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Expression and Production of Human Interleukin-7 in Insect Cells Using Baculovirus Expression Vector System (BEVS)

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Abstract Interleukin-7 (IL-7) is a glycoprotein cytokine with significant clinical and biomedical potential, such as cancer therapy and HIV infections. Earlier it has been cloned and expressed in various protein expression systems; however, they are not efficient for large-scale production. To address this inadequacy, we report in this paper the production of recombinant human interleukin-7 (hIL-7) in insect cells. A recombinant bacmid containing hIL-7 was constructed, purified, and characterized. It was used to infect *Trichoplusia ni* (BT1-TN-5B1/High Five™) insect cells. Result shows that *T. ni* cells successfully produce hIL-7 in shake flask cultures. A scale up to 2.5-L laboratory batch bioreactor showed the efficacy of this system for large-scale production. Our results offer a highly efficient, inexpensive, and convenient system for the large-scale expression and production of recombinant hIL-7.

Keywords Interleukin-7 · Insect cells · *Trichoplusia ni* cells · Glycoprotein cytokine production · Baculovirus vector system · Batch bioreactor

Introduction

Human interleukin-7 (hIL-7) is a cytokine with a molecular weight of 25 kDa [1–3]. It is mainly produced by the thymus, bone marrow, intestinal epithelium, and skin, and plays an

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important role for lymphocyte development and survival [4, 5]. There are several biomedical and therapeutic applications for hIL-7 such as enhancement of lymphopoiesis, promotion of stem cell engraftment, and antitumor activity. It is also known to have great potential in the treatment of HIV infections and cancer therapy or as an adjuvant to vaccines [5–7]. Therefore, the large-scale production of hIL-7 is desirable. The production of biologically active hIL-7 has been demonstrated by other investigators in *Escherichia coli* [5, 8, 9]. Although it was expressed at high levels, the product was unprocessed and deposited as an insoluble inclusion body, which made its purification difficult and time consuming. Thus, the investigation of alternative methods for the production of hIL-7 is desirable. Indeed, hIL-7 has been produced in yeast and mammalian expression vectors in COS 7 cells [1, 3]. However, there are limitations in large-scale productions. The yeast expression system has limitation in the case of glycosylation. For the mammalian expression system, large-scale production is difficult and time consuming. Also, this system needs complex nutrient with low productivity [10].

In this study, we have designed a baculovirus vector system for the infection and subsequent expression of hIL-7 in insect cells. This has advantages over currently used methods, as the baculovirus insect expression vector systems can produce larger quantities of recombinant protein which can be processed by the eukaryotic protein processing systems. In addition, this system has high capacity expression efficiency, lack of endotoxin contamination, is inexpensive, and a convenient system for the expression of recombinant protein. Furthermore, as insect cells can grow to high densities [10–12] in suspension, it provides a relative reduction in the cost of hIL-7 production. This article describes the expression, large-scale production, and purification of hIL-7 cells in insect cells using baculovirus expression vector system (BEVS).

Materials and Methods

Cells and Media

Spodoptera frugiperda, Sf9 and Sf21 (Invitrogen, Carlsbad, CA, USA) were maintained at 27 °C in stationary T-flasks and shake flasks. *Trichoplusia ni* (BT1-TN-5B1/High Five™) cells (Invitrogen, Carlsbad, CA, USA) were maintained at 27 °C in shake flasks (20–30 ml in 125-ml flask; Corning, Corning, NY, USA) with agitation at 110–120 rpm. SF900-II media (Gibco, Life Technologies, Grand Island, NY, USA) and Excell 405 media (JRH Biosciences, Lenexa, KS, USA) were used for Sf9, Sf21, and High Five cells, respectively.

