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Extracellular Secretion of Recombinant Human Epidermal Growth Factor by Using Trimethylamine N-Oxide Reductase A (TORA) Signal Peptide in *Escherichia Coli* BL21 (DE3)

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Abstract

Human Epidermal Growth Factor (hEGF) is a protein that helps in the healing process of wounds, in the process of proliferation, migration and cell differentiation. hEGF composed by 53 amino acid residues, with a molecular weight of 6.2 kDa and has a single chain conformation, and resistant to high temperatures up to 70˚C. hEGF is already very widely produced in an industrial scale using Escherichia coli as a host cell, but the majority of hEGF recombinant protein is produced intracellularly, so it has a lot of disadvantages such as; the accumulation of non-functional protein, the amount of protein inclusion bodies are formed as a result of misfolding, and the high rate of degradation due to intracellular protease enzymes. Therefore the purpose of this study was to express recombinant proteins in the extracellular hEGF using a signal peptide TorA, via the Twin Arginine translocation (TAT) pathway, measure the levels of hEGF recombinant protein secreted into the culture medium with the use of a signal peptide TorA via TAT. This research using Escherichia coli BL21 as the host cell to be transformed by using recombinant plasmid pD881-TorA the consensus already containing the signal peptide TorA and also hEGF gene, then expressed by L-rhamnose induction, then the recombinant protein hEGF purified by heat treatment and then the results were characterized using SDS-PAGE, and ELISA. The result is hEGF protein can be secreted into culture medium and periplasm with concentration 0.9625 µg/ mL.

Keywords: Recombinant Protein hEGF, TorA signal peptide, extracellular secretion

INTRODUCTION

Human Epidermal Growth Factor (hEGF) is a protein that helps in wound healing, in terms of the process of proliferation, cell migration and cell differentiation. HEGF protein composed of 53 amino acid residues, with a molecular weight of 6.2 kDa and has a single-chain conformation[1], hEGF produced by the human duodenum and by the salivary glands[2], hEGF is often used in medical and cosmetic fields, for hEGF has the ability to stimulate the growth of various types of cells and accelerate the phase of cell differentiation in tissues. hEGF have broad biological reactions, and quickly spur growth which a variety of skin layers. In general, skin care products just add liquid skin to help moisturize the skin and slow the aging, but it does not improve. Instead hEGF can penetrate the dermis to stimulate the growth of new cells to replace old cells that have been damaged, and serves to control the growth of skin cells. hEGF can help form a protein that can stimulate other tissues to regenerate the cells, so that hEGF is very effective for use as a wound healer protein[3].

In the industrial world, the production of recombinant proteins is commonly used *E.coli* as a host cell. Gram-negative bacteria have a very fast growth process, so as to minimize production costs. *E.coli* is also relatively easy for the recombinant plasmid is inserted into it, so that the gene hEGF can be easily inserted into the plasmid vectors of *E.coli*. As well as other advantages are, *E.coli* can reach high cell density rapidly[4].

But besides these advantages, the use of *E.coli* as a host cell has several disadvantages, including: (1) the accumulation of protein secretion is non-functional, (2) high chances for the formation of inclusion bodies as a result of incorrect folding (misfolding), and (3) the high rate of protein degradation due to their intracellular protease enzymes[5].

To overcome these drawbacks, the most effective solution is to produce hEGF in extracellular proteins[6] by means of a membrane secreting into periplasm or to the culture medium. By using a secretion system in extracellular, can simplify the purification process, avoiding attacks protease enzyme to denature hEGF, and can minimize mis-folding for protein hEGF directed to folding of the right to the presence of protein chaperones before secreted extracellular[7].

To be able to secrete recombinant protein into the membrane hEGF periplasm or to the culture medium to be used the signal peptide which is fused with the target protein to be passing lane or lanes sec TAT (Twin Arginine Translocation)[8]. Protein secretion pathways that can make production much more effective, as well as to further optimize the secretion of recombinant proteins used hEGF TorA signal peptide with through TAT. Because the signal peptide TorA and TAT way can secrete proteins are great as Green Fluorescent Protein (GFP) very well, so if the signal peptide TorA and TAT way is used to secrete hEGF small size, it is expected to be much better, because hEGF

generates will be folded perfectly, has a good activity and the amount of the maximum secreted. If hEGF produced numbers up and have a good activity, then hEGF can be used for a wide variety of active ingredients in a wide range of cosmetics, serum autologous to cure the tears dry, wound healing drug for diabetic patients are difficult to heal, and therapeutic proteins other, and therefore are expected to use the signal peptide TorA with TAT pathway can secrete recombinant protein production hEGF with high activity[9].

MATERIALS AND METHODS

Materials

The equipment used in this study include glassware commonly used in the Research Laboratory of the Department of Chemistry. Also used other ancillary equipment such as a AKTA START (GE Healtcare), means of agarose gel electrophoresis and SDS-PAGE (Biorad Power PacTM Basic), autoclave sterilizer (Hirayama Autoclave HVE-50), EZ Read 400 Mikroplate Reader (Biochrom), ice bath , ice maker (GPERGA), concentrator (Eppendorf), magnetic stirrer (HMS-79 Magenetic Heated), milipore, mini spin (Eppendorf), balance scales (Mettler Toledo AL204), oven (Memmert), pH meter (Mettler Toledo InLab pH combination polymer elecrodes), micro pipette (Eppendorf), sentrifugator (Tomy MX-305), shaking incubator (N-Biotech), sonicator (Vibra Cell 20 kHz), spectrophotometer (Ultrospec 3000 pro UV / Vis), tubes falcon, micro-tubes (Biologix), tube mikrosentrifugasi (Biologix), micro pipette tip, tool λ_{312} nm ultraviolet light (Vilber Lourmat TCP-20M), vortex (Boeco V-1 plus), and the water bath (Poly Science).

This study uses pD881-TorA plasmid containing the gene hEGF (DNA2.0), enzyme SapI, 1 kb DNA marker, marker proteins Amresco and BioRad, tris-base, L-rhamnose, glycerol, EDTA, yeast extract, tryptone, bakto, sodium chloride, akuabides, tetracycline, kanamycin, Escherichia coli BL21, Bromfenol blue, Comassive brilliant blue, SDS running buffer 1x SDS sample buffer 5x, SDS 10%, TEMED, ammonium persulfate, methanol, ethanol, potassium thiocyanate, Tianprep Mini Plasmid Kit.

