Expression and Purification of Proliferating Cell Nuclear Antigen in Spodoptera frugiperda Insect Cells (Post-Print)

Authors: Chen Junjun, Lou Ying, Zhang Yuanxing, Liu Qin, Liu Xiaohong

Date: 2018-05-16T00:00:00+00:00

Abstract

Objective: To produce recombinant human proliferating cell nuclear antigen (PCNA) using an insect cell expression system, and to purify the protein and characterize its antibody binding properties. Methods: Using reverse-transcribed cDNA from HeLa cells as template, the human PCNA gene was amplified and inserted into the baculovirus vector AcMNPV. Recombinant baculovirus carrying the PCNA gene was obtained using insect cells. The virus-infected cells expressed the protein, and high-purity recombinant human PCNA protein was obtained through a combination of nickel-affinity chromatography and ion-exchange chromatography. Antibody binding specificity was determined by ELISA. Results: The gene sequence obtained using HeLa cell cDNA as template was identical to the human PCNA gene sequence in GenBank. The optimal multiplicity of infection (MOI) and infection time for expression of recombinant human PCNA (rPCNA) in Spodoptera frugiperda (Sf9) cells were 0.05 and 144 h, respectively. The yield of rPCNA reached 110 mg/L of cells, with purity >95%. Indirect ELISA detection of antibody binding characteristics showed that the sensitivity and specificity of rPCNA were 93.3% and 85.0%, respectively. Conclusion: An expression and purification method for rPCNA was established, obtaining PCNA protein with high-level expression and high antibody binding specificity. This protein can be further developed as an in vitro diagnostic kit for PCNA-related diseases and has significant application value.

Full Text

Expression and Purification of Proliferating Cell Nuclear Antigen in *Spodoptera frugiperda* Insect Cells

Junjun Chen[1], Ying Lou[1], Yuanxing Zhang[123], Qin Liu[123], Xiaohong Liu[12]*

[1]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China
[2]Shanghai Engineering Research Center of Maricultured Animal Vaccines, Shanghai 200237, China
[3]Shanghai Collaborative Innovation Center for Biomanufacturing, Shanghai 200237, China

Corresponding author: Xiaohong Liu
Tel.: +86-21-64253065; Fax: +86-21-64253025; E-mail: liuxiaohong@ecust.edu.cn
Address: 130 Meilong Road, Xuhui District, Shanghai 200237, China

Abstract

Objective: To produce recombinant human proliferating cell nuclear antigen (PCNA) using an insect cell expression system, purify the protein, and characterize its antibody binding properties. **Methods:** The human PCNA gene was amplified from reverse-transcribed HeLa cell cDNA and inserted into the baculovirus vector AcMNPV. Recombinant baculovirus carrying the PCNA gene was generated using insect cells. Protein expression was achieved through viral infection, and high-purity recombinant human PCNA (rPCNA) was obtained via a combination of nickel affinity chromatography and ion exchange chromatography. Antibody binding specificity was determined by ELISA. **Results:** The gene sequence obtained from HeLa cell cDNA was identical to the human PCNA gene sequence in GenBank. The optimal multiplicity of infection (MOI) and infection time for rPCNA expression in *Spodoptera frugiperda* (Sf9) cells were 0.05 and 144 h, respectively. The rPCNA yield reached 110 mg/L of cell culture with purity exceeding 95%. Indirect ELISA demonstrated that rPCNA possessed a sensitivity of 93.3% and specificity of 85.0% for antibody binding. **Conclusion:** We established a robust method for rPCNA expression and purification, yielding highly expressed protein with strong antibody binding specificity. This rPCNA protein shows significant potential for development into in vitro diagnostic kits for PCNA-associated diseases.

Keywords: Proliferating cell nuclear antigen; Baculovirus; Insect cells; Protein purification; Antibody binding specificity

Introduction

Proliferating cell nuclear antigen (PCNA) serves as an auxiliary protein for mammalian DNA polymerase and plays crucial roles not only in DNA synthesis and repair but also in cell cycle regulation and apoptosis [1]. Additionally, PCNA acts as an autoantigen that triggers autoantibody production in systemic lupus erythematosus (SLE) patients [2], making it an important disease marker for SLE diagnosis in vitro [3].

