Construction, Cloning, and Overexpression of Staphylococcal Enterotoxin B Gene Synthetic (SEBsyn) in pET-28a(+): Pre-development Bacterial-Toxin Therapy for Cancer

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ABSTRACT
Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* could be considered as a therapeutic agent to eliminate cancer cells. SEB can activate immune response, which furthermore could induce apoptosis of various cancer cells. This study was proposed to design a SEBsyn coding sequence suitable for *Escherichia coli* BL21(DE3) expression system for recombinant protein production. This research was conducted by designing the inserted gene that included codon optimization. The obtained synthetic gene was transformed into *E. coli* BL21(DE3) and the transformants were verified by PCR with T7 promoter and T7 terminator primers. The results showed that the SEBsyn encoding gene could be synthesized and cloned into pET-28a(+). This recombinant plasmid that carried the SEB encoding gene (pET-28a_SEBsyn) was successfully transformed into *E. coli* BL21(DE3). Amplicon of colony PCR visualized in 2% agarose gel showed that transformants carrying the recombinant plasmid had an inserted gene length about 1,000 bp. In contrast, the amplicon from colonies that carries control plasmid (pET-28a(+) without insert) had a gene length about 300 bp. The protein verification by SDS-PAGE also confirmed that the plasmid carrying the gene insert was successfully overexpressed in *E. coli* BL21(DE3).

Keywords: *Bacterial toxin, Cancer, pET-28a(+), SEB, Staphylococcus aureus.*

1. INTRODUCTION

Cancer is a group of diseases resulting from abnormal cells growth. The mechanisms of cancer development are very complex. Generally, it comes from cell cycle gene mutations, carcinogenic compound exposure, and unhealthy lifestyles [1]. Recently, data from the global cancer observatory (Globocan) reported that in 2020 the incidence of cancer worldwide reached 19.3 million cases with 10.0 million cancer deaths [2]. In Indonesia, the case of cancers in both sexes and all ages reached 396,914, with a death toll of 234,511 people from a total population of 273,523,621 people. Breast cancer has the highest incidence and mortality of 44.0% and 15.3%, respectively [3].

The high prevalence of cancer could be overcome with effective treatment to prolong the patient's life. Current cancer treatments seem to be significantly harmful for patients, like chemotherapy that also attacks healthy cells and sometimes triggers metastasis event [4], [5]. Bacterial-toxin therapy is currently developed for cancer treatment because it eliminates cancer cells by several mechanisms, which could induce apoptosis. Several studies are using bacterial toxins for the development of cancer therapy. For instance, azurin could induce apoptosis in osteosarcoma U2OS cells [6], and other toxins from various species had been proven to be able to kill cancer cells [7].

Staphylococcal enterotoxin B produced by *Staphylococcus aureus* could induce apoptosis, which is essential for cancer therapy development [8]. This

toxin can activate the immune system followed by secretion of apoptosis signals, like TNF-α and Fas Ligand [9], [10]. SEB has an ideal size for a recombinant protein, and it is easily expressed in bacteria. The availability of recombinant DNA technology combined with massive developments in bioinformatics can be used for protein engineering to increase the sensitivity of SEB proteins in target cells. Recombinant DNA technology has contributed to medicine-related fields through the production of pharmaceutical proteins (biopharmaceuticals) and therapeutic proteins [11]. The production of SEB recombinant protein would be an essential step in the development of cancer therapy using bacterial-toxin to kill cancer cells.

2. METHODS

2.1 Vector construction and gene synthesis

The commercial plasmid, namely pET-28a(+) from Novagen (cat: 69864-4), was the cloning and expression vector used in this study. SEB encoding sequence deposited in NCBI with acc. id M11118.1 was downloaded with *.fasta* format. The virtual recombinant plasmid was constructed with SnapGene 1.1.3 Software. This SEB gene was edited virtually by adding restriction sites NdeI at the 3' and XhoI at the 5', followed by cloning simulation into pET-28a(+) at the NdeI and XhoI cloning sites. Next, the final construct was exported into a *.jpg* file. Before the inserted gene was synthesized, it was optimized firstly with codon optimization tools present in Synbio technologies gene synthesis services (https://www.synbio-tech.com/gene-synthesis/).

