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Expression of a Synthetic Staphylococcal Enterotoxin B in *Escherichia coli* BL21(DE3) for Cancer Therapy Development

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***Abstract*— Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* is a potential therapeutic agent for cancer. SEB can activate the immune response, which could induce apoptosis of various cancer cells. This study aimed to design and produce a recombinant SEB protein using *Escherichia coli* BL21(DE3) expression system. We designed and optimized the codon of the inserted gene for *E. coli* and then transformed it into *E. coli* BL21(DE3). The transformants were verified by selective media containing kanamycin followed by colony PCR with T7 promoter and T7 terminator primers and DNA sequencing. The results showed that the SEBsyn encoding gene could be synthesized and cloned into pET-28a(+) expression plasmid. This recombinant plasmid that carried the SEB encoding gene (pET-28a_SEBsyn) was successfully transformed into *E. coli* BL21(DE3). The amplicons of colony PCR visualized in 2% agarose gel showed that transformants carrying the recombinant plasmid had an inserted gene length of about 1 kb. Gene inserts sequence verification by DNA sequencing resulted in 1,148 bp sequence consensus. The blastx analysis showed that it had the best hit with enterotoxin B in the NCBI database (accession id CAC6284025.1). The recombinant SEB protein was also successfully overexpressed in *E. coli* BL21(DE3) with 0.1 mM IPTG. Our recombinant SEB protein was dominantly insoluble in the inclusion body; however, some SEB protein was expressed as soluble. The molecular weight of the target protein is about 29 kDa.**

Keywords*—Enterotoxin; cancer; Staphylococcal enterotoxin B; *Staphylococcus aureus.

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I. INTRODUCTION

Cancer is a group of diseases resulting from abnormal cell growth. The mechanisms of cancer development are very complex. Generally, it comes from cell cycle gene mutations, carcinogenic compound exposure, stress, and unhealthy lifestyles [1], [2], [3]. 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 [4]. In Indonesia, the case of cancers in both sexes and all ages reached 396,914, with a death toll of 234,511 people from the total population of 273,523,621 people. Breast cancer has the highest incidence and mortality of 44.0% and 15.3%, respectively [5].

The high prevalence of cancer could be overcome with effective treatment to prolong the patient's life. With molecular tumor profiling (MTP), new agents or compounds for treating cancer could be effectively developed [6]. Current cancer treatments seem to be significantly harmful to patients, like chemotherapy that also attacks healthy cells and sometimes triggers metastasis events [7], [8]; also, it was associated with chemotherapy-induced myelosuppression (CIM) that impacts to patient's daily lives [9]. So, patients after chemotherapy must enhance the awareness and tackled the side effects via counselling with the doctor or health professionals for better self-care [10], [11].

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 glioblastoma cells [12], and other toxins from various species were proven to kill cancer cells [13]. This novel approach of bacterial-toxin therapy has great promise in treating tumors or cancer cells [14], [15].

Staphylococcal enterotoxin B or enterotoxin B (SEB) produced by *Staphylococcus aureus* could induce apoptosis, which is essential for cancer therapy development [16]. This toxin can activate the immune system, followed by the secretion of apoptosis signals, like TNF-α and Fas Ligand [17]. Compared to other enterotoxins from *S. aureus*, SEB has antitumor activity in animals model[18]. To prevent the side

effects of this toxin, a previous study [19] reported that infused antibody therapy Ig121 or c19F1 could be used to neutralize that toxin.

SEB is an ideal recombinant protein size, 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 improvement to enhance the sensitivity of SEB proteins to target cells. Recombinant DNA technology has contributed to medicine-related fields by producing pharmaceutical proteins (biopharmaceuticals) and therapeutic proteins. This study aims to produce SEB recombinant with *E. coli* BL21(DE3) as a host for protein expression. The production of this protein recombinant would be an essential step in developing cancer therapy using bacterial toxins to kill cancer cells.

II. Materials and Methods

A. Vector Construction and Gene Synthesis

The commercial plasmid, namely pET-28a(+) from Novagen (cat: 69864-4), was the expression vector used in this study. The wild type of SEB encoding gene deposited in NCBI with acc. no. M11118.1 [20] was downloaded with *.fasta* format. This reference gene was needed to construct the SEB synthetic gene that could be expressed optimally in *E. coli* BL21(DE3). The 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. The sequence of the edited SEB gene was sent to Synbio technologies (https://www.synbio-tech.com/gene-synthesis/), a gene synthesis company. After codon optimization by the Synbio technologies, the synthesized SEB gene was inserted into the pET-28a(+), and the final construct was called pET-28a_SEBsyn.

