Efficient Production of an Engineered Apoptin from Chicken Anemia Virus in a Recombinant *E. coli* for Tumor Therapeutic Applications

Meng-Shiou Lee[1], Fang-Chun Sun[2], Chi-Hung Huang[3], Yi-Yang Lien[4], Shin-Huei Feng[5], Guan-Hua Lai[5], Meng-Shiunn Lee[6], Jung Chao[7], His-Jien Chen[8], Jason T C, Tzen[5*], Hao-Yuan Cheng [9*]

[1]School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan. [2]Department of Bioresources, Dayeh University, Chunghwa, Taiwan. [3]Graduate School of Biotechnology, Hung Kuang University, Taichung, Taiwan. [4]Dept. of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan. [5]Graduate Institute of Biotechnology, College of Agriculture and Natural Resources, National Chung Hsing University, Taichung, Taiwan. [6]Department of Medical Research, Tung's Taichung MetroHarbor Hospital, Taichung,Taiwan. [7] Institute of Pharmacology, National Yang-Ming University, College of Medicine, Taipei, Taiwan. [8]Dept. of Safety, Health and Environmental Engineering, Mingchi University of Technology, Taipei, Taiwan. [9]Department of Nursing, Chung Jen College of Nursing, Health Sciences and Management, Chia-Yi, Taiwan

* Author for Correspondence:

Fax: +886-5-2658913 (HYC); Fax: +886-4-22853527 (JTCT).

E-mail:

MSL[1]: leemengshiou@mail.cmu.edu.tw

FCS: fcsun@mail.dyu.edu.tw

CHH: huangchihung@gmail.com

YYL: yylien@mail.npust.edu.tw

SHF: jessica.0726@yahoo.com.tw

GHL: just_dfm@hotmail.com

MSL[6]: mengshiunn@yahoo.com.tw

JC: rich720925@yahoo.com.tw

HJC: hjchen@mail.mcut.edu.tw

JTCT: TCTZEN@dragon.nchu.edu.tw

HYC: m098026@cjc.edu.tw

Abstract

Background:

Apoptin, a nonstructural protein encoded by the VP3 gene of chicken anemia virus (CAV), has been shown to not only induce apoptosis when introduced into the precursors of chicken thymocytes, but has been found to specifically kill human cancer cells, tumor cell and transformed cells without affecting the proliferation of normal cells. This tumor-specific apoptotic characteristic of the protein potentially may allow the development of a protein drug that has applications in tumor therapy. However, several major problems, which include poor expression and poor protein solubility, have hampered the production of apoptin in bacteria.

Results:

Significantly increased expression of recombinant full-length apoptin that originated from chicken anemia virus was demonstrated using an *E. coli* expression system. The CAV VP3 gene was fused with a synthetic sequence containing a trans-acting activator of transcription (TAT) protein transduction domain (PTD). The resulting construct was cloned into various different expression vectors and these were then expressed in various *E. coli* strains. The expression of the TAT-Apoptin in *E. coli* was significantly increased when

TAT-Apoptin was fused with GST-tag rather than a His-tag. When the various rare amino acid codons of apoptin were optimized, the expression level of the GST-TAT-Apoptin$_{opt}$ in *E. coli* BL21(DE3) was significantly further increased. The highest protein expression level obtained was 8.33 g/L per liter of bacterial culture after induction with 0.1 mM IPTG for 4 h at 25^{o}C. Moreover, approximately 90% of the expressed GST-TAT-Apoptin$_{opt}$ under these conditions was soluble. After purification by GST affinity chromatography, the purified recombinant TAT-Apoptin$_{opt}$ protein was used to evaluate the recombinant protein's apoptotic activity on tumor cells. The results demonstrated that the *E. coli*-expressed GST-TAT-apoptin$_{opt}$ showed apoptotic activity and was able to induce human premyelocytic leukemia HL-60 cells to enter apoptosis.

Conclusions:

On expression in *E. coli*, purified recombinant TAT-Apoptin$_{opt}$ that has been fused to a GST tag and had its codons optimized, was found to have great potential. This protein may in the future allow the development of a therapeutic protein that is able to specifically kill tumor cells.

Background

Chicken anemia virus (CAV), is a non-enveloped virus and the causative agent of chicken anemia disease. The disease results in aplastic anemia, lymph organ atrophy and immunosuppression in chickens [1-3]. The virus transcribes and translates three viral proteins, VP1, VP2 and VP3, which are respectively encoded by ORF3, ORF1 and ORF2 of the CAV genome. VP1 protein (51 kDa) is the sole structural protein of CAV and is responsible for capsid assembly [4]. The VP2 protein (30 kDa) is a nonstructural protein that possesses a dual-specificity protein phosphatase (DSP) [5]. The VP3 protein (13 kDa), also called apoptin, is a strong inducer of apoptosis in precursor chicken thymocytes and various human transformed and tumor cell lines but not in normal cells [6]. At present, the full anti-tumor mechanism of apoptin remains unclear. However, it is worth mentioning that apoptin-induced apoptosis in tumor cells is p53-independent and also is not suppressed by Bcl-2 or BCR-ABL protein [7]. Thus, apoptin is thought to be a good candidate for use as a therapeutic protein and has potential to be developed as a cancer treatment, including those cancers that lack p53.

To develop apoptin as an anti-cancer drug, efficient transduction tools such as recombinant virus and a recombinant plasmid within liposomes have been used to deliver apoptin into tumor cells [8]. However, these approaches have

restrictions in terms of therapeutic application including a size limitation of the genes that can be delivered, insertional mutagenesis and transient expression [9-11]. Novel cell-penetrating peptides, such as the trans-acting activator of transcription (TAT) protein transduction domain (PTD), which consists of 11 amino acid residues (aa 47-57, YGRKKRRQRRR) have therefore been used as vectors for protein delivery [8, 12]. With respect to apoptin, previously studies have been demonstrated that apoptin fused with a TAT peptide shows apoptotic activity against tumor cells because the TAT peptide's delivery system is able to move apoptin into cells [13,14].

Up to the present, a number of different expression systems have been used to express apoptin, including *E. coli*, baculovirus-insect cells and plant cells [15-18]. However, production of recombinant full-length apoptin/VP3 protein has generally been possible only in *E. coli* [17]. Several production problems involving the expression efficiency and protein solubility of apoptin in *E. coli* have been encountered [13,17,18]. Thus, there is a need to overcome these difficulties in order to scale-up production of full-length apoptin protein using an *E. coli* expression system. If successful, this would not only allow the efficient development of a therapeutic protein that is able to actively kill cancer cells specifically but the recombinant protein would also be potentially useful when

developing diagnostic kits for the clinical detection of CAV infection [17].

It is clear that *E. coli* has a number of limitations and disadvantages in terms of the production of apoptin protein. However, expression of apoptin protein in *E. coli* is still an attractive alternative to the current production system when assessed in terms of cost, time and operational considerations. In this study, the CAV VP3 gene was fused to a synthetic gene containing the trans-acting activator of transcription (TAT) protein transduction domain (PTD). This protein, named TAT-Apoptin, was expressed using various different expression vectors in order to evaluate TAT-Apoptin expression and production by a number of different *E. coli* strains. Two expression vectors were used, one harboring a glutathione-S-transferase (GST) tag and the other a 6xHis tag; these were investigated to explore the effect of these fusion tags on the expression of TAT-Apoptin in the various *E. coli* strains. In addition, changes in codon usage for various amino acids within the VP3 gene were also assessed in terms of their effect on expression of TAT-Apoptin$_{opt}$. Rare codons for *E. coli* within the VP3 gene of the TAT-Apoptin protein were optimized using prediction software and the preferred codon usage changed to that of *E. coli*. After this optimization, the expression levels of the modified VP3 genes were examined in various *E. coli* strains and the various production parameters for TAT-Apoptin$_{opt}$ protein

expression assessed. To the best of our knowledge, the yield of *E. coli* expressed recombinant TAT-Apoptin$_{opt}$ in this study after codon optimization of the VP3 gene is the highest known to date.

Results

A GST fusion tag improves the expression of recombinant TAT-Apoptin protein in *E. coli*

To develop apoptin protein as anti-tumor drug, a composite cDNA of the CAV VP3 gene fused synthetic gene of TAT peptide was used to create two distinct expression constructs of TAT-Apoptin (Figure 1A, a and b). These constructs, pET-TAT-VP3 and pGEX-TAT-VP3, were transformed into three different *E. coli* strains in order to investigate the effect of the fusion tags on the expression of TAT-Apoptin. The 6×His and GST tags were fused with TAT-Apoptin at the N-terminus using the expression vectors pET28a and pGEX-4T-1, respectively. The expression of these two TAT-Apoptin constructs in various *E. coli* strains was explored (Figure 2). When *E. coli* BL21(DE3) was used, significant amounts of full-length TAT-Apoptin protein were present in the whole cell lysate after IPTG induction for 4 h with either pET-TAT-VP3 or pGEX-TAT-VP3 (Figure 2B, SDS-PAGE and Western-blotting). The 16 kDa His-TAT-Apoptin and the 42 kDa GST-TAT-Apoptin were detected using monoclonal anti-His antibody and anti-GST antibody, respectively (Figure 2B, lane 4 of Western-blot). Densitometric analysis of the blots showed that total expressed GST-TAT-Apoptin protein, both soluble and insoluble, from BL21(DE3) was approximately 19 fold greater than that of His-TAT-Apoptin produced under

identical conditions (Figure 3A and 3C). When His-TAT-Apoptin and GST-TAT-Apoptin were induced for between 1 and 4 h, the expression of both proteins continued to increase significantly over the 2-4 h induction range (Figure 3A and 3C) with the highest amount of His-TAT-Apoptin protein produced being 0.34 mg/ml at 4 h of IPTG induction. In contrast, the highest amount of GST-TAT-Apoptin produced was 7.13 mg/ml at 4 h. A similar pattern of expression was observed for the two proteins in BL21(DE3)CodonPlus-RP and BL21(DE3)pLysS strains (Figure 2). The highest amounts of GST-TAT-Apoptin produced by BL21(DE3)CodonPlus-RP and by BL21(DE3)pLysS were 2.43 and 1.15 mg/ml, respectively, after 4 h of IPTG induction (Figure 3A and 3C). The parallel results for His-TAT-Apoptin were 0.09 and 0.82 mg/ml, respectively (Figure 3A and 3C). Thus the GST fusion tag improved protein expression significantly and allowed large amounts of intact TAT-Apoptin protein to be produced.

VP3 gene encoding apoptin protein of CAV is rich in *E. coli* rare codons

CAV VP3 gene that encodes the apoptin protein consists of 121 amino acid codons. The rare codons for *E. coli* within the VP3 gene were pinpointed using the GenScript Rare Codon Analysis Tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). The deduced amino acid sequence of apoptin was found to contain 15% basic amino acid residues such as arginine (R) and lysine (K) (Figure 1B). Overall, approximately 21% rare *E. coli* codons were present in the VP3 gene, including arginine, leucine, isoleucine, proline, cysteine, threonine, serine and glycine codons (Figure 1B).

The presence of these codons is one possible reason for the relatively poor expression of apoptin in *E. coli*.

