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Original Article

THE EXPRESSION AND PURIFICATION OF OCTA-ARGININE APOPTIN AND ITS ABILITY TO KILL CANCER CELLS

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ABSTRACT

Objective: In this research, *chicken anemia virus* apoptin optimized genetically for expression in *Escherichia coli* and also modified using $(His)_6$ tag, $(Arg)_8$ tag, and HlyA tag intended for purification needs, penetration enhancement, and secretion from bacterial host to the growth media.

Methods: The modified apoptin gene was optimized using an Integrated DNA Technology (IDT). The gene (606 bp) then ordered and synthesized by Eurofins. The apoptin gene was expressed using *E. coli* BL21 CodonPlus as host, in cultivation temperature of 37 °C, and 25 °C and purified using Ni-NTA agarose beads. The addition of $(His)_6$ tag enabled the apoptin to be purified in only one step by using nickel column. The expression and purification data analyzed qualitatively as well as quantitatively using SDS-PAGE. MTT assay was used to identify the antitumor effect of octa arginine-apoptin to two kinds of cancer cells, cervix HeLa cancer cell and colon Widr cancer cell. The viability of cell was analyzed when the cell incubated in the variation concentration protein for 72 h.

Results: The constructed apoptin gene were expressed in *E. coli* successfully. The MTT assay indicated that Octaarginin-Apoptin was able to induce apoptosis of HeLa and Widr cells lines in a dose-dependent manner. The recombinant apoptin without tagging with octa-arginine, have no ability to induce apoptosis of HeLa and Widr cells lines.

Conclusion: This octa arginine-apoptin may in the future allow the development of a therapeutic protein that is able to kill cancer cells specifically.

Keywords: *Recombinant apoptin,* Escherichia coli, *Anti- cancer*

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INTRODUCTION

Cancer is a term usually used to represent a group of diseases which affect various parts of the body. According to WHO, cancer is defined as the production of abnormal cells which is able to invade another part of the body and spread to other organs. This process is called metastasis, the largest cause of death by cancer. Based on WHO statistical data, cancer is one of the most deadly diseases in the world, with 8, 2 millions of deaths out of 14 million of cases in 2012. More specifically in South East Asia, WHO data showed 1, 72 million cases with 1, 17 million of deaths. As a developing country, Indonesia also has to face cancer problem. In Indonesia, cancer is ranked as the sixth most fatal disease. The most common cancer case in Indonesia are cervical and breast cancer where about 170–190 new cases are predicted to be found every 100,000 people [1].

A lot of methods have been developed to treat cancer. Generally, a treatment method for cancer can be classified into 6 groups, including chemotherapy, immunotherapy, radiotherapy, targeted therapy and transplantation. The most common cancer treatments in Indonesia consist of radiotherapy (70%), medical operation (20-25%) and chemotherapy (5-10%) [1]. According to National Cancer Institute, National Institute of Health United States, radiotherapy treatment uses high-energy radiation to kill cancer cells. Unfortunately, this method does not target cancer cells specifically. In turn, it would also harm normal cells and cause several side effects such as fibrosis, memory loss, and impotency.

In order to overcome those problems, cancer treatment which specifically target cancer cells and leave the normal cells unharmed is being developed. One of the potential candidates is the use of apoptin as a therapeutic protein. Apoptin consist of 121 amino acids and is known to be able to induce apoptosis specifically in cancer cells [2]. This mechanism is related to the specific kinase which is only available in cancer cells [2]. Apoptin production in bacterial host has been conducted by using various modifications, such as $(His)_{12}$ tag and $(Arg)_8$ tag [3]. The presence of related tags enables the protein purification process using chromatography column to be done in an easier way. On the other hand, the presence of related tags might also affect bioactivity of apoptin itself. It has been known that $(his)_6$ tag at C-terminus of heparin cofactor increases protein activity [3]. On the other hand, $(his)_{10}$ and $(his)_7$ at N-terminus of tumor necrosis factor alpha decrease its activity drastically [4]. Hence, tag removal is considered necessary after protein purification in order to maintain the protein activity.

