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Extracellular secretion recombinant of human epidermal growth factor (hEGF) using pectate lyase B (PelB) signal peptide in escherichia coli BL21(DE3)

Sriwidodo[1], Iman P. Maksum[2], Riswanto N[1], Tina Rostinawati[1], Toto Subroto[2]*

[1]Pharmaceutic & Pharmaceutical Technology Department, Faculty of Pharmacy, Universitas Padjadjaran, Bandung, Indonesia

[2]Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia

ABSTRACT

The extracellular expression of high valued therapeutic proteins such as Human Epidermal Growth Factor (hEGF) from Escherichia coli has become a challenge yet give some advantages. It ensures the correct folding of recombinant hEGF (rhEGF) so the therapeutic activity and immunogenicity profile will be similar with the native hEGF. In addition, extracellular expression eliminates the host cell disruption and simplifies the purification process, although other study has shown the protein yield from E. coli extracellular expression is lower tha n the intracellular expression. Various factors such as codon usage, inducer concentration, induction time, and harvest time can be optimized to increase the rhEGF secretion. The aim of this research is to express rhEGF extracellularly from E. coli BL21 (DE3). The expression system was supported by optimized codon usage to E. coli codon preference, pectate lyase B (PelB) signal peptide in the pD881 -PelB expression vector, and L-rhamnose as an inducer. Growth curve of E. coli was made to determine the L-rhamnose induction time. Induction was performed with 4mM L-Rhamnose at OD600 0.7. rhEGF in the soluble fraction, periplasmic extraction, and culture medium was characterized by tricine SDS-PAGE. Quantification of rhEGF concentration was performed by ELISA. The codon optimization showed that the Codon Adaptive Index (CAI) of rhEGF gene was 1, GC percentage was 50.93% and relative adapts was 100% after codon usage optimization. SDS-PAGE showed 6.2 kDa band of rhEGF band from soluble fraction, periplasmic extraction (after 18 hour induction), and culture medium. The quantification of rhHEGF by ELISA showed the rhEGF concentration was 310.8 µg/mL.

Keywords: Recombinant human EGF; Codon optimization; PelB signal peptide; Extracellular expression of *E. coli* BL21 (DE3); Periplasmic extraction.

INTRODUCTION

Epidermal Growth Factor is a polypeptide containing 53 amino acids and having 3 disulfide bonds. It was firstly discovered on the submaxillary gland of rats in 1962 by Cohen et al.while Human Epidermal Growth Factor (hEGF) was first isolated from urine in 1975 by Starkey et al. (Gainza et al., 2015). hEGF was known as a potent mitogenic factor for maturation on epidermal cell. Many studies showed the ability of hEGF in stimulating the cell proliferation in tissue culture (Dinh et al., 2015). Researchers agree that hEGF is strongly related to the released thrombocyte and growth factor during blood clotting. The experiment on animal test has showed the treatment of EGF on the condition animal was proved to help the increase of wound healing (Zhang et al., 2015).

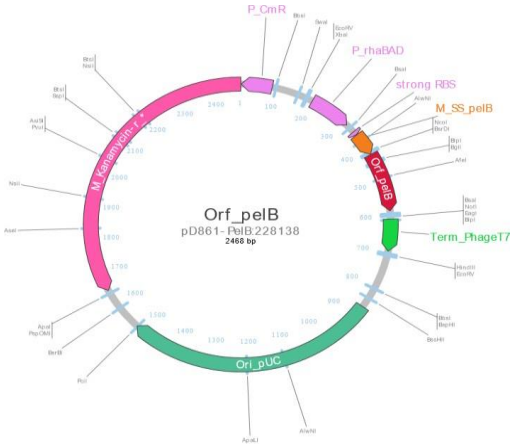

Figure 1: Plasmid map of pD881-PelB

Some studies that have been done since 1975 by Cohen & Capenter showed hEGF gave significant result in healing chronic wounds, corneal injuries, and gastric ulcers (Huang et al., 2001). The increasing needs for hEGF in clinical applications, many studies have attempted to increase the production of recombinant hEGF (rEGF) as terapeutic protein by recombinant DNA

* Corresponding Author
Email: t.subroto@unpad.ac.id
Contact: +62 (022) 7307522 Fax: +62 (022) 7307522
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technology (Lee et al., 2003; Sharma et al., 2008). Unfortunately, the expression of small peptides in Escherichia coli is ineffective due to rapid peptide degradation by intracellular protease, hence its fusion with another protein become an alternative way to hinder proteolityc degradation. Nevertheless, cleaving the fusion protein with enzyme is a costly process (Razis et al., 2006).