Methods

Escherichia coli BL21 Transformation Using pD881-TorA

One colony of Escherichia coli BL21 sampled and transferred into 5 mL of liquid LB and incubated overnight at 37 °C with shaking 200rpm. 1 mL of overnight culture were transferred into 100 mL LB liquid without sodium chloride. The cultures were then incubated with shaking 200 rpm at 37 °C until OD_{600} reached 0.8-1.0. Having achieved the desired OD_{600}, the culture was cooled on ice for 15 minutes. The execution is then performed on ice. The culture was centrifuged at 4 °C, the speed of 5000 rpm, for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 100 mL sterile cold aquabidest then centrifuged again at 4 °C, the speed of 5000 rpm, for 5 minutes. The supernatant was discarded and the pellet resuspended in 50 mL sterile cold aquabidest then centrifuged again at 4 °C, the speed of 5000 rpm, for 5 minutes. The supernatant was discarded and the cell pellet resuspended with 2 mL of cold sterile aquabidest then centrifuged again at 4 °C, the speed of 5000 rpm, for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 200 mL of glycerol 100%. Electrocompetent transplanted cells 50 uL in 1.5 mL micro tube. Electrocompetent cells ready for use or stored at -20 °C.

Escherichia coli BL21 Transformation using pD881-TorA

A total of 5 mL recombinant plasmid is inserted into micro-tubes containing 50 mL electrocompetent cells. The mixture was resuspended and transferred into a 0.1 cm cuvette cold. Let stand in ice for 5 minutes. Dry the outside wall cuvette with a tissue and place the cuvette in eporator tool. Set electroporation at a voltage of 1500 volts. After electroporation, 1 mL of SOC liquid is added to the cuvette. Cells resuspended in cuvette then transferred into a 2 mL micro tube and then incubated at 37 °C for one hour with agitation speed of 200 rpm. After incubation, 100 mL culture propagated in solid LB media containing 75 ug / mL kanamycin. Furthermore, a single colony transformants were replicated.

Isolation of pD881-TorA Transformation Result

Isolation of cloned using plasmid isolation kit Tianprep Mini Plasmid Kit. E.coli BL21 transformant containing plasmid pD881-TorA grown in 5 mL of liquid LB media which has been added 5 mL kanamycin (75 mg / mL) and in*cubated for 16-18 hours at 37* °C, with shaking speed of 200 rpm. Cell cultures of E.coli BL21 put in 1.5 mL micro tube and centrifuged at 13,400 rpm for 3 minutes to collect pellets. The cell pellet was added 250 mL of buffer P1 (added RNase) and then reconstituted using a vortex until dissolved. Then added 250 mL of buffer P2, and micro-centrifuge tube inverted 8 times. A total of 350 mL of buffer P3 is added to the micro-centrifuge tube and inverted 8 times. Micro-centrifuge tubes centrifuged at 13,000 rpm for 10 minutes. A total of 800 mL of the supernatant was transferred to a spin column CP3 calibrated. Column calibration is done by adding 500 mL column buffer BL to CP3. CP3 column and then centrifuged for 1 minute at a speed of 12,000 rpm and then the resulting solution was discarded laundering. Supernatant that has been incorporated into a column that has been calibrated subsequently centrifuged for 1 minute at a speed of 12,000 rpm and then centrifuged supernatant was discarded. Column PD CP3 then added 500 mL buffer and centrifuged at 12,000 rpm for 4 minutes and then the supernatant was discarded. Furthermore, the column was added buffer PW 600 mL and centrifuged at 12,000 rpm for 1 minute. Results centrifugation removed and centrifuged again with the speed of 12,000 rpm for 2 minutes. Container results next column is replaced with the micro tube. Furthermore, EB buffer is added as much as 70 mL and incubated at a temperature of 600C for 2 min and centrifuged for 2 minutes at a speed of 12,000 rpm. EB back buffer is then added and incubated at 600C for 2 minutes and centrifuged at 12,000 rpm for 2 minutes. The results of the elution mikrotube can then be characterized by agarose gel electrophoresis.

Characterization of E.coli BL21 pD881-TorA Isolation Result

Isolation result is characterized by restriction enzyme cutting in one side. A total of 5 mL isolates were incorporated into micro-tubes and add 1 mL of a SapI for restriction enzymes with the addition of 1 mL buffer smart cut and 2 mL of nuclease-free water and then incubated for 4 hours at 37 °C. The restriction results were characterized using agarose electrophoresis 1% (w / v). Marker contains a mixture of 4 mL of nuclease-free water, 1 mL of loading dye, gel 1µL red, and 1µL 1 kb DNA ladder. Then 1 mL of loading dye, red gel 1 mL, and 5 mL of the isolated plasmid to plasmid without cuts while cutting results included all the volume of the reaction product of restriction. Furthermore, markers and mixed plasmid lowered into agarose gel 1% (w / v). Electrophoresis was performed with a voltage of 80 V for ± 40 minutes and using 1x TAE buffer as current conductor media. DNA bands seen with the aid of ultraviolet light on λ_{312} nm.

Expression of hEGF Coding Gene in Escherichia coli BL21

Transformant *E.coli* BL21 were grown in 5 mL of liquid LB medium which has been added kanamycin for 16-18 hours, at 37 ° C, with shaking speed of 200 rpm. *E.coli* BL21 cell culture incorporated into the 1000 mL Erlenmeyer flask containing 100 mL LB liquid and the antibiotic kanamycin. Cultures are grown in an incubator shaker for 12 hours at 37 ° C, with shaking speed of 200 rpm and culture OD600 measurements were taken every hour.

Transformant *E.coli* BL21 were grown in 5 mL of liquid LB medium which has been added kanamycin for 16-18 hours, at 37 ° C, with shaking speed of 200 rpm. *E.coli* BL21 cell culture incorporated into the 1000 mL Erlenmeyer flask containing 100 mL LB liquid and the antibiotic kanamycin. Cultures are grown in an incubator shaker at 37 ° C, with shaking speed of 200 rpm until OD600 0.7 were then added 4 mM L-rhamnose and regrowth in a shaker incubator at 37 ° C, with shaking speed of 200 rpm for 20 hours and the culture OD600 was measured every hour.