The baculovirus expression vector system (BEVS) efficiently produces recombinant baculovirus and has been widely used to express heterologous proteins in various insect cell lines [4-7]. This system offers numerous advantages, including accommodation of large DNA inserts, appropriate post-translational modifications, and high biosafety. To date, many autoantigens associated with autoimmune diseases have been successfully expressed using BEVS, such as thyroid peroxidase for autoimmune thyroid disease diagnosis [8] and zymogen granule glycoprotein 2 for Crohn' s disease [9]. Although PCNA has been expressed in *Escherichia coli* [10-11] and *Saccharomyces cerevisiae* [12], a production process for recombinant PCNA using the baculovirus expression system has not yet been established.

In this study, we developed a complete process for expressing human PCNA protein in Sf9 insect cells. Through a two-step purification procedure, we obtained high-purity recombinant PCNA with a yield of 110 mg/L. The antibody binding properties of rPCNA were validated by ELISA, providing a solid foundation for subsequent development of in vitro diagnostic kits.

Materials and Methods

1.1 Strains, Plasmids, and Reagents

E. coli DH5 and *Spodoptera frugiperda* ovarian cells (Sf9) were maintained in our laboratory. The transfer vector pFastBac HT-B and *E. coli* DH10Bac™ strain were purchased from Invitrogen. Restriction enzymes were obtained from NEB. Plasmid miniprep kits, DNA gel extraction kits, and protein markers were from Tiangen Biotech. Plasmid purification kits were from QIAGEN. Ampicillin, gentamicin, kanamycin, and tetracycline were from Inalco. TRIzol was from Thermo Fisher, and reverse transcriptase was from Takara. Sf900™ II SFM, fetal bovine serum, cell culture antibiotics, Cellfectin transfection reagent, 1.3× medium for virus titer determination, and low-melting-point 4% agarose gel were from Invitrogen. Ni Bestarose 6 FF and Q Bestarose FF were from 博格隆 (Shanghai) Biotechnology. Imidazole and glycerol were from Sangon Biotech (Shanghai). PCNA-positive sera from autoimmune disease patients and healthy control sera were donated by hospitals. 96-well microplates were from Corning, HRP-conjugated rabbit anti-human IgG was from Boster Bioengineering, and reference PCNA protein was from Diarect. Other common reagents were from

Sangon Biotech (Shanghai) or Shanghai Lingfeng, all of analytical grade unless otherwise specified.

1.2 Construction of Recombinant Baculovirus

Total RNA was extracted from HeLa cells and reverse-transcribed into cDNA. Using this cDNA as template and PCNA-specific primers (F: 5' -ATGTTCGAGGCGCGCCTGGTCCAG-3', R: 5'-CTAAGATCCTTCTTCATCC-3'), the full-length human PCNA gene was amplified. The pFastBac HTB plasmid was digested with BamHI and HindIII, and the PCNA gene with a C-terminal 6×His tag was subcloned into the vector. Insertion was verified using pFastBac standard primers (F: 5' -TCCGGATTATTCATACCGTCCC-3' and R: 5' -CCTCTACAAATGTGGTATGGCTG-3'). The correctly recombinant pFastBac HTB-rPCNA plasmid was transformed into *E. coli* DH10Bac for transposition to generate recombinant baculovirus, which was confirmed by PCR and sequencing using pUC/M13 standard primers (F: 5' -CCCAGTCACGACGTTGTAAAACG-3' and R: 5' -AGCGGATAACAATTTCACACAGG-3'). The recombinant baculovirus genome (AcMNPV-rPCNA) was transfected into Sf9 insect cells using Cellfectin II reagent, and culture supernatant was collected after 3 days. Two rounds of viral amplification were performed at MOI 0.05 to obtain high-titer virus stock, with titer determined by plaque assay.

1.3 Insect Cell Culture and Maintenance

Sf9 cells were cultured as previously described [13]. Cells were inoculated into medium containing 2.5% fetal bovine serum and cultured in suspension at 27°C with shaking at 135 rpm. Initial cell density was 6-8×10 cells/ml with >95% viability. Cells were counted and passaged every 2-3 days. For counting, cell suspension was diluted with PBS and stained with trypan blue, and viable cells were counted under a microscope.

1.4 Determination of Optimal MOI and Infection Time

To optimize protein expression parameters, Sf9 cells were seeded at 1×10 cells/well in 6-well plates. After overnight attachment, cells were infected with recombinant baculovirus AcMNPV-rPCNA at different MOI values (0, 0.05, 0.2, 1, 4, 10). One 6-well plate was harvested daily. Cells were separated from supernatant by centrifugation at 4°C, 1000×g for 10 min. After removing supernatant, 1× loading buffer was added to each well for sample preparation. Relative protein expression levels were detected by western blot using anti-His primary antibody.

