JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2014. Vol. **8**(3), p. 1837-1844

Expression and Enzymatic Activity Analysis of Deoxycytidine Kinase in Silk worm Larvae using Dual Promoters BmNPV/Bac-to-Bac System

Juan Gao[1], Yan Zhang[2], Xue-yang Wang[3], Xin Yang[4], Hai-zhong Yu[5] and Jia-ping Xu[1,*]

School of Life Sciences, Anhui Agricultural University, China.

(Received: 07 August 2013; accepted: 31 October 2013)

Deoxycytidine kinase (DCK) is required for the phosphorylation of several deoxyribonucleosides and their nucleoside analogs. Deficiency of DCK is associated with resistance to antineoplastic chemotherapeutic agents. We developed a baculovirus expression system to express the DCK protein directly in silkworm. A recombinant dual expression vector containing his-tagged dck and polyhedrin (polh) was constructed, and baculovirus polyhedra were generated by transfecting the recombinant plasmid into BmN cells and used to orally inoculate the silkworm larvae. The results showed that the His-tagged DCK fusion protein was successfully expressed in the silkworm larvae and purified by Ni - NTA affinity chromatography, and ELISA quantative assay showed that recombinant DCK protein enzymatic activity up to 106.79U/L. These results proved that it is feasible and convent expression system to express DCK protein in silkworm through oral feeding.

Key words: DCK, pFastBacDual, His-tag, Recombinant baculovirus, Bac-to-Bac system.

Deoxycytidine kinase (DCK) is required for the phosphorylation of several deoxyribonucleosides and their nucleoside analogs. It is a deoxynucleoside kinase with broad substrate specificity[1]. The enzyme plays a significant role in the initial phosphorylation of some nucleoside analogs that are of therapeutic interes[2]. The phosphorylation process is also important in the development of compounds that could be used in the treatment of Human immune deficiency virus (HIV)[3]. Inefficient DCK activity in the first phosphorylation step could influence on the antineoplastic activity of some nucleoside analogs. Conversely, increased deoxycytidine kinase activity is associated with increased activation of these compounds to cytotoxic nucleoside triphosphate derivatives. DCK is clinically important because of its relationship to drug resistance and sensitivity[2,4].

Some researchers have demonstrated that the phosphate group on the nucleoside analogs has the potential to be a new antineoplastic drug[4]. Thus some emphasis has been placed on developing a commercial and simple way of synthesizing the monophosphate derivative of the nucleoside analogs[5,6]. However, no protocol or reference that related to the enzymatic synthesis of the nucleoside analog derivatives had been reported. Since DCK is able to catalyze the phosphorylation of nucleoside analogs, it's possible that DCK will catalyze the synthesis of

* To whom all correspondence should be addressed.
Tel.:/Fax: +86 551 5786691;
E-mail: jiapingxu@163.com

the nucleoside analog's monophosphate. Therefore, in order to synthesize new types of antineoplastic nucleoside analogs, it is essential to develop an efficient and streamlined purification method for DCK[7].

The substrate specificity and clinical application of the human DCK have been widely investigated in terms of molecular biology, biochemistry, separation, and relevance to chemotherapeutics[8]. However, the methods to separate and characterize the DCK from mammals except humans have been attached less attention. Here, Our researche provide a simple, reliable and economic way for the large-scale production of human Deoxycytidine kinase with a obvious enzyme activity and high purity.

With the development of biotechnology, *B. mori* has been used as an important bioreactor for the production of recombinant proteins through baculovirus expression system (BES)[9] . Since the Bombyx mori nuclearpolyhedrosis virus (BmNPV) expression system utilizes an insect of great economic interest, such as silkworm, reared on a large scale, would reduce the cost of recombinant protein production, and more importantly, make large scale industrial production possible[10,11] . Recently, the bacmid BES had been developed for BmNPV baculovirus expression system[12]. It greatly reduces both technical difficulty and time to purify and select recombinant viruses within 10 days.