Enhancement of recombinant TAT-Apoptin protein expression in *E. coli* by optimizing the codon usage of the VP3 gene

We have shown that expression of full-length TAT-Apoptin in *E. coli* is improved by fusing a GST tag to the N-terminus of the TAT-Apoptin protein. However, adding a His tag to give His-TAT-Apoptin did not produce such a yield improvement even when the optimal host strain BL21(DE3)pLysS is used (Figure 2A and 3A). Therefore, we next explored the effect on TAT-Apoptin productivity of optimizing the codon usage of the VP3 gene for *E. coli*. The TAT-Apoptin gene was engineered such that AGA/CGA/CGG were changed to CGT or CGC (R), CCC/CCT were changed to CCG or CCA (P), CTC/CTA/TTG were changed to CTG (L), ATA was changed to ATC (I), GGA/GGG were changed to GGT (G), ACT/ACA were changed to ACC (T), and CAA was changed to CAG (Q) (figure 1B). The codon optimized VP3 gene was then linked to the TAT sequence at 5' end of the VP3 gene to give an intact open reading frame. The new codon-optimized TAT-VP3 gene, denoted TAT-$VP3_{opt}$, was then cloned into pGEX-4T-1 and pET28a to give pGEX-TAT-$VP3_{opt}$ and pET-TAT-$VP3_{opt}$, respectively (Figure 1A, d and c). These plasmids were then individually expressed in *E.* coli. When expression in *E. coli* strain BL21(DE3) using whole cell lysate was analyzed by SDS-PAGE and Western blotting, both GST-TAT-$Apoptin_{opt}$ and His-TAT-$Apoptin_{opt}$ were successfully expressed after IPTG induction (Figure 4A and 4C) with a yield after 4 h IPTG induction of about 9 mg/ml and 0.65 mg/ml, respectively. The yield of GST-TAT-$Apoptin_{opt}$ was

almost 13 fold higher than His-TAT-Apoptin$_{opt}$ (0.65 mg/ ml) (Figure 5C and 5A). BL21(DE3)-pLysS, similarly, gave increased expression of both His-TAT-Apoptin$_{opt}$ and GST-TAT-Apoptin$_{opt}$ (Figure 4A and 4C). The highest yields of GST-TAT-Apoptin$_{opt}$ and His-TAT-Apoptin$_{opt}$ in BL21(DE3)pLysS were obtained after 4 h IPTG induction and were 6.38 and 1.2 mg/ml, respectively (Figure 5C and 5A). These results confirm that the codon-optimized of TAT-Apoptin improved protein expression significantly and allowed large amounts of intact TAT-Apoptin$_{opt}$ protein to be produced in either *E. coli* BL21(DE3) or BL21(DE3)pLysS with either fusion tag.

Effect of cultivation temperature and IPTG concentration on the protein solubility of *E. coli*-expressed GST-TAT-Apoptin$_{opt}$

In addition to the expression level of TAT-Apoptin$_{opt}$, the solubility of TAT-Apoptin$_{opt}$ protein is also needs to examine and improved. To this end, the cultivation parameters and their effect on the TAT-Apoptin$_{opt}$ protein solubility were explored, namely cultivation temperature and the IPTG concentration for induction; GST-TAT-Apoptin$_{opt}$ was used as the model protein. Protein solubility and the expression levels of GST-TAT-Apoptin$_{opt}$ were determined using *E. coli* BL21 (DE3) at 25^{o}C and 37^{o}C. As shown in Figure 6A, an increased amount of soluble GST-TAT-Apoptin$_{opt}$ was obtained after IPTG induction at 25^{o}C. Approximately 15% and 65% of the GST-TAT-Apoptin$_{opt}$ in the whole cell lysate of the BL21(DE3) strain was soluble at 37^{o}C and 25^{o}C, respectively. In addition, the amounts of GST-TAT-Apoptin$_{opt}$ protein expressed in BL21(DE3) were 8.33 mg/ml and 8.99 mg/ml, respectively, at 25^{o}C and 37^{o}C after 4 h IPTG induction (Figure 7A, and 5C); thus the total amount of GST-TAT-Apoptin$_{opt}$ protein

obtained at 25°C compared to 37°C was only very slightly lower (Figure 7A & 5C).

When various IPTG induction concentration from 0.1 to 1 mM were explored for protein induction, the highest amount of GST-TAT-Apoptin$_{opt}$ protein obtained was with 1 mM IPTG induction using the BL21(DE3) strain (Figure 6B) and this gave about 65% soluble protein at 25°C (Figure 6C). The expression level of GST-TAT-Apoptin$_{opt}$ at 0.1 and 0.5 mM IPTG was lower than for 1 mM IPTG (Figure 6B), but the amount of soluble protein was highest at 0.1 mM (95%) IPTG (Figure 6C). Thus a lower cultivation temperature of 25°C and an IPTG concentration of 0.1 mM IPTG were able to produce the higher amount of soluble GST-TAT-Apoptin$_{opt}$ protein.

Purification of recombinant GST-TAT-Apoptin$_{opt}$ protein using GST affinity chromatography

Using the approximately 95% soluble GST-TAT-Apoptin$_{opt}$ expressed by *E. coli* at 25°C and 0.1 mM IPTG, the lysate was subjected to purification using a GST affinity column. After affinity chromatography, the eluted soluble GST-TAT-Apoptin$_{opt}$ protein was confirmed antigenically using anti-GST antibody and CAV-infected positive serum (Figure 8A, 8B and 8C). A typical elution profile of the protein fractions collected from a GST column is shown in Figure 8A after separation by SDS-PAGE. Fraction 4 contains the most eluted protein, has a significant absorbent peak at OD_{280} and was eluted at 12 min (data not shown). The specific 42 kDa band eluted in fraction 4 was almost purified to homogenicity (Figure 8A, lane 4 of elution). When the purified GST-TAT-Apoptin$_{opt}$ protein was examined by MALDI-TOF, seven peptides from GST-TAT-Apoptin$_{opt}$ could be

identified after trypsin digestion and these demonstrated good alignment and a high score when compared to the predicted protein (data not shown). The longest peptide fragment, VNELKESLITTTPSRPR, consists of 17 amino acid residues and overall the coverage was 35.7% of the published amino acid sequence of apoptin (Accession No. AF212490) without any miss-match (Figure 8D). These MALDI-TOF results confirmed that the purified 42 kDa protein is GST-TAT-Apoptin$_{opt}$ and that the *E. coli* preferred codon usage optimization within the VP3 gene has not altered either the amino acid sequence (Figure 8C; Figure 8B, lane 4) or the antigenicity (Figure 8C) of the protein.

Recombinant GST-TAT-Apoptin$_{opt}$ protein has apoptotic activity and induces apoptosis in **HL-60**

To investigate whether GST-TAT-Apoptin$_{opt}$ protein expressed by *E. coli* has apoptotic activity when introduced into tumor cells, purified GST-TAT-Apoptin$_{opt}$ protein was used to examine the protein's apoptotic activity when it was used to treat human premyelocytic leukemia HL-60 cells. As illustrated in Figure 9, flow cytometry analysis by Annexin-V FITC and propidium iodide staining showed an approximately 10% increase in the level of apoptotic levels among HL-60 cells that were co-cultured with GST-TAT-Apoptin$_{opt}$ compared to HL-60 cells only, HL-60 co-cultured with GST or chromatographic elution buffer. However, compared to the control, HL-60 cells co-cultured with GST-TAT-Apoptin$_{opt}$ induced less apoptosis than when HL-60 cells were induced into apoptosis by treatment with cyclohexamide (CHX). Nevertheless, GST-TAT-Apoptin$_{opt}$ was able to induce apoptosis in HL-60 cells to a meaningful degree. These results indicate that *E.coli* expressed GST-TAT-Apoptin$_{opt}$ retains the protein's original

apoptotic activity when used to treated HL-60 cells even after the modifications carried out in this study.

Discussion

Apoptin from chicken anemia virus has been demonstrated to have apoptotic activity and to be able to specifically kill several types of tumor cells including the cell lines HeLa, Saos-2, lung cancer cells H1299 and HepG2 [9-11,13,14,19]. Apoptin is not only p53 and Bcl^- independent, but also does not require specific post-modification in order to be able to induce apoptosis [7]. Therefore, apoptin has great potential to be developed into a protein drug that will be useful as part of the cancer therapy armory. In this context, previously studies have shown that fusing the TAT peptide to apoptin, in order to create a recombinant protein vehicle, which allows protein uptake by cells, improves protein translocation into the cell and increases Saos-2 cell killing [19]. This approach is a useful was of overcoming problems associated with the delivery of apoptin into cells without affecting the anti-cancer activity of the protein. However, up to the present, few studies have investigated anti-cancer activity using such a recombinant TAT-Apoptin protein. The main reason for this lack of progress is the poor expression and low protein solubility of TAT-Apoptin. Previous studies have shown that the large-scale production of recombinant TAT-Apoptin using a prokaryotic expression is difficult and therefore this has become a bottle-neck [17,18]. Even today, in spite of the fact that many eukaryotic expression systems are well established, various factors, such as cost-effectiveness, insertional mutagenesis and transient expression, still need to be resolved for these systems. Taking the above into consideration, the effective production of TAT-Apoptin

protein using a prokaryotic system is a crucial key step in developing this protein drug as an anti-tumor therapy. Therefore, in this study, the specific aim was to develop a prokaryotic expression system that allowed the efficient production of recombinant TAT-Apoptin protein. Using a prokaryotic expression system to express heterologous recombinant protein has several advantages including time-savings, cost-effectiveness, ease of production, simplified characterization and others. It is for these reasons that *E. coli* is the most used expression system when evaluating the expression of a foreign protein [20]. Indeed, the above points are some of the critical factors associated with choosing a suitable production system when developing a protein drug. To this end, problems associated with having a truly effective system for the large-scale production of apoptin protein still needed to be explored.

To improve protein expression and to enhance the solubility of any protein produced in an *E. coli* expression system, a number of strategies are available. These include cultivation parameters, the effect of fusing the protein to an affinity tag and the optimization of codon usage of foreign gene for *E. coli*. All of these have been frequently employed to improve the amount of recombinant protein recovered [17,18,21-23]. In the present study, we first explored the effect of two different fusion tags on TAT-Apoptin expression, although others remain available, are untested as yet and may further improve the yield in the future. The presence of a GST fusion tag was found to significantly improve the yield of TAT-Apoptin compared to a 6×His tag (Figure 2 and Figure 3). In a previous study, Liu *et al* successfully overcame the problem of less efficient expression in *E. coli* of porcine circovirus (PCV) by fusing the maltose-binding protein (MBP) to an 8xHis tag [23]. The main mechanism by which the MBP-8xHis tag improved

protein expression remains unclear. However, one possibility is improved protein solubility [24]. Similarly, in our previous study, the addition of a GST tag to the CAV VP1 protein also improved expression in *E. coli* significantly compared to a His×6 tag [21]. Thus it would seem that some fusion tags are able to improve expression of soluble protein in *E. coli* compared to other tags, perhaps by aiding the correct folding of their fused partner [21, 24].