1.5 Small-Scale Expression, Isolation, and Purification of rPCNA

Log-phase Sf9 cells with >95% viability were diluted to 1×10 cells/ml in 200 ml volume and cultured for 24 h until density reached 1.8-2.4×10 cells/ml. Cells

were then infected with AcMNPV-rPCNA at MOI 0.05. After 6 days of infection, Sf9 cells were harvested at 40-50% viability. Cell pellets were separated from supernatant by centrifugation at 4°C, 1000×g for 10 min and stored at -80°C until purification. To test extraction and purification, cell pellets from 200 ml culture were resuspended in 50 ml lysis buffer (40 mM sodium phosphate, pH 7.4, 500 mM NaCl, 5% (v/v) glycerol, 1 mM PMSF), homogenized by high-pressure lysis, incubated with shaking for 30 min, and centrifuged at 12,000×g for 40 min. The supernatant was filtered through a 0.45 m PVDF membrane and loaded onto a Ni-NTA affinity column. All steps were performed at 4°C with PMSF protease inhibitor to minimize recombinant protein degradation.

1.6 Large-Scale Expression, Isolation, and Purification of rPCNA

Large-scale expression was performed in a 5 L bioreactor using optimal parameters. After homogenization, rPCNA in the lysate was purified by nickel affinity chromatography followed by ion exchange chromatography (Q Sepharose). Ion exchange purification employed a strategy of stable pH with NaCl concentration gradient elution. Purified rPCNA in elution buffer was transferred to storage buffer (16 mM HEPES pH 8.0, 400 mM NaCl, 20% (v/v) glycerol) using ultrafiltration. Protein concentration was determined by Bradford assay, and purity was assessed by Coomassie brilliant blue R-250 staining.

1.7 Indirect ELISA for Serum Samples

Purified rPCNA or bacterially expressed PCNA reference protein (designated PCNA1, purchased from Diarect) was coated onto microplates at 2 g/ml (100 l/well) and incubated overnight at 4°C. After washing, plates were blocked with 5% skim milk for 2 h. Serum samples were diluted 1:100 in PBST, and 100 l was added per well, incubating at 37°C for 1 h. HRP-conjugated rabbit anti-human IgG (Jackson, USA) was diluted 1:4000, and 50 l was added per well. After 1 h, TMB substrate (100 l/well) was added and incubated for 5-10 min. The reaction was stopped with 2 M H SO (50 l/well), and absorbance was read at 450 nm [14].

For diagnostic protein evaluation, sensitivity and specificity of rPCNA were calculated [15]. Sensitivity (%) = (ELISA-positive samples/positive sera) × 100%; Specificity (%) = (ELISA-negative samples/negative sera) × 100%. The cut-off value was defined as twice the mean value of negative control sera [16].

Results

2.1 Preparation of Recombinant Baculovirus

The full-length PCNA gene obtained from HeLa cells matched the theoretical sequence from GenBank (accession NM_002592.2) (Fig. 1 [Figure 1: see original paper]A). The gene was subcloned into pFastBac HTB with a C-terminal

6×His tag (Fig. 1B). The recombinant plasmid was transformed into *E. coli* DH10Bac for transposition to generate recombinant bacmid, verified by PCR and sequencing using pUC/M13 primers. The recombinant baculovirus genome (AcMNPV-rPCNA) was transfected into Sf9 cells to generate P1 virus stock, which was further amplified to produce P2 and P3 stocks.

Typically, P3 virus stock is used for recombinant protein production. Three days post-infection with P3 virus, Sf9 cells showed signs of lysis, budding, and detachment from plates, while control cells remained tightly attached and grew to high density (Fig. 1C). Plaque assay determined the P3 stock titer at 2×10 pfu/ml, demonstrating efficient proliferation of rPCNA baculovirus in Sf9 cells. Western blot analysis of cell pellets and culture supernatant 3 days post-infection revealed that rPCNA protein was primarily retained in the cell pellet and was not effectively secreted into the supernatant (Fig. 1D).

2.2 Effect of MOI and Infection Time on Protein Expression

To maximize PCNA protein yield, we optimized MOI and infection time. Since rPCNA was not secreted into the culture medium, only cell pellets were analyzed by western blot to compare expression levels under different conditions. High protein yields were achieved with low MOI (0.05) at 144 h or high MOI (10) at 48 h (Fig. 2 [Figure 2: see original paper]). However, under high MOI conditions, rPCNA yield decreased sharply with prolonged infection time, as high MOI severely compromises insect cell viability, leading to low recombinant protein production due to (1) short growth period and low cell density, and (2) release of proteases from damaged cells that degrade recombinant products [8]. Therefore, low MOI (0.05) with extended infection time (144 h) was selected as the optimal expression parameter.