However, generally the polyhedrin (*polh*) gene is replaced by a foreign gene to produce recombinant baculovirus in baculovirus expression vector system, in which the powerful polh promoter drives high expression of the foreign gene[13,14]. As a result, the recombinant baculoviruses, without the protection of the polyhedrin protein, are needed to inoculate silkworm larvae through subcutaneous injection, which results in low efficiency. Therefore, the expression of both foreign gene and *polh* gene in one expression system is necessary to generate high titer baculoviruses in large scale[15].

In this study, using the pFastBacDual vector, we successfully constructed *polh* gene and target *dck* (*Deoxycytidine kinase*) gene under polyhedron and p10 promoters, respectively, through oral infection. In addition, we attached his-tag into *dck* gene. Recombinant baculovirus polyhedrin was generated by transfecting the recombinant plasmid into BmN cells and used to orally inoculate the silkworm larvae. As a result, DCK was successfully expressed in the silkworm larvae. These results imply that it is feasible to express target genes through oral feeding by Bac - to-Bac baculovirus expression system. And then in non-denaturing conditions, the His-Fusion protein was purified using Ni-NTA agarose. The Enzymatic activity of DCK was examined by ELISA. It will provide important material foundation for anticancer study.

MATERIALS AND METHODS

Materials

Disease-free eggs of the silkworm DaZao were hatched and reared under standard condition at 26±2°C, 70–85% RH and 12L:12D photoperiod with mulberry leaves. After the fourth ecdysis, larvae were divided to three groups and used for infection.

The *E. coli* DH10Bac/BmNPV and pFastBacDual vector was preserved in our laboratory. FuGENE 6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). The Human Deoxycytidine kinase (DCK) ELISA kit was purchased from Jijin chemical technology Co., Ltd.(Shanghai, CHN). Ni-NTA was purchased from GenScript (Nanjing) Co., Ltd.

Bm cell line, BmN, was originally derived from ovary and supplied by Laboratory of Life Science, Jiangsu University and cultured at 27°C with TC-100 medium containing 10% fetal bovine serum (FBS).

Construction of dual expression vector

The *polh* gene was amplified by PCR used wild BmNPV DNA as template under primers. The forward primer:5'-CGCGGATCCATGCCGAA TTATTCATACA-3', and reverse primer:5'-CCCAAGCTTTTAATACGCCGGACCAGTGAACAG-3' (restriction enzyme sites *Bam*H I and *Hin*d III are underlined). The PCR product inserted into the multiple cloning site (MCS) of the pFastBacDual under Pph promoter.

The partial sequence of *dck* gene (NCBI login: NM_000788.2) was amplified by PCR used pFastBac1-*dck* as template under primers by attached a His-tag. The forward primer:5'-CCGCTCGAGATGTCGTACTACCA-3', and

reverse primer:5'-CATGCCATGGTAACAAA GTACTCAAAAAC-3' (restriction enzyme sites *Xho* I and *Nco* ™ are underlined) were designed to amplify the open reading frame (ORF) of *dck* gene by PCR.The PCR product was inserted into the multiple clone site (MCS) of the pFastBacDual under p10 promoter.

The map of constructed plasmid pFastBacDual-*dck*/*polh* (pFD-*dck*/*polh*) was shown in Fig.1 and transformed to *E. coli* DH10Bac/ BmNPV.

Construction and isolation of recombinant bacmid baculoviruses

The *E. coli* DH10Bac containing recombinant bacmid was propagated in LB medium containing antibiotics (50 µg/mL kanamycin, 7 µg/ mL gentamicin and 10 µg/mL tetracycline), 100 µg/ ml X-gal and 40 µg/mL IPTG. The white colonies were selected for further amplification. The pFD-*dck*/*polh* plasmids was isolated using the alkaline lysis method, and transfected into BmN cells using FuGENE 6.The recombinant P1 viral solution was collected from BmN cells 120 h post-transfection and stored at 4°C protected from light. The P1 viral stock was further used to infect BmN cells to generate high-titer P2 stock that was used to infect the silkworm larvae.

Production of recombinant DCK in silkworm larvae

The silkworm larvae on the first day of fifth instar were fed with 8 µl P2 pFD-*dck*/*polh* recombinant baculovirus solution which concentration of polyhedron to 2×10^7 NPB/mL by oral, while the same amount of water and wild type BmNPV solution was licked by the control silkworm larvae. The infection on silkworm larvae was monitored every day, and the blood of silkworm larvae was collected to purify the polyhedrins on days of 6 and 7 after infection. The DNA from polyherins was isolated to verify the expression of DCK by PCR amplication.