Next we investigated which of three different *E. coli* strains, BL21(DE3), BL21(DE3)pLysS and BL21(DE3)Codonplus-RP, was able to improve protein production and yield. With both GST-TAT-Apoptin and His-TAT-Apoptin, BL21(DE3) was preferred and produced more TAT-Apoptin protein than either BL21(DE3)pLysS or BL21(DE3)Codonplus-RP (Figure 2A and 2C). It is worth noting that BL21(DE3)pLysS has a higher growth rate than BL21(DE3) or BL21(DE3)Codonplus-RP when expressing His-TAT-Apoptin (Figure 2B and 2D). This discrepancy may involve either poor protein stability or the cytotoxic nature of His-TAT-Apoptin when present in BL21(DE3) and BL21(DE3)Codonplus-RP. These results are similar to those obtained for the production of GST-VP1 protein from CAV in *E. coli* at low expression levels [21]. BL21(DE3)pLysS superiority may be due to the presence in the strain of the *pLysS* plasmid during protein induction [17,21]. This difference may enable BL21(DE3)pLysS to tolerate cytotoxicity associated with the expression of T7 lysozyme by attenuating the transcription leakage by T7 RNA polymerase [17,21]. However, this phenomenon was not important when GST-TAT-Apoptin was expressed and the growth profiles of the three strains producing GST-TAT-Apoptin were almost identical on induction by IPTG. In this area, it is possible that the cytotoxicity of GST-TAT-Apoptin may be less that that of His-TAT-Apoptin in BL21(DE3) and

BL21(DE3)Codonplus-RP [21]. Furthermore, the phenomenon of "protein burden" may not have been encountered when BL21(DE3) was used to express GST-TAT-Apoptin [25]. It was concluded that BL21(DE3) is the preferred choice for expression of GST-TAT-Apoptin.

Rosenberg *et al* have proposed that the abundance of a rare codon near the 5'-end of the gene might affect the efficiency of protein translation [26]. If this is true, then the approach used in the present study to modify the codon usage in the gene ought to improve the expression of full-length apoptin protein. When Genscript OptimumGene^{TM} bioinformatic software was used to identify rare codons in *E. coli* that exist in the wild-type CAV VP3 gene, the pinpointed amino acid residues included arginine, leucine, proline and lysine; these are commonly found in the N-terminus and C-terminus regions of the apoptin protein. After codon optimization of CAV VP3 gene, TAT-Apoptin$_{opt}$ was fused with both tags and successful expressed in *E. coli* (Figure 4A, 4C). The amount of expressed GST-TAT-Apoptin$_{opt}$ in *E. coli* was substantially higher than that of GST-TAT-Apoptin and His-TAT-Apoptin$_{opt}$ when BL21(DE3) or BL21(DE3)pLysS were used (Figure 5A, 5C and Table 1). Thus codon optimization within VP3 gene of the rare codons in *E. coli* was able to improve the expression level of TAT-Apoptin; specifically translation efficiency was improved without the need to supply extra copies of the rare tRNA genes [27,28]. Interestingly, in terms of growth, BL21(DE3) and BL21(DE3)pLysS performed almost identically when expressing GST-TAT-Apoptin (Figure 3D). However, the growth rate of BL21(DE3)pLysS was significantly slower than that of BL21(DE3) when expressing GST-TAT-Apoptin$_{opt}$ (Figure 5D). This might be explained in terms of the "protein burden" within BL21(DE3)pLysS when it is producing

GST-TAT-Apoptin$_{opt}$ at a relatively high level early during induction and this protein burden may eventually result in BL21(DE3)pLysS undergoing growth arrest. The growth profile of BL21(DE3) expressing GST-TAT-Apoptin$_{opt}$ may involve the balancing of the steady-state growth conditions for the strain with the yield and also suggests that this strain had reached the maximum possible growth rate; as a result the growth profile of BL21(DE3) during the expression of GST-TAT-Apoptin$_{opt}$ did not significantly change (Table 1, Figure 3D and 5D). In contrast, when producing His-TAT-Apoptin$_{opt}$, the growth profiles of BL21(DE3) and BL21(DE3)pLysS were very similar to the situation when the strains were producing His-TAT-Apoptin (Figure 5B and 3B). One possible explanation for this is that the level of produced His-TAT-Apoptin or His-TAT-Apoptin$_{opt}$ did not reach the threshold value where there was a protein burden and therefore no growth arrest occurred.

The presence of a fusion tag and optimization of the codon usage within the gene were both useful strategies for improving the production of TAT-Apoptin (Table 1). When compared, the increase level in yield obtained when a fusion tag was used seems to be greater than that derived from codon optimization (Table 1). Specifically, the yield was only increased from 7.1 mg/ml (GST-TAT-Apoptin) to 8.9 mg/ml (GST-TAT-Apoptin$_{opt}$) was expressed in BL21(DE3).

In order to improve the protein stability of TAT-Apoptin, various cultivation parameters were adjusted. Although a cultivation temperature of 37^{o}C was able to produce a higher growth rate and greater protein yield, this temperature did not produce a high yield of soluble protein. When 25^{o}C was used or when a lower IPTG concentration was used (0.1 mM of IPTG), it was not only possible to obtain a similar expression level of TAT-Apoptin within 6 hrs (compared to 4 hrs),

but there was also a significant improvement in protein solubility (Figure 7A, 6A). Recovering soluble TAT-Apoptin using the GST tag as an affinity ligand during the purification process is highly convenient in terms of downstream processing. After GST affinity chromatography, approximately 80% of the GST-TAT-Apoptin produced by the *E. coli* was recovered and recovery of GST-TAT-Apoptin even reached over 90% with one batch (Figure 8). The purified target protein can then be subjected to further purification steps in order to obtain a protein drug with high purity.

In this study, human premyelocytic leukemia HL-60 cells were used to evaluate the apoptotic activity of the *E. coli*-expressed TAT-Apoptin. Apoptosis was induced when the HL-60 cells were co-cultured with 90 ug/ml of GST column purified GST-TAT-Apoptin$_{opt}$. There was a 10% increase in apoptosis compared to the non-apoptosis control (Figure 9A, 9B and 9C). However, this is less apoptosis than that induced by CHX, which is the positive apoptosis control. Previous studies have reported that approximately 70% of tumor cells undergoing apoptosis in the presence of apoptin [11]. This discrepancy may be a result of differences in the tumor cell lines used, which may have different levels of tolerance with respect to apoptin. In addition, the TAT-Apoptin carried the GST fusion tag had not undergo protease cleavage before use and the presence of the tag may have affected the protein drug's apoptotic activity during co-culture with the tumor cells. Nevertheless, it is clear that the *E. coli*-expressed GST-TAT-Apoptin$_{opt}$ produced in the present study does retain its anti-tumor cell activity and the ability to induce apoptosis.

Conclusions

The expression of recombinant full-length TAT-Apoptin protein was established successfully herein using a prokaryotic system, and the yield was significantly improved by fusing the protein with an affinity tag and by optimizing the codon usage of the polypeptide for expression in *E. coli*. Direct engineering of the apoptin protein was a convenient and cost-effective strategy of increasing the expression of TAT-Apoptin protein in *E. coli*. This paves the way for the large-scale production of TAT-Apoptin protein using this approach. In the future, this will also allow TAT-Apoptin to be used for the development of a protein drug that has anti-cancer activity or as part of a CAV diagnostic test.

Methods

Bacterial strain and cells inoculation

Three *E. coli* strains, BL21(DE3) (Invitrogen, Carlsbad, CA), BL21(DE3)CodonPlus-RP (Stratagene, La Jolla, CA) and BL21(DE3)pLysS (Stratagene, La Jolla, CA) were used and maintained at 37^{o}C using 10 ml Luria-Bertani (LB)medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) in 50 ml flasks. For strain activation, 0.5 ml of an overnight culture were inoculated into 50 ml LB medium and grown at 37^{o}C for around 3 h, by which time the optical density of culture had reach 0.5 of OD_{600}. These bacterial cells could then be used for transformation or for protein expression.

Construction of the recombinant plasmids

A 420 bp of synthetic cDNA encoding the full-length CAV apoptin protein that was fused to a trans-acting activator of transcription (TAT) protein transduction domain (PTD) at its N-terminus, the latter being synthesized by Genemark Biosci & Tech Co. (Taichung, Taiwan) and then ligated into pBluescript II SK(-) using *Bam* H1 (Takara, Japan) and *Xho* I (Takara, Japan) restriction sites. The resulting recombinant plasmid was designated pB-TAT-VP3. The *TAT-VP3* gene then was subcloned into either pET28a (Novagen, Madison, WI) or pGEX-4T-1 (GE Healthcare, Piscataway, NJ) using *Bam* H1 and *Xho* I. The resulting constructs were designated pET-TAT-VP3 and pGEX-TAT-VP3 (Figure 1A, a and b), respectively. To generate the *VP3* gene of CAV harboring the codon optimized nucleotide sequence, a codon optimized fragment of *VP3* gene fused with the *TAT* PDT was also synthesized by Genemark Biosci & Tech Co. and then ligated into the pBluescript II SK(-) using *Bam* H1 and *Xho* I restriction sites. The *TAT-VP3*$_{opt}$ gene was then further subcloned into either pET28a or pGEX-4T-1 as described above and the resulting constructs were designated pET-TAT-VP3 $_{opt}$ and pGEX-TAT-VP3$_{opt}$, respectively (Figure 1A, c and d). The constructs described above were then transformed into One Shot® Top10 (Invitrogen, CA) chemically competent *E. coli* for maintenance of the recombinant plasmids and for protein expression. Transformants that contained a

gene of the correct size by PCR were then confirmed as correct by restriction enzyme digestion and by DNA sequence analysis.

Expression of TAT-Apoptin protein and codon optimized TAT-Apoptin$_{opt}$ protein in recombinant *E. coli*

To express the His-TAT-Apoptin, GST-TAT-Apoptin, His-TAT-Apoptin$_{opt}$ and GST-TAT-Apoptin$_{opt}$ proteins, the four constructed recombinant plasmids, pET-TAT-VP3, pGEX-TAT-Apoptin, pET-TAT-VP3$_{opt}$ and pGEX-TAT-VP3$_{opt}$, were transformed into *E. coli* strain to allow evaluation of protein expression. Three *E. coli* host strains, BL21(DE3), BL21(DE3)CodonPlus-RP and BL21 (DE3)pLysS, each being a different recombinant constructions, were used for protein induction and expression. The recombinant strains were grown in LB medium in the presence of kanamycin (50 μg/ml), ampicillin (50 μg/ml) or chloramphenicol (34 μg/ml) as appropriate at 37^{o}C. When the culture had reached an optical density (OD_{600}) of 0.5, isopropyl-β-D-thiogalactopyronoside (IPTG) at different concentrations was added to induce protein expression and then growth was continued for 4 h. After IPTG induction, samples of the cells were harvested and analyzed for protein expression. Protein concentration was determined using the procedure described in a previous study [21]. Samples containing the various

expressed proteins, His-TAT-Apoptin or His-TAT-Apoptin$_{opt}$ and GST-TAT-Apoptin or GST-TAT-Apoptin$_{opt}$, were analyzed by 12.5% SDS-PAGE and Western-blotting using a monoclonal anti-His antibody (Invitrogen, Carlsbad, CA) or a monoclonal anti-GST antibody (GE healthcare, Piscataway, NJ).

