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Escherichia coli BL21(DE3) expression system using TorA signal peptide for Recombinant Human Albumin (rHA) secretion

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ABSTRACT

Human serum albumin (HSA) is the most abundant protein in blood plasm. This protein consisted of 585 amino acids with a molecular weight of 66 kDa and 17 disulfide bonds. HSA obtained from conventional technique allow viral or prion contamination. For that reason, recombinant DNA technology becomes a promising alternative. Because of its well-known genetic, simplicity, and capacity to accommodate many foreign protein, *Escherich ia coli* remains the most widely used in the production of recombinant proteins. But, overproduction of protein may lead to the formation of inclusion bodies and proteolytic degradation. These problems can be overcome by using protease-deficient strain and protein secretion into periplasmic space. The objective of this research is to secrete recombinant HA on *E. coli* BL21(DE3) using TorA signal peptide and proved using SDS-PAGE. This research method begins with the preparation of competent cell and transformation of *E. coli* BL21(DE3), expression of recombinant HA in *E. coli* BL21(DE3), and characterization of expression result by using SDS-PAGE. The result of this study was rHSA can be secreted into extracellular medium using TorA signal peptide with a molecular weight of ± 66.5 kDa.

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INTRODUCTION

HSA is the most abundant protein in human plasma blood, which constitutes about 60% of the serum proteins. According to (He *et al.*, 2011), many other novels use for HSA in biological applications have recently been explored, such as the carrier of oxygen, nano delivery of drugs, and fusion of peptides. However, albumin, as it is currently obtained by conventional techniques involving the fractionation of plasma obtained from blood donors is with the risk of transmitting possible viral/prion contaminants. In this respect, recombinant DNA technology offers an important new source of biomaterials that are suitable for use as pharmaceuticals without any need for pooled human blood products (Chuang *et al.*, 2002).

Among the many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (Baneyx, 1999). It is, however, not uncommon that over expressed recombinant proteins fail to reach a correct conformation and undergo proteolytic degradation or associate with each other to form insoluble aggregates of non-

native proteins known as inclusion bodies (Baneyx and Mujacic, 2004).

Some of approachment can be done to increase target protein expression, including the use of protease-deficient strain and periplasm expression. BL21 is deficient in ompT and lon, two proteases that may interfere with the isolation of intact recombinant protein (Sørensen and Mortensen, 2005). Based on research conducted by (Latta *et al.*, 1987), the major part of the HSA polypeptide (usually more than 90%, depending on the promoter and strain) is synthesized in *E. coli* cells as an insoluble aggregate. In addition, it shows that nonnative N-terminal HSA (Methionyl-HSA or Met-HSA) is produced. To overcome this problem, this research uses the periplasm expression method. Secretory production of recombinant proteins has several advantages over intracellular production. These advantages include solving inclusion bodies because it increases protein folding, decreasing proteolytic degradation, the N-terminal amino acid residue of the secreted product can be identical to the natural gene product, and facilitate purification (Maksum *et al.*, 2017; Mergulhão *et al.*, 2005; Bloois, 2012; Choi and Lee, 2004).

In Gram-negative bacteria, at least three different types of protein secretion systems have been described—Type I, Type II, and Type III (Pugsley, 1993), with type II being the most widely used. The general secretory pathway is a two-step process for the extracellular secretion of proteins mediated by periplasmic translocation (Koster *et al.*, 2000). Three pathways can be used for secretion across the bacterial cytoplasmic membrane: the Sec-dependent pathway, the signal recognition particle (SRP), and the twin-arginine translocation (TAT) pathways (Mergulhão *et al.*, 2005).

Peptide signal selection is the first option for recombinant protein secretion efficiency (Yoon *et al.*, 2010). Trimethylene N-oxide Reductase A (TorA) is TAT pathway signal peptide. It is proved that green fluorescent protein (GFP) secretion via TAT pathway is better than via Sec-dependent pathway, which reaches 46% secretion efficiency and the totality of protein secreted has been in folding form or totally active. Moreover, TAT pathway can export high molecular weight heterologues protein until 142 kDa and has disulfie bonds in its active form better than Sec-dependent pathway (Thomas *et al.*, 2001; Mergulhão *et al.*, 2005). This makes HSA to be possibly secreted, considering its molecular weight that is quite high.