Regardless of its modification, protein expression in a bacterial host might fail due to the differences of protein expression system between the original host and the new bacterial host. One of the approaches to increase protein expression in a bacterial host is codon optimization. Codon optimization has been successfully done to increase the expression level of halohydrin dehalogenase from *Agrobacterium radiobacter* in *E. coli* [5].

In this research, apoptin was modified in order to secrete the protein of interest, enhance its affinity for nickel chromatography column, and optimize the codon to be expressed in *Escherichia coli*. The recombinant protein was monitored their ability to kill cancer cells by MTT assay to HeLa and Widr Cells.

MATERIALS AND METHODS

Gene construction

The information of apoptin gene was taken from gene bank with code AY171617.1. Some tags added into the apoptin sequence to gain protein. The modified apoptin gene was optimized using an online application from integrated DNA technology (IDT) accessed at http://sg.idtdna.com/CodonOpt. The gene (606 bp) then ordered and

produced synthetically by Euro fins. The gene of interest placed inside pTAKN2 (3345 bp) and then moved from that plasmid into a new vector chosen, pET9a (4341 bp). The cut and ligate method used by NdeI and BamHI sites. The gene construction confirmed by agarose electrophoresis 0, 8 % (w/v) and DNA sequencing. DNA Sequencing primer was designed using Gene Runner.

Gene expression

The gene which has been constructed then transformed into and expressed using *Escherichia coli*. The expression is done using temperature variation (25 °C, 37 °C), and host cell variation (*E. coli* DH5a, BL21 pLysS, BL21 CP). The expression was done using Terrific Broth Hi-Media for 4 hours of cultivation and 4 hours of induction using IPTG 1 mmol.

Protein analysis

The protein analyzed using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Gel concentration used are 15%. The protein from supernatant fraction of the cell culture precipitated by acetone precipitation method [6]. 250 µl of the supernatant mixed with 1 ml cold acetone, incubated for 2 h in-20 °C then centrifuged 12,000 rpm for 10 min. The pellets suspended in 1 time of phosphate saline buffer.

Detection of the inhibition of HeLa and widr cell by the MTT assay

The MTT method was used to study the effect of Apoptin 8 Arg on the growth of cancer cells [7]. HeLa and Widr cells were used for this assay. The viability of cell was analyzed when the cells incubated in the variation concentration protein for 48 h for HeLa cell and 72 h for Widr cell.

RESULTS AND DISCUSSION

Gene construction

The gene construction confirmed using agarose gel electrophoresis and the result shown in fig. 1. The sequencing result of the gene constructed gave an exactly identic apoptin gene sequence as ordered to Eurofins. There are 3 N (could be any bases) read in the apoptin gene from Apoptin_R primer, confirmed to be the correct base by the reading from T7 Fw primer.

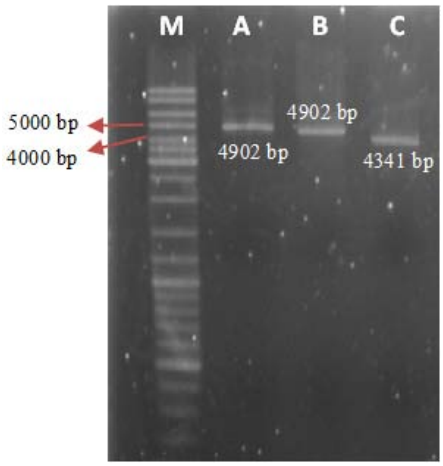

Fig. 1: Isolated plasmids were digested by restriction enzyme BamHI of isolated DNA. M refers to Marker, A and B refer to *E. coli* DH5α and as host of the plasmid, respectively, C refers to plasmid of pET9a. red arrow showed the molecular weight of the marker

Gene expression and protein analysis

The gene expression gave protein samples. The SDS-PAGE result for *E. coli* BL21 pLysS and DH5a cell pellets fraction are not showing overexpression of modified apoptin (Data not shown). On the other hand, expression in *E. coli* BL21 CP shows an overexpression around 20 kDa as shown in fig. 2. Overexpression shown both in empty vector and inserted vector. The purification of the protein from 500 µl lysates done using Ni-NTA agarose beads shown in fig. 3 and fig. 4. Purified protein seen in SDS-PAGE of apoptin lysate, but not seen in pET9a lysate.