Purification of recombinant GST-TAT-Apoptin$_{opt}$ protein using GST affinity chromatography

To purify the recombinant GST-TAT-Apoptin$_{opt}$ proteins, cells were spun down from 50 mL of culture supernatant and resuspended in GST resin binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). The mixture was then sonicated on ice three times for 3 minutes with a 20% pulsed activity cycle (MISONIX Sonicator® 300). Next, the lysate was centrifuged for 10 min at 10,000 rpm to remove the cell debris. The resulting cell supernatant was loaded onto a GSTrap FF affinity column (GE healthcare, Piscataway, NJ) for protein purification using the standard procedure described in a previous study [21]. The total protein concentration of each collected fraction from the column was determined using a Micro BCA kit (Pierce, Rockford, IL) with bovine serum albumin acting as the reference protein. The purity of the protein from each fraction was analyzed by 12.5% SDS-PAGE and then the

resulting gels were Western blotted using monoclonal anti-GST antibody (GE healthcare, Piscataway, NJ).

Mass spectrometry

To confirm the identity of the recombinant apoptin protein, *E. coli* expressed GST-TAT-Apoptin was purified by GSTrap FF column, and then the eluted proteins containing in collected fraction were separated by 12.5% SDS-PAGE. The relevant band was then cut out from the 12.5% SDS-PAGE gel after Coomassie blue staining and digested with trypsin. The resulting samples were subjected to the MALDI-TOF-MS mass spectrometry (ESI-QUAD-TOF) to allow amino acid sequence identification of the protein, as described in a previous study [17].

Apoptosis assay

Human promyelocytic leukemia HL-60 cells (CCRC 60043) were purchased from the Food Industry Research and Development Institute (FIRDI) (Hsichu. Taiwan). These cells were used to evaluate the apoptotic activity of GST-TAT-Apoptin$_{opt}$ using an Annexin-V FITC apoptosis kit (BD Phaemingen, San Diego, CA). The cell was grown in the FIRDI-suggested medium, and all experiments were performed in 6-well culture plates. The cultured HL-60 cells were stained with Annexin-V FITC and propidium iodide (PI) according to manufacturer's guidelines. Briefly, 2×10^5 cells were co-cultured with 90 ug/ml of GST-TAT-Apoptin$_{opt}$ for 24 hrs, and then resuspended in 100 ul of 1× binding buffer, which was followed by incubation with 2.5 ul of Annexin-V FITC and PI

at room temperature for 30 min. Finally the cells were added to 200 ul of 1× binding buffer and assayed immediately using FACSCanto flow cytometry (BD Biosciences, San Jose, CA). A positive control was included and this consisted of treating the cells with 100 mM cycloheximide (CHX) (Sigma-Aldrich, USA) for 24 hrs. The negative controls were either cells treated with protein buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) or cells treated with 90 ug/ml glutathione-S-transferase (GST) protein for 24 hrs.

Acknowledgments

This work was supported by the grant from China Medical University of Taiwan (CMU100-TS-05) and the National Science Council (NSC 95-2313-B-039-004-, NSC96-2313-B-276-001-MY3), Taiwan.

Authors' contributions

MSL participated in this study design, performed the experiments and helped with the writing of the manuscript. SHF, GHL and FCS performed the experiments and participated in the construction of the plasmids. YYL participated in the experiments on protein antigenicity and GHL, CHH and JC participated in the protein purification. HJC and MSL[6] participated in the data analysis and the writing of the manuscript. MSL, HYC and JTCT coordinated the study and participated in the writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

All of authors declare no competing interests.

References

1. Ducatez MF, Owoade AA, Abiola JO, Muller CP: **Molecular epidemiology of chicken anemia virus in Nigeria.** *Arch Virol* 2006, **151**:97-111.

2. Meehan BM, Todd D, Creelan JL, Earle JA, Hoey EM, McNulty MS: **Characterization of viral DNAs from cells infected with chicken anemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome fragments.** *Arch Virol* 1992, **124**:301-319.

3. Noteborn MH, de Boer GF, van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG, Eurissen SHJ, Hoeben RC, Zantema A, Koch G: **Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle.** *J Virol* 1991, **65**:3131-9.

4. Yuasa N, Imai K, Watanabe K, Saito F, Abe M, Komi K: **Aetiological examination of an outbreak of haemorrhagic syndrome in a broiler flock in Japan.** *Avian Pathol* 1987, **16**: 521-526.

5. Peters MA, Jackson DC, Crabb BS, Browning GF: **Chicken anemia virus VP2 is a novel dual specificity protein phosphatase.** *J Biol Chem* 2002, **277**: 39566-73.

6. Noteborn MH, Todd D, Verschueren CA, de Gauw HW, Curran WL, Veldkamp S, Douglas AJ, McNulty MS, van der EA, Koch G: **A single chicken anemia virus protein induces apoptosis.** *J Virol* 1994, **68**: 346-51.

7. Habib NA: **Cancer gene therapy- past achievements and future challenges.** Kluwer Academic/ Plenum Publisher, New York. 2000, pp.155.

8. Mae M**,** Langel U: **Cell-penetrating peptides as vectors for peptides, protein and oligonucleotide delivery.** *Curr Opin Pharmacol* 2006, **6:** 509-514.

9. Li X, Jin N, Mi Z, Lian H, Sun L, Zheng H: **Antitumor effects of a recombinant fowlpox virus expressing Apoptin in vivo and in vitro.** *Int J Cancer* 2006, **119**: 2948-57.

10. Pietersen AM, van der Eb MM, Rademaker HJ, van den Wollenberg DJ, Rabelink MJ, Kuppen PJ, van Dierendonck JH. Van Ormondt H, Masman D, van de Velde CJ, van der Eb AJ, Hoeben RC, Noteborn MH: **Specific tumor-cell killing with adenovirus vectors containing the apoptin gene.** *Gene Ther* 1999, **6**: 882-92

11. Los M, Panigrahi S, Rashedi I, Mandal S, Stetefeld J, Essmann F, Schulze-Osthoff K: **Apoptin, a tumor-selective killer.** *Biochim Biophys Acta* 2009, **1793**: 1335-42.

12. Green M, Loews. enstein PM: **Autonomous functional domain of chemically synthesized human immunodeficiency virus tat trans-activator protein.** *Cell* 1988, **55**: 1179-88.

13. Guelen L, Paterson H, Gäken J, Meyers M, Farzaneh F, Tavassoli M: **TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells**. *Oncogene* 2004, **23**:1153-65.

14. Han SX, Ma JL, Lv Y, Huang C, Liang HH, Duan KM: **Secretory Transactivating Transcription-apoptin fusion protein induces apoptosis in hepatocellular carcinoma HepG2 cells.** *World J Gastroenterol* 2008, **14**: 3642-9.

15. Iwata N, Fujino M, Tuchiya K, Iwata A, Otaki Y, Ueda S: **Development of an enzyme-linked immunosorbent assay using recombinant chicken anemia virus proteins expressed in a baculovirus vector system.** *J Vet Med Sci* 1998, **60**: 175-80.

16. Lacorte C, Lohuis H, Goldbach R, Prins M: **Assessing the expression of chicken anemia virus proteins in plants.** *Virus Res* 2007, **129**: 80-6.

17. Lee MS, Chou YM, Lien YY, Lin MK, Chang WT, Lee HZ, Lee MS, Lai GH, Chen HJ, Haung CH, Lin WH: **Production and diagnostic application of a**

purified, *E. coli*-expressed, serological specific chicken anemia virus antigen VP3. *Transbound Emerg Dis* 2011, 58: 232-9.

18. Nogueira-Dantas EO, Ferreira AJ, Astolfi-Ferreira CS, Brentano L: **Cloning and expression of chicken anemia virus VP3 protein in *Escherichia coli*.** *Comp Immunol Microbial Infect Dis* 2007, **30**: 133-42.

19. Flinterman M, Farzaneh F, Habib N, Malik F, Gäken J, Tavassoli M: **Delivery of therapeutic proteins as secretable TAT fusion products.** *Mol Ther* 2009, **17**: 334-42.

20. Makrides SC: **Strategies for achieving high-level expression of genes in *Escherichia coli*.** *Microbiol Rev* 1996, **60**: 512-538.

21. Lee MS, Hseu YC, Lai GH, Chang WT, Chen HJ, Huang CH, Lee MS, Wang MY, Kao JY, You BJ, Lin W, Lien YY, Lin MK: **High yield expression in a recombinant *E. coli* of a codon optimized chicken anemia virus capsid protein VP1 useful for vaccine development.** *Microb Cell Fact* 2011, **10**: 56.

22. Trundova M, Celer V: **Expression of porcine circovirus 2 ORF2 gene requires codon optimized *E. coli* cells.** *Virus Genes* 2007, **34**: 199–204.

23. Liu Q, Willson P, Attoh-Poku S, Babiuk LA: **Bacterial expression of an immunologically reactive PCV2 ORF2 fusion protein.** *Protein Expr Purif* 2001, 21: 115-120.

24. Sun SQ, Guo HC, Sun DH, Yin SH, Shang YJ, Cai XP, Liu XT: **Development and validation of an ELISA using a protein encoded by ORF2 antigenic**

domain of porcine circovirus type 2. *Virol J* 2010, **7**: 274-280.

25. Kurland CG. Dong H: **Bacterial growth inhibition by overproduction of protein.** *Mol Microbiol* 1996, **21**: 1-4.

26. Rosenberg AH, Goldman E, Dunn JJ, Studier FW, Zubay G: **Effects of consecutive AGG codons on translation in *Escherichia coli*, demonstrated with a versatile codon test system.** *J Bacteriol* 1993, **175**: 716-22.

27. Rosano GL, Ceccarelli EA: **Rare codon content affects the solubility of recombinant proteins in a codon bias-adjusted *Escherichia coli* strain.** *Microb cell fact* 2009, **8**: 41-49.

28. Gvritishvili AG, Leung KW, Tombran-Tink J: **Codon preference optimization increases heterologous PEDF expression**. *PLoS One* 2010, **5**: e15056.

Figure Legends

Figure 1. Schematic diagram of the constructs used for TAT-Apoptin protein expression. (A) Schematic representation of the TAT-Apoptin protein fused with different affinity tags together with the expression vectors used in this study. The designations of the TAT-Apoptin protein and its expression vectors are indicated, (a), (b), (c) and (d). The constructs, (a) and (b), contain the full-length TAT-VP3 gene cloned into the vectors pET28a and pGEX-4T-1; these were used for expression of TAT-Apoptin protein with either a six-histidine (6×His) tag or a glutathione-s-transferase (GST) tag at the N-terminus, respectively. Constructs (c) and (d) containing the TAT-VP3 gene that was codon-optimized; this was derived from construct (b) by replacing rare codons without altering the amino acid sequence. The codon-optimized TAT-VP3gene, TAT-$VP3_{opt}$, was then cloned into pET28a and pGEX-4T-1. (B) Sequence comparison between the TAT-VP3 gene and the TAT-$VP3_{opt}$ gene. The nucleotide sequences were compared between the original TAT-VP3 gene (wild type TAT-VP3) and the sequence of codon-optimized TAT-VP3 gene (TAT-$VP3_{Opt}$) over the whole coding region. An asterisk (*) represents the fact that the aligned nucleotides are identical.

Figure 2. Expression of recombinant TAT-Apoptin protein in three different *E. coli* strains. The TAT-Apoptin protein expression in three *E. coli* strains, BL21(DE3), BL21(DE3)pLysS and BL21(DE3)CodonPlus-RP, which were transformed with either pET-TAT-VP3 or pGEX-TAT-VP3 and cultivated at 37^{o}C. His-TAT-Apoptin and GST-TAT-Apoptin protein were examined and detected using SDS-PAGE (A, C) and Western-blotting (B, D). Anti-His and anti-GST tag monoclonal antibodies was respectively used to recognize the His-TAT-Apoptin

protein and GST-TAT-Apoptin. Lane M, pre-stained protein marker; the symbols "−" and "+" represented pre-induction and post-induction with 1mM of IPTG over 4 hrs of cultivation in *E. coli*, respectively.