2.2 Competent cells preparation and Transformation

E. coli BL21(DE3) was used as an expression machine in this study. Bacterial cells from the glycerol stock were cultured in Luria Bertani (LB) agar by streak plate method to obtain single colonies. The obtained single colony was cultured overnight in a shaker incubator at 37◦C. On the next day, the cells were harvested by centrifugation at 4000 rpm, 4◦C for 10 minutes. The pellets were resuspended with cold-$CaCl_2$ 0.1 M then placed in ice for 30 minutes. The suspension treated with cold-$CaCl_2$ was centrifuged at 4000 rpm, 4◦C for 10 minutes. The pellets were resuspended with cold-$CaCl_2$ + 15% Glycerol. The competent cells were stored in a -80◦C freezer before the transformation.

The heat-shock method was used for cell transformation. The competent cells were incubated first in ice for 20 minutes. Plasmids pET28a(+) (500 ng/µL, 3 µL) and recombinant pET28a_SEBsyn (500 ng/µL, 3 µL) were added into a tube containing competent cells. These suspensions were incubated on ice for 30 minutes. Heat shock treatment was conducted in 42◦C water bath for 90 seconds, followed by an ice bath for five minutes. The LB broth (850 µL) was added to the transformation mixture and the mixture was incubated in a shaker incubator at 200 rpm for 1.5 hours. After incubation, the transformation mixture was centrifuged at 14,000 rpm for two minutes at room temperature and supernatant was discarded. The pellets containing 50 uL of LB broth supernatant were resuspended by pipetting then the cells were spread onto LB agar + 50 mg/mL kanamycin. The plate containing transformants was incubated at 37◦C overnight.

2.3 Verification of transformants

Amplification of the cloning region that carried the inserted gene was performed by colony PCR using primer T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') were used for amplification the cloning region that carries the inserted gene. The PCR reaction was conducted with Ez PCR 5x mastermix from miniPCR (cat: RG-1000-01). The PCR reaction was set as follow: Initial denaturation at 95◦C for 3 minutes, 30 cycles of denaturation (95◦C for 45 seconds) - annealing (55◦C for 30 seconds) – (elongation 72◦C for 45 seconds), then final elongation at 72◦C for 5 minutes and hold at 4◦C. The amplicon was checked on 2% agarose (cat: R0491) gel electrophoresis with a running program of 70 volts for 40 minutes with 100 bp gene marker from miniPCR (RG-1001-01). This gel was stained with ethidium bromide then visualized with a UV-transilluminator.

2.4 Protein expression

The positive transformants, namely pET-28a_SEBsyn T1 and pET-28a_SEBsyn T2 were induced expression with isopropyl β-D-1-thiogalactopyranoside (IPTG). First, the selected transformants were pre-cultured in 1 mL LB broth + 50 mg/mL kanamycin and they were incubated in shaker at 37◦C, 200 rpm for overnight. On the next day, approximately 0.5 mL of the pre-culture transformants were cultured in 4.5 mL LB broth + 50 mg/mL kanamycin. Those cultures were incubated approximately for 2-3 h to reach optical density about 0.6-0.8. These bacterial cultures were then induced expression by IPTG from Thermofisher (Cat: R1171) with final concentration at 0.1 mM. Then, they were incubated in shaker at 37◦C, 200 rpm for overnight.

2.5 Target protein verification by SDS-PAGE

The induced cultures were then centrifuged to obtain pellets with two replications. The obtained pellets were resuspended with protease inhibitor buffer (50 mM Tris-Cl pH 7.4, and phenylmethanesulfonyl fluoride or PMFS 100mF) followed by centrifugation 6000 rpm at 4◦C for 5 min, the supernatants were discarded. The pellets from this step could be stored in -80◦C freezer. The pellets from first replication were resuspended with lysis buffer containing 50 mM Tris-Cl pH 7.4, and Triton X-100. These pellets treated with lysis buffer were used to detect target protein in the form of soluble or insoluble fraction. The freezing thawing method with 10 times were used to treat both pellets that have been treated in the previous step. The suspensions after freezing thawing were centrifuged at 12000 rpm, 4◦C for 15 min. The supernatants were collected to detect target protein in the soluble fraction. However, the pellets, as the form of in-soluble protein fraction were washed with washing-solubilization buffer (urea, glycine, and β-mercaptoethanol) for three times following by the centrifugation at 12,000 rpm, 4◦C for 15 min, supernatant discarded.