Plasmid Construction and Bacmid Preparation

The plasmid pORF9 containing the *IL-7* gene was purchased from Invivogen, San Diego, CA, USA. A forward [GCCTACCTGGGATCCGGTCAAC] and a reverse [TCATCAATG TATGCGGCCGCCTTATCATGTCGAG] PCR primer were designed to allow cloning of the *Bam*HI and *Not*I digested amplified hIL-7 product into the *Bam*HI and *Not*I sites of a pFastBacHT-A a fusion vector. The constructed plasmid was amplified in *E. coli* DH5α. Positive colonies were cultured in LB medium; the plasmids were purified and sequenced to ensure the correct insertion of the gene. It was then transformed into *E. coli* DH10Bac, which is an efficient site-specific transposition system (Bac-to-Bac Baculovirus Expression System). The resulting large recombinant bacmid baculoviruses (rbacmid/hIL-7) were purified using 15 mM Tris–HCl, pH 8.0, 10 mM EDTA, supplemented with 100 μg/ml

RNase A and 0.2 N NaOH/1% SDS and 3 M potassium acetate, pH 5.5 according to the manufacturer's protocol.

Insect Cell Transfection and Baculovirus Stock Preparation

For baculovirus stock preparation, approximately 10^6 Sf9 cells were seeded unto six-well plates. The transfection mixture comprised of 1 μg of the constructed bacmid and 6 μl of the transfection reagent Cellfectin diluted in 200 μl media (Invitrogen Life Technologies, Carlsbad, CA, USA). The mixture was incubated for 45 min at room temperature before combining with the cells and was incubated with the cells for 5 h at 27 °C. The transfection medium was then replaced with fresh medium (SF900II). These cultures were further incubated at 27 °C, and the supernatants were collected at 96 h post transfection, centrifuged (4 °C, 1,000×*g*, 10 min), filtered through a 0.22-μm membrane, and stored at 4 °C (P1 Virus Stock). Larger volumes of virus stock were made by scale-up amplification in shaker flask cultures of Sf9 cells. Briefly, Sf9 cells were grown to midexponential phase and diluted to $2–2.5\times10^6$ cells/ml with fresh SF900II medium. The cultures were infected with P1virus stock (VS) at a multiplicity of infection (MOI) of 0.1–1. During the infection process, the total and viable cell densities and cell size were measured using the automated Trypan Blue exclusion method (Cedex, Innovatis, Bielfeld, Germany). The supernatant containing the recombinant baculovirus (rbac/hIL-7) was harvested by centrifugation when cell viability was less than 80–90%.

Quantification of Viral Titer

The quantification of total viral particle concentration is performed by flow cytometry according to a protocol developed by Shen et al. [13]. Flow cytometry analyses for Sybr Green I stained viral particles was performed on a Coulter EPICSTM XL-MCL flow cytometer (Beckman-Coulter, FL, USA) equipped with 15 mW at 488 nm argon ion laser as an excitation source. The green fluorescence emission was detected using a 550-nm dichroic long pass and a 525-nm band pass filter set.

Optimization of Recombinant Human Interleukin-7 Expression

For optimization assays, rbac/hIL-7 at different MOIs between 5 and 0.5 was used to infect Sf9, Sf21, and High Five cells. Sf9 cells were cultured in 125 shaker flasks (Corning, Corning, NY, USA) and were incubated at 27 °C with agitation at 115 rpm. Samples were taken at regular intervals (24, 48, 72 and 96 h post infection [hpi]). The automated Trypan Blue exclusion method was used to estimate the total, viable cell densities and cell size during the infection process. Supernatant and pellets were harvested by centrifugation after 96 hpi. Optimization of protein production in the High Five cell line was carried out using five different MOIs (5, 0.5, 1, 0.1, and 0.01) for infection of the cell line.

Analytical Methods

The total protein assays were performed using a protein kit based on the Lowry protocol (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as standard. The amount of hIL-7 in supernatants and cell lysate was analyzed by sandwich ELISA using rabbit antihuman IL-7 antibody (RDI, MA, USA) as the capture antibody and a biotinylated

monoclonal antihuman IL-7 (R&D Systems, ON, Canada) as the detection antibody. Streptavidin-horseradish peroxidase (R&D Systems, ON, Canada) was used for conversion of a substrate mixture containing stabilized hydrogen peroxide and stabilized tetramethylbenzidine (R&D Systems, ON, Canada). The reactions were terminated by adding H_2SO_4, and the absorbance was measured at 450 nm using 570 nm as the reference wavelength. Commercially available purified hIL-7 (R&D Systems, ON, Canada) was used as standard.