Polyhedrin observation

The larval hemolymph was checked for the presence of polyhedrins under light microscope at 96 h post-infection.

Purification of DCK in silkworm larvae

The blood of diseased silkworm larvae were collected, and used for SDS-PAGE analysis and DCK purified. His-tagged DCK expressed in silkworm larvae was purified using Ni-NTA agarose beads (GenScript (Nanjing) Co., Ltd.). Eluted fractions were analyzed on 12% SDS-PAGE, followed by Coomassie blue staining.

Western blot analysis

Electrophoresis was performed by loading samples onto a 12% SDS-PAGE gel. The separated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% w/v nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) for 30 min at room temperature and then incubated with anti-His monoclonal antibody (BEIJING TRANSGEN BIOTECH Co., Ltd.) for 1 hour at room temperature. After washed by TBST 10 min/time for 3 times, the membrane was incubated with goat anti-mouse IgG (BEIJING TRANSGEN BIOTECH Co., Ltd.) for 1 hour and washed with TBST 3 times for 10 min. Enhanced HRP-DAB chromogenic substrate Kit (TIANGEN Biotech (Beijing)Co., Ltd.) was used for signal generation.

Measurement of DCK enzyme activity

The hemolymph of infected silkworm larvae were mixed with LE Buffer (50mM Na_2PO_4, 300mM Nacl, pH 8.0), and undergone ultrasonic disruption. The supernatant collected after centrifugation was measured by Human Deoxycytidine kinase (DCK) ELISA kit according to the manufacture's instructions.The OD values for all samples in triplicates and standard curve was measured at 450 nm.

RESULTS

Construction of pFD-*dck*/*polh* plasmid

The fragments of *dck* and *polh* genes were amplified from the plasmids pFastBac1-*dck* and wild type BmNPV, respectively. The sizes of PCR products were consistent with the length of *dck* DNA (874 bp, Fig. 2A) and *polh* DNA (756 bp, Fig. 2B) respectively. The PCR products were purified and inserted into pFastBacDual vector to generate the pFD-*dck*/*polh* plasmid. The accuracy of pFD-*dck*/*polh* was confirmed by two sets of double enzymes digestion, *Xho* I /*Nco* I for *dck*(Fig. 2C) and *BamH* I/*Hind* III for *polh* (Fig. 2D). The digested fragments were purified and sequenced, and the result of sequencing showed the inserted fragments were consistent with the sequences of *dck* and *polh* genes.

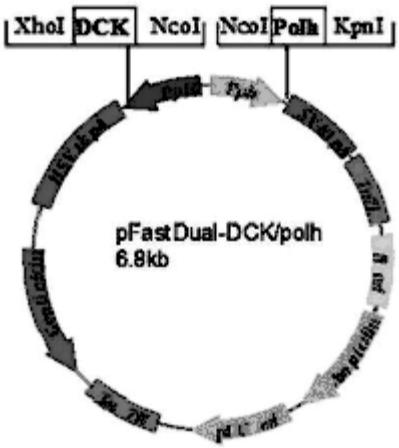

Fig. 1. Map of the donor plasmid pFastBacDual. The polyhedron gene and target *dck* (Deoxycytidine kinase) gene under polyhedron and p10 promoters, respectively

Transfection of recombinant plasmids into BmN cells

The recombinant plasmids were transfected into BmN cells using lipofectamine. After 72 h transfection, BmN cells showed the obvious infection symptom Most of cells became round and lots of polyhedra were observed under microscope (Fig. 3B), while normal control BmN cells showed polygonal morphology (Fig. 3A).