The supernatants from lysis step and the pellets after washing were mixed with sample buffer SDS 2X (4xTris-Cl/SDS pH 6.8, 20% glycerol, 4% SDS, 0.2% β-mercaptoethanol, 0.001% bromphenol blue) for protein analysis with SDS-PAGE. Approximately 20 uL of the mixed sample was used for SDS-PAGE with separating gel 12% and stacking gel 5%. The PageRuler^{TM} Prestained Protein Ladder 10 to 180 kDa from Thermofisher (Cat: 26617) was used in the SDS-PAGE. This gel was run in the electrophoresis SDS-PAGE chamber with 120 volts for 90 min. The gel was stained with Coomassie blue G-250 staining solution for 15 min, then this gel was distained with hot water for 15 min in microwave.

3. RESULTS AND DISCUSSION

The construction of pET-28a_SEBsyn with SnapGene software is shown in Figure 1. The cloning site used in this construct was NdeI which carried a start codon, and XhoI. pET plasmid series, including pET-28a(+), are commercial plasmids widely used for recombinant protein production in *E. coli*. The inserted gene that are cloned into pET-28a(+) are controlled by bacteriophage T7 promoter, and its transcription is performed by T7 RNA polymerase present in the host cell [12], [13]. A hexa histidine tag (6xHis-Tag) is present in this vector to facilitate protein purification with standard protocols like the Ni-NTA chromatography column. The features of this vector include kanamycin-resistance gene, 6xHis-Tag at the C-terminal and N-terminal, and thrombin protease cleavage site. Protein production by the host cell when using the expression vector pET-28a(+) could be improved by restoring the conserved T7 promoter and translation initiation region (TIR) 1 or TIR-2 synthetically to avoid design flaws caused by ad hoc genetic fusion [14].

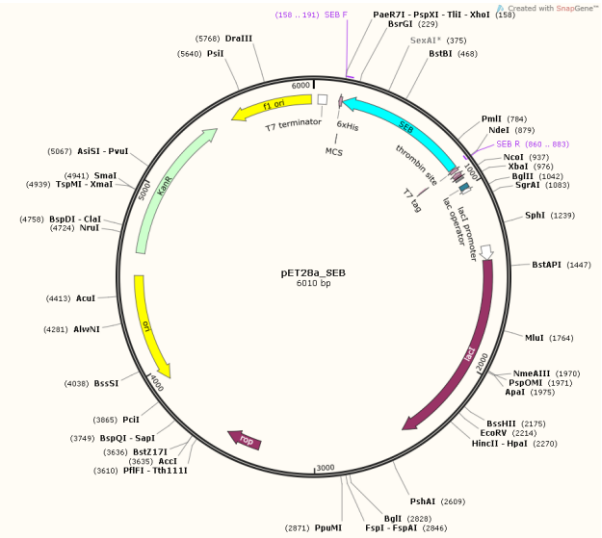

Figure 1. Vector design of pET-28a(+) inserted with SEBsyn.

Sequence alignment to compare the wildtype SEB encoding gene with the optimized codon of SEBsyn for *E. coli* host is shown in figure 2. This optimized codon was proposed to increase the production level of the protein of interest. This method generally replaced the rare codons in the target gene with commonly used codon in the host without modifying the target protein [15]. This codon usage is a critical parameter to achieve adequate protein expression levels in *E. coli* [16]. This technique had been proven to improve the production of various recombinant proteins expressed by *E. coli* [17]–[19].

E. coli BL21(DE3) was used as a host for the host expression because this strain contains DNA that carries the gene for T7 RNA polymerase [20], [21]. This bacterial strain is widely used in various research related to recombinant protein production. Our results showed that the transformation of wildtype pET-28a(+) and pET-28a_SEBsyn was successfully transformed into *E. coli* BL21(D3). The presence of colonies that grew in LB medium containing 50 mg/mL kanamycin was indicated the successful of transformation. The total number of transformants carrying pET-28a(+) was more than 300 single colonies (Figure 3a), transformants carrying pET-28a_SEBsyn was 81 colonies (Figure 3b), the plate control as a negative control had no colony (Figure 3c),