Western Blot Analysis

The expression of hIL-7 was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed using a polyclonal rabbit antihuman IL-7 (RDI, Flanders, NJ, USA) as the primary antibody. The blots were developed using a chemiluminescence kit (Boehringer Mannheim, Mannheim, Germany) and visualized with the Kodak manager system. SeeBlue protein marker (Invitrogen, Carlsbad, CA) was used as standard marker. Commercially available purified hIL-7 (R&D Systems, ON, Canada) was used as standard.

Large-scale Production of Recombinant hIL-7

The production of hIL-7 using High Five cells was carried out in a Chemap 3.5-L type SG bioreactor (Mannedorf, Switzerland). Scale-up production of recombinant hIL-7 was performed according to the procedure described previously [14]. Briefly, exponentially growing cultures from High Five cells grown in shake flasks were seeded into the bioreactor at initial densities of 5×10^5 cells/ml. Cells were grown to a density of about 1.5×10^6 cells/ml before infection with a recombinant baculovirus expressing hIL-7 at a MOI of 5. Samples were taken every 12 h and stored at −80 °C until further analyses. The culture was harvested at 48 hpi, and then centrifuged for 15 min at 2,200×*g* using a J-6B centrifuge (Beckman Instruments, USA).

Purification of Human IL-7

The histidine-tagged hIL-7 was purified by immobilized metal affinity chromatography (IMAC) using a Ni^{2+} immobilized resin (Bio-Rad, Hercules, CA, USA). Harvested cells were resuspended in lysis buffer (50 mM Na H_2PO_4, 300 mmM NaCl, 5 mM imidazole enzyme grade, IgePal CA-630; Sigma) containing protease inhibitor cocktail tablets (Roche, Penzberg, Germany), and incubated on ice for 30 min. Sonication was performed on ice for 3×10 s per 10 ml lysate at 40% of maximum energy output (Misonix, Farmingdale, NY, USA). The lysate was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant (crude extract) was filtered through a 0.45-μm cellulose acetate (CA) low protein binding membrane and poured into a column (10 ml) which was packed with Profinity IMAC Ni-Charged Resin (Bio-Rad) that was equilibrated with equilibration buffer (50 mM Na H_2PO_4, 300 mmM NaCl, 5 mM imidazole adjusted at pH 7.5). Chromatography was performed on a Pharmacia FPLC system, and the absorption (280 nm) and conductivity of the eluent was monitored. The column was washed with wash buffer (50 mM Na H_2PO_4, 300 mmM NaCl, 5 mM imidazole pH 7.5) at a flow rate of 2 ml/min at 5–10 column volumes. The protein was eluted with 200 mM imidazole in elution buffer (50 mM Na H_2PO_4, 300 mmM NaCl, 200 mM imidazole pH 7.5) at a flow rate of 0.5 ml/min. The purified protein was dialyzed at 4 °C using the ultrafiltration membrane YM3 (Amicon, Milipore) against a solution containing 50 mM NaH_2PO_4,

300 mmM NaCl, 5 mM imidazole, and 10% glycerol adjusted at pH 7.5. Aliquots were taken and stored at −80 °C.

Results and Discussion

Plasmid Construction and Baculovirus Stock Preparation

Experiments were designed to use BEVS in insect cells for the expression and production of hIL-7. A detail of the cloning strategy is described in Fig. 1a and b. pFastBacHT-A is a fusion vector with the N-terminal 6xHis tag and the TEV protease cleavage site (Invitrogen, Carlsbad, CA, USA) to facilitate protein purification. The plasmid construct was successfully transfected into *E. coli* DH10Bac, which resulted in the generation of the bacmid. For the production of the viral stock, the bacmid was constructed in the Bac-to-Bac baculovirus expression system, which allows rapid and efficient generation of recombinant baculovirus [15]. The P1 viral stock was then used to infect Sf9 cells for generating high-titer P2 and P3 viral stock. The total viral particle concentration in the P3 viral stocks was 1.2×10^9 pfu/ml as determined by flow cytometry.