This research aim is to secrete HSA in *E. coli* BL21(DE3) using TorA signal peptide and characterize it using SDS-PAGE method.

MATERIALS AND METHODS

Construction of pD881-torA-HSA Plasmid

The Expression method of *HSA* gene in E. coli BL21(DE3) has referred to (Sriwidodo *et al.*, 2017). HSA amino acid sequence was obtained from PDB 1AO6, then it is back-translated into its nucleotide sequence. *HSA* gene codon was input to GCUA web *server* and a variety degree of codon adaptiveness graphic was obtained. *HSA* gene codon optimization was done by changing rare codon into optimal (high preferences) codon in *E. coli* so optimized codon was obtained, it is shown by codon preference that reach 100%. GC precentage and *codon adaptiveness indices* (CAI) was calculated in www.endmemo.com . Optimized *HSA* gene codon then was synthesized by DNA 2.0.

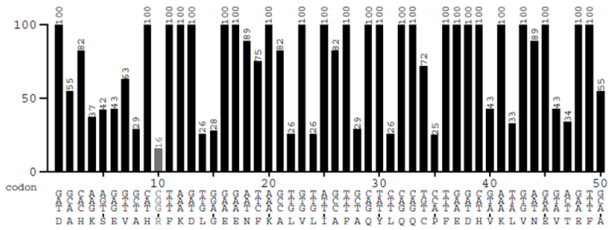

Figure 1: Frequency percentage graphic of HSA codon gene (0 till 50 from 585) before it was optimized using GCUA

Expression and characterization of *the H SA* gene in *E. coli* BL21(DE3)

One μL of the recombinant plasmid (1 ng/1 μL) was added into a microtube containing 50 μL of BL21(DE3) electrocompetent cells. The mixture was resuspended and moved into 0.1 cm cold cuvette. The cuvette left on ice for 5 minutes. The outer side of the cuvette was wiped with a tissue and cuvette was placed in the electroporator. Electroporation was set on 1,500 volts. After electroporation, 1 mL of super optimal broth (SOC) medium was added into the cuvette. Cells were resuspended in the cuvette and then moved into 15 mL tube. It was then shaken with 200 rpm and incubated at 37°C for one hour. 100 mL of culture were spread into Luria-Bertani (LB) agar containing 75 μg/mL of kanamycin.

A single colony of transformant was grown in 5 mL of LB medium containing kanamycin at 37°C for 16-18 hours (200 rpm). 1 mL of culture was then moved into 25 mL LB medium containing kanamycin and was incubated at 200 rpm, 37°C until OD_{600} 0.8. 1 mL from culture was separated into microtube as pre-induction sample (t_o) for SDS-PAGE analysis. Expression was started by inducing *E. coli*

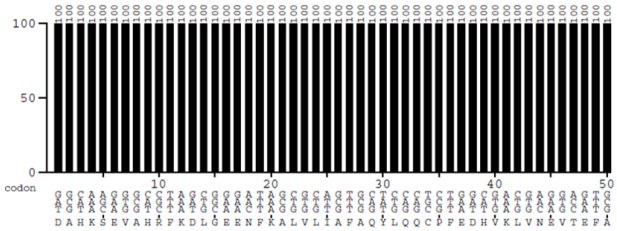

Figure 2: Frequency precentage graphic of HSA codon gene (0 till 50 from 585) after it was optimized using GCUA

BL21(DE3) culture with 4 mM L-Rhamnosa. Supernatant and pellet from culture was separated by centrifuge at 3000 *g*, 4^{o}C for 20 minutes. Pellet was used for lysis cell process and periplasm protein fraction extraction. The supernatant was used for medium protein fraction analysis using SDS-PAGE.