Figure *3*. Productivities of TAT-Apoptin protein and the growth curves of the three recombinant *E. coli* strains. The productivities of His-TAT-Apoptin (A) and GST-TAT-Apoptin (C) for the three *E. coli* strains, BL21(DE3), BL21(DE3)pLysS, and BL21(DE3)CodonPlus-RP containing pET-TAT-VP3 or pGEX-TAT-VP3 are shown over the time course of cultivation at 37^{o}C after IPTG induction. The growth curves of BL21(DE3), BL21(DE3)CodonPlus-RP and BL21(DE3)pLysS expressing His-TAT-Apoptin (B) and GST-TAT-Apoptin (D), respectively, in LB medium post-induction.

Figure 4. Expression of recombinant TAT-Apoptin$_{opt}$ protein in the different *E. coli* strains. The TAT-Apoptin$_{opt}$ protein was expressed in the *E. coli* strains BL21(DE3) and BL21(DE3)pLysS, which contained either pET-TAT-VP3$_{opt}$ or pGEX-TAT-VP3$_{opt}$, at 37^{o}C. His-TAT-Apoptin$_{opt}$ and GST-TAT-Apoptin$_{opt}$ protein were examined and detected using SDS-PAGE (A, C) and Western-blotting (B, D). Anti-His and anti-GST tag monoclonal antibodies was respectively used to recognize the His-TAT-Apoptin$_{opt}$ protein and GST-TAT-Apoptin$_{opt}$. Lane M, pre-stained protein marker; " − " and " + " represented pre-induction and post-induction with 1mM of IPTG over 4 hrs of cultivation in *E. coli*, respectively.

Figure 5. Productivities of TAT-Apoptin$_{opt}$ protein and the growth curves of two recombinant *E. coli* strains. The productivities of His-TAT-Apoptin$_{opt}$ (A) and GST-TAT-Apoptin$_{opt}$ (C) using two *E. coli* strains, BL21(DE3) and BL21(DE3)pLysS containing either pET-TAT-VP3$_{opt}$ or pGEX-TAT-VP3$_{opt}$, respectively, are shown over a time course after IPTG induction at 37^{o}C. The growth curves of BL21(DE3) and BL21(DE3)pLysS expressing His-TAT-Apoptin$_{opt}$ (B) and GST-TAT-Apoptin$_{opt}$ (D) in LB medium post-induction.

Figure 6. Solubility of *E. coli*-expressed GST-TAT-Apoptin$_{opt}$ protein under using various cultivation parameters during protein induction. The solubility of GST-TAT-Apoptin$_{opt}$ was determined in the BL21(DE3) strain at different cultivation temperatures (A) and in the presence of various concentrations of IPTG (B, C).

Figure 7. Productivities of TAT-Apoptin$_{opt}$ protein and the growth curves in two recombinant *E. coli* strains. The productivities of GST-TAT-Apoptin$_{opt}$ using two *E. coli* strains, BL21(DE3) and BL21(DE3)pLysS containing pGEX-TAT-VP3$_{opt}$ are shown over time after IPTG induction at 25^{o}C (A). Growth curves of BL21(DE3) and BL21(DE3)pLysS expressing GST-TAT-Apoptin$_{opt}$, respectively, in LB medium post-induction (B).

Figure 8. Purification of recombinant GST-TAT-Apoptin$_{opt}$ protein. SDS-PAGE(A) and Western-blot (B) analysis of the GST-TAT-Apoptin$_{opt}$ protein contained in various elution fractions collected from the GSTrap FF affinity column. The

cytosolic extract of *E. coli* strain BL21(DE3) expressing GST-TAT-Apoptin$_{opt}$ protein was loaded onto a GSTrap FF column and the bound protein was eluted with elution buffer as described in Material and Methods. The eluted protein from the GSTrap FF affinity column was analyzed by SDS-PAGE and Western blotting using monoclonal anti-GST antibody. (C) Antigenicity analysis of GST-TAT-Apoptin$_{opt}$. The purified GST-TAT-Apoptin$_{opt}$ was assayed by Western blotting using positive CAV-infected chicken serum. Lane M, pre-stained protein marker; lane 1, flow through; lane 2, fraction obtained after column washing, lane 3 and 4, eluted fraction 1 and 2, respectively, collecting after column elution.(D) Identity of the GST-opt-VP1 protein determined by MALDI-TOF. The bold letters represent actual amino acid matches to published amino acid sequence (Accession No. AF212490) .

Figure 9. Induction of apoptosis in HL-60 cells by GST-TAT-Apoptin$_{opt}$. An apoptotic assay of HL-60 cells was performed by flow cytometry after 90 ug/ml of the purified *E. coli*-expressed recombinant GST-TAT-Apoptin$_{opt}$ protein was co-cultured with HL-60 cell for 24 hours (D). Non-apoptosis controls, (A), (B) and (C), were assayed. These represent respectively HL-60 cells only, HL-60 cells co-cultured with protein buffer and HL-60 cells co-cultured with GST protein. The apoptosis induced by CHX was used as a positive apoptosis control.

Table 1. Summary of the productivities of the various TAT-Apoptin proteins expressed in the range of *E. coli* strains.

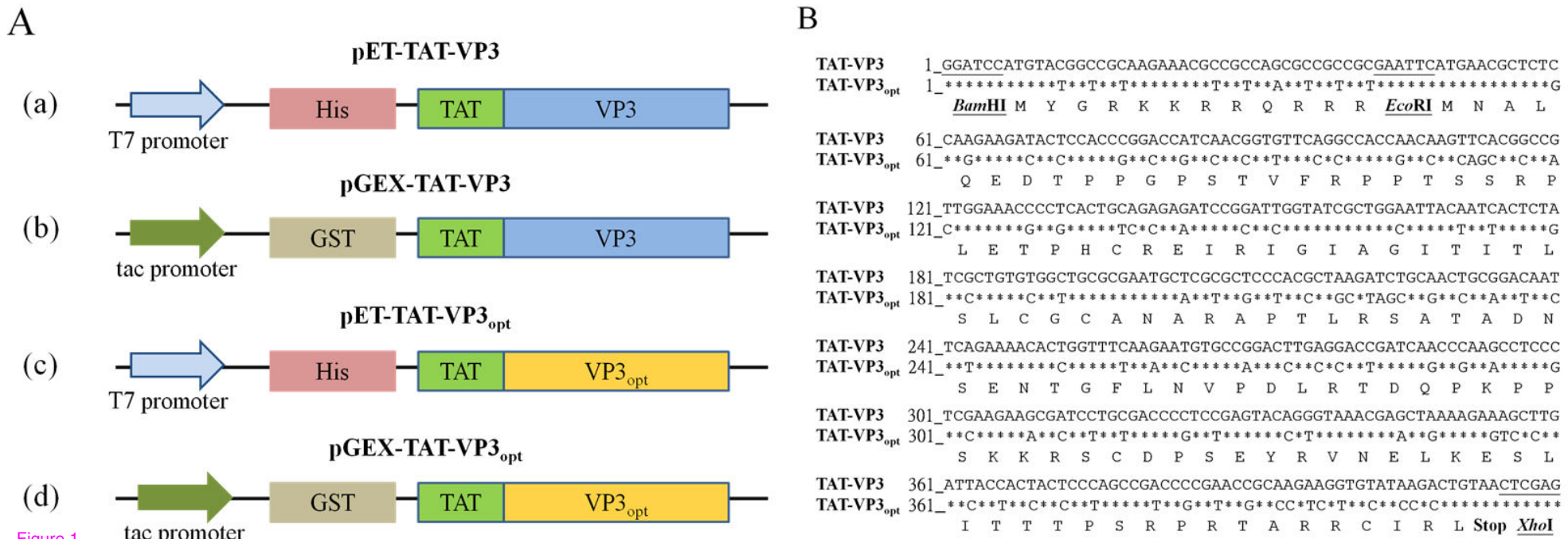

A
pET-TAT-VP3
(a)
His
TAT
VP3
T7 promoter
pGEX-TAT-VP3
(b)
GST
TAT
VP3
tac promoter
pET-TAT-VP3opt
(c)
His
TAT
VP3opt
T7 promoter
pGEX-TAT-VP3opt
(d)
GST
TAT
VP3opt
tac promoter
B
TAT-VP3 1_GGATCCATGTACGGCCGCAAGAAACGCCGCCAGCGCCGCCGCGAATTCATGAACGCTCTC
TAT-VP3opt 1_**********T**T**T********T**T**A**T**T**T***************G
BamHI M Y G R K K R R Q R R R EcoRI M N A L
TAT-VP3 61_CAAGAAGATACTCCACCCGGACCATCAACGGTGTTCAGGCCACCAACAAGTTCACGGCCG
TAT-VP3opt 61_**G*****C**C*****G**C**G**C**C**T***C*C*****G**C**CAGC**C**A
Q E D T P P G P S T V F R P P T S S R P
TAT-VP3 121_TTGGAAACCCCTCACTGCAGAGAGATCCGGATTGGTATCGCTGGAATTACAATCACTCTA
TAT-VP3opt 121_C*******G**G*****TC*C**A*****C**C**********C*****T**T*****G
L E T P H C R E I R I G I A G I T I T L
TAT-VP3 181_TCGCTGTGTGGCTGCGCGAATGCTCGCGCTCCCACGCTAAGATCTGCAACTGCGGACAAT
TAT-VP3opt 181_**C*****C**T**********A**T**G**T**C**GC*TAGC**G**C**A**T**C
S L C G C A N A R A P T L R S A T A D N
TAT-VP3 241_TCAGAAAACACTGGTTTCAAGAATGTGCCGGACTTGAGGACCGATCAACCCAAGCCTCCC
TAT-VP3opt 241_**T********C*****T**A**C*****A***C**C*C**T*****G**G**A*****G
S E N T G F L N V P D L R T D Q P K P P
TAT-VP3 301_TCGAAGAAGCGATCCTGCGACCCCTCCGAGTACAGGGTAAACGAGCTAAAAGAAAGCTTG
TAT-VP3opt 301_**C*****A**C**T**T*****G**T******C*T********A**G*****GTC*C**
S K K R S C D P S E Y R V N E L K E S L
TAT-VP3 361_ATTACCACTACTCCCAGCCGACCCCGAACCGCAAGAAGGTGTATAAGACTGTAACTCGAG
TAT-VP3opt 361_**C**T**C**C**T*****T**G**T**G**CC*TC*T**C**CC*C**********
I T T T P S R P R T A R R C I R L Stop XhoI