Transformant *E.coli* BL21 were grown in 5 mL of liquid LB medium which has been added kanamycin for 16-18 hours, at 37 ° C, with shaking speed of 200 rpm. Cell culture of *E.coli* BL21 incorporated respectively into a 1000 mL Erlenmeyer flask containing 6 each 100 mL of liquid LB and the antibiotic kanamycin. Cultures are grown in an incubator shaker at 37 ° C, with shaking speed of 200 rpm until OD600 reached 0.7 and then added L-rhamnose with various concentrations of L-rhamnose (40µM, 1 mM, 2 mM, 4mm and 6 mM) in each culture then grown back on the shaker incubator at 37 ° C, with shaking speed of 200 rpm for 20 hours, culture supernatants were harvested and taken.

HEGF recombinant proteins expressed in Escherichia coli BL21. A single colony was incubated in 5 mL of liquid LB containing antibiotic kanamycin (75 mg / mL) and incubated at 37 °C with a speed of 200 rpm until OD600 reached 0.7. Then 1 mL of culture was expressed in 30 mL liquid LB medium containing the antibiotic kanamycin (75 mg / mL) and incubated at 37 °C until OD600 reached 0.7 back. A total of 1 mL of culture were sampled prior to induction (To) for SDS-PAGE analysis. Induces the expression starts with *E.coli* cultures with a final concentration of 2 mM L rhamnose after reaching OD_{600} of 0.7 and incubated at 37 °C with a speed of 200 rpm. A total of 1 mL of culture were sampled after induction (Ti) for SDS-PAGE analysis and incorporated into micro-tubes. Media and pellets of cultures separated by centrifugation at 3,000 g at 4 °C for 20 minutes. Pellets are used for cell lysis and extraction processes periplasmic membrane and media can be analyzed using SDS-PAGE.

Cell Lysis and Preparation Laundering / Dissolution of Inclusion Body

A total of 500 mL of buffer Tris-Cl EDTA added, do lysis using sonicator. Lysis process 2 seconds on and 2 seconds off. Lysis supernatant centrifuged at 10,000 g at 4 °C for 25 minutes. Supernatant was transferred to a new micro tube as a fraction of dissolved (soluble fraction) and analyzed by SDS-PAGE. Pellet was added 80 mL of 8M urea and then heated at a temperature of 95 °C for 15 min and centrifuged at 10,000 g at 4 °C for 10 minutes. Supernatant incorporated into a new micro tube as an insoluble fraction (insoluble fraction) and analyzed by SDS-PAGE. For to and ti diluted with 50 mL of 20 mM glycine buffer pH 8.6.

Periplasm Membrane Extraction

Pellets of the separation of culture was added 100 uL Buffer TSE and resuspended slowly. After the pellets dissolve, the solution was incubated in an ice bath for 30 minutes. The suspension solution is then transferred into a micro tube and centrifuged at 16,000 g for 30 minutes at a temperature of 40 ^{o}C. Supernatant separated into micro-tubes and characterized by SDS PAGE.

Characterization Expression Result by Using SDS-PAGE

Samples were taken as the result of expression of 15 uL and mixed with 5µL 5x SDS sample buffer in the micro tube. The mixture is then heated at a temperature of 95 °C for 15 minutes in the water bath. Each sample and SDS sample buffer 5x as much as 20 uL and 5 uL total protein markers inserted into the well. Electrode wires then paired with a device electrophoresis gel electrophoresis at 30 volts to limit stacking gel and increased the voltage to 100 volts when the sample is at resolving gel for ± 120 minutes. Gel is then immersed in staining solution (Commasie Brilliant Blue R-250 0.25%, 45% methanol, 10% acetic acid) for 1 h with slow shaking or for overnight. Gels are then stored in destaining solution until the gel excess dye is lost. The positive results of SDS-PAGE indicated by the presence of recombinant hEGF measuring band ± 6.2 kDa.

Determination of recombinant hEGF levels by ELISA

Enter each 100 uL EGF standard / sample into each well on the ELISA plate, then incubated for 16-18 hours at a temperature of 4^{0}C with low speed agitation. Discard the solution in the wells and wash by adding 300 uL of Wash Buffer into each well, remove the wash solution, then drain well by placing the plate on absorbent paper. Perform washing 4 times. Enter 100 uL Biotinylated EGF Antibody Detection added to each well and incubating the plate for 1

hour at a temperature of 25^{0}C with shaking speed is low. Remove solution from the wells and wash 4 times with Wash Buffer. Enter 100 uL Streptavidin HRP solution into each well, and plates were incubated for 45 minutes at a temperature of 25^{0}C with shaking speed is low. Remove solution from the wells and wash 4 times with Wash Buffer. Enter 100 uL reagent TMB One Step Substarte added to each well, and plates were incubated for 30 minutes at a temperature of 25^{0}C with shaking speed is low. Then add immediately 50 uL Stop Solution to each well and absorbance was measured at a wavelength of 450 nm.

RESULTS AND DISCUSSION

hEGF protein is a protein that can be a signal that can trigger other proteins to activate cell growth activities such as cell proliferation, cell migration, and so forth. HEGF coding genes have been widely expressed in *E.coli* host cells and produced intracellular or extracellular. Extracellular hEGF production method offers several advantages, including to facilitate the purification process, to secrete extracellular hEGF it takes the signal peptide. Gene encoding hEGF constructed beforehand on a plasmid which has a signal peptide TorA, genes for resistance to kanamycin as agents selectors, and so forth, plasmids which have constructed successfully synthesized by DNA 2.0 directly using the expression vector pD881-TorA-hEGF, plasmid is designed in the form circular with the size of 2557 bp.

Construction of pD881-TorA Expression Vector

To perform the transformation in *E.coli* BL21 needed plasmid construction should be done beforehand. Construction of this plasmid includes several things, such as codon optimization hEGF derived from Homo sapiens to be transcribed into a language that is recognized by *E.coli* in order to obtain hEGF protein which has a high homology and activity should be. The process of transcription or translation is done using a web server www.gcua.de with the results as shown in Figure 1(a) and Figure 1(b).

Figure 1 (a) shows the hEGF codons which have been analyzed by the web server www.gcua.de and it can be seen that there are several codons encoding a particular amino acid is not good to use when expressed in *E.coli* host cells as it has a level of less adaptivity of 100%, so it must be optimized in advance by replacing certain nitrogenous bases in the codon. In Figure 1 (b) shows that the level of adaptivity whole codon encoding hEGF gene has to be 100% full, it shows that codons are ready to be expressed in *E.coli* host cells. Furthermore, the overall construction of the plasmid vector. Plasmids constructed an expression vector, as compared to the cloning, expression vectors have the ability to express proteins with optimum ability, but has the capability of cloning that is much lower than cloning vector.