Optimization of Recombinant hIL-7 Production

To optimize the production of hIL-7 in the BEVS system, a preliminary experiment was designed using different MOIs of the recombinant virus containing hIL-7 (rbac/hIL-7) to infect Sf9, Sf21 cell lines from *S. frugiperda* [16] and High Five from *T. ni* [17] in suspension culture. Sf9 and Sf21 are very similar; however, Sf21 has greater range in size and there are some reports showing that they can produce proteins in higher yield compared to Sf9 [12]. High Five insect cells were able to produce a higher level of the secreted hIL-7 glycoprotein. Insect cells infected with baculovirus stop dividing, and after 24 hpi, the cell size starts increasing because of cytoplasmic effects [10, 14]. Samples were collected every 24 h for further study. Results also showed that the three cell lines are able to produce hIL-7, although the High Five cells were able to secrete the protein at higher level than the other two cell lines tested.

To evaluate the expression characteristics of the recombinant hIL-7, Western blot assays were used. Figure 1c shows hIL-7 is produced and processed normally in BEVS using High Five insect cells with molecular weights similar to that produced in mammalian cells. The protein was found in the supernatant and in the cell lysates. However, the intracellular protein was found to have multiple molecular weights. These different molecular forms are likely a result of incomplete glycosylation which may occur in this system [18, 19]. The expression of hIL-7 has been achieved in *E. coli* with a net molecular weight of about 17 kDa compared to the glycosylated hIL-7 with a molecular weight of 25 kDa, indicating that the protein is not glycosylated in *E. coli* [3, 20] and that glycosylation of hIL-7 is carried out strictly in eukaryotic cells.

To optimize the production of hIL-7 in High Five insect cells, cells were infected with different MOIs (0.01, 0.1, 0.5, 1, and 5). As expected, the final viable cell density of the uninfected cells (control) was higher than those of cells infected by recombinant virus (Fig. 2a). The number of viable cells increased to more than 4×10^6 cells/ml in the control samples (Fig. 2b) and decreased to less than 1×10^6 cells/ml in cells infected at MOI 5, 1, and 0.5. The viability of infected cells in the case of MOI 5, 1, and 0.5 rapidly decreased after 48 hpi to less than 60%. For infected cells with MOI 0.1 and 0.01, the viability