B. Competent cells preparation and Transformation

This study used E. coli BL21(DE3) as an expression host. Bacterial cells from the glycerol stock were cultured in Luria Bertani (LB) agar by streak plate method to obtain a single colony. A colony was cultured overnight in a shaker incubator at 37 ◦C. The cells were harvested by centrifugation at 4,000 rpm, 4 ◦C for 10 minutes. The pellets were resuspended with cold-$CaCl_2$ 0.1 M and 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 pET-28a(+) (500 ng/µL, 3 µL) and recombinant pET-28a_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 incubation in 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 mixtures were centrifuged at 14,000 rpm for two minutes at room temperature, and supernatants were removed. The pellets containing 50 µL of LB broth supernatant were resuspended by pipetting then the cells were spread onto LB agar + 50 µg/mL kanamycin. The plate containing transformants was incubated at 37 ◦C overnight.

C. Verification of Transformants and pET-28a_SEBsyn Recombinant Plasmid

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 follows: 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 and visualized with a UV-transilluminator.

The pET-28a_SEBsyn was validated with DNA sequencing reads with T7 promoter and T7 terminator primers. The sequences read from DNA sequencing were trimmed with BioEdit 7.0.4.1 software (https://bioedit.software.informer.com/), and the files with .ab format from DNA sequencing were visualized with FinchTV 1.5.0 software (www.digitalworldbiology.com/FinchTV). These trimmed sequences were then used to build a consensus sequence using DNA Baser V4 software (www.dnabaser.com). The consensus sequence was aligned with the Blastx program from the NCBI web server (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

D. Protein Expression

The positive transformants, namely pET-28a SEBsyn T1-T4 were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). The selected transformants were pre-cultured in 1 mL LB broth + 50 µg/mL kanamycin and incubated in shaker at 37◦C, 200 rpm overnight. On the next day, approximately 0.5 mL of the pre-culture transformants were cultured in 4.5 mL LB broth + 50 µg/mL kanamycin. Those cultures were incubated for approximately 2-3 h to reach an optical density of about 0.6-0.8. These bacterial cultures were then induced with 0.1 mM IPTG from Thermofisher (Cat: R1171). Then, they were incubated in a shaker incubator at 37◦C, 200 rpm for overnight (18 h).

E. 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 phenylmethanesulfonylfluoride or PMFS 100mF) followed by centrifugation 6000 rpm at 4 ◦C for 5 min, the supernatants as soluble protein fraction was collected. The supernatants and pellets from this step were stored in -80◦C freezer. The pellets were resuspended with lysis buffer containing 50 mM Tris-Cl pH 7.4 and Triton X-100. These pellets were treated with lysis buffer to solubilize the

insoluble protein fraction. The freezing-thawing method with 10 repetitions was used to treat pellets for completing cell lysis. After freezing and thawing, the suspensions were centrifuged at 12,000 rpm, 4 ◦C for 15 min, and the supernatants were collected. The pellets, in the form of insoluble protein fraction, were washed with washing-solubilization buffer (urea, glycine, and β-mercaptoethanol) three times, followed by centrifugation at 12,000 rpm, 4 ◦C for 15 min, supernatants from washing were collected. The pellets were then solubilized with lysis buffer.

After washing, the supernatants from the lysis step and the solubilized pellets 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 µL of the mixed sample was used for SDS-PAGE, separating gel 15% 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 a microwave.

III. Results and Discussion

The construction of pET-28a_SEBsyn with SnapGene software is shown in Fig. 1a and 1b. The cloning sites used in this construct were NdeI (5'-CA/TATG-3') which carried a start codon, and XhoI (5'-C/TCGAG-3'). pET plasmid series, including pET-28a(+), are commercial plasmids widely used for recombinant protein production in *E. coli*.