High yield of rhEGF expression has been reported by many studies in the form of intracellular precipitation or inclusion bodies aggregate. Renaturetion need to be performed on inclusion bodies aggregate which can lead to proper protein refolding failure. Despite of the availability of refolding protocols such as dialysis and dilution, those protocols is time-consuming, inefficient, and expensive because the needs of additional chemicals and laminar flow technology on a micro fluid chip (Yamaguci & Miyazaki, 2014). Therefore, some issues in expression still remain despite the recombinant protein can be prevented from proteolytic degradation and accumulated into higher level (Razis et al., 2006).

Recently, many techniques are developed to overcome the inclusion bodies, extracellular expression of recombinant protein from E. coli cell factory is one of the technique. The advantage of the extracellular expression from E. coli over intracellular expression and mammalian cell culture are the possibility of proper refolding, the accumulation of recombinant protein in culture medium, and the simplification of isolation and purification in downstream process (Yoon et al., 2010). These systems has given an interesting approach in extracellular rhEGF production for an efficient commercial use. (Razis et al., 2006).

The strategy for production development through the introduction of E. coli membrane signal peptide on Sec dependent pectate lyase b (pelB) pathway becomes an interesting strategy to produce secreted rhEGF with proper refolding to generate high yield of active hEGF (Choi & Lee, 2004). This study gives information to the development of efficient hEGF extracellular expression from hEGF synthetic gene by using pelB signal peptide. The amount of rhEGF in culture medium in this study was 31.81 µg/mL.

MATERIALS AND METHODS

Reagent and chemicals

Material used in this study were bacto agar bacto (Oxoid), agarose (Sigma-Aldrich), ammonium persulfat (Bio Basic INC), Bromphenol blue (Merk), Comassive briliant blue (SERVA), EDTA(1'st Base), E.coli BL21(DE3), Gel Red (Biotium), hEGF synthetic gene inserted in pD881-PelB plasmid (synthesized by DNA 2.0, California, USA), glycerol (1'st Base), Kanamycin Sulfat (Sigma-Aldrich), L-rhamnosa (Sigma-Aldrich), protein marker (Biorad), DNA 1 kb marker (Thermo Fisher Scientific), sodium chloride (Merk), SDS (Merk), tetracyclin, TEMET, TIAN prep Rapid Mini Plasmid Kit (TianGen), tris base (Merk), tricine (Sigma-Aldrich), tripton (1'st Base), Urea (Merk), yeast extract (1'st Base).

hEGF gene optimization and plasmid construction

The HEGF gene sequence was obtain from http://ncbi.nlm.nih.gov with accession number gq214314.1. The hEGF gene sequence was optimized by online software available at http://genomes.urv.es/OPTIMIZER/ and was substituted by the optimized sequence with E. coli preference codon by online software http://gcua.schoedl.de/. The amino acid and gene sequences were verified using online software http://www.biologicscorp.com/tools/RareCodonAnalyzer#. Expression plasmid construction was purchased from www.dna20.com.

E. coli BL21(DE3) competent cell preparation

One colony of E.coli BL21(DE3) was transferred into 5 mL LB medium containing suitable antibiotics, then was incubated overnight at 37°C with shaking at 200 rpm. After incubation, 1 mL culture was transferred into 100 mL LB medium containing suitable antibiotics without sodium chloride. The culture was incubated at 37°C with shaking at 200 rpm until it reached OD600 0,8-1,0. Subsequently, the culture was cooled in ice bath for 15 minutes. The culture was centrifuged 5000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 mL of cold aqua bidestilata. The suspension was centrifuged 5000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 50 mL of cold aqua bidestilata. The suspension was centrifuged 5000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2 mL of cold aqua bidestilata. The suspension was centrifuged 5000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 µL gliserol 10%. Alliquote of 50 µL electro competent cell was made in 1.5 mL micro tube to use or to store at -20°C.