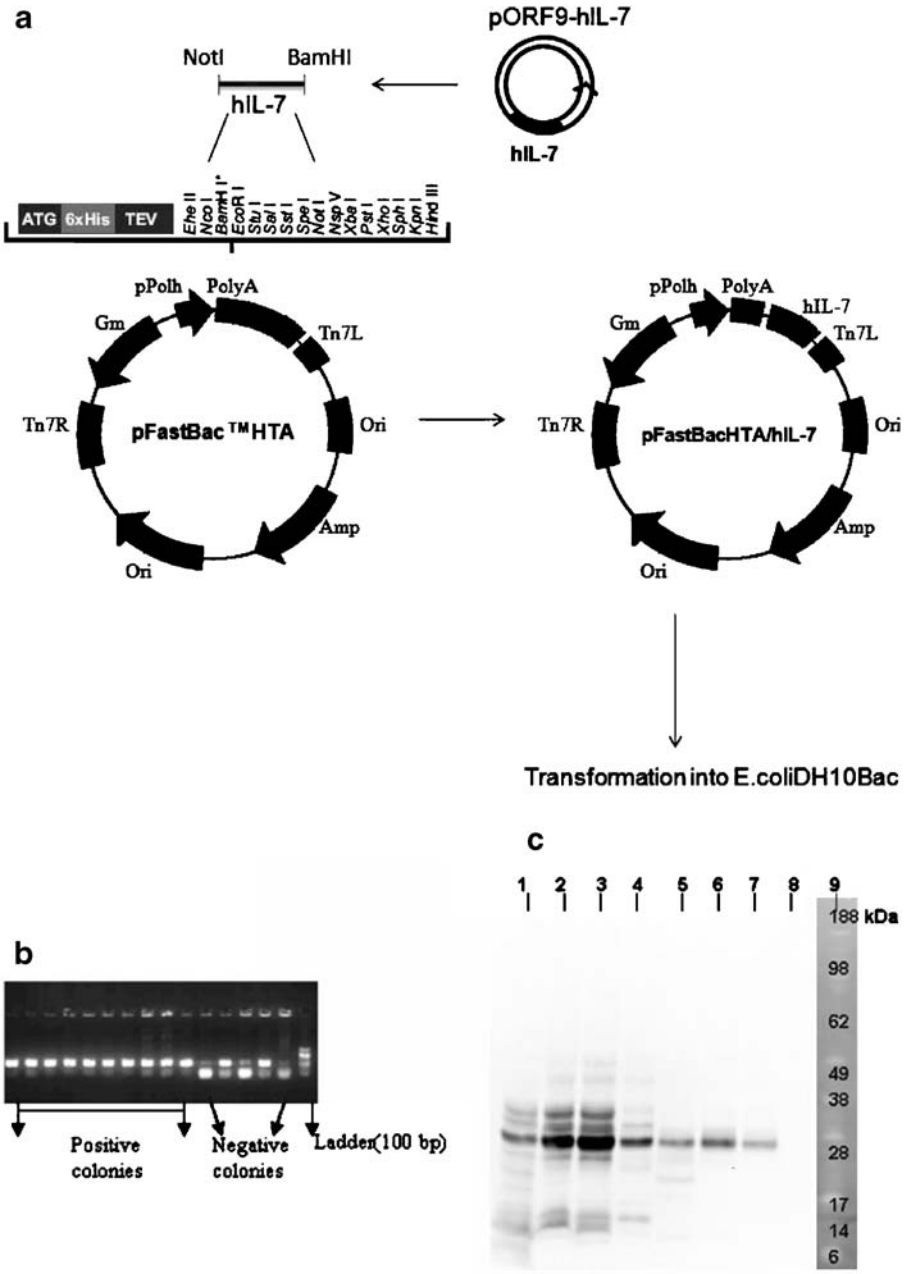

Fig. 1 Schematic of the cloning strategies and constructed pFastBacHT-A/hIL-7 vector containing 6xHis at the multiple cloning sites (*MCS*). **a** The *IL-7* gene was obtained from the pORF9-hIL-7 vector inserted into the pFastBacHT-A cloning system, and the constructed plasmid was transformed into *E. coli* DH10Bac containing bacmid. **b** PCR screening of the positive *E. coli* DH10Bac colonies containing the pFastBacHT-A/hIL-7 plasmid. **c** Expression of hIL-7 in High Five cells using rbac/hIL-7. *Lanes 1–4* cell lysate after 96, 72, 48, and 24 hpi, respectively; *lanes 5–8* supernatant after 96, 72, 48, and 24 hpi, respectively; *lane 9* protein marker