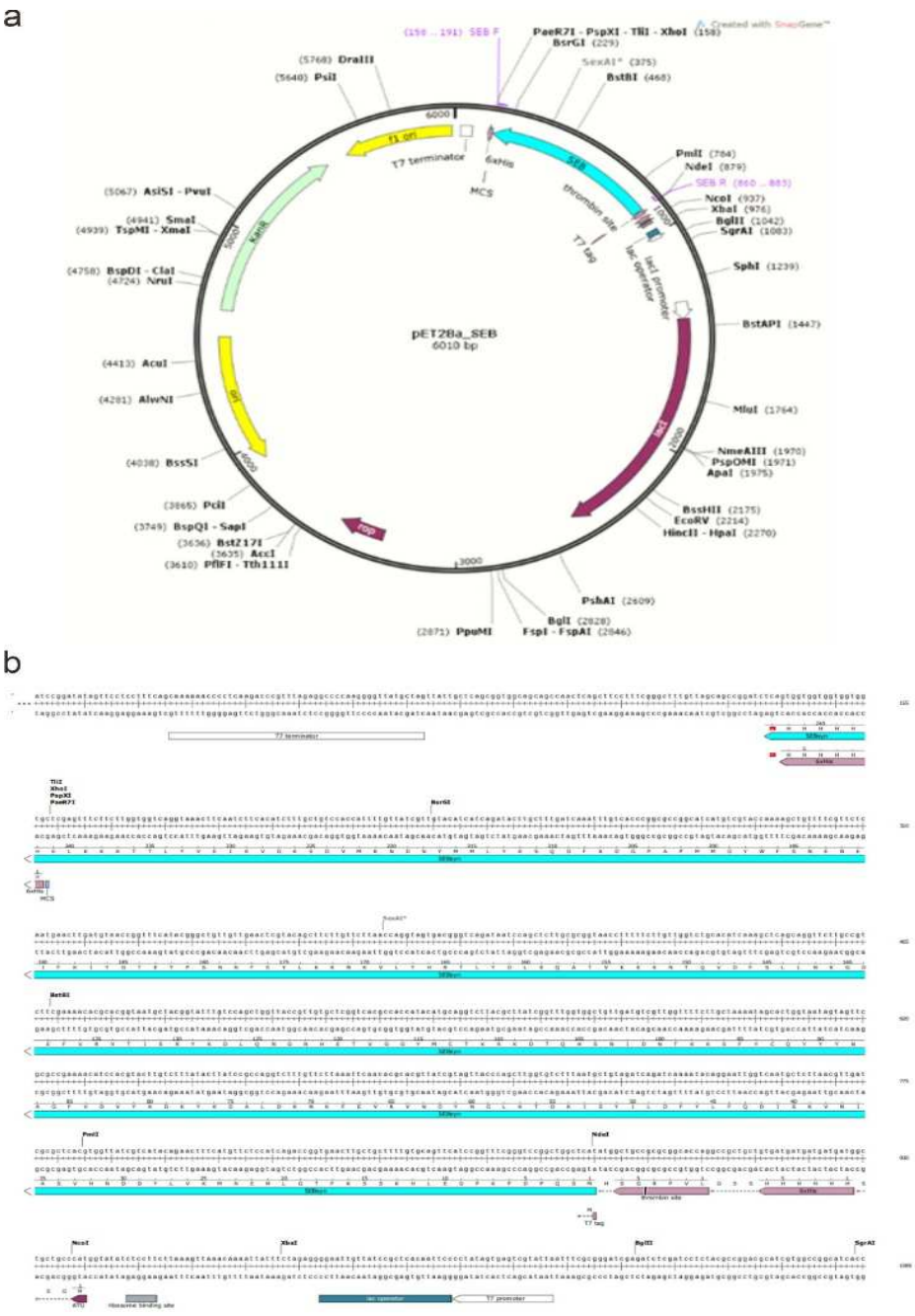

Fig. 1 a) Vector map of pET-28a(+) inserted with SEBsyn, b) Sequence construct of SEBsyn in pET-28a(+)

The inserted gene that are cloned into pET-28a(+) are controlled by bacteriophage T7 promoter, and its transcription system is performed by T7 RNA polymerase present in the host cell [21], [22]. A hexahistidine 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 the kanamycin-resistance gene, 6xHis-Tag at the C-terminal and N-terminal, and the 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 [23].