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                                 10        20        30        40        50        60        70        80        90       100
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    AGTCAACCAGATCCTAAACCAGATGAGTTGCACAAATCGAGTAAATTCACTGGTTTGATGGAAAATATGAAAGTTTTGTATGATGATAATCATGTATCAG
                          S  Q  P  D  P  K  P  D  E  L  H  K  S  S  K  F  T  G  L  M  E  N  M  K  V  L  Y  D  D  N  H  V  S
Optimized SEB            AGTCAACCGGATCCGAAACCGGACGAACTGCACAAAAGCAGCAAGTTCACCGGTCTGATGGAGAACATGAAGGTCCTGTACGACGACAACCACGTCAGCG
                          S  Q  P  D  P  K  P  D  E  L  H  K  S  S  K  F  T  G  L  M  E  N  M  K  V  L  Y  D  D  N  H  V  S
                                 110       120       130       140       150       160       170       180       190       200
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    CAATAAACGTTAAATCTATAGATCAATTTCTATACTTTGACTTAATATATTCTATTAAGGACACTAAGTTAGGGAATTATGATAATGTTCGAGTCGAATT
                         A  I  N  V  K  S  I  D  Q  F  L  Y  F  D  L  I  Y  S  I  K  D  T  K  L  G  N  Y  D  N  V  R  V  E  F
Optimized SEB            CGATTAACGTCAAAAGCATCGACCAGTTCCTGTACTTCGACCTGATCTACAGCATCAAGGACACCAAGCTGGGTAACTACGACAACGTCCGCGTCGAATT
                         A  I  N  V  K  S  I  D  Q  F  L  Y  F  D  L  I  Y  S  I  K  D  T  K  L  G  N  Y  D  N  V  R  V  E  F
                                 210       220       230       240       250       260       270       280       290       300
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    TAAAAACAAAGATTTAGCTGATAAATACAAAGATAAATACGTAGATGTGTTTGGAGCTAATTATTATTATCAATGTTATTTTTCTAAAAAAACGAATGAT
                           K  N  K  D  L  A  D  K  Y  K  D  K  Y  V  D  V  F  G  A  N  Y  Y  Y  Q  C  Y  F  S  K  K  T  N  D
Optimized SEB            CAAGAACAAGGACCTGGCGGACAAGTACAAAGACAAGTACGTGGACGTCTTTGGCGCGAACTATTACTACCAGTGCTACTTCAGCAAGAAGACCAACGAC
                           K  N  K  D  L  A  D  K  Y  K  D  K  Y  V  D  V  F  G  A  N  Y  Y  Y  Q  C  Y  F  S  K  K  T  N  D
                                 310       320       330       340       350       360       370       380       390       400
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    ATTAATTCGCATCAAACTGACAAACGAAAAACTTGTATGTATGGTGGTGTAACTGAGCATAATGGAAACCAATTAGATAAATATAGAAGTATTACTGTTC
                          I  N  S  H  Q  T  D  K  R  K  T  C  M  Y  G  G  V  T  E  H  N  G  N  Q  L  D  K  Y  R  S  I  T  V
Optimized SEB            ATCAACAGCCACCAGACCGATAAGCGCAAAACCTGTATGTACGGCGGCGTAACCGAACATAACGGTAACCAGCTGGACAAATATCGCAGCATCACCGTTC
                          I  N  S  H  Q  T  D  K  R  K  T  C  M  Y  G  G  V  T  E  H  N  G  N  Q  L  D  K  Y  R  S  I  T  V
                                 410       420       430       440       450       460       470       480       490       500
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    GGGTATTTGAAGATGGTAAAAATTTATTATCTTTTGACGTACAAACTAATAAGAAAAAGGTGACTGCTCAAGAATTAGATTACCTAACTCGTCACTATTT
                         R  V  F  E  D  G  K  N  L  L  S  F  D  V  Q  T  N  K  K  K  V  T  A  Q  E  L  D  Y  L  T  R  H  Y  L
Optimized SEB            GCGTTTTCGAAGACGGCAAAAACCTGCTGAGCTTTGACGTTCAGACCAACAAGAAGAAAGTCACCGCGCAGGAACTGGATTATCTGACCCGTCACTACCT
                         R  V  F  E  D  G  K  N  L  L  S  F  D  V  Q  T  N  K  K  K  V  T  A  Q  E  L  D  Y  L  T  R  H  Y  L
                                 510       520       530       540       550       560       570       580       590       600
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    GGTGAAAAATAAAAAACTCTATGAATTTAACAACTCGCCTTATGAAACGGGATATATTAAATTTATAGAAAATGAGAATAGCTTTTGGTATGACATGATG
                           V  K  N  K  K  L  Y  E  F  N  N  S  P  Y  E  T  G  Y  I  K  F  I  E  N  E  N  S  F  W  Y  D  M  M
Optimized SEB            GGTCAAGAACAAGAAGCTGTACGAGTTCAACAACAGCCCGTACGAAACCGGCTACATCAAGTTCATCGAGAACGAGAACAGCTTCTGGTACGATATGATG
                           V  K  N  K  K  L  Y  E  F  N  N  S  P  Y  E  T  G  Y  I  K  F  I  E  N  E  N  S  F  W  Y  D  M  M
                                 610       620       630       640       650       660       670       680       690       700
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
M11118.1 SEB Wildtype    CCTGCACCAGGAGATAAATTTGACCAATCTAAATATTTAATGATGTACAATGACAATAAAATGGTTGATTCTAAAGATGTGAAGATTGAAGTTTATCTTA
                          P  A  P  G  D  K  F  D  Q  S  K  Y  L  M  M  Y  N  D  N  K  M  V  D  S  K  D  V  K  I  E  V  Y  L
Optimized SEB            CCGGCACCGGGCGATAAATTTGATCAGTCTAAGTACCTGATGATGTACAACGACAACAAGATGGTCGACAGCAAGGACGTCAAGATCGAAGTCTACCTGA
                          P  A  P  G  D  K  F  D  Q  S  K  Y  L  M  M  Y  N  D  N  K  M  V  D  S  K  D  V  K  I  E  V  Y  L
                                 710       720
                         ....|....|....|....|
M11118.1 SEB Wildtype    CGACAAAGAAAAAGTGAAAT
                         T  T  K  K  K  *  N
Optimized SEB            CCACCAAGAAGAAGCTCGAG
                         T  T  K  K  K  L  E
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Figure 2. Alignment of wild type SEB with SEBsyn. There is no variation in the protein sequence. Amino acid L and E in carboxyl end is due to the presence of the XhoI restriction site.