2.3 Small-Scale Expression and Purification of rPCNA

Based on optimal MOI and infection time, small-scale expression and purification were performed. Sf9 cell lysate (50 ml) was loaded onto a Ni-NTA column, washed with binding buffer (20 mM imidazole), and eluted with an imidazole gradient (50, 100, 200, 500 mM). High-purity rPCNA was obtained in the 200 mM and 500 mM imidazole fractions (Fig. 3 [Figure 3: see original paper]A). These fractions were pooled, concentrated by ultrafiltration, and analyzed by SDS-PAGE and western blot. Compared to the standard PCNA protein (P1, 36 kDa), recombinant PCNA (P2) was approximately 37 kDa (Fig. 3B), with the additional 1 kDa corresponding to the His-tag and TEV recognition site. The 37 kDa band was specifically detected by anti-PCNA antibody (Fig. 3C), confirming successful small-scale production and purification of rPCNA.

2.4 Large-Scale Expression and Purification of rPCNA

For large-scale production, rPCNA was purified by Ni-NTA chromatography followed by silver staining analysis (Fig. 4 [Figure 4: see original paper]A).

Fractions containing a single band were pooled and concentrated, showing >90% purity and a yield of 140 mg/L cell culture by Coomassie blue staining (Fig. 4B). To further enhance purity, strong anion exchange chromatography (Q Sepharose) was employed based on preliminary experiments (data not shown). rPCNA was completely eluted with 400 mM NaCl (Fig. 4C), achieving >95% purity (Fig. 4D) and specific recognition by anti-PCNA antibody (Fig. 4E). We established a 5 L-scale production and purification method yielding 110 mg/L of >95% pure rPCNA. For comparison, yeast PCNA expressed in *E. coli* yielded only 60 mg/L [10], while human PCNA produced in *E. coli* yielded merely 10 mg/L [17], demonstrating that Sf9 cells are superior for human PCNA production.

2.5 Antibody Binding Specificity of rPCNA

To evaluate antibody binding characteristics, indirect ELISA—a standard method for antigen-antibody interaction assessment [18-20]—was employed. Fifteen positive and twenty negative human SLE serum samples from hospitals were tested (Table 1). Based on negative serum results, cut-off values were determined as 0.208 for PCNA1 and 0.196 for rPCNA (Fig. 5 [Figure 5: see original paper]). The antibody binding specificity of rPCNA was significantly higher than that of *E. coli*-expressed PCNA1, with sensitivities of 93.3% versus 73.3%, respectively. Specificities were 85.0% for rPCNA and 80.0% for PCNA1. These results demonstrate that insect cells are more suitable than *E. coli* for PCNA expression, and that rPCNA possesses excellent antibody binding properties for potential application in PCNA-associated disease diagnostics.

Discussion

In 1983, human interferon- was first expressed in insect cells using recombinant baculovirus [21], marking the inaugural report of heterologous protein expression using the baculovirus expression vector system (BEVS). Over three decades of development [22,23], BEVS has become an effective and commonly used tool for research and production of active proteins from diverse species. Compared to *E. coli* systems, BEVS provides post-translational modifications similar to mammalian cells, yielding recombinant proteins with structures closer to their native counterparts. Moreover, BEVS offers simpler operation and lower costs than mammalian cell expression systems. Through decades of adaptation, many insect cell lines have become fully adapted to suspension culture, enabling easy scale-up for industrial protein production.

This study employed a two-step purification strategy to obtain high-purity PCNA protein with a yield of 110 mg/L. Compared to *E. coli*-produced PCNA, rPCNA demonstrated superior antibody binding capacity, efficiently recognizing antibodies in SLE patient sera. Thus, insect cells represent a more suitable host for human PCNA production. We have, for the first time, established a complete process for recombinant human PCNA expression in Sf9 cells and a

corresponding purification strategy. The high-purity rPCNA protein shows potential for development into in vitro diagnostic kits and may significantly reduce diagnostic costs for PCNA-associated diseases.