Figure 1

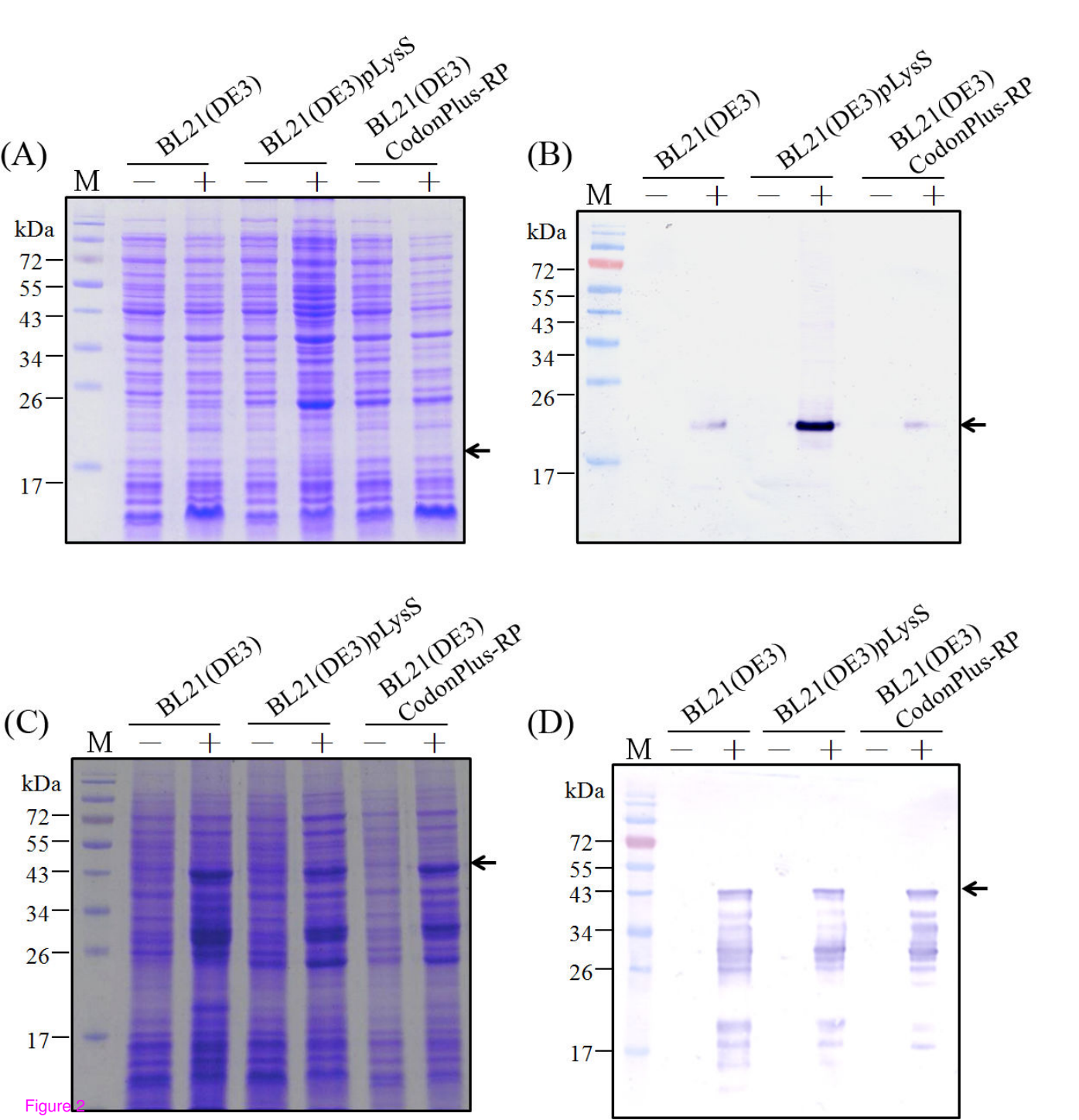

(A)
BL21(DE3)
BL21(DE3)pLysS
BL21(DE3) CodonPlus-RP
M
kDa
72
55
43
34
26
17
(B)
BL21(DE3)
BL21(DE3)pLysS
BL21(DE3) CodonPlus-RP
M
kDa
72
55
43
34
26
17
(C)
BL21(DE3)
BL21(DE3)pLysS
BL21(DE3) CodonPlus-RP
M
kDa
72
55
43
34
26
17
(D)
BL21(DE3)
BL21(DE3)pLysS
BL21(DE3) CodonPlus-RP
M
kDa
72
55
43
34
26
17

Figure 2

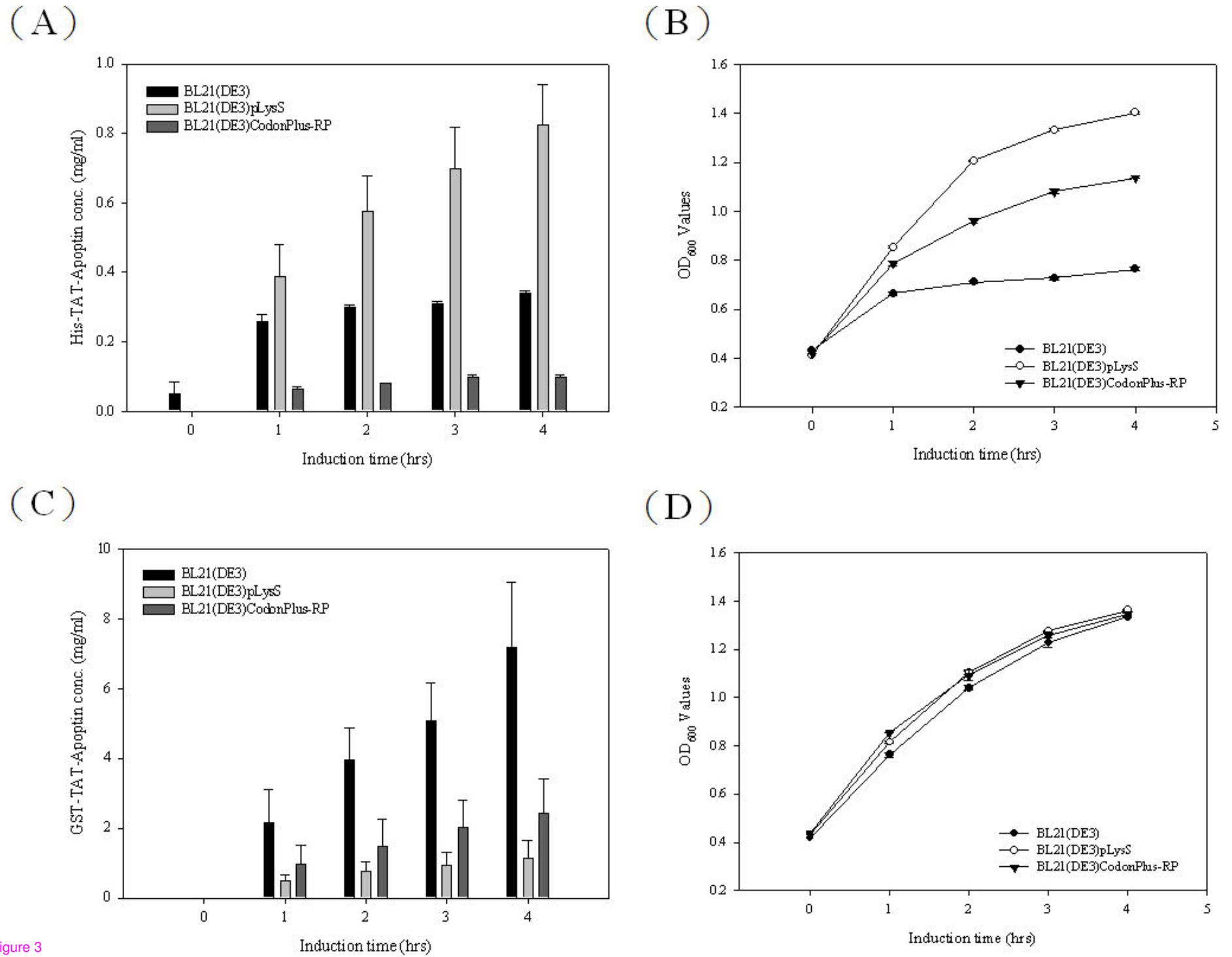

(A)
BL21(DE3)
BL21(DE3)pLysS
BL21(DE3)CodonPlus-RP
His-TAT-Apoptin conc. (mg/ml)
Induction time (hrs)
(B)
OD600 Values
Induction time (hrs)
(C)
GST-TAT-Apoptin conc. (mg/ml)
Induction time (hrs)
(D)
OD600 Values
Induction time (hrs)

Figure 3

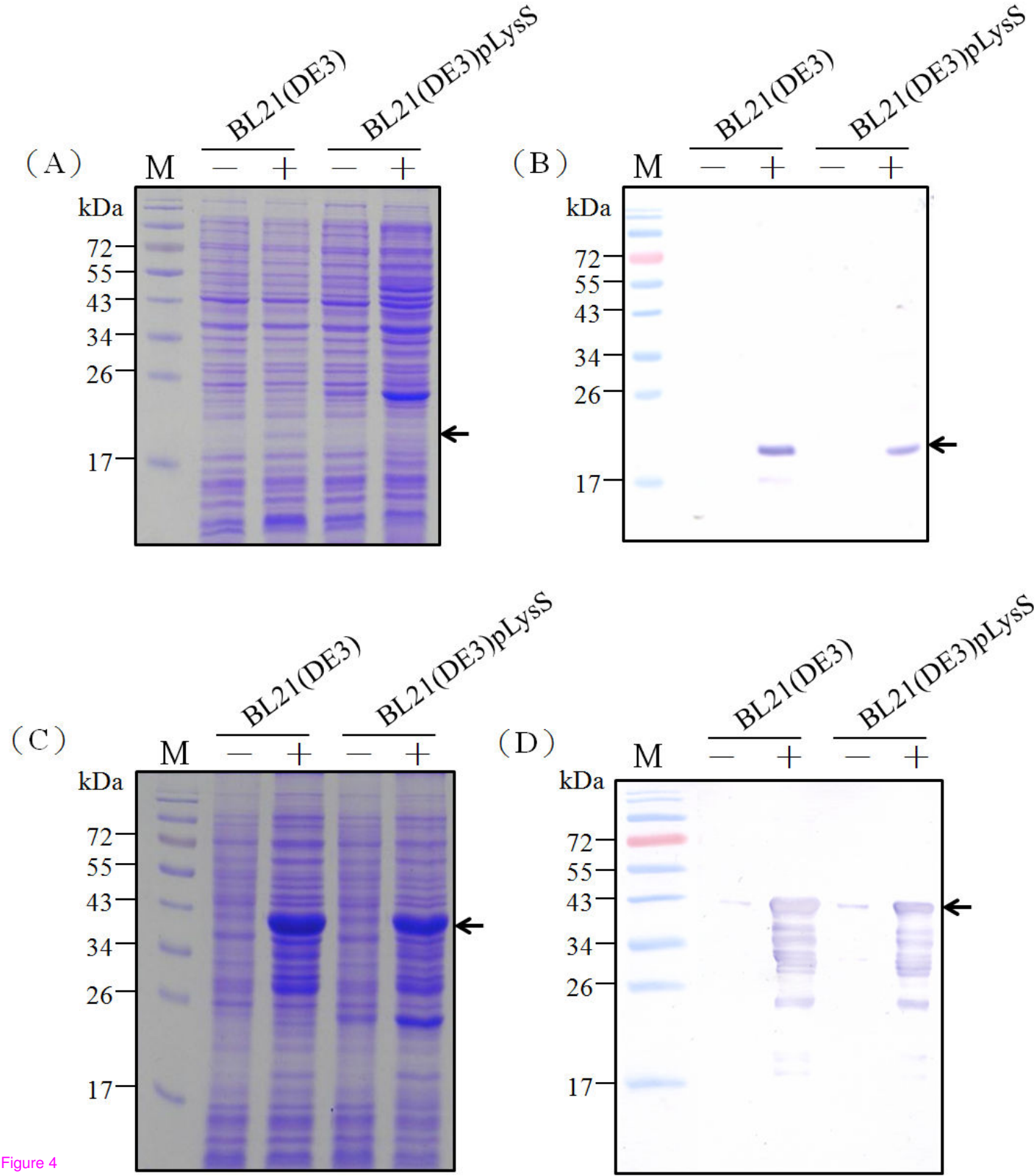

(A)
M
BL21(DE3)
BL21(DE3)pLysS
— + — +
kDa
72
55
43
34
26
17
(B)
M
BL21(DE3)
BL21(DE3)pLysS
— + — +
kDa
72
55
43
34
26
17
(C)
M
BL21(DE3)
BL21(DE3)pLysS
— + — +
kDa
72
55
43
34
26
17
(D)
M
BL21(DE3)
BL21(DE3)pLysS
— + — +
kDa
72
55
43
34
26
17