500 μL of Tris-Cl EDTA buffer was added into one microtube containing cells pellet, resuspended, and lysed using sonicator. Lysis process includes 2 minutes on and 4 minutes off. Supernatant from lysis process was then centrifuged at 10,000 *g*, 4°C for 30 minutes. Supernatan was moved into new microtube as a soluble fraction (SF). Pellet was added with 80 μl of 8 M urea, then heated at 95°C for 15 minutes and centrifuged at 10,000 *g*, 4°C for 10 minutes. Supernatant was moved into new microtube as an insoluble fraction (IF).

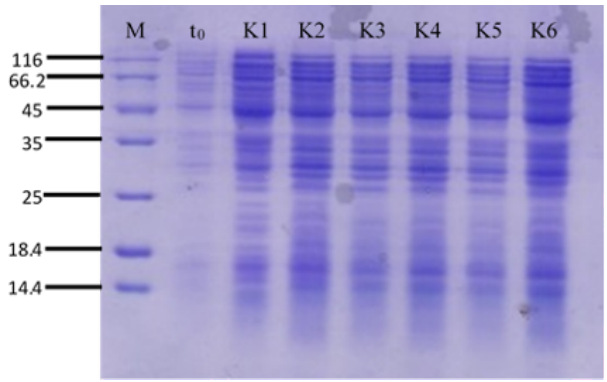

Figure 3: SDS-PAGE electrophoregramof HSA soluble fraction in cytoplasm after induction of 4 mM L-rhamnose from E. coli BL21(DE3)[pD881-torA-HSA] colonies. (M) protein marker; (t_0) fraction before L-rhamnose induction; (K_i) colony transformant 1-6 after induction L - rhamnose induction

Periplasm extraction was done by adding 100 μL of TSE buffer (0.2 M Tris-Cl pH 8.0; 0.5 M sucrose, 1 mM EDTA) into a new microtube. Cells pellet were resuspended and incubated on ice for 30 minutes, then centrifuged at 20,000 *g*, 4°C for 30 minutes. Supernatant was moved into new microtube as periplasm protein extract. Protein fraction in the medium was concentrated using the freeze-drying method, and then dilluted with 100 μL of aqua dest.

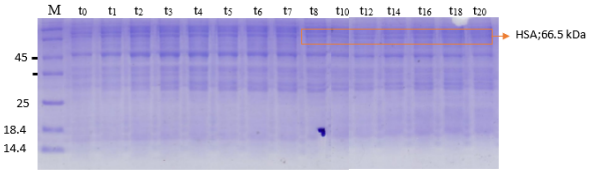

Figure 4: SDS electrophoregram of HSA soluble protein in periplasm after induction of 4 mM L-rhamnose. (M) protein marker; (t_0) fraction before L-rhamnosa induction; (t_i) fraction after 1-20 hours of L-rhamnose induction

ample from expression result (35 μl), was mixed with 5 μl of SDS sample buffer (5X) in a microtube. The mixture then was heated at 100^{o}C for 15 minutes in a water bath. The mixture was centrifuged at 15,000 *g* for 1 minute at 4^{o}C. Each of 20 μl sample and 5 μl marker protein was input to the well. SDS-PAGE was done at 30 volts for 100 minutes. The gel was then placed overnight in staining solution, and gel dye was washed off by destaining solution.

RESULTS AND DISCUSSION

pD881-torA-HSA Plasmid Construction

HSA amino acid sequence was obtained from PDB with number 1AO6, and then it was back-translated into its nucleotide sequence that contains 1755 bp or 585 amino acids. Gene codon *HSA* was optimized using GCUA web server. This optimization was done by inputing gene codon *HSA*, then changed it based on *E. coli* codon preference. After *HSA* sequence entered in GCUA, codon with varied adaptiveness degree was obtained.