The plasmids were constructed selected by kanamycin resistance gene for the marker in the selection process, then have the signal peptide Tora because the signal peptide can carry hEGF protein leading to the periplasm and recognized by the TAT pathway, then there is also the Ribosomal Binding Site (RBS). RBS is used because it can speed up the process so that expression of the protein translation initiation hEGF can be done quickly and optimum. In this plasmid there is also a promoter regulated by L-rhamnose, when there are molecules L rhamnose the legislation negatively at the promoter will not run, because L rhamnose will trigger repressor binding so that it becomes inactive, and the promoters that are regulated by L-rhamnose can help express the protein hEGF with optimum. Plasmid pD881-Tora ori_p15a also have a side that can help the process of replication of this plasmid in the host cell, this ori_p15a side can make up to 10 copies at up to 12 copies in the host cell. Complete construction of this plasmid contained in Figure 2 and synthesized by DNA 2.0.

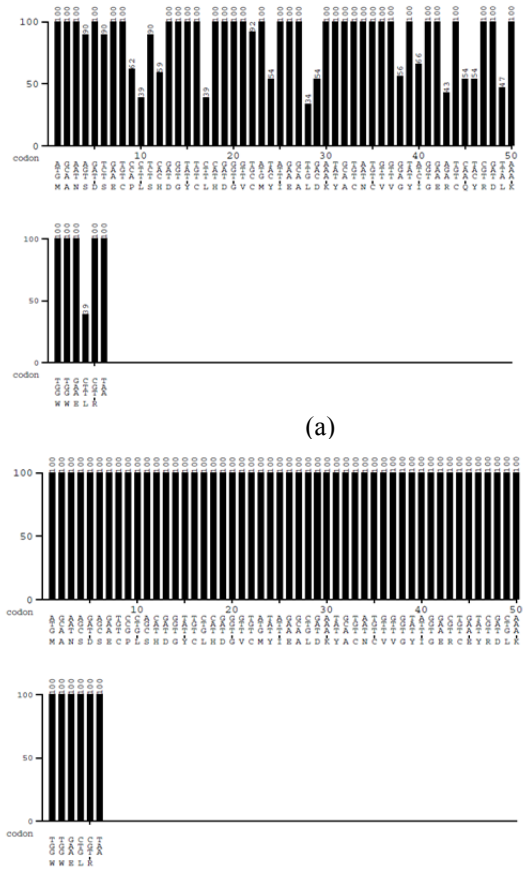

Figure 1 hEGF codon before optimized (a) and hEGF codon after optimized (b)

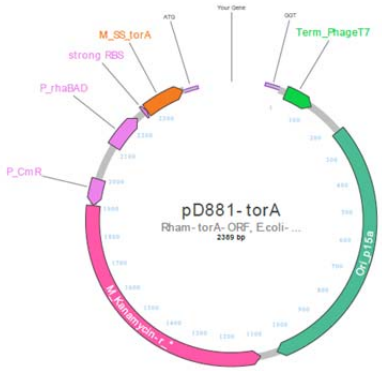

Figure 2 Plasmid pD881-TorA construction

***E.coli* BL21 Transformation Using pD881-TorA**

Plasmid pD881-TorA is a plasmid that can be used directly by the host cell expression, and therefore the plasmid pD881-TorA can be directly used to transform *E.coli* BL21. The transformation can be seen in Figure 3.

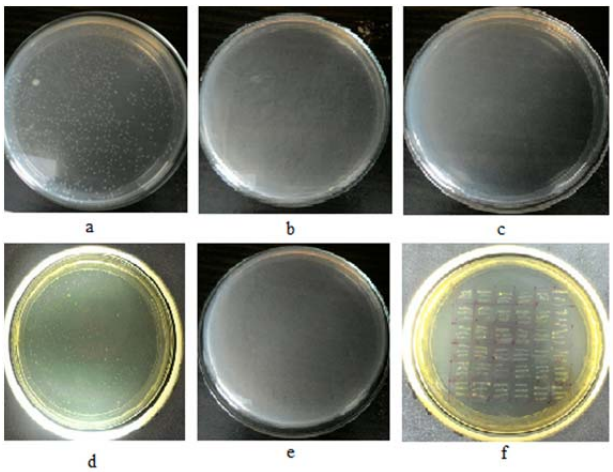

Figure 3 Transformants *E.coli* BL21 (a) Control transformant pPICZ-□β (b) *E.coli* BL21 competent cells positive (c) *E.coli* BL21 competent cells negative (d) *E.coli* BL21 [pD881-TorA] transformant after dilution 20x (e) Concentrated *E.coli* BL21 [pD881-TorA] transformant (f) *E.coli* BL21 [pD881 TorA] replication.

The transformation method used is the method electroshock or electroporation. Electroporation is a method that utilizes electrical current that could destabilize the bacterial cell membrane. The electroporation method should use *E.coli* BL21 cells are already competent, therefore do making *E.coli* BL21 competent cells so that the cells can be inserted plasmid pD881-TorA. Competent cells are cells that have undergone changes in permeability so it can be inserted recombinant DNA. *E.coli* bacteria are not equipped with proteins on the surface of the outer layer of cells to bind the foreign DNA molecules from the surrounding environment, so that the competent *E.coli* must be made in advance for use in a transformation process[10].

Competent cell making is done by growing *E.coli* BL21 in liquid LB medium without NaCl as NaCl or other salts can conduct electric current and the electric voltage increases when electroporation. The voltage is too high can make the cell death that may decrease the effectiveness of electroporation method. The process of growing *E.coli* BL21 performed until OD_{600} reached 0.8-1. This is done because the OD_{600} 0.8-1, the bacteria were in the exponential phase, which in this phase the cells were actively dividing, so that it can be easily inserted foreign DNA.