The expression also includes the supernatant fraction. If the protein designed successful, the protein should be secreted into the medium because of the HlyA tag. The precipitated protein from supernatant fraction analyzed by SDS-PAGE. Unfortunately, the supernatant fraction do not show a significant protein band around 20 kDa.

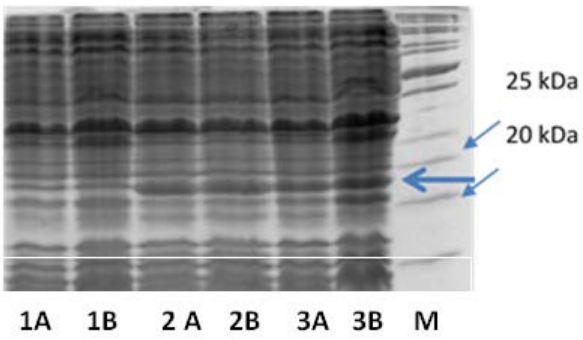

Fig. 2: SDS PAGE of *E. coli* strain BL21 CP pellets non-induced and induced by 1 mmol IPTG 1 mmol before (A) and after (B) induction. 1 refers to E. coli BL21 CP only, 2 refer to transformed by pET9a, and 3 refer to transformed of B by pET9a contained the Apoptin gene. M refer to marker; blue arrow showed the expressed of apoptin

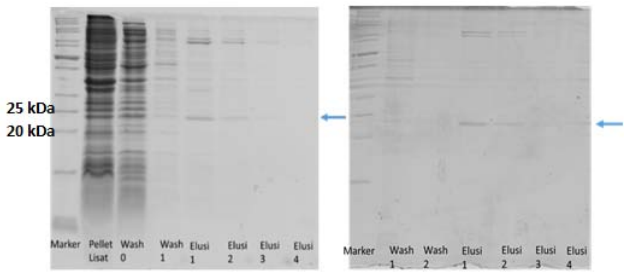

Fig. 3: SDS-PAGE of *E. coli* BL21 CP purification result (left) and BL21 pLysS (right). Blue red shown the molecular weight of apoptin

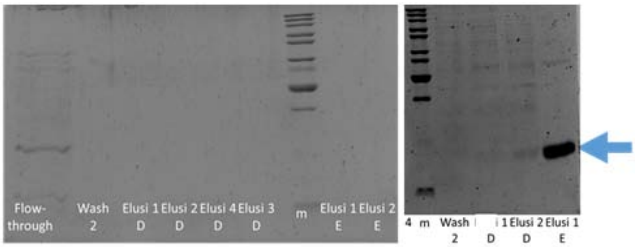

Fig. 4: Protein purification in denaturing condition of *E. coli* BL21 CP contained pET9a (left) and pET9a-apo (right)

The purification result shows that his-tagged apoptin successfully expressed. However, it's not significantly overexpressed in the cells. This might happen due to the secretion of protein accompanied by HlyA-tag or not optimal expression condition. The expression result shows that even without overexpression, his-tagged apoptin can be purified from BL21 pLysS lysate. Compared with BL21 pLysS, the purification and expression result of apoptin in BL21 CP is qualitatively better. On the other hand, a different result was reported by Lee *et. al* which shown that expression of modified apoptin in BL21 pLysS is better than BL21 CP [8]. This might happen due to different DNA construction for protein expression. Compared with that construction,

the current research DNA construction uses His-tag instead of GST tag which is intensively used for large scale protein purification. Furthermore, the current construction also includes cell penetrating peptide poly-arginine-tag and secretion signaling peptide HlyA-tag which separated by Thrombin site for tag removal. Those tags not only increase the penetrating ability of apoptin, but also its purification. However, further comparison needs further analysis, such as western blotting and Bradford assay for quantitative analysis.