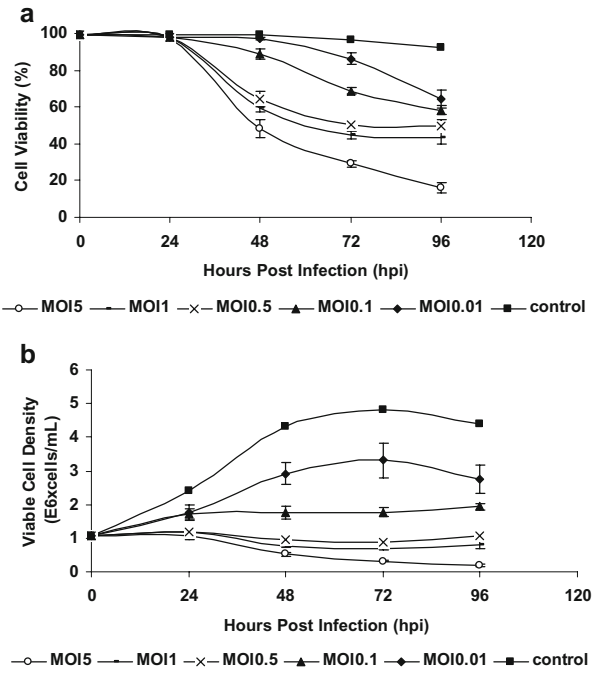

Fig. 2 Growth curves of High Five insect cell infected with rbac/hIL-7. **a** Cell viability and **b** viable cell density of High Five cell line. Cells were infected with different MOIs. Samples were taken at different hpi. The cultures were inoculated at 1×10^6 cells/ml and infected with MOIs of 5, 1, 0.5, 0.1, and 0.01, respectively (n=3)

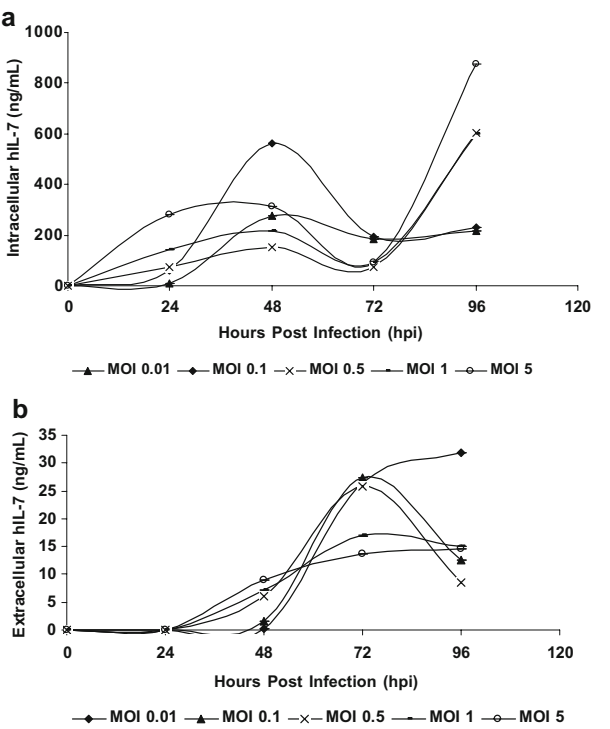

Fig. 3 The hIL-7 production profile of High Five insect cell infected with rbac/hIL-7. **a** Extracellular and **b** intracellular concentration of hIL-7 measured using sandwich ELISA at hpi of cultures infected at different MOIs. Rabbit antihuman IL-7 antibody, as the capture antibody, and a biotinylated monoclonal antihuman IL-7, as the detection antibody, were used. Streptavidin-horseradish peroxidase was applied for the conversion of the substrate mixture, and the absorbance was measured at 450 nm using 570 nm as the reference wavelength. Commercially available purified hIL-7 was used as standard (n=3)

