Efficient Expression of Recombinant Soluble Apoptin in *Escherichia coli* and *Bacillus subtilis*

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***Abstract*—**Apoptin, a small protein encoded by chicken anemia virus (CAV), has great attention in last decade because of its ability to induce apoptosis of tumor cells selectively. This protein was expressed as a recombinant protein by employing *Escherichia coli* and *Bacillus subtilis* as hosts for expression. Gateway cloning system was used in this study to clone Apoptin gene, while plasmid destination vectors pOGW and pOXGW were used for expression of apoptin gene in *E. coli* and *B. subtilis*, respectively. In the former plasmid vector IPTG was employed as an inducer for protein expression whereas in the latter xylose, cheaper inducer than IPTG, was employed. A 12 his tag was added to the C-terminal, whilst a Shine Dalgarno (SD) promoter sequence and an ATG start codon were added to the N-terminal region preceeding the Apoptin structural gene. The expressed recombinant Apoptin was purified by performing affinity chromatography column of immobilized Ni^{2+}. Results from SDS-PAGE show that the recombinant protein was well expressed, and furthermore, the protein was produced in soluble form. We were also able to demonstrate that recombinant Apoptin can also be produced in soluble form by adding cell-penetrating peptide, octaarginine. This soluble recombinant Apoptin open the way to confirm biophysical, biochemical properties and detail mechanism of Apoptin inducing-apoptosis, *in vitro*, and producing recombinant protein apoptin efficiently.

***Keywords*—** apoptin, *Bacillus subtilis*, 12 his tag, soluble protein.

I. Introduction

Apoptin is a protein of 121 amino acids and 14 kDa which is isolated from chicken anemia virus (CAV). This protein has an ability to induce apoptosis in various human tumors and transformed cells [1-3] , and it is potential to be used in medical application for the treatment of cancers because they appear to recognize the process of cancerous normal cell and to induce apoptosis when the process occur [4]. It was reported that apoptin selectively induces apoptosis process in various cancer cell including osteosarcoma, hepatoma, cholangiocarcinoma, melanoma, colon carcinoma, lung cancer, breast cancer, prostate and cervix cancer and so on, and it has no effect in normal cells [5-6]. Unfortunately, the mechanism of selective apoptin-induced-tumor cell apoptosis are still being understood [6]. One of the reasons is biophysical properties and structure is not elucidated yet causing difficulties to express recombinant protein apoptin in native form [6].

The Apoptin was expressed for the first time as reported by Leliveld and coworkers in bacterial and mammalian cells. They constructed several apoptin recombinant. They fused N-terminal of apoptin with maltose binding protein (MBP) and fused the C-terminal of it with six histidine and were transformed in mammalian and bacterial cells. Unfortunately, the recombinant Apoptin formed insoluble aggregates even tagged with maltose binding protein. To treat insoluble recombinat apoptin, the apoptin resuspended in the buffer containing 6 M urea was refolded in phospate buffer [7]. Guelen and coworkers, 2004, tried to fuse Apoptin with HIV-1 trans-activator protein (TAT) that have ability to deliver protein across the cell membrane and express it in *Escherichia coli*. Similarly, the recombinant protein formed inclusion body, and then treated by denaturing agent 8 M urea. The urea was removed by through the desalting column [8]. Furthermore, the recombinant Apoptin was expressed as a fusion with more efficient peptide that deliver protein across the cellular membrane, protein transduction domain 4 (PTD4) by Sun *et al.* (2009) and purified it with similar method as Guelen et al. [9]. The last, Yan and coworkers (2010) also tagged Apoptin with carcinoembryonic antigen called CAtin and produced inclusion body recombinant protein and was then refolded by treated with denaturing agent, 8 M urea followed by refolding process incubation in the refolding buffer [10].