Growth inhibition effect of apoptin 8 Arg on the HeLa and widr cell lines

Following incubation with different concentration of Apoptin 8 Arg for 48 or 72 h, the proliferation of HeLa and Widr Cell were significantly inhibited in a concentration-dependent manner (see fig. 5 and fig. 6), the apoptin without a tag was used as a control. In this study, we conducted an experiment on *in vitro* cultured HeLa and Widr cells this experiment was also used to confirm apoptin activity for other cell lines such as EJ cells. Our results on HeLa and Widr cells confirmed similarly with Apoptin which tagged by TAT. Following treatment with Apoptin 8 Arg for 1 µg/ml had significantly effect on the HeLa cell and Widr cell [9].

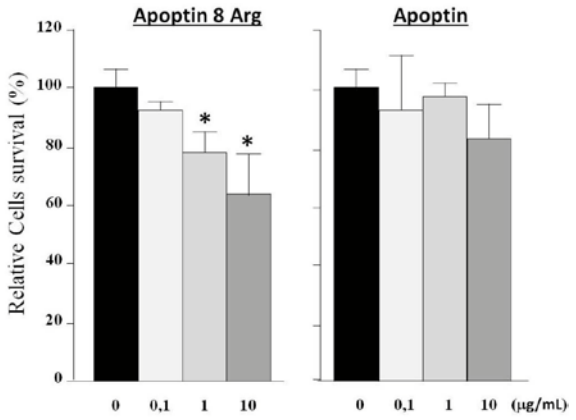

Fig. 5: Growth inhibition of HeLa cells treated with Apoptin 8 Arg and its control at different concentrations. *P<0.05 compared with apoptin group

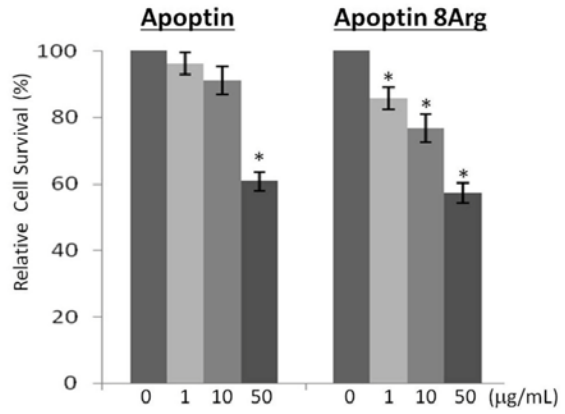

Fig. 6: Growth inhibition of widr cells treated with apoptin 8 Arg and its control at different concentrations. *P<0.05 compared with apoptin group

Apoptin was confirmed induces tumor cell-specific cell death and a potential future anti-cancer therapeutic. Several strategies were developed, based on gene therapy or protein therapy. Gene therapy approach more developed compares with protein therapy that virus and cell is the common vectors [10,11]. Unfortunately, therapy that using protein are limited, it's because of the cost of expression and purification of protein are high compared with gene therapy.

Our study showed new strategy to expressed extracellular protein, by tagged the apoptin by HlyA the protein could exported to extracellular. To increase the protein expression in *E. coli* we optimized and synthesized apoptin gene. The apoptin was well expressed in *E. coli*, and have similar activity compared with original gene. This extracellular protein opens the continuous method to produce protein more cheap compare batch method.

CONCLUSION

Gene construction of modified apoptin has been done and confirmed by sequencing. Modified apoptin obtained and overexpressed on the BL21 CP cells. However, the apoptin is not obtained as an overexpressed protein in *E. coli* BL21 pLysS and DH5a cells pellet and a supernatant fraction. On the other hand, the possibility of Apoptin overexpression in media fraction still can be analyzed using a higher concentration of media precipitated.

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CONFLICTS OF INTERESTS

Declared none

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