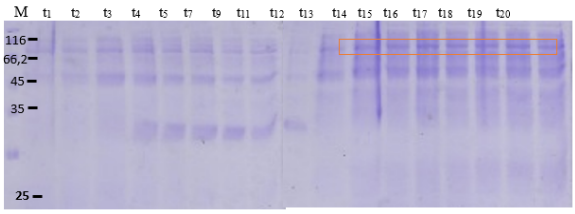

Figure 5: SDS-PAGE electrophoregram of soluble protein in extracellular medium after 4 mM L-rhamnose induction. (M) marker protein; (t_0)fraction before L-rhamnosa induction; (t_i) fraction after 1-20 hours of L-rhamnose induction

Figure 1 shows that there are still several codons

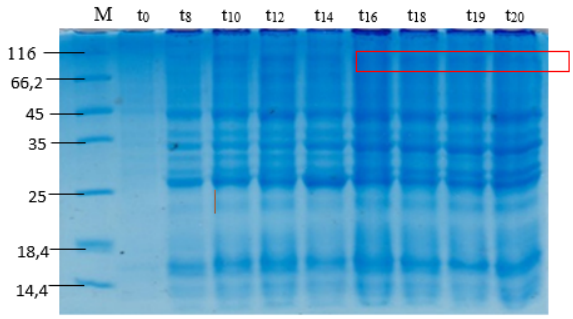

Figure 6: SDS-PAGE electrophoregram of insoluble HSA incytoplasm 4 mM L-rhamnosa induction. (M) marker protein; (t_0) fraction before L-rhamnosa induction; (t_i) fraction after 1-20 hours of L-rhamnose induction

with low adaptiveness degree (less than 100%). Thus the change of rare codon to optimal codon was needed. GC% obtained from nonoptimized codon was 42.9%, and CAI was 0.353.

Figure 2 shows that all of the adaptiveness degrees from *HSA* codon had been changed (100%). This sequence was obtained by changing codon before with preferred codon. The optimized sequence has GC percentage as much as 52.5%, and CAI was 0.838. When optimization was done, GC content should be in the range of 40-60%. This was done in order to double helix DNA won't be easily opened, because hydrogen bond between G and C is strong. If GC content less than 40%, it is feared that double helix DNA will be easily opened,

because DNA stability is lessen. Higher CAI value usually enables the target gene to be highly expressed.

Kanamycin resistance gene was chosen as a selection process marker. Then TorA signal peptide that can secrete HSA into the periplasm and can be recognized by TAT pathway was chosen. Strong RBS was chosen in order to increase initiation translation speed (Yoon *et al.*, 2010). pD881-torA plasmid has *ori_p15a* that can be used to make copy until 10-12 plasmid copy in one cell. Other than that, this plasmid use $rhaP_{BAD}$ promotor that is known can express the high amount of protein, tight-regulated by addition of D-glucose, and can express the functional host-toxic protein (Chuang *et al.*, 2002). Then it was synthesized by DNA 2.0.

Expression and characterization of *the HSA* gene in *E. coli* BL21(DE3)

This step started with growing *E. coli E. coli* BL21(DE3) [pD881-torA-HSA] transformant as starter culture at 200 rpm, 37°C for 16-18 hours. Then starter culture was moved as much as 1% into 25 mL Luria-Bertani medium containing kanamycin as a selection marker. *E. coli* BL21(DE3) cell culture was grown until OD_{600} reach 0.8 for induction. Before induction was done, 1 mL sample from culture was separated as a protein fraction before induction (t_0). Induction was done by adding 4 Mm L-rhamnose as final concentration into the expression medium. To obtain protein fraction in the cytoplasm, the sonication method was used. Lysate from six *E. coli* BL21(DE3) [pD881-torA-HSA] transformant colonies showed that HSA was expressed in the cytoplasm, it was characterized with the presence of $\pm$67.0 kDa and in the SDS-PAGE electrophoregram Figure 3.

Expressed HSA was fused with TorA signal peptide that would secrete HSA protein expressed in the cytoplasm into periplasm through TAT pathway. To know whether HSA protein can be secreted or not into the periplasm, SDS-PAGE characterization was done on periplasm fraction that is shown in Figure 4.