and the control without kanamycin showed that the competent cells without plasmids could grow well (Figure 4d). The final length of pET-28a_SEB was about of 6,010 bp, while pET-28a(+) had a length of about 5,369 bp. The difference in the number of transformants between pET-28a(+) and pET-28a_SEBsyn was probably due to the difference size of the plasmids. Studies reported that increasing plasmid size would decrease the transformation efficiency [22], [23].

Colony PCR is a fast approach to determine the presence of the gene of interest in the host. For bacteria, this is a simple method where the colony that contains the template DNA is added to the PCR mastermix [24], [25]. Colony PCR to verify the presence of the inserted gene amplified the region between the T7 promoter and the T7 terminator, which covered the gene for SEBsyn in the recombinant pET-28a(+). The correct amplicon size was about 300 bp for colonies carrying pET-28a(+). In

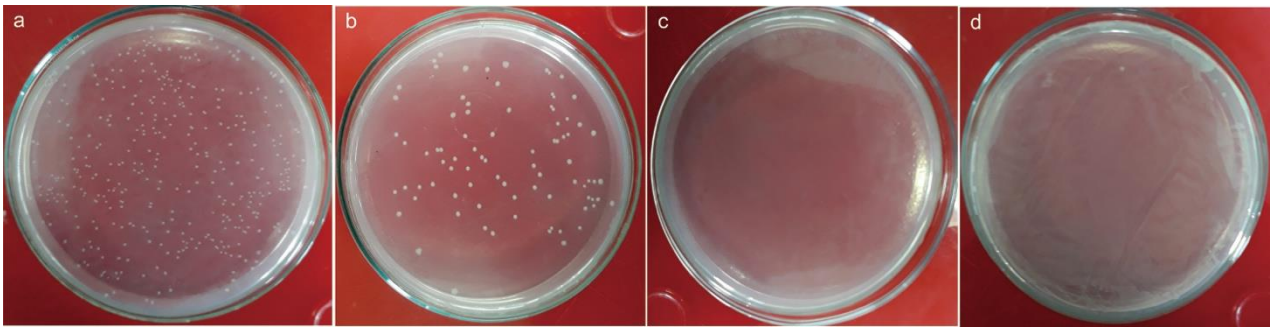

Figure 3. Colonies growing on LB agar + kanamycin after transformation. a) transformants carrying pET-28a(+), b) transformants carrying pET-28a_SEBsyn, c) control (competent cells), d) control (competent cells on LB agar without kanamycin.

contrast, colonies with pET28a_SEBsyn produced an amplicon size about 1,000 bp.