Expression of DCK gene in silkworm larvae

The larvae at first day of the fifth instar were fed orally with recombinant virus (rBacmid/BmNPV/*dck*). The silkworm larvae oral infection with baculovirus solution from wild type BmNPV were set as positive control, while the normal silkworm larvae were used as negative control. Five days (120 h) after infection, the silkworm larvae fed with baculovirus solution containing wild

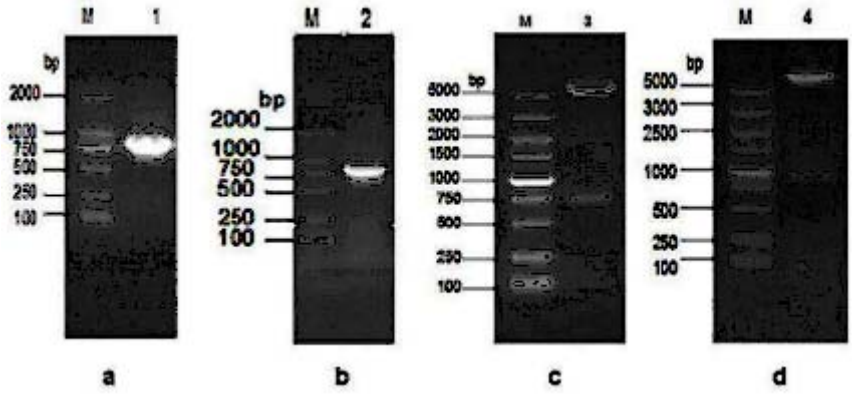

Fig. 2. Conctruction and identification of *polh* and *dck* DNA in the pFD-*dck*/*polh* plasmid. M. DL2000 DNA Marker (a,b), DL5000 DNA Marker (c,d); 1. PCR product of *polh* gene; 2. PCR product of *dck* gene; 3. *BamH* I/*Hind* III digestion of pFD-dck/polh plasmid; 4. *Xho* I /*Nco* I digestion of pFD-dck/polh piasmid

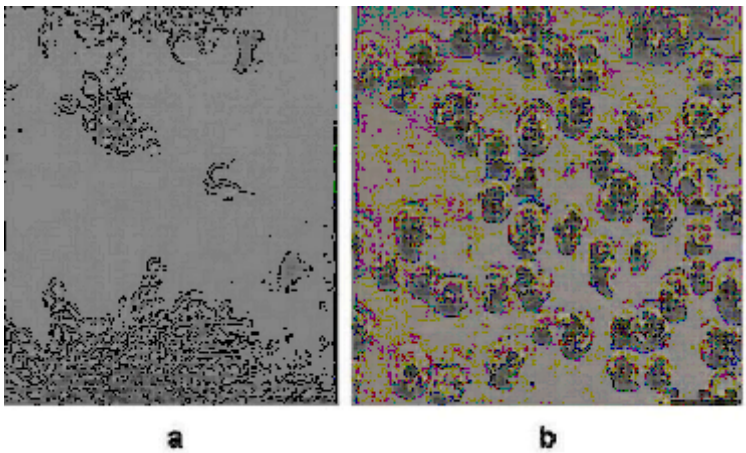

Fig. 3 . Cell morphology of normal BmN (a) and BmN cells infected by recombinant Bacmid(b)