The pET-28a(+) and pET-28a_SEBsyn plasmids were successfully transformed into *E. coli* BL21(D3) with $CaCl_2$ heat-shock procedure. The presence of colonies that grew in an LB medium containing 50 µg/mL kanamycin indicated the success of the transformation. The total number of transformants carrying pET-28a(+) was more than 300 single colonies (Fig. 2a), and transformants carrying pET-28a_SEBsyn were 81 colonies (Fig. 2b). The negative control of transformation using *E. coli* BL21(DE3) without plasmid had no colony in the selective medium (Fig. 2c), while the other control plate without kanamycin showed that the competent cells without plasmid could grow well (Fig. 2d).

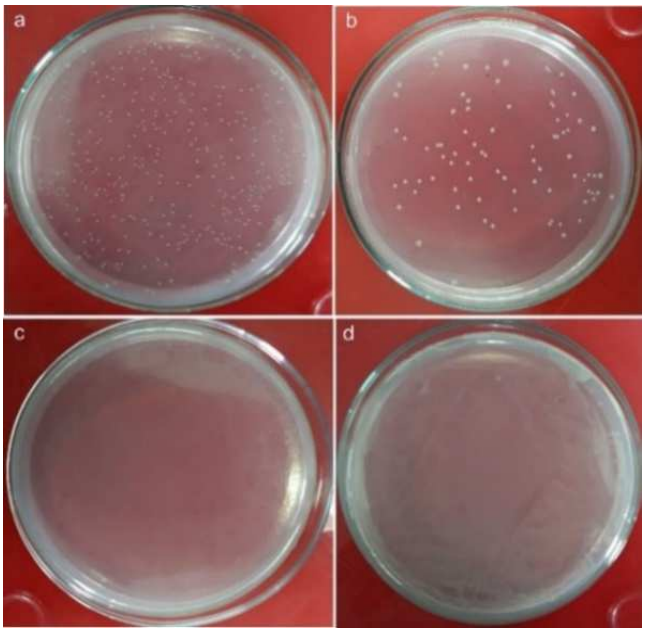

Fig. 2 Colonies are growing on LB agar + kanamycin after transformation. a) transformants carrying pET-28a(+), b) transformants carrying pET-28a_SEBsyn, c) control (competent cells), d) control (competent cells on LB agar without kanamycin.

The difference in the number of transformants between pET-28a(+) and pET-28a_SEBsyn was probably due to the different size of the plasmids. The final length of pET-28a_SEB was about 6,010 bp, while pET-28a(+) had a length of about 5,369 bp. Studies reported that increasing plasmid size would decrease the transformation efficiency [24], [25], [26].

PCR colony is used to verify the target transformants carrying SEBsyn gene. The colonies PCR method is a fast approach to confirm the presence of the gene of interest in the host. This is a simple method for bacteria where the colony containing the template DNA is added to the PCR master mix [27], [28]. Colony PCR to verify the presence of the inserted gene was amplified by T7 promoter and T7 terminator primers, which covered the SEBsyn in the pET-28a(+). The correct amplicon size obtained was about 1 kb for colonies carrying pET-28a_SEBsyn. In contrast, the colonies carrying pET-28a(+) produced an amplicon size of about 300 bp (Fig. 3).

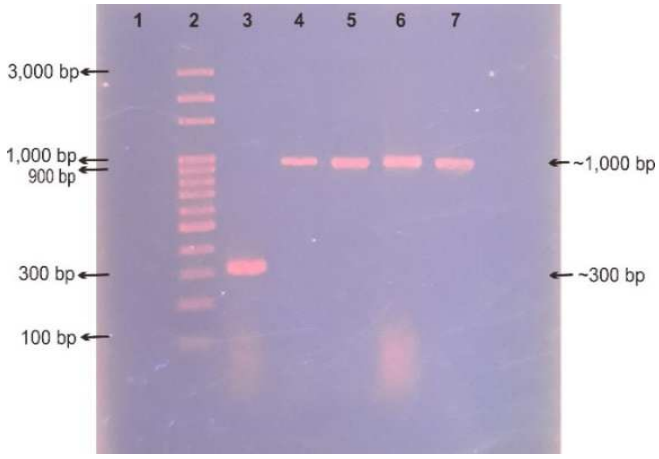

Fig. 3 Electropherogram of amplicons from colony PCR of transformants visualized in 2% agarose gel. Lane 1: No template control (NTC), lane 2: Marker 100 bp, lane 3: pET-28a_SEBsyn transformants, lane 4-7: Recombinant transformants (T) number 1-4. Amplicon from transformant number 2 was used for further verification with DNA sequencing.