Figure 4

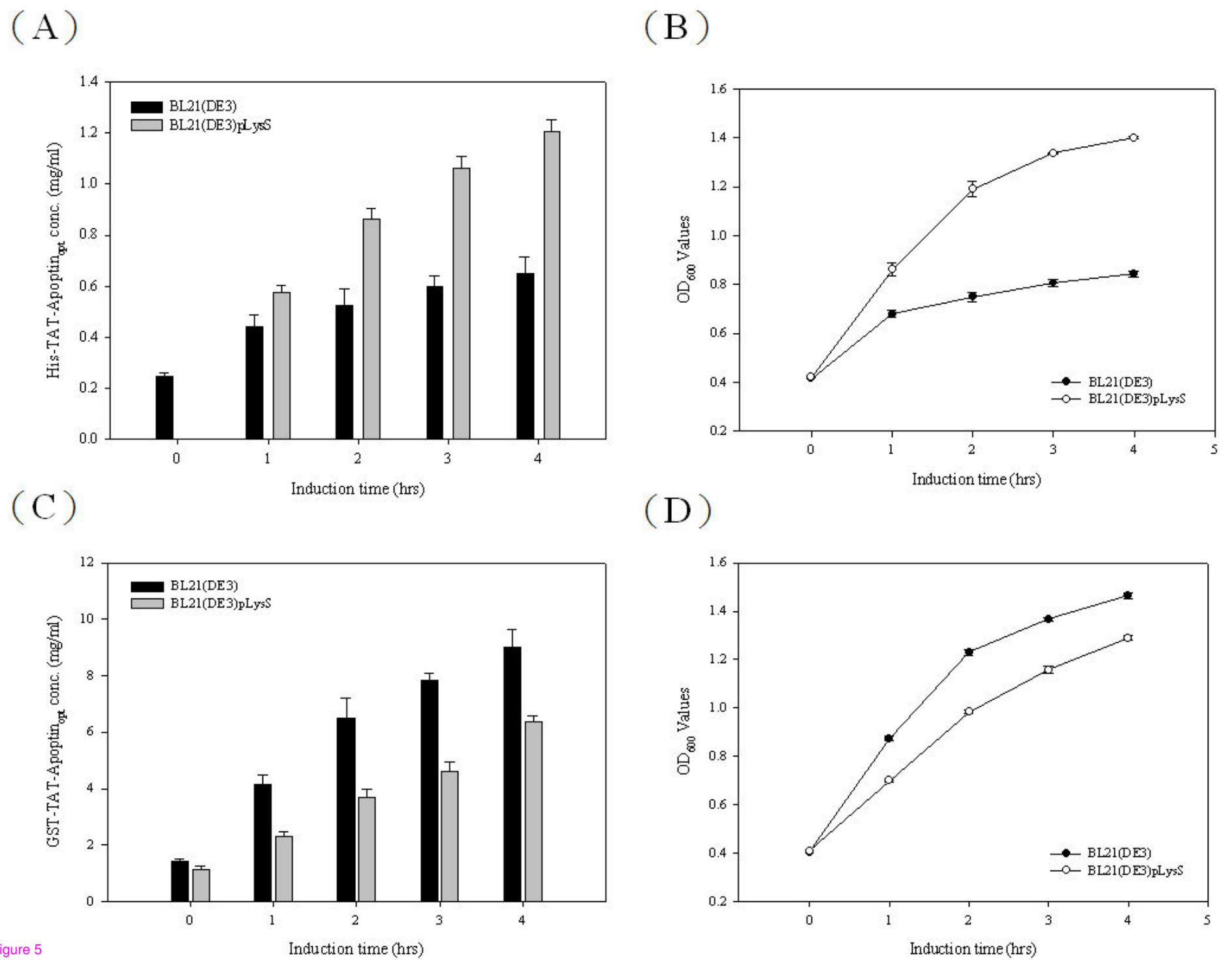

(A)
BL21(DE3)
BL21(DE3)pLysS
His-TAT-Apoptin$_{opt}$ conc. (mg/ml)
Induction time (hrs)
(B)
OD$_{600}$ Values
BL21(DE3)
BL21(DE3)pLysS
Induction time (hrs)
(C)
BL21(DE3)
BL21(DE3)pLysS
GST-TAT-Apoptin$_{opt}$ conc. (mg/ml)
Induction time (hrs)
(D)
OD$_{600}$ Values
BL21(DE3)
BL21(DE3)pLysS
Induction time (hrs)

Figure 5

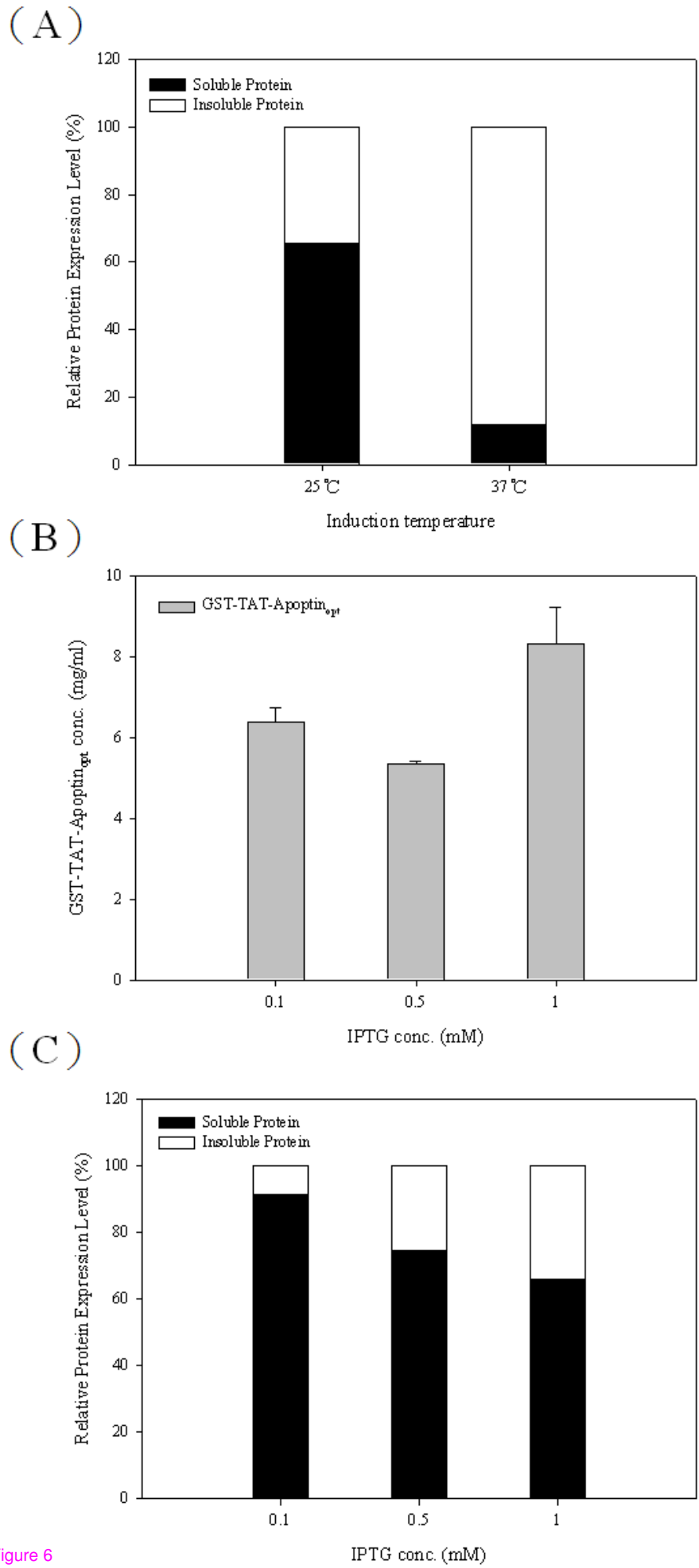
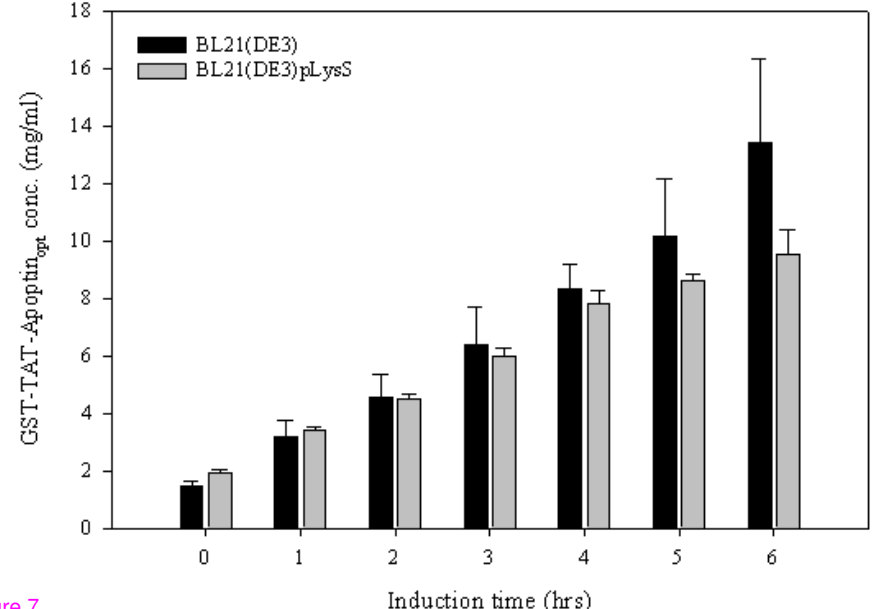

(A)
Soluble Protein
Insoluble Protein
Relative Protein Expression Level (%)
120
100
80
60
40
20
0
25℃
37℃
Induction temperature
(B)
GST-TAT-Apoptin
GST-TAT-Apoptin conc. (mg/ml)
10
8
6
4
2
0
0.1
0.5
1
IPTG conc. (mM)
(C)
Soluble Protein
Insoluble Protein
Relative Protein Expression Level (%)
120
100
80
60
40
20
0
0.1
0.5
1
IPTG conc. (mM)

Figure 6

(A)


BL21(DE3)
BL21(DE3)pLysS
GST-TAT-Apoptin$_{opt}$ conc. (mg/ml)
Induction time (hrs)
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5
6

(B)