Protein fraction from periplasm was obtained by using the periplasm extraction method. By using this method, protein from periplasm would be secreted into the extracellular medium by adding TSE buffer. The addition of this buffer aim was to help the protein secretion process from the periplasm into the extracellular medium. According to (Quan *et al.*, 2013), sucrose in the extraction buffer will increase extracellular osmolality, resulting in cell shrinkening and release water and periplasm content into the medium. EDTA facilitate periplasm extraction by chelating divalenion that is normally stabilized lipopolysaccharide (LPS) in outer membrance, causing LPS released and increasing outer membran permeability. Figure 4 shows that HSA protein successfully translocated into the periplasm, characterized by $\pm$67.0 kDa band from t8 and became slighten on t_{18}. There was no significant amount of protein that secreted into periplasm after 8 hours (t_8) after induction, showed with no thickening band in the electrophoregram.

This very thin band indicated that the amount of HSA protein secreted was a little. This was probably because most of the protein in periplasm wasn't correctly folded, resulting protein couldn't be secreted into periplasm through TAT pathway whereas TAT pathway can only secrete correctly folded protein into the periplasm. Moreover, according to (Mergulhão *et al.*, 2005), tatABC operon co-expression is needed for high-scale production of high molecular weight protein. However, only TAT pathway that can secrete protein into the medium in its folded form through the inner membrane and freed from its

fusion form. Therefor additional protease wouldn't be needed to obtain native protein or secrete protein folding machinery.

HSA band from periplasm fraction became slighten until 18 hours after induction (t18), it indicated that HSA protein in periplasm had been secreted into the extracellular medium. Then isolated protein fraction from the extracellular medium was characterized. In order to make protein became clearly seen, the freeze-drying method was done. This method was chosen because it can maintain protein structure stability. This is because this method was done in a very low temperature and only utilize high pressure to relieve water molecule so that it won't degrade protein structure. In addition, the protein will hardly be contaminated since it uses an isolated operating room.

SDS-PAGE electrophoregram of extracellular medium Figure 5 shows the presence of HSA protein in the extracellular medium, characterized with the presence of $\pm$67,0 kDa band. Band in 18 hours after induction (t_{18}) was seen really thin but slightly thicken after 19 hours after induction (t_{19}). It showed that HSA protein wasn't optimally secreted from the periplasm into the extracellular medium. The least amount of HSA in periplasm expected to cause the least amount of HSA that was secreted into the extracellular medium. On the other hand, the least amount of secreted HSA into extracellular probably because of periplasm space limited size so it can't accommodate a high amount of HSA. With limited periplasm space, the protein will be pushed out into membrane will a little too.

From what have been explained earlier, the small amount of secreted HSA protein into the periplasm and extracellular media was because of the selection process by Tat pathway. Protein that wasn't folded similar to its native structure would be returned to the cytoplasm as insoluble protein (Alanen *et al.*, 2015). Therefore, an expression test was done to obtain the insoluble protein fraction in the cytoplasm.

Figure 6 shows the presence of a thick band in $\pm$67.0 kDa from after 8 until 20 hours after induction. This band thickness showed that HSA protein was expressed in the cytoplasm, but wasn't translocated into the periplasm or extracellular medium. It was because BL21(DE3) lacked the ability to produce soluble protein so that HSA protein couldn't be folded similar to its native structure, rejected by Tat pathway, returned to the cytoplasm, and accumulated in the cytoplasm as an insoluble fraction. Based on marker protein molecular weight standard curve, it was obtained linear equation of y = -1,0819x + 4,9617 and R^2= 0,9362. Recombinant HSA molecular weight that was obtained from the extracellular medium from the equation was 67.0 kDa, resemble with native HSA molecular weight that is 66.5 kDa.

CONCLUSION

Recombinant HSA protein can be secreted using TorA signal peptide in *E. coli* BL21(DE3) through TAT pathway that characterized using SDS-PAGE.

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