Here, we present for the first time, soluble recombinant apoptin expressed in *E. coli* and *Bacillus subtilis*. In this study we describe four strategies to obtain native form Apoptin by recombination. In previous report, a His(6)-tag was added at C-terminal of Apoptin, and this did not disturb the apoptosis activity [10], Here we first constructed a 12 × Histidine tagged Apoptin in C-terminal and inserted in pOGW which IPTG -inducing expression vector. Second, to produce Apoptin in large scale, use of cheaper inducer than IPTG would be realistic for application purpose. Thus, we constructed a new destination vector, pOXGW, which can be converted to expression vector in which cloned gene can be induced by xylose. We used SD promoter in both

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constructions as this is a universal promoter for bacteria. Arginine-rich cell-penentrating peptides residues including oligoarginine, have been reported to penentrate various types of cells efficiently in comparison with other peptides, without causing significant cytotoxicity. It was also reported that octaarginine accumulated in high levels in tumor tissues compared with other oligoarginine. Here, we also linked apoptin with octaarginine and the recombinant also produce soluble protein.

II. Materials and Methods

A. Bacterial strains and plasmids

Escherichia coli strain DH5α (Takara, JP), was used for plasmid preparation. The *E. coli* and *B. subtilis* were used for the expression of the recombinant protein. DNA manipulations were carried out by the Gateway® cloning system (Invitrogen) [11]. The pDONR201 and pOGW or pOXGW were used for cloning of the PCR products and expression, respectively. To construct pOXGW, xylose-controlable expression cassette containing *xyl*A promoter and *xyl*R (repressor for *xyl*A promoter) derived from *Bacilllus megaterium* was amplified from pX plasmid with xylR-F-XhoI and xylR-R-SphI primers, which contain XhoI and SphI sites at the 5' ends respectively [12]. pOGW contains a IPTG-controlable expression cassette containing lacI and spac promoter and XhoI and SphI sites are positioned at the outside [13]. Thus, pOGW was digested by XhoI and SphI and the IPTG-controlable expression cassette was replaced with PCR-amplified xylose-controlable expression cassette. The apoptin gene was amplified from the plasmid pHM6-apoptin [14].

B. Construction of the pENTR-apoptin Vectors

In gateway cloning reactions, DNA sequences are flanked by *att*B sites which assist recombination mediated by BP clonase enzyme by two step PCR reactions. The first PCR reaction was performed in 50 µl volume mixture with specific primers (5’ primer : CGAAGGGAGGTGTCATAAATG AACGCTCTCCAAGAAGATACTC; 3’ primer : GATGGTGATGGTGATGCGATCCCAGTCTTATACACCT TCTTGCGG) by PCR cycling conditions of denaturing at 94^{O}C for 15 s, annealing at 62^{O}C for 30 s, and extension at 68^{O}C for 30 s for 5 cycle followed by condition of denaturing at 94^{O}C for 15 s and extension at 68^{O}C for 30 s for 25 cycle. In the second PCR reaction, Apoptin gene was extended with 12 histidine and 8 arginine in C-terminal. The reaction was performed in 10 µl volume mixture with specific primers (5’ primer : GGGGACAAGTTTGTACAAAAAA GCAGGCTCGAAG GGAGGTGTCATAAATG; 3’ primer : GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATCT ATTTAATGGTGATGATGGTGGTGGTGATGGTGATGG TGATGCGATCC for extention 12 histidine only and 3’ primer:
GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATCT ATTTAACGGCGTCGCCGACGGCGTCGCCGATGGTGA TGATGGTGGTGGTGA for 12 histidine and 8 arginin) by PCR cycling conditions of denaturing at 94^{O}C for 15 s, and extension at 68^{O}C for 30 s for 25 cycle. The individual fragments were subjected to recombination reaction with pDONR201, in a 5 µl reaction containing the PCR product, 0.5 µl (150 ng/µl) of pDONR201 vector and 1 µl BP clonase enzyme. The reaction was performed at 25^{O}C overnight and terminated by proteinase K (0.5 µl). The reaction mixture was used to transform competent cells of *E. coli* (DH5α) and transformed colonies were selected on LB agar plates containing kanamycin (50 µg/ml). The modified vectors were confirmed by DNA sequenching and designated as *pENTR-apoptins*..

C. LR Recombination Reaction to Costruct pG-apoptin-12his, pXG-apoptin-12his, pG-apoptin-12his-8arg and pXGW-apoptin-12his-8arg

The resulting recombinant entry vectors were subjected to recombination reaction with modified destination vector pOGW and pOXGW in a 10 µl reaction mix containing of 2 µl of LR clonase enzyme mix (Invitrogen). The reaction was performed at 25^{O}C overnight and terminated by proteinase K (0.5 µl).The recombined product was used to transform *E. coli* (DH5α) and recombinant clones were selected on LB agar plate containing tetracycline (5 µg/mL).

