In contrast, lower concentrations of glucose and glutamine could not provide enough carbohydrate for cell metabolism and propagation, and higher concentrations of glucose and glutamine were not beneficial to cell growth due to the production of extra lactic acid or ammonia. A stable oxygen level is essential in insect cell culture. Good DO control ensures that cell growth is not limited by oxygen. Many studies have proven that different culture system including cell lines and medium have various demand of oxygen. Here, cell growth could be inhibited by oxygen at the middle and late phases of fermentation when the apparent concentration of DO was more than 40%. Maybe, excessive DO conditions can bring the formation of nascent oxygen, superoxide and peroxide which destroy cellular components and then destroy the cell growth.

At the optimal conditions, the yield of recombinant gD2 protein was up to 192 mg/L cell culture, which was dozens of times higher than the expression level in previous reports (Landolfi et al. 1993). Therefore, it is the first report to express a large amount of recombinant gD2 using the fed-batch culture technique.

The guinea pig vaginal infection, a close model of human genital herpes, can be used to determine the protective capability of recombinant gD2 against HSV-2 infection. Our results indicated that the immunization of recombinant gD2 greatly increased neutralizing antibody level and highly reduced lesion scores in guinea pigs infected with HSV-2 when compared with the animals treated with PBS alone. Compared with the results by Fotouhi et al. (2008), the neutralizing antibody titers were higher in our study, which might be due to the purified recombinant gD2 with stronger immunogenicity in our study and the crude cell culture with lower immunogenicity to immunize guinea pigs in Fotouhi et al. (2008) study. The immunity between the recombinant gD2-immunized group and the commercially gD2-immunized group did not exhibit a significant difference, which suggested that bioactive recombinant gD2 was expressed successfully.

In conclusion, recombinant gD2 protein expression conditions were successfully optimized to improve expression level and attenuating its degradation. The baculovirus expression system and fed-batch cultivation provided a valuable and reproducible source to produce substantial amounts of purified, immunogenic and protective vaccine candidate of recombinant gD2 for genital herpes model of guinea pigs infected with HSV-2. Further studies focused on optimizing the vaccine protection of the gD2 with various adjuvants and doses are underway.

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