Cuvettes are used for electroporation using a 0.1 cm cuvette with a power supply voltage of 1.5 kV. The treatment is done by the protocol BioRad, namely for electroporation of *E.coli* in general if using a 0.1 cm cuvette can use a voltage of 1.8 kV and 1.5 kV- if using a 0.2 cm cuvette can use a voltage of 2.5 kV[11]. The result of the transformation was grown on solid LB media with appropriate antibiotics for selection process. The success of this transformation process can be determined by comparing the control transformant, and for the presence of contaminants at the time of making competent cells can be compared with the positive control and the negative controls were carried out simultaneously with the work of transformation *E.coli* BL21 cells [pD881-TorA].

After electroporation, transformants were grown in SOC media (Super Optimal Broth with Catabolic Repressor) in 1 mL for 2-3 hours at 37 °C. Growth of cells *E.coli* BL21 on media SOC to accelerate the recovery and growth of the cells after getting extreme conditions during the process of electroporation, as media SOC is a growth medium that is rich in carbon source derived from glucose and salts of Mg^{2+} and K^{+} which serves to maintain the stability of cells and ease of cell metabolism.

After all transformants were grown in SOC media, and transformants *E.coli* BL21 [pD881-Tora] was grown on solid LB medium containing the antibiotic kanamycin, due to *E.coli* BL21 naturally do not have any antibiotic resistant genes, but because it has been inserted plasmid pD881-Tora then the cells own genes for resistance to kanamycin, because plasmid-Tora pD881 there are genes for resistance to kanamycin. Therefore, as shown in Figure 3 (d) and (e) *E.coli* BL21 cells can grow. Transformants *E.coli* BL21 [pPICZ-β] is used as an indicator of whether or not the process of electroporation runs, when *E.coli* BL21 cells [pPICZ-β] grow well, then the process of electroporation can be said to be going well, and any desired plasmid should be is inserted properly. In Figure 3 (a) shows that the cells of *E.coli* BL21 [pPICZ-β] can grow well with antibiotic selection zeosin, because plasmid pPICZ-□β have zeosin resistance gene used for the selection process. It can be concluded that the electroporation process take its course and the plasmid was successfully inserted into *E.coli* BL21 cells.

Making competent cells positive and negative competent cells aims to look at the quality of *E.coli* BL21 competent cells that have not inserted any plasmid. At competent cells positive should the cell can grow well, because the solid LB media was not given any antibiotic, and as well as the results shown in Figure 3 (b), whereas for competent cells negative there should not be any cell grown on solid LB media that, for solid LB media was added antibiotic kanamycin, kanamycin antibiotic use is to ensure that the competent cells actually do not carry kanamycin resistance gene as well as on-TorA pD881 plamsid be inserted. In Figure 3 (c) indicates the absence of cells grown in the media indicating that the absence of contaminants in *E.coli* BL21 competent cells, so the cells are made competent *E.coli* BL21 has good quality and there are no contaminants therein.

Through these controls can be known if the cause of the transformation process is not running properly is whether the making of competent cells, transformation process or the selection of antibiotics used. This transformation process is considered successful because all the controls grown in accordance with properly.

In the *E.coli* BL21 transformant [pD881-TorA] (Figure 3 (e)) turns colony grows very thick and overlap each other, so it can not take a single colony to do replication. Single colonies should have in theory a single colony is a collection of cells that have the same genetic code are derived from a single stem cell that replicates repeatedly. Selection of a single colony will produce a pure culture more possibilities than colonies that accumulate because of the overlap there is a possibility colonies of cells that have mutations or contaminants trapped in it, so the dilution of 100x and 20x (Figure 3 (d)). The result in dilution of 100X very few single colonies were generated, and it is feared the contaminant, while the 20x dilution seen in Figure 3 (d) to produce a single colony enough to do replication.

Single colonies were grown subsequently sampled and rejuvenated through the replication process on a new solid LB media which already contains antibiotics kanamycin. The objectives of replication of a single colony is to multiply each single colony produced, and the result can be seen in Figure 3 (f).

Plasmid pD881-TorA Isolation and Characterization

Once the colonies are replica grow well, the colony picked out and grown in liquid LB medium for 18 hours at 37 °C, which is expected to grow until early stationary phase. Early stationary phase is the condition of bacterial cells are in high quantities and constant before bacterial growth slowed because the nutrients will begin to run out and start the product of secondary metabolites inhibit bacterial growth. Furthermore, every culture that made its glycerol stock (200 uL and 800 uL of glycerol bacterial culture) to make it easier if you want regrowth and can be stored for longer at -20 °C.

After the transformation necessary to isolate plasmid which aims to extract the plasmid and separate it from the cellular components of bacteria, particularly chromosomal DNA. Results plasmid isolation pD881-TorA further characterization using restriction enzymes SapI. Results plasmid isolation and restriction then visualized by 1% agarose electrophoresis. The resulting electropherogram is shown in Figure 4

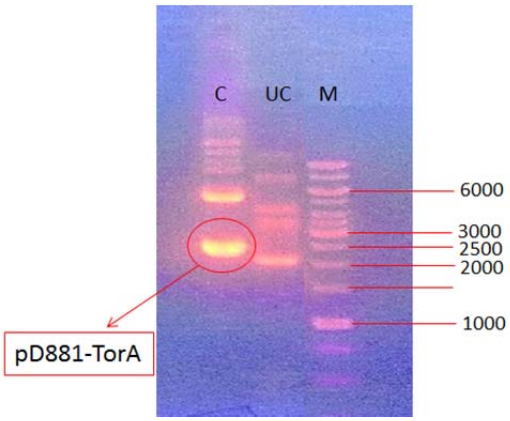

Figure 4 Electropherogram plasmid isolated from *E.coli* BL21 and digestion with enzymes restiksi SapI. (C) Plasmid pD881-TorA / SapI (cut). (UC) Plasmid pD881-TorA (uncut). (M) Marker 1 kb.

In lane C (cut) it can be seen that the lane has a band to be around 2500 bp markers that can be inferred that these bands indicate pD881 plasmid-TorA successfully truncated by restriction enzyme cutting a SapI on one side which causes changes in plasmid form pD881- TorA those of conformation initially circular be linear, but on the lane C is still visible there are some bands are visible above plasmid pD881-TorA, these bands are bands of plasmid pD881-TorA uncut by the enzyme SapI , so it is still shaped circular has some conformation that led to many bands. It uncut plasmid pD881-TorA by restriction enzyme SapI can be caused because of not optimum cutting done by the SapI enzyme.