In further verification, amplicon from transformant number 2 is used for DNA sequencing because it has a good quality amplicon compared to others. The SEBsyn sequence from DNA sequencing was showed that the nucleotides have a good read marked with a single peak signal (Fig. 4). Nucleotides trimming from DNA sequencing result was carried out based on the *Phred* score threshold marked with the blue line in the Fig 4. The estimation of base calling quality using *Phred* score is critical parameter for genetic analysis [29]. The *Phred* score range from 2 to 40 is generally accepted for confidence result in nucleotides read [30]. Based on that parameter, the reverse sequence was trimmed about 41 bases and 273 bases at the end (Fig. 4a), while the forward sequence was trimmed about 40 bases at the first and 209 bases at the end (Fig. 4b).

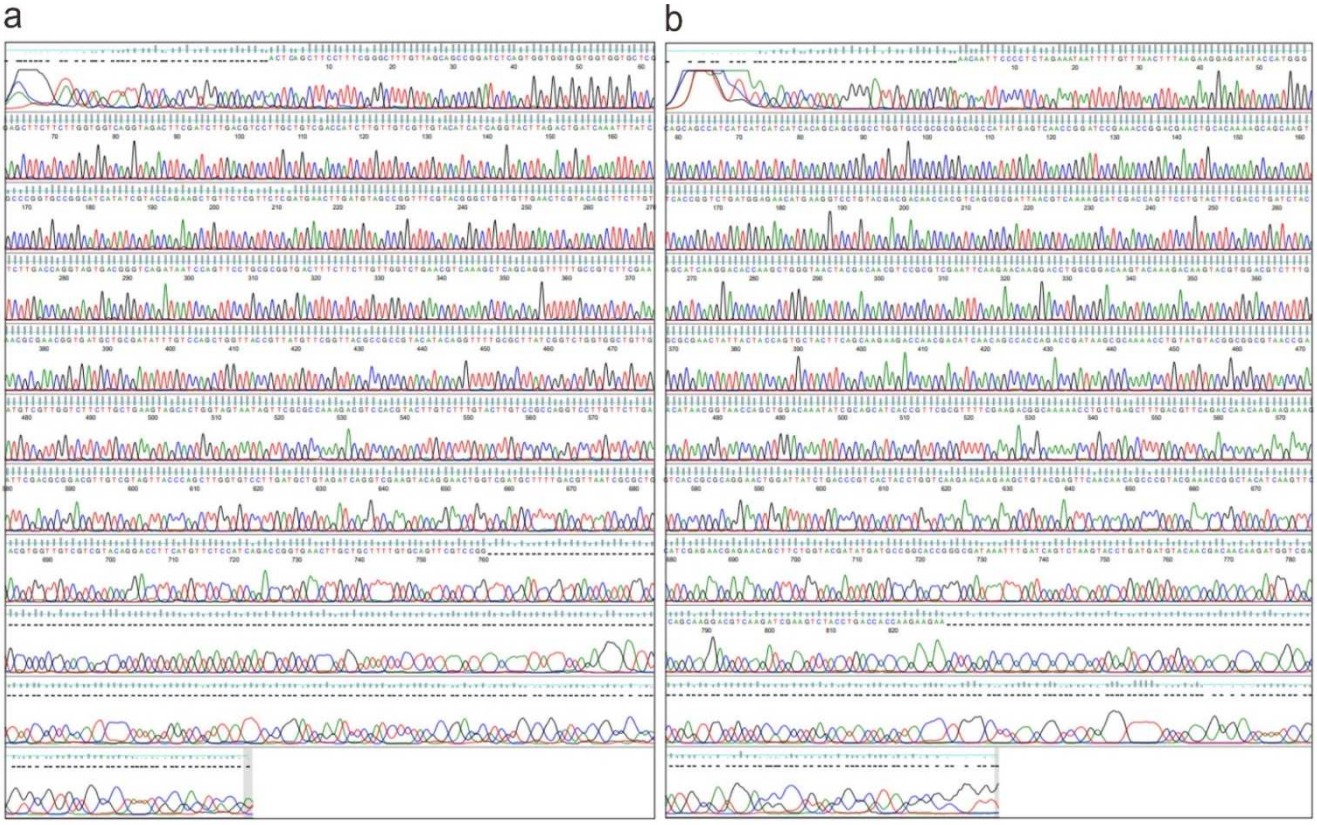

Fig. 4 Visualization of the trimmed-DNA sequencing result from pET-28a_SEBsyn. a) Reverse sequence read, and b) Forward sequence read.