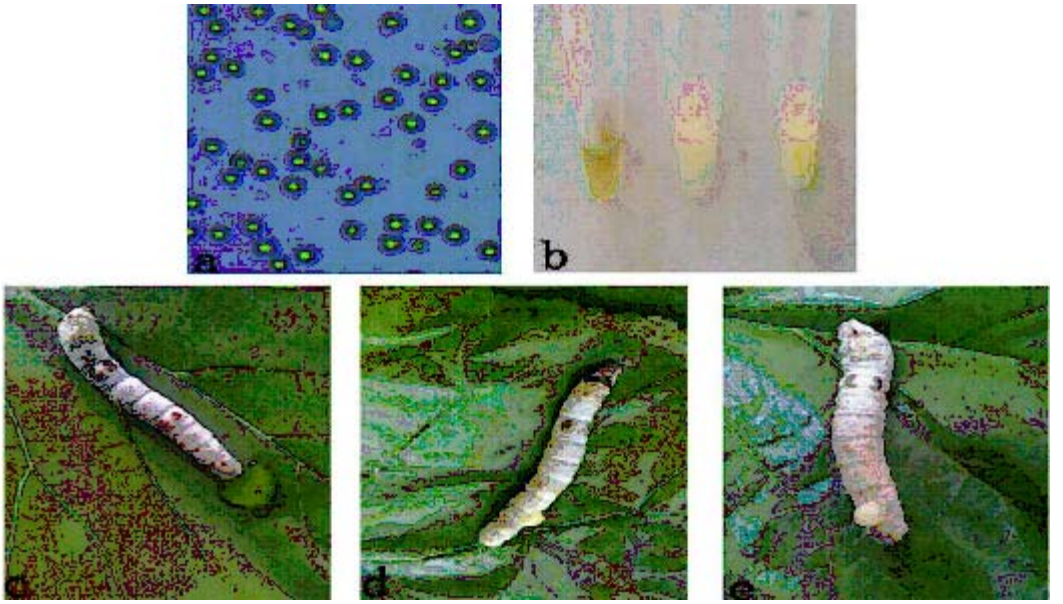

Fig. 4. Photos of polyhedra, hemolymph and larvae after infection of recombined baculoviruses. a: Polyhedra in larvae hemolymph under microscope. The larvae was fed orally with hemolymph which was collected from the first batch of infected larvae; b: The larvae hemolymph which was collected from silkworm larve and the lanes from left to right are as follows : normal silkworm, wild type baculovirus virus, recombined pFD-dck/polh baculovirus. The color of tainted blood was ivory white; c: Normal silkworm; d: Larvae infected wild type baculovirus; e: Larvae infected pFD-dck/polh baculovirus

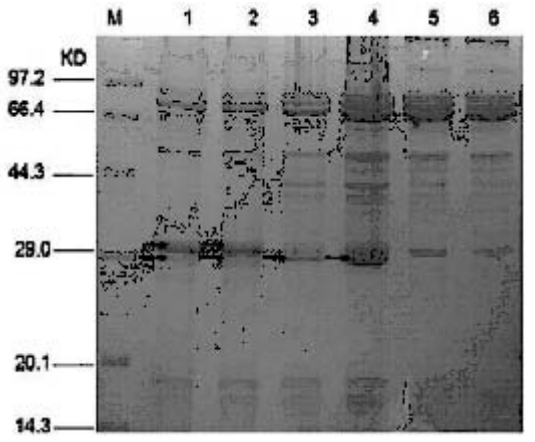

Fig.5. SDS-PAGE analysis of *polh* and *dck* expression products. M. Protein molecular mass standard ;1~2: hemolymph of the fifth instar 6th07th days of the larvae fed orally with recombinant virus (rBacmid/BmNPV/*dck*); 3~4: hemolymph of the fifth instar 6th07th days of the larvae fed orally with wild type baculovirus; 5~6: Hemolymph of the fifth instar 6th07th days of normal silkworm; The arrows point out the polyhedrin protein (29 KDa) and DCK protein (32 KDa)

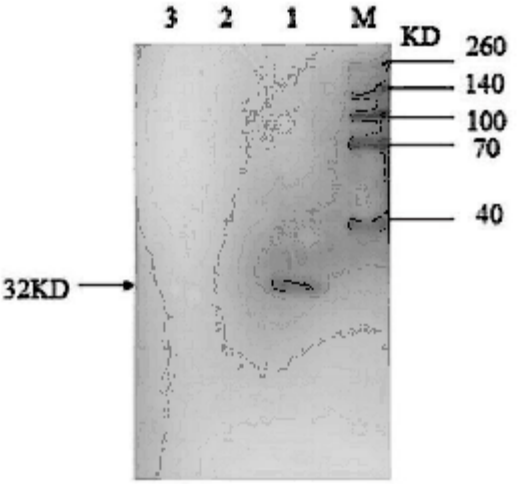

Fig. 6. Western blotting analysis for the generation of the recombinant BmNPV viruses in silkworm larvae M indicates the positions in KDa of protein markers. Lane 1 the larvae fed orally with recombinant virus; Lane2 the larvae fed orally with wild type baculovirus; Lane3 normal silkworm