D. Expression the recombinant proteins

B. subtilis and *E. coli* (DH5α) were transformed by pG-apoptin-12his, pXG-apoptin-12his, pG-apoptin-12his-8arg and pXGW-apoptin-12his-8arg respectively and grown to OD600 of 0.6 – 0.8 at 37 ^{O}C with shaking at 200 rpm in 500 ml LB medium supplemented with 10 and 5 µg/mL tetracycline. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration 1 mM for cells transformed by pG-apoptin-12his and pG-apoptin-12his-8arg, for cells tranformed by pXG-apoptin-12his and pXG-apoptin-12his-8arg is added by xylose to a final concentration 1%. The culture has incubated for additional 2 h. Then the cells were harvested by centrifugation and resuspended in buffer A (100 mM Tris-Cl pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM DTT and 20 mM Imidazole) and lysed by sonication in ice water bath. Inclusion bodies was separated by centrifugation at 10,000g for 10 min at 4 ^{O}C. The recombinants apoptin were purified by MagneHis^{TM} protein purification system (Promega) with buffer A as binding and washing buffer and buffer B (100 mM Tris-Cl pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM DTT and 500 mM Imidazole) as elution buffer. The purified recombinant apoptin was confirmed by 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

III. Result and Discussion

A. Construction of Plasmid Apoptin

The expression vectors pG-apoptin-12his, pXG-apoptin-12his, pG-apoptin-12his-8arg and pXGW-apoptin-12his-8arg

constructed by Gateway® cloning system [11]. The gene of apoptin was amplified by PCR from pHM6-apoptin and fused with 12 × histidine tag to the C-terminal. We also fused Apoptin gene with octaarginine and 12 × histidine to C-terminus and inserted them to pDONR201 produced *pENTR*-apoptins (see Fig. 1). Final DNA sequenching of the construction confirmed that the sequenches matched with apoptin, 12 histidine and 8 arginine genes (data not shown). Finally, by using LR reactions the expression plasmids was produced.

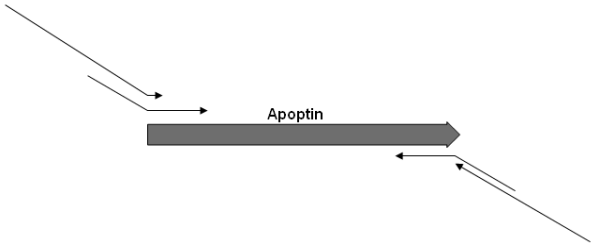

Fig 1. **Schematic illustration of the plasmid construction for the expression of Apoptin.** pHM6-apoptin was amplified by two step PCR. The first PCR the primers contain annealing region with apoptin gene, the second PCR extend the first fragments PCR with *att*B1 and *att*B2, see material and method for detail description

B. Expression and Purification of Recombinant Apoptin

To study the constructed expression plasmids, first, we transformed pG-apoptin-12his into the *E. coli* strain DH5α and selected on LB plates containing tetracycline. The selected colony were cultured in 1 litre LB medium, induced by 1 mM IPTG and treated as described above. Previously reported results showed that recombinant protein apoptin produced inclusion bodies. Suprisingly, we found that the apoptin fused with 12×His tag to C-terminal produced in supernatant on lysate buffer without refolding processes. The protein was efficiently purified by immobilized metal ion-affinity chromatography (Ni^{2+}-chelating) column. This construction of pG-apoptin-12his should produce a recombinat protein of approximately 16 kDa (about 14 kDa of apoptin and 2 kDa of 12 histidine). As shown in Fig. 2A, the band approximately 36 kDa shipted up with the predicted full-length fusion protein of apoptin-12×his. This phenomena occasionally occur in polyhistidine linked recombinant protein [15]. Second, we also transformed pG-apoptin-12his-8Arg into the *E. coli* cells, and the recombinat protein was produced in soluble form and SDS-PAGE analysis shows the band shipted up with the prediction (Fig. 2B).