The consensus sequence from the contig analysis was resulting 1,148 bases, and it was deposited to GenBank with accession number ON330464. Alignment of the SEBsyn consensus sequence with the wildtype SEB encoding gene showed that the sequence of the genes is not totally similar; nevertheless, the amino acid sequences are similar (fig. 5). After codon optimization, this SEBsyn sequence was proposed to increase the production of the protein of interest.

The codon optimization method generally replaced the rare codons in the target gene with the commonly used codon in the host cell without modifying the target protein [31]. Codon usage is critical to achieving adequate protein expression levels in *E. coli* [32], [33]. This technique can improve the production of various recombinant proteins expressed by *E. coli* [34], [35], [36].

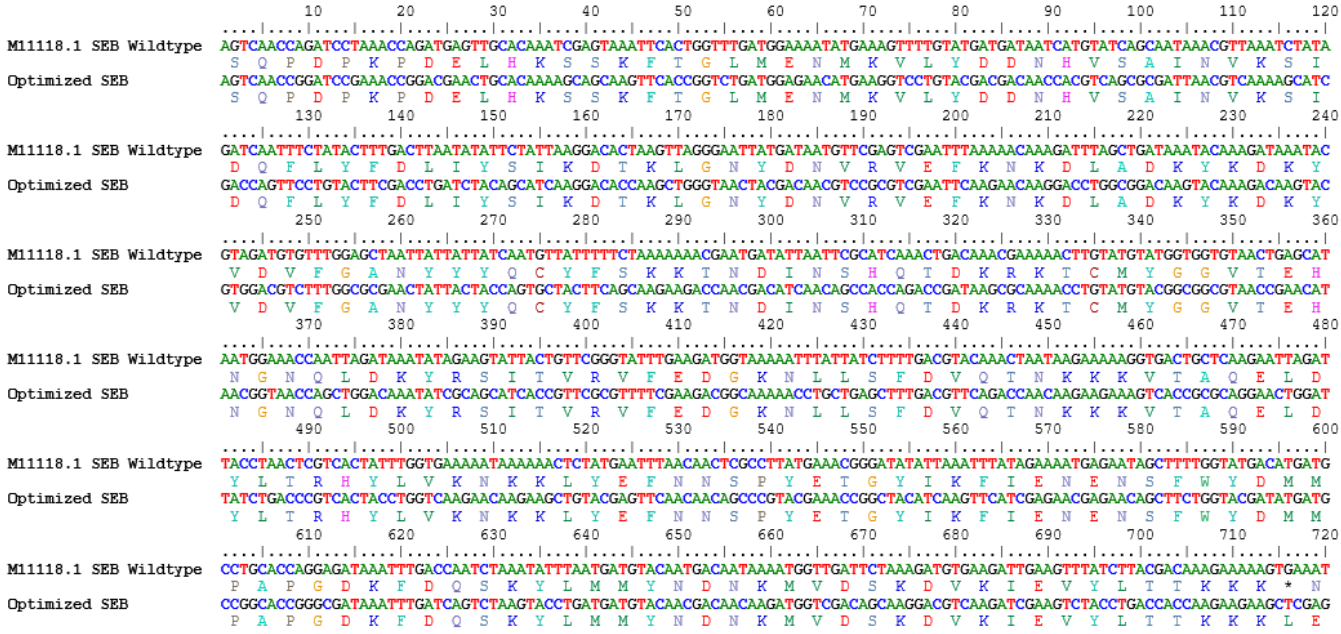

Fig. 5 Graphic view alignment of wild type SEB with SEBsyn. There was no variation in the protein sequence. Amino acid L and E at the carboxyl end is due to the presence of the XhoI restriction site.