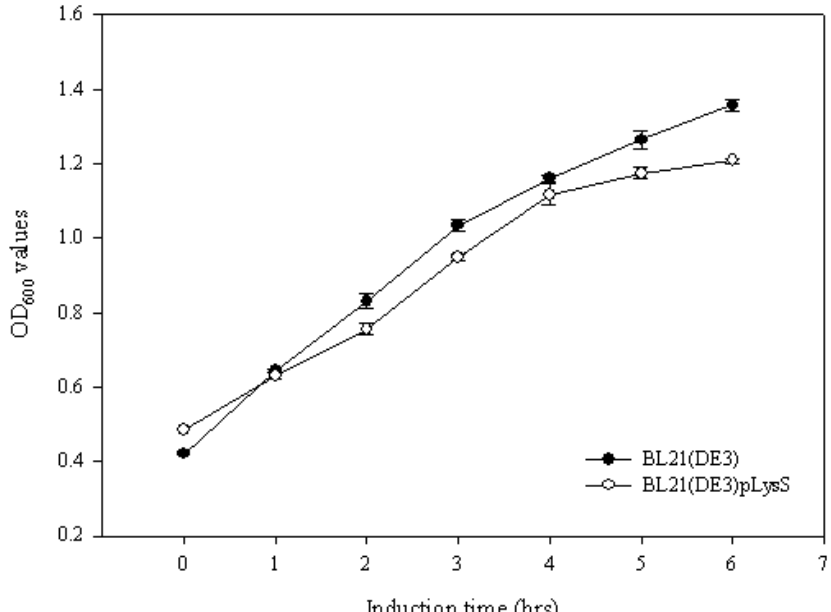
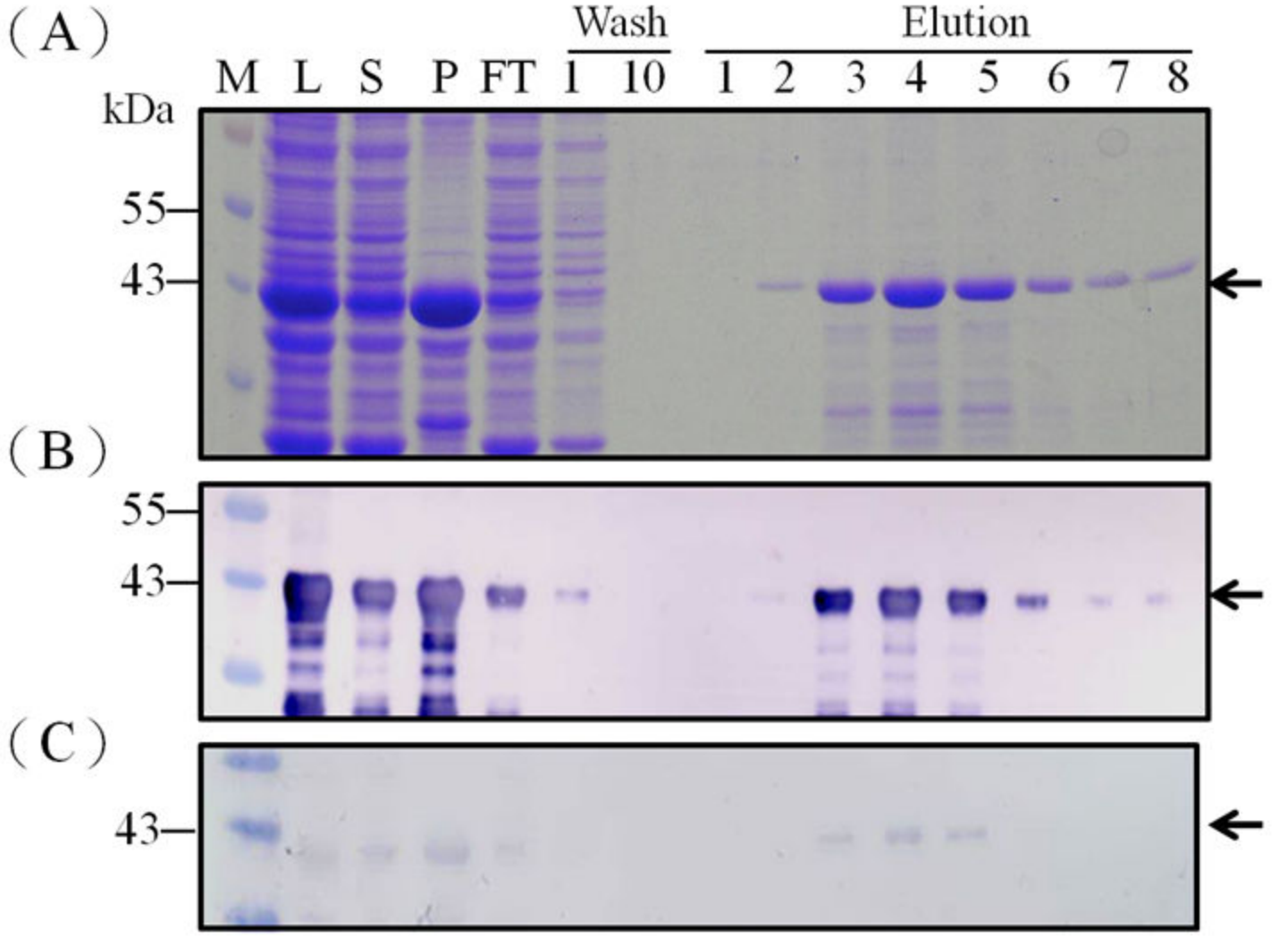

OD$_{600}$ values
Induction time (hrs)
BL21(DE3)
BL21(DE3)pLysS
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
1
2
3
4
5
6
7

Figure 7



(D)

```
  1  MNALQEDTPP GPSTVFRPPT SSRPLETPHC REIRIGIAGI
 41  TITLSLCGCA NARAPTLRSA TADNSENTGF KNVPDLRTDQ
 81  PKPPSKKRSC DPSEYRVNEL KESLITTTPS RPRTARRCIR
121  L
```

Figure 8

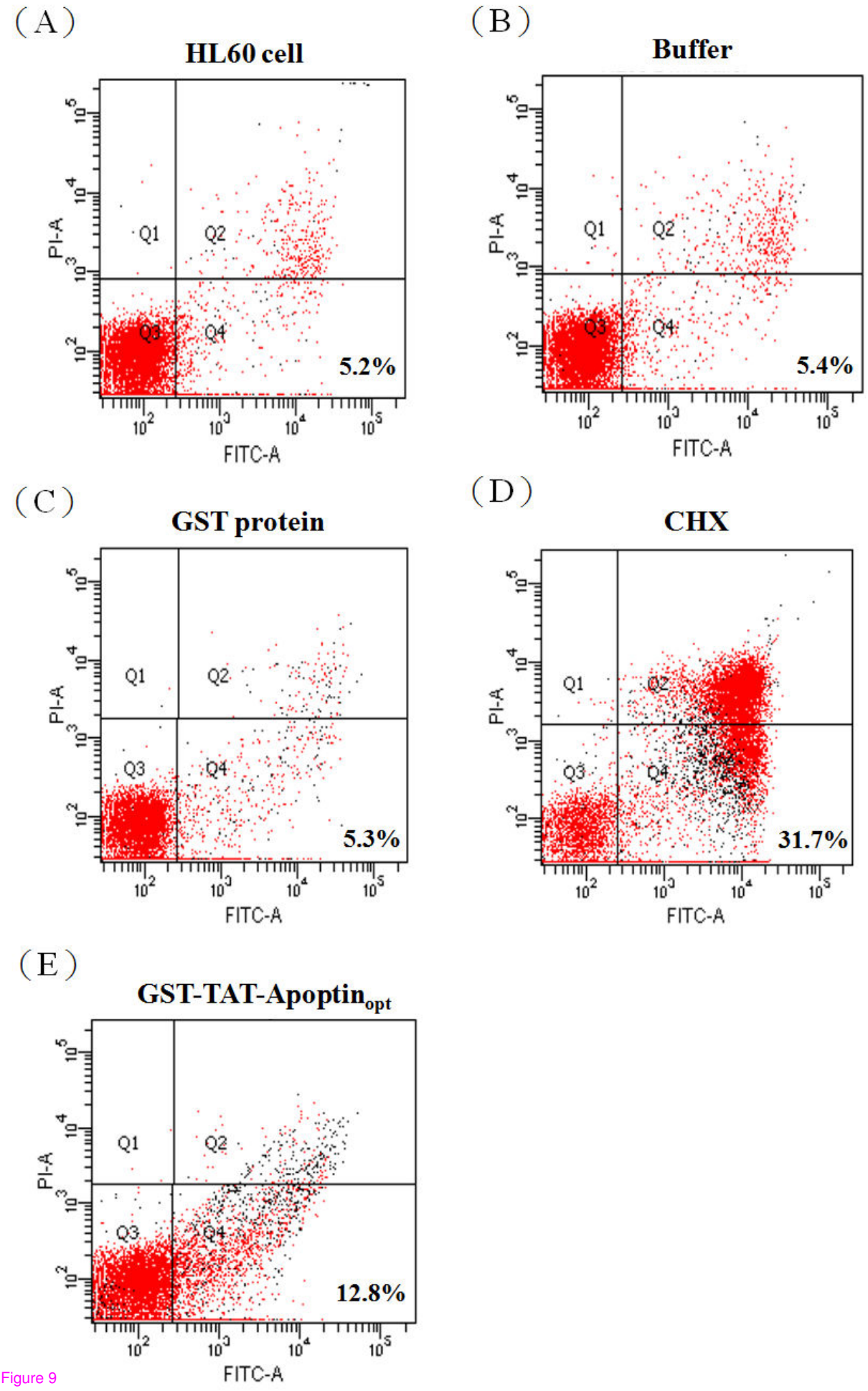

(A)
HL60 cell
Q1
Q2
Q3
Q4
5.2%
PI-A
FITC-A
(B)
Buffer
Q1
Q2
Q3
Q4
5.4%
PI-A
FITC-A
(C)
GST protein
Q1
Q2
Q3
Q4
5.3%
PI-A
FITC-A
(D)
CHX
Q1
Q2
Q3
Q4
31.7%
PI-A
FITC-A
(E)
GST-TAT-Apoptin$_{opt}$
Q1
Q2
Q3
Q4
12.8%
PI-A
FITC-A

Figure 9

Protein	Productivity of *E. coli* strains (mg/ml)			Plasmid
	BL21(DE3)	pLysS	CodonPlus-RP	
His-TAT-Apoptin	0.34 ± 0.01	0.82 ± 0.11	0.09 ± 0.01	pET-TAT-VP3
GST-TAT-Apoptin	7.13 ± 1.88	1.15 ± 0.49	2.43 ± 0.98	pGEX-TAT-VP3
His-TAT-Apoptin$_{opt}$	0.65 ± 0.06	1.20 ± 0.05	-	pET-TAT-VP3$_{opt}$
GST-TAT-Apoptin$_{opt}$[a]	8.99 ± 0.65	6.38 ± 0.18	-	pGEX-TAT-VP3$_{opt}$
GST-TAT-Apoptin$_{opt}$[b]	8.33 ± 0.89	7.84 ± 0.44	-	pGEX-TAT-VP3$_{opt}$

[a] *E. coli* Strains were cultured at 37 ℃

[b] *E. coli* Strains were cultured at 25 ℃

Figure 10