BmNPV and pFD-*dck*/*polh* showed obvious symptom of infection (Fig. 4C-E), and lots of polyhedra were observed in the hemolymph of silkworm larvae under microscope and the color of the hemolymph was ivory white (Fig. 4A and B). Moreover, we analyzed the expression of DCK and Polh proteins in the hemolymph cells by SDS-PAGE. As shown in Fig. 5, the predicted band of Polh protein (29KDa) was found in the blood of silkworm larvae after infection with pFD-*dck*/*polh* and wild BmNPV baculoviruses (Fig. 5, as lower arrows shown), while the predicted band of DCK protein (32KDa) was only observed in the blood cells from silkworm larvae infected by pFD-*dck*/*polh* recombinant baculoviruses (Fig. 5, as upper arrows shown). We further verified whether *dck* gene was expressed in the recombinant baculoviruses from silkworm larvae blood after pFD-*dck*/*polh* baculoviruses infection by PCR amplification. A single band of 874bp was detected

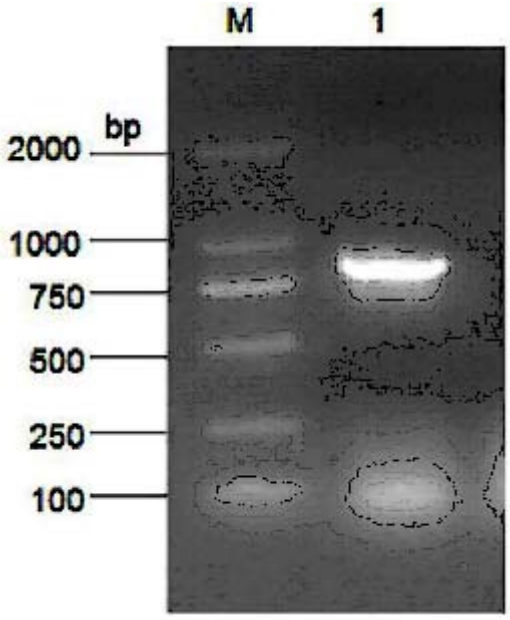

Fig. 7. Electrophoresis of *dck* PCR product from recombinant baculoviruses. Using silkworm larvae blood after pFD-*dck*/polh recombinant baculoviruses infection by PCR amplification. It is confirmed the baculoviruses were pFD-dck/polh recombinant baculoviruses; M. DL2000 DNA Marker 1. PCR product of dck gene

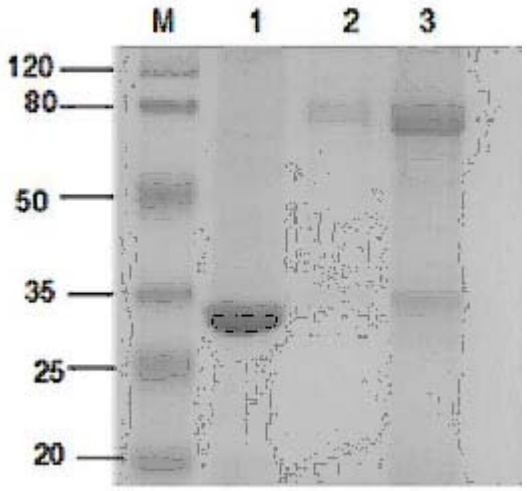

Fig. 8. SDS-PAGE analysis of His-DCK purificated by Ni-NTA agarose column.The lanes from left to right are as follows: M, Protein molecular mass standard; 1, eluted fractions; 2, washing fractions; 3, low-through; The arrow point out the position of His-DCK

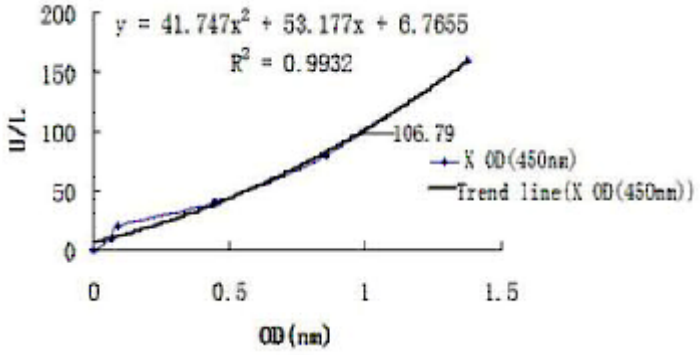

Fig. 9. Standards linear regression curve. DCK ELISA kit to qualitative the protein of recombinant DCK in silkworm larva. The OD values for all samples in triplicates and the standard were measured at 450nm

(Fig. 7), thus we confirmed the baculoviruses were the DCK recombinant baculoviruses. Furthermore, recombinant protein was confirmed by western blot analysis with anti-His antibody (Fig.6).