References

[1] Moldovan G L, Pfander B, Jentsch S. PCNA, the Maestro of the Replication Fork. Cell, 2007, 129(4): 665-679.
[2] Miyachi K, Fritzler M J, Tan E M. Autoantibody to a nuclear antigen in proliferating cells. J Immuno, 1978, 121(6): 2228-2234.
[3] Sherer Y, Gorstein A, Fritzler M J, et al. Autoantibody explosion in systemic lupus erythematosus: More than 100 different antibodies found in SLE patients. Semin Arthritis Rheu, 2004, 34(2): 501-537.
[4] van Oers M M. Opportunities and challenges for the baculovirus expression system. J Invertebr Pathol, 2011, 107(Suppl): 3-15.
[5] Liu L, Zhong S, Yang R Z, et al. Expression, purification, and initial characterization of human alanine aminotransferase (ALT) isoenzyme 1 and 2 in High-five insect cells. Protein Expres Purif, 2008, 60(2): 225-231.
[6] Shay B, Gruenbaum-Cohen Y, Tucker A S, et al. High yield expression of biologically active recombinant full length human tuftelin protein in baculovirus-infected insect cells. Protein Expr Purif, 2009, 68(1): 90-98.
[7] Wilde M, Klausberger M, Palmberger D, et al. Tnao38, high five and Sf9–evaluation of host-virus interactions in three different insect cell lines: baculovirus production and recombinant protein expression. Biotechnol Lett, 2014, 36(4): 743-749.
[8] Grennan J F, Wolstenholme A, Fowler S, et al. High-level expression of recombinant immunoreactive thyroid peroxidase in the High Five insect cell line. J Mol Endocrinol, 1996, 17(2): 165-174.
[9] Roggenbuck D, Reinhold D, Wex T, et al. Autoantibodies to GP2, the major zymogen granule membrane glycoprotein, are new markers in Crohn' s disease. Clin. Chim. Acta., 2011, 412(9-10): 718-724.
[10] Biswas E E, Chen P H, Biswas S B. Overexpression and rapid purification of biologically active yeast proliferating cell nuclear antigen. Protein Expr Purif, 1995, 6(6):
[11] Byrne-Steele M L, Hughes R C, Ng J D. Recombinant production, crystallization and preliminary X-ray analysis of PCNA from the psychrophilic archaeon *Methanococcoides burtonii* DSM 6242. Acta Crystallogr Sect F Struct Biol Cryst Commun, 2009, 65(11):
[12] Bauer G A, Burgers P M. Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene. Nucleic Acids Res, 1990, 18(2): 261-265.
[13] Naneh O, Zavec A B, Pahovnik D. An optimized protocol for expression and purification of murine perforin in insect cells. J Immunol Methods, 2015(1), 426: 19-28.

[14] Bao H, Yu T, Jin Y, et al. Purification of HRSV F protein from a eukaryotic expression vector and establishment of a sandwich ELISA method. Mol Med Rep, 2012, 6(1):
[15] Zheng Y, He R, He M, et al. Characterization of *Sarcoptes scabiei* cofilin gene and assessment of recombinant cofilin protein as an antigen in indirect-ELISA for diagnosis. BMC Infect Dis, 2016, 16(1): 16-21.
[16] Zhao Y, Ma T, Ju X, et al. Expression of E2 gene of bovine viral diarrhea virus in *Pichia pastoris*: a candidate antigen for indirect Dot ELISA. J Virol Methods, 2015, 212(1):
[17] Zhang P, Zhang S J, Zhang Z J, et al. Expression and physicochemical characterization of human proliferating cell nuclear antigen. Biochemistry, 1995, 34(34): 10703-10712.
[18] Haubruck H, Mauch L, Cook N J, et al. Expression of recombinant human thyroid peroxidase by the baculovirus system and its use in ELISA screening for diagnosis of autoimmune thyroid disease. Autoimmunity, 1993, 15(4): 275-284.
[19] Pavlidis P, Shums Z, Koutsoumpas A L, et al. Diagnostic and clinical significance of Crohn' s disease-specific anti-MZGP2 pancreatic antibodies by a novel ELISA. Clin Chim Acta, 2015, 441(1): 176-181.
[20] Mitchell M C, Tzelos T, Handel I, et al. Development of a recombinant protein-based ELISA for diagnosis of larval cyathostomin infection. Parasitology, 2016, 143(8):
[21] Smith G E, Summers M D, Fraser M J. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol, 1983, 3(12):
[22] van Oers M M. Opportunities and challenges for the baculovirus expression system. J Invertebr Pathol, 2011, 107(Suppl): 3-15.
[23] Li S F, Wang H L, Hu Z H, et al. Genetic modification of baculovirus expression vectors. Virol Sin, 2012, 27(2): 71-82.

Note: Figure translations are in progress. See original paper for figures.