In lane UC (uncut) it can be seen that the lane has some band. This is the same plasmid DNA but because they form circular DNA plasmid has a different conformation as supercoiled monomer, dimer supercoiled and nicked circular (nc)[12]. According Edvotek 2001, DNA with a supercoiled conformation has the fastest rate of migration and more numerous than other plasmid DNA conformation and the conformation of DNA to nicked circular (nc) has a slower rate of migration.

Restriction enzymes can be used to characterize because it is specific, which recognize short nucleotide sequences in a DNA molecule and cut at a certain point in the nucleotide sequence[13]. SapI restriction enzymes recognize specific nucleotide sequence 5 'GCTCTTCT ↓ A 3' listed on the point that it can be inserted gene encoding hEGF with the introduction of the same restriction.

Plasmid isolation procedures are conducted using Tianprep Plasmid Mini Kit is based on a modified alkaline lysis method in bacterial cells followed by adsorption of DNA to silica by using high concentrations of salt. There are three basic steps of this method is the process of bacterial lysis, DNA adsorption on Tianprep membrane, and washing and elution of plasmid DNA.

Bacterial lysis process takes place in alkaline conditions (base) and then neutralized lysate from alkaline conditions and adjusted to the conditions binding with the membrane in a buffer containing a high concentration of salt in one step. P1 buffer containing glucose with a high concentration that when it enters into cells capable of damaging the cell membrane. P2 buffer containing SDS and NaOH. SDS serves to damage the cell membrane and remove components that are in the cell and serve NaOH to denature the DNA plasmid and chromosomal DNA into single strands. Buffer N3 serves to renaturation of DNA double-strand DNA plasmid into. Thread-single-stranded circular DNA plasmid that is small, air can form a double-stranded complete renaturation which remain in solution, while the chromosomal DNA are much bigger than plasmid can not air the complete renaturation and precipitated along with other cell components. Tianprep column using silica membranes for selective adsorption of plasmid DNA in a buffer containing a high salt concentration and elution in a buffer containing a low concentration of salt. Tiangen products using buffers that have been optimized in lysis procedures and combined with a unique silica membrane to ensure that only the DNA adsorbed while RNA, cellular proteins and metabolites are not retained on the membrane.

Washing and elution process is performed to remove salts from the previous buffer and to eliminate endonuclease when using bacterial strains containing endA+ to prevent the degradation of plasmid DNA.

Agarose gel electrophoresis is a separation technique based on the charge and molecular size by using an electric field. In electrophoresis there is an electric current flowing in TAE buffer. The existence of a phosphate group (PO_4) in the DNA cause all the DNA is negatively charged so that DNA can migrate from the cathode to the anode. Migration speed of each DNA differs depending on the size of the DNA. If a large-sized DNA then migration will be slow, and vice versa. This migration speed difference causes each DNA of different sizes can be separated.

Expression Test and Protein hEGF Characterization

To test the expression and obtain an optimum condition needs to create growth curve and the production curve. Making this growth curve based on turbidimetry method is to measure the amount of bacterial cells by turbidity of a medium, wherein the absorbance will be inversely proportional to the number of cells and the number of cells will be directly proportional to OD. Making the growth curve is intended to determine the right time to do induction in *E.coli* BL21 cells in order to produce protein hEGF, because the time is right for induction is when the early exponential phase. At the beginning of the exponential phase is the phase in which the cells divide in an active state at a constant speed. Induction conducted in this phase will increase the yield of the desired target protein. *E.coli* BL21 growth curve shown in Figure 5

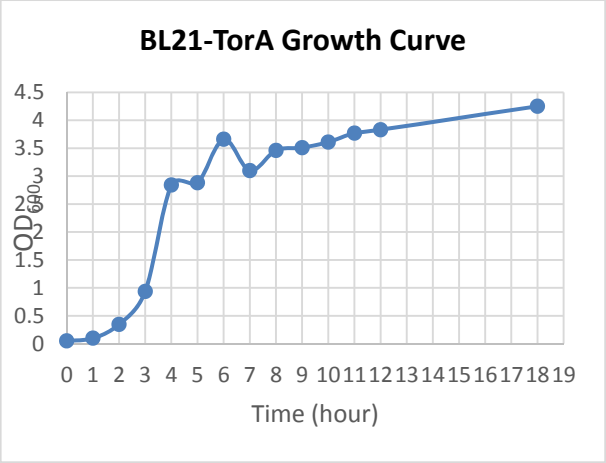

Figure 5 The growth curve of *E.coli* BL21. Exponential phase took place at the 2nd till the 4th. Stationary phase took place at the 5th until the 18th hour.

Now we know the exact time and OD_{600} to do induction, ie between the hours of 2 and 3 hours all the time OD_{600} reached 0.6-0.8. Then created a production curve to determine the right time to produce hEGF protein that can be harvested in the media. Making the production curve is accomplished by growing culture by involving the induction L-rhamnose on hour-2.5. The results are shown in Figure 6

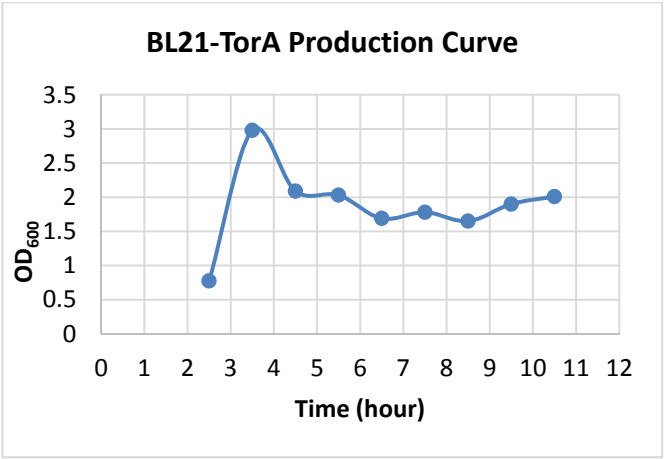

Figure 6 Production curve *E.coli* BL21. Maximum cell growth occurs on the first hour after induction

Expression and Characteroization

Gene expression is the process by which information from a gene contained in nucleotides is transcribed and translated into proteins or RNA until appropriate. Based on the results of the growth curve, the induction time is when OD_{600} reached a range between 0.6 to 0.8. Therefore, when the culture OD_{600} reached 0.6-0.8 induction using L-rhamnose to a final concentration of 2 mM. After expression, to determine whether the protein can be secreted extracellular hEGF or not, were characterized by SDS-PAGE on a periplasm fraction shown in Figure 7