Blastx analysis from the SEBsyn consensus sequence was showed that this sequence has specific hits with SEB protein sequences in the database with identities ranging about 97-100 %. The top five blastx hits are shown in Table 1. Based on this result, the SEBsyn sequence could be confirmed as a SEB encoding gene.

TABLE I
TOP FIVE HITS FROM BLASTX ANALYSIS

No	Description	Organisms	Accession	E-value	Identity (%)
1	Enterotoxin B	*Staphylococcus aureus*	CAC6284025.1	8e-173	97.55
2	Enterotoxin B	*Staphylococcus aureus*	AAW21709.1	9e-175	97.55
3	Staphylococcal enterotoxin type B	*Staphylococcus aureus*	MBU5037927.1	9e-173	97.55
4	Staphylococcal enterotoxin type B	*Staphylococcus aureus*	WP_000278085.1	9e-173	97.95
5	X-ray structure enterotoxin type B	*Staphylococcus aureus*	4C56_C	1e-172	100

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 [37]. This bacterial strain is widely used in various research related to recombinant protein production. *E. coli* BL21(DE3) expression system carrying pET-28a_SEBsyn plasmid could overexpress the SEB protein. The molecular weight of our SEB protein, which was fused with 6x Histidine, was about 29.4 kDa. This fused 6x Histidine is essential for protein purification and detection with Ni-NTA resin under native as well as denaturing conditions [38]. Figs. 6a and 6b showed that the plasmid carrying SEBsyn (pET-28a_SEBsyn) could express the density of the target protein band.

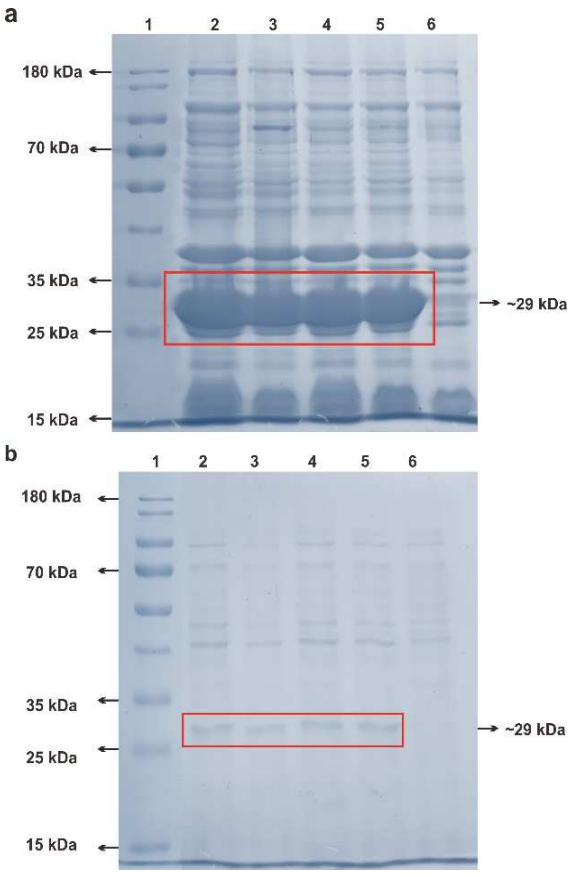

Fig. 6 SDS-PAGE of induced transformants. a) Insoluble protein fraction (inclusion bodies), b) Soluble protein fraction. Lane 1: protein ladder, lane 2-5: pET-28a_SEBsyn T1-T4, respectively, lane 6: pET-28a(+) transformant (negative control).

Our overexpressed protein was dominantly found in inclusion bodies (Fig. 6a). Nevertheless, some soluble protein target could be found in the supernatant (Fig. 6b). Previous study showed that most recombinant proteins produced *in E. coli* expression system were mostly found in the inclusion bodies. Only about 30% of recombinant proteins were expressed in soluble forms [39]. Other studies also reported that the recombinant protein SEB is accumulated in the cytoplasm, and most of this protein was found in the inclusion bodies [40].

IV. CONCLUSION

In conclusion, the SEBsyn encoding gene could be synthesized and cloned into pET-28a(+) in *E. coli* BL21(DE3). The recombinant SEBsyn could be overexpressed as soluble and insoluble protein. The molecular weight of the recombinant SEB was about 29 kDa.

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