To produce apoptin in large scale, use of cheaper inducer than IPTG would be realistic for application purpose. Thus, we constructed a new destination vector, pOXGW, which can be converted to expression vector in which cloned gene can be induced by xylose. To construct pOXGW, xylose-controlable expression cassette containing xylA promoter and xylR (repressor for xylA promoter) derived from *Bacilllus megaterium* was amplified from pX plasmid with xylR-F-XhoI and xylR-R-SphI primers, which contain XhoI and SphI sites at the 5' ends respectively [12]. Then, we inserted apoptin gene to it, which produced pXG-apoptin-12his and pXG-apoptin-12his-8arg. For confirming our constructed plasmid, we transformed pXG-apoptin-12his-8arg into *B. subtilis* cells and cultured in 1 litre LB medium, induced by xylose and treated as described above. The results shows that 1% xylose could control expression system in *B. subtilis*, and produce soluble apoptin similar with *E. coli* IPTG-induced expression system. SDS-PAGE analysis also shows the band shipted up with the prediction Fig. 3.

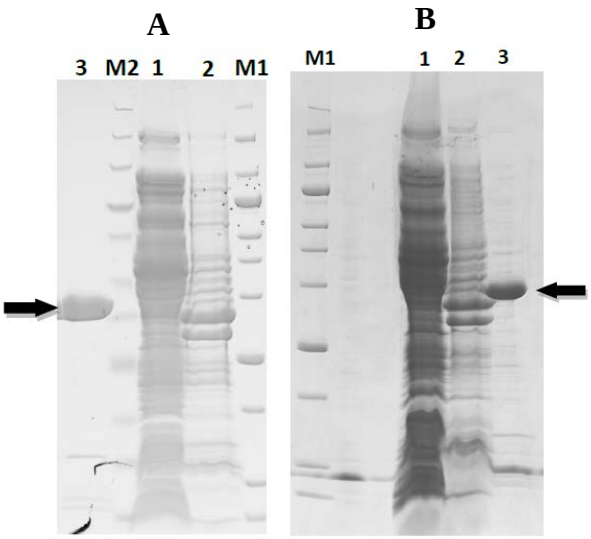

Fig 2. **SDS-PAGE analysis of the expression of apoptin-12×His (a) and apoptin-12×His-8×Arg (b) in *E. coli*.** M1: Molecular mass marker 1 (from bottom to top: 10, 15, 20, 30, 40, 60, 80,100, 150, 200 kDa); M2: Molecular mass marker 2 (from bottom to top: 8, 15, 18, 23, 31, 36, 44, 55, 75, 105, 150 kDa); Lane 1: supernatant of cell lysate; Lane 2: pellet of cell lysate; Lane 3: eluted fraction containing apoptin-12×His (a) and apoptin-12×His-8×Arg (b). The arrow indicates the band corresponding to the Apoptin.

In this work we presented for the first time the soluble recombinant apoptins expressed in *E. coli* and *B. subtilis*, without resuspending and refolding processes. The results are different from other resulting that fused apoptin with six histidine, MBP, TAT, PTD4 and carcinoembryonic antigen, fusing apoptin with 12 histidine improve the solubility of apoptin in buffer. Furthermore, the expression system we developed is efficient because of the xylose-dependent system and one step purification.

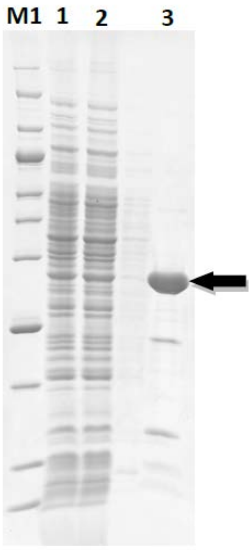

Fig 3. **SDS-PAGE analysis of the expression of apoptin-12×His-8×Arg (b) in *B. subtilis.*** M: Molecular mass marker (from bottom to top: 10, 15, 20, 30, 40, 60, 80,100, 150, 200 kDa); Lane 1 and 2: supernatant of cell lysate; Lane 3: eluted fraction containing apoptin-12×His-8×Arg. The arrow indicates the band corresponding to the Apoptin

IV. Conclusion

A conclusion this soluble recombinant apoptins produced in the present work, open the way to confirm biophysical, biochemical characterization and study detail mechanism of apoptin inducing-apoptosis, *in vitro*. .

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