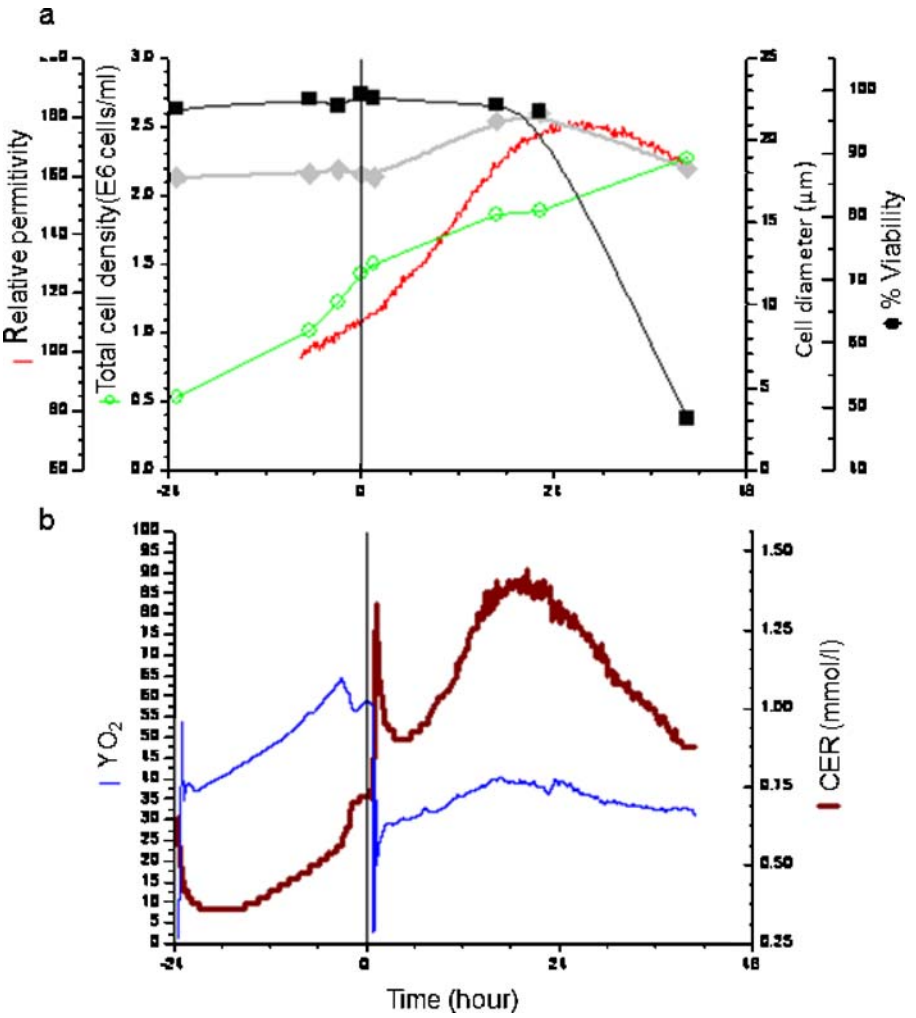

Fig. 4 Production of hIL-7 in a batch bioreactor using High Five insect cells using rbac/hIL-7. Online and offline analysis was used to monitor cell growth. **a** Profiles of relative permittivity (ϵ'), total cell densities, cell diameter (μm), and percentage of cell viability. **b** CER, Y_{O2}, and percentage of O_2 in total gas flow Q_{TOT}

decreased to 60% after 72 hpi. There was no significant difference in viability between control cells over a period of 96 hpi. At high MOI, all cells are infected immediately and synchronously; cell growth is also stopped and the cell density is close to that at the time of infection. At lower MOIs, a fraction of the cells are infected and the rest continue to grow as noninfected cells. These cells and their progeny are infected later, so the cell density is higher than the cell density at the time of infection.

Quantification of Small-scale hIL-7 Production

The expression profile of the protein at different MOIs was determined by ELISA. Secretion of the protein (Fig. 3a) increased significantly after 48 hpi and reached a maximum (over 35 ng/ml) at 80 hpi in the case of MOI 0.01; in other cases, the maximum

amount was achieved at 72 hpi and decreased slightly to 15 ng/ml at 96 hpi. In these cases, the decrease in the level of recombinant protein after 80 hpi was likely because of proteolysis [21]. Figure 3b shows the production profile of hIL-7 in cell lysate at different hpi. Cells had identical profiles with maximal production at 48 hpi and at 96 hpi. The maximum concentration of hIL-7 expressed in the cell lysate was about 1 μg/ml. Small-scale results showed that the highest amount of protein can be achieved from the cell lysate using rbac/hIL-7 at MOI 5.