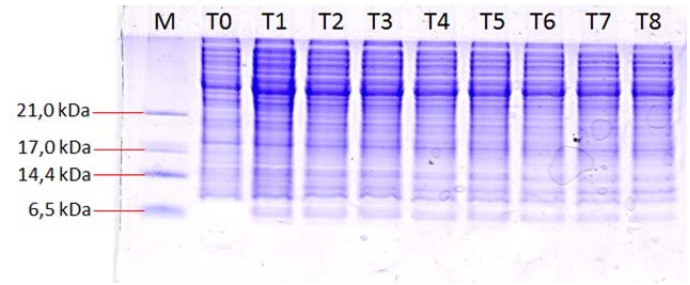

Figure 7 SDS-PAGE electropherogram of *E.coli* BL2 periplasmic fraction. (M) Amresco marker, (T0) h-0 before induction (T1) h-1 after induction, (T2) h-2 after induction, (T3-T8) hours 3rd until 8th after induction.

Figure 7 shows that there is no band in the adjacent lane markers T0 6.5 kDa, but on the lane T1 to T8 seen the band adjacent to the 6.5 kDa markers. This indicates that the band adjacent to the lane markings 6.5 kDa T1 to T8 is hEGF protein bands, because the hour-0 before induction of protein hEGF will not be expressed, and therefore does not appear the band on the track T0.

Calculation of molecular weight of protein samples are also performed by using linear regression analysis. First made in the manufacture of standard curve of protein markers by extrapolating the molecular weight markers as the Y axis and Rf as the X axis, it was found that the standard curve was linear equation y = -1,02x + 1.811, so it was found that the molecular weight of the recombinant hEGF of ± 6 , 18 kDa.

Inducer L-rhamnose serves as compounds that can activate the gene encoding inducer which can bind to the repressor, so that the regulation negatively can not be executed because of the absence of repressor binds to the promoter gene and the expression can take place, so that the protein hEGF can only be generated when it has induction with L-rhamnose.

Based on the results electropherogram contained in Figure 4.7 also shows that the protein band hEGF fraction of periplasmic increasingly thin, it is suspected that the protein hEGF is located in the cytoplasm are secreted into the media, so it is necessary to characterize SDS-PAGE fraction of the media, the result is shown in Figure 8

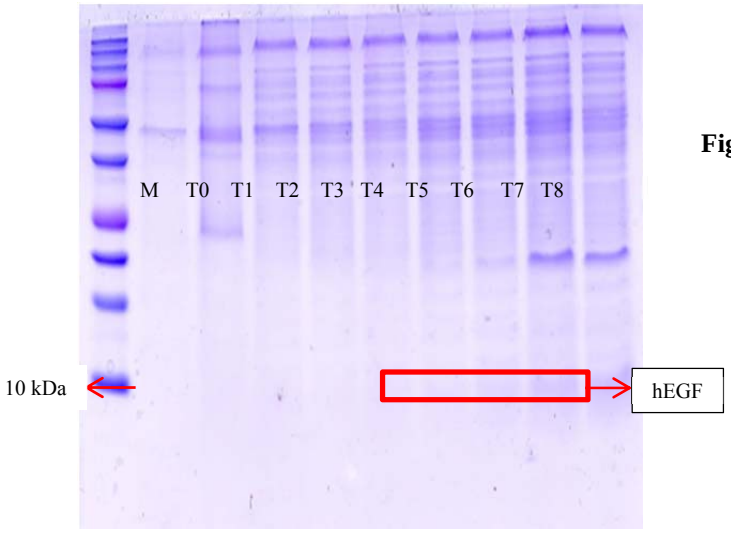

Figure 8 SDS-PAGE electropherogram of *E.coli* BL21 media fraction. (M) Amresco marker , (T0) h-0 before induction (T1) h-1 after induction, (T2) h-2 after induction, (T3-T8) hours 3rd until 8th after induction.

In Figure 8 visible band size is less than 10 kDa that thicken over time, the longer the time after induction of the emerging band that is getting thicker on the fraction of this media, and on lane T0 which shows the fraction of the media prior to induction is not seen the band size less than 10 kDa, so it can be presumed that the band size is less than 10 kDa protein bands are hEGF. Thickening of the band from time to time to indicate that the protein hEGF in periplasmic start secreted into the media. Thus, the longer the protein hEGF will increasingly accumulate on the media.

Calculation of molecular weight of the recombinant hEGF also done by making a standard curve of protein markers with linear regression method as was done in the periplasm fraction. Based on the extrapolation of the standard curve, the molecular weight of the recombinant hEGF in the fraction of the media has a weight of ±6,21kDa.

To convince the hEGF protein translocation process needs to be carried out on the characterization of the dissolved fraction of the cytoplasm, periplasm fraction, and the fraction of media simultaneously to the mobility of the protein hEGF. Electropherogram to show the mobility can be seen in Figure 9

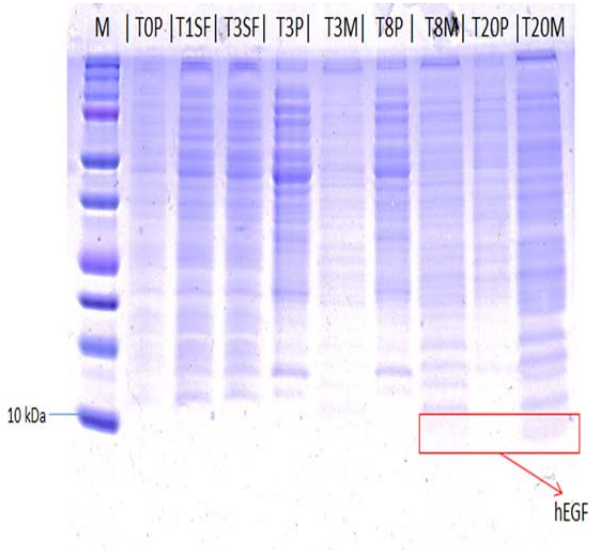

Figure 9 SDS-PAGE electropherogram mobility hEGF protein in *E.coli* BL21. (M) BioRad Marker, (T0P) h-0 before induction fraction periplasm, (T1SF) h-1 after the induction fraction dissolved, (T3SF) hours to 3 hours after induction fraction dissolved, (T3P) hours to 3 hours after induction fraction periplasm, (T3M) hours to 3 hours after induction fraction of the media, (T8P) hours to 8 after the induction fraction periplasm, (T8M) hours to 8 after the induction fraction of the media, (T20P) hours of the 20th after the induction fraction periplasm, (T20M) 20th hour after the induction fraction of the media