Purification of DCK

To facilitate the purification of His-DCK expressed in silkworm larvae, we developed a procedure for purification on Ni-NTA agarose beads in combination with ion-exchange chromatography. The blood of silkworm larvae were mixed with LE Buffer, and undergone ultrasonic disruption. Lysates of transfected blood cells were cleared by high-speed centrifugation, and the supernatant mixed with LE Buffer was applied to Ni-NTA column, and then washed with wash buffer. It was found that 10 mM imidazole is optimal for removing most contaminants while retaining the majority of DCK protein. The proteins were eluted with elution buffer (50 mM Na_2PO_4, 300 mM Nacl, 250 mM imidazole, pH 8.0) and the eluted fractions were analyzed by 12% SDS-PAGE (Fig. 8).

Enzymatic activity identification of DCK

Assay for DCK activity was performed by Human deoxycytidine kinase (DCK) ELISA kit. A specifc DCK ELISA kit was employed for qualitative analysis of the content of recombinant DCK in silkworm larva. According to this kit, the coefficient R value between the linear regression of standard and expected concentration is above 0.99, and it is very sensitive to detect as low as 1.0 U/L. The calibration standards were assayed at the same time as the samples and allowed the operator to produce a standard curve of Optical Density (OD_{450nm}) versus DCK activity (Fig. 8). The enzyme activity of sample was determined by the linear regression equation from the standard. According to the linear regression equation, the protein of recombinant DCK after infection with pFD-*dck*/*polh* baculoviruses was detected and the enzyme activity was up to 106.79 U/L in the hemoloph.

DISCUSSION

With the development of biotechnology, *B. mori*, as an important economical animal, has been used as an important bioreactor for the production of recombinant proteins through BES. In the present study, we successfully constructed the *dck* and *polh* genes into pFastBacDual vector and expressed the recombinant His-DCK protein in the silkworm larvae using Bac-to-Bac baculovirus expression system. Here we inserted the *polh* gene behind the Pph promoter and installed the *dck* gene behind the p10 promoter and generated the recombinant baculovirus packaged by the Polh protein. The results of SDS-PAGE and Western blotting analysis suggested that fusion protein His-DCK was successfully expressed in silkworm larvae. And then in non-denaturing condition, the His-Fusion protein was purified using Ni-NTA agarose. The enzyme activity of DCK was examined by ELISA, which was up to 106.79 U/L, showing a relatively high Enzymatic activity[16].

Traditionally, the recombinant virus infects the larvae through individual dorsal injection. The infection is a time- and labor-consuming procedure. This drawback has become a bottleneck for practical and industrial utilization of baculovirus expression system in silkworm bioreactor. In order to resolve this application bottleneck, we employed a dual expression baculovirus to express *polh* and target *dck* gene under polyhedron and p10 promoters, respectively. Then we infected the silkworm larvae with recombinant baculoviruses by licking because the strong alkaline environment in the intestine of silkworm larvae can release the proteinases to digest polyhedrins[17] . Compared with traditional injection of recombinant virus, the oral administration should be more rapidly and simpler in practice.

The protein purification of baculovirus expression system has always been a difficult problem to solve. In this paper, we attached his-tag into *dck* gene,which is convenient to purify recombinant protein. While the fusion protein expressed, but 6 * his-tag is small, and stable at physiological pH. Therefore it does not change the domain of recombinant protein fold and biochemical properties, almost has no effect on protein[18]. And also we need to further investigate how to remove this his-tag.

In summary, we used baculovirus expression system to expressed and purified protein that having biological activity successfully. Further, we'll research the concentration of recombinant proteins expressed in the silkworm larvae. This will lay the foundation for further study of DCK in

tumor occurrence, development of the function and mechanisms.

ACKNOWLEDGEMENTS

The research was supported by grants from the National 863 plans projects of China (No. 2011AA100306).

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