Large-scale Production of Recombinant hIL-7

We further investigated the scale up of the production process using a batch bioreactor for the large-scale production of hIL-7. Figure 4 shows the results of those investigations indicating the suitability of the batch bioreactor for large-scale production of hIL-7. Growth in the bioreactor was monitored online using relative permittivity (ϵ'). Growth was also monitored by the routine cell counting methods. Cells were infected at a density of 1.5×10^6 cells/ml; cells appeared to be infected as seen by the increase in diameters after 24 hpi. The time course of the relative permittivity (Fig. 4a) which is a measure of the total biovolume follows the cell (total and viable) density profiles closely until the time of infection. Upon infection, a further increase is seen in the permittivity profile, which can be attributed to the increase in cell diameter. Later, the permittivity profile follows the cell diameter profile. Furthermore, it can be seen that once cell diameter reaches a maximum value there is no further increase in the permittivity followed by a decrease related to the decrease in cell diameter and viable cell density. These data are similar to previous work in our laboratory [22]. Relative permittivity was already used for measuring cell growth up to densities as high as 52×10^6 cells/ml and could also be used to monitor infection in high cell density cultures [22–24]. The oxygen demand (Y_{O2}) is expressed as the fraction of O_2 in the gas fed to the bioreactor headspace. The dissolved oxygen (DO) level was controlled at 40% of air saturation. It has been shown that oxygen consumption in infected insect cells with baculovirus increases during the first 48 hpi [25]. The CO_2 evolution rate (CER) was measured as an indicator of the physiological status of the culture. The CER profile followed $\acute{\epsilon}$, viable and total cell density (Fig. 4b). This CO_2 production pattern after infection was similar to the previous work [24].

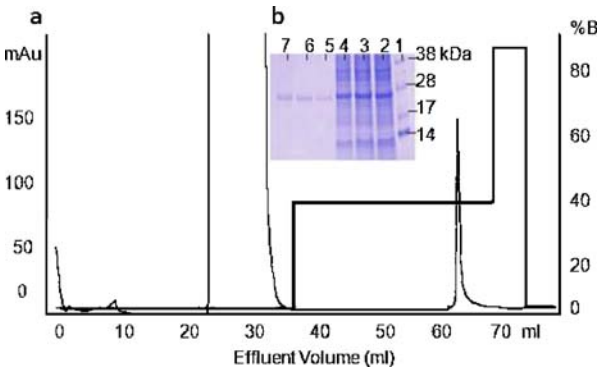

Fig. 5 hIL-7 purification using IMAC. Profiles of A_{280} showed the separation of histidine affinity of recombinant hIL-7 (**a**) and SDS-PAGE analysis (**b**) of hIL-7 purification. *Lane 1* protein marker, *lane 2* cell lysate, *lanes 3–4* wash fractions, *lanes 5–7* elution fractions with 200 mM imidazole

Purification of Recombinant hIL-7

After the large-scale production of hIL-7, the product, which contains a histidine affinity ligand at the N-terminus, was subjected to purification. It was purified in one step using immobilized metal affinity chromatography (IMAC) as shown in Fig. 5. Fractions collected during the IMAC elution were measured for absorbance at 280 nm (A_{280}). Weakly bound proteins were washed and proteins were found to elute at an imidazole concentration between 100 and 200 mM. A single band with an apparent molecular weight of about 24 kDa was obtained after nickel affinity chromatography. The results from the protein assay after purification showed that the amount of protein in the crude extract was about 1.765 mg/ml; 0.124 mg/ml was recovered in the elution step with 100–200 mM imidazole, which was further concentrated and diafiltered to a final yield of 0.343 mg/ml. The protein may also be purified directly from the supernatant after concentration.

Conclusion

BEVS are the most widely used systems for the production of recombinant proteins in insect cells [26]. Construction of the baculoviral vector was found to be relatively convenient. Recombinant hIL-7 protein was expressed under the control of the viral polyhedrin promoter, an especially strong promoter for high protein expression in Sf9, Sf21, and High Five inset cells. We have found that insect cells perform most of the processing steps of recombinant protein which occur in mammalian cells. As a result, it is much more probable that a protein expressed in insect cells will have normal biological activity than in the case of production of the protein in *E. coli.* This study suggests that BEVS is a useful tool to facilitate the large-scale production of hIL-7 for biophysical and pharmacological investigations. However, more work is needed to determine its glycosylation pattern and compare it with the mammalian expression system.

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