Determination of the sampling clock to-0 before induction in the fraction of periplasmic to prove that the protein hEGF really not expressed in the absence of inducer L-rhamnose, and in Figure 9 proved that on the lane T0P there is no band hEGF size is less than 10 kDa. Lane T1SF taken as based on the production curve, on the hour all 1 shows the number of cells after induction at most and indicated that the protein hEGF had already been formed in the cytoplasm, and therefore do cell lysis in *E.coli* BL21 and dissolved fractions taken to characterize the presence of protein hEGF. h-3rd after induction samples taken at the fraction of the dissolved fraction of periplasmic and fractions media, because it is assumed on the hour to 3 hours after induction of protein hEGF had been produced on the clock to1 in the cytoplasm will be transported out of the cytoplasm, either into the periplasm or to the media.

Based on the results electropherogram SDS-PAGE in Figure 7 and Figure 8 shows that the protein hEGF on hour-8 is evident that thinning protein bands hEGF fraction periplasm whereas in the fraction media thickening, and therefore the samples taken in the hours to 8 fractions periplasm and media to see results, and the results in Figure 9 managed to confirm that occur hEGF from periplasmic protein translocation to the media which is indicated by the presence of protein bands hEGF on media fraction, whereas there is no band in the periplasmic fraction of the 8th hour.

Figure 9 shows the mobility of hEGF protein on the host cell *E.coli* BL21, overall bands that look very thin, but it

can be seen that the 20th hour after the induction of the periplasmic fraction no visible band size is less than 10 kDa, but at a fraction media seen the band is less than 10 kDa. This indicates that all proteins are located in the periplasmic hEGF has been successfully secreted into the media, and on the hour to -20 after the induction is suitable for harvesting time in the media.

Calculation of molecular weight of recombinant hEGF that appear in Figure 9 were calculated using linear regression and obtained hEGF molecular weight of ± 6.16 kDa. Thin band seen on SDS-PAGE electropherogram can be caused by processes that lead to lysis of denatured protein, the use of rare codons of a particular amino acid, mRNA and protein was not stable so easily degraded or their unexpected stop codon as mutation. In addition, because the protein in very small hEGF ie 6.2 kDa and the amount of production that very little can cause the hEGF protein unreadable on the SDS-PAGE.

hEGF Protein Assay Using ELISA

Determination of protein hEGF performed by ELISA kit protocol (abcam ab100504 - EGF). ELISA kit abcam use the system sandwich ELISA or also called ELISA plated for using more than one antibody involved in the process, namely the primary antibody as catcher and secondary antibodies as detection, as well as enzymes additional function to convert the substrate to a product that can absorb light at a wavelength of 450 nm.

The principle of testing for protein content by ELISA, hEGF is by measuring the light at a wavelength of 450 nm is absorbed by the product of the conversion of the substrate by the enzyme.

In ELISA kit abcam ab100504 - EGF has been equipped with the well that already has a primary antibody serves to capture hEGF standard or sample, and then after standard / sample hEGF attached to the primary antibody, was added to the wells that have been biotinylated secondary antibody. This Biotin acts as a ligand that binds to a protein-bound streptavidin with HRP enzyme. This protein streptavidin binding biotin on the secondary antibody with high affinity approximately $1x10^{-15}$ M. HRP enzyme accelerates the oxidation process of a substrate, such as benzidine and other aromatic amine groups. The enzyme substrate TMB will change into a blue product, TMB chosen as a substrate for the level of danger is lower than other benzidine group. Once added H2SO4 solution as a reagent which lay the reaction, the substrate will change color to yellow product, where the situation is stable at acidic pH[14]. Furthermore, the ELISA plate is scanned with DAR800 ELISA reader at a wavelength of 450 nm. The intensity of the resulting yellow color according to the absorbance and concentration of hEGF produced. Based on the results of the ELISA readings obtained hEGF concentration of 0.9625 ug /mL.

SDS-PAGE of hEGF as an Inclusion Body

Based on the data which has been described previously that hEGF protein expression always has the result that little is secreted into periplasm or media. This can be caused by the presence of the selection owned by TAT pathway. The proteins can not fold like It native structure in the cytoplasm, will be brought to the complex TatBC by the signal peptide will then be returned to the cytoplasm as insoluble protein[15]. Protein is not dissolved extracted from cytoplasmic cell lysis, analyzed and then characterized using SDS-PAGE and the resulting electropherogram as in Figure 10

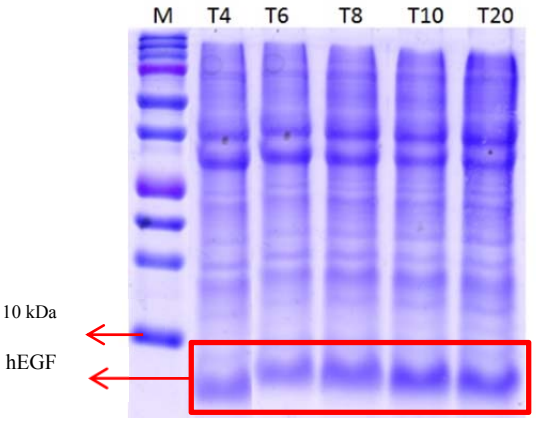

Figure 10 hEGF protein electrophoregram as an inclusion body in cytoplasm

Based on Figure 10 it can be seen that at the 4th, 6th, 8th 10th, and 20th hours after the induction of an increasingly visible presence of a thickened band size is less than 10 kDa. It shows that the longer, the more protein hEGF expressed but not transported to the periplasm and the media, because the *E.coli* strain BL21 there are no cytosolic chaperones to help the protein hEGF third form disulfide bridges that should have protein hEGF, then from the hEGF protein that can not fold approach It native structure will be returned to the cytoplasm and will accumulate as insoluble protein.

CONCLUSION

hEGF recombinant can be secreted extracellularly by using TorA signal peptide via TAT pathway that proved by SDS-PAGE method, and hEGF recombinant assay that successfuly secreted into culture medium using TorA signal peptide via TAT pathway using ELISA method is 0.9625 ug/mL.

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