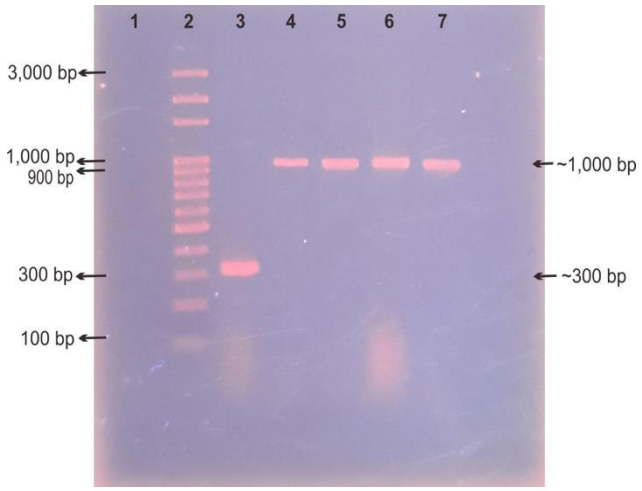

Figure 4. Electropherogram of amplicons from colony PCR of transformants visualized in 2% agarose gel. Lane 1: No template control (NTC), lane 2: Marker 100 bp, lane 3: pET-28a(+) transformants, lane 4-7: Recombinant transformants (T) number 1-4.

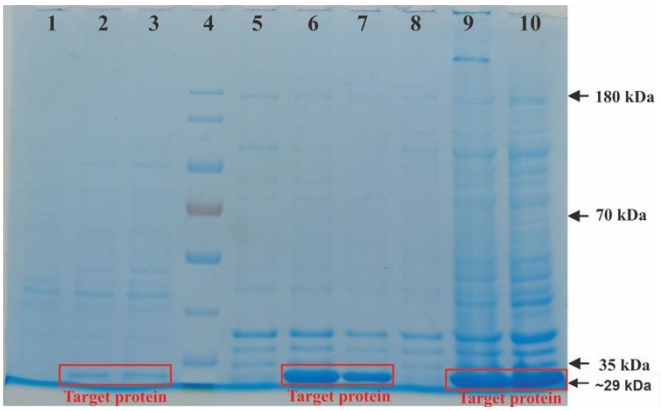

Figure 5. SDS-PAGE of induced transformants. Lane 1: pET-28a(+) (supernatant), 2: pET-28a_SEBsyn T1(supernatant), 3: pET-28a_SEBsyn T2(supernatant), 4: Protein ladder 15-180 kDa, 5: pET-28a(+) (pellet), 6: pET-28a_SEBsyn T1(pellet), 7: pET-28a_SEBsyn T2(pellet), 8: pET-28a(+) (crude cell), 9: pET-28a_SEBsyn T1(crude cell), 10: pET-28a_SEBsyn T2 (crude cell).

The *E. coli* BL21(DE3) expression system carrying expression plasmid pET-28a(+) and plasmid pET-28a_SEBsyn has been shown the overexpression activity. Our inserted gene that it translates into protein attached with 6xHistidine has the molecular weight about 29 kDa. This fused 6xHistidine are essential for protein purification and detection with Ni-NTA resin approach, both under native or denaturing conditions [26]. In the result of SDS-PAGE, it clearly confirms that the plasmid carrying SEBsyn (pET-28a_SEBsyn) has the dense of target protein band. However, the plasmid control without insertion of a gene target (pET-28a(+)) was not expressed; so, at the same position, there were not found band of protein (Figure 5).

Our overexpressed protein seemly most dominant could be found in inclusion bodies. Nevertheless, some soluble protein target could be found in the supernatant. Most recombinant proteins using *E. coli* expression system were mostly resulted in inclusion bodies, only about 30% of recombinant protein were expressed in the soluble forms [27]. In other studies, it also reported that development of recombinant SEB is localized in the cytoplasm and most of this protein was found in inclusion bodies [28].

In conclusion, the SEBsyn encoding gene could be synthesized and cloned into pET-28a(+) expression vector. PCR colony also verified that the transformants *E. coli* BL21(DE3) carrying the inserted gene with an amplicon length about 1,000 bp. However, the amplicon length from transformants that carried pET-28a(+) with no insert was about 300 bp. The target protein could be expressed induced by IPTG. The prospective protein band for SEBsyn could be detected in SDS-PAGE with protein mass about 29 kDa.

AUTHORS' CONTRIBUTIONS

AR carried out the laboratory work. AR and MIT wrote the manuscript. MIT and HN supervised this study.

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