Plasmid transformation

Five µL of plasmid was inserted into micro tube containing 50 µL electro competent cells. The mixture was resuspended and was transferred into 0.1 cm cuvette then was put in an ice bath for 5 minutes before electroporating it. Electroporation voltage was set to 1500 volts. One mL SOC was added after the electroporating the mixture. The mixture was transferred into 2 mL microtube than was incubated at 37°C for an hour wi th shaking at 200 rpm. After incubation, 100 µL of culture was spread on LB agar medium containing 100 µg/mL tetracycline and 25 µg/mL kanamycine.

Plasmid isolation

One to five mL E. coli BL21(DE3) [pD881-PelB] culture was grown for 18 hours in LB medium containing kan-

amycine. CP3 column was equilibrated by putting it in a collection tube. BL buffer 500 µL was added in the CP3 column. The column was centrifuged for 1 minute at 12,000 rpm then the supernatant was discarded and the CP3 column was put back in collection tubes. The cell was harvested from the medium by centrifuging at 12,000 rpm for 1 hour at room temperature. The cell pellet was resuspended by buffer P1 250µL containing RNAse. Buffer P2 250µL was added and mixed gently by flipping the tube 6-8 times. Buffer P3 350µL was added and mixed well by gently flipping the tube 6 -8 times. The tube was centrifuged at 12,000 rpm for 10 minutes. Supernatant was transferred into CP3 column, then the column was put in collection tube, then was centrifuged at 12,000 rpm for 30-60 seconds. CP3 column was washed 2 times with buffer PW 600µL that had been added absolute ethanol. The column was centrifuged at 12,000 rpm for 30-60 seconds. After the flow-through was discarded the column was centrifuged again at 12,000 rpm for 2 minutes to remove the PW buffer residue. CP3 column was put in 1.5 mL microtube than was eluted with 50-100 µL of EB buffer, then was incubated for 2 minutes and was centrifuged at 12,000 rpm for 2 minutes. The isolated plasmid was in the flow-through and was characterized by agarose electrophoresis.

Growth curve and Production curve

Transformant was grown in LB medium 5 mL containing kanamycin about 16-18 jam at 37^{o}C with shaking at 200 rpm. 1,000 µL of culture was transferred into LB medium 100 mL in shake flask containing kanamycin for 12 hours at 37^{o}C with shaking at 200 rpm. OD600nm was measured every hour. Starter culture 1000 µL was transferred into LB medium 100 mL containing kanamycin and was grown to OD600nm reach 0,7. Inducer L-rhamnosa 4 mM final concentration was added. The culture was incubated for 20 hours at 37°C with shaking at 200 rpm. OD600nm was measured every hour.

Induction optimization

Transformant was grown in LB medium 5 mL containing kanamycin about 16-18 hours at 37^{o}C with shaking at 200 rpm. 1000 µL of the culture was transferred into 6 LB 100 mL medium in shaken flask containing kanamycin. The culture was incubated at 37^{o}C with shaking at 200 rpm until OD600nm reach 0,7 then L-rhamnosa was added to final concentration variation 40 µM, 1 mM, 2mM, 4mM, and 6 mM. Incubation was continued for 20 hrs .

Recombinant hEGF expression

E. coli BL21 (DE3) [pD881-PelB] that has been characterized was grown in 5 mL LB medium containing kanamycin about 16-18 hours at 37oC with shaking at 200 rpm. E. coli BL21 (DE3) [pD881-PelB] culture 1000 µL was transferred into 100 mL LB medium containing kanamycin in shaken flask. Native E. coli BL21 (DE3) was also grown and treated like the transformant. The culture was incubated until OD600nm reach 0,7. 1 mL E. coli BL21 (DE3) [pD881-PelB] culture was taken as a sample for t0 (before L-Rhamnosa induction) and transferred into microtube then was centrifuged at 3,000 g, 4^{o}C for 20 minutes. L-Rhamnosa was added into E. coli BL21 (DE3) [pD881-PelB] and native E. coli BL21 (DE3) culture at final concentration 2 mM. Incubation was continued at 37^{o}C for 20 hours with shaking 200 rpm. 2 mL of E. coli BL21 (DE3) [pD881-PelB] culture was taken as a sample ti (after L-Rhamnosa induction) and transferred into 2 microtubes then was centrifuged at 3000 g, 4^{o}C for 20 minutes.

Soluble and insoluble fraction

500 µL Tris-Cl EDTA buffer was added into microtube containing cell pellet then was resuspended an lysed using sonicator. Lysis process was performed in 3 cycles. One cycle was 2 minute process with 2 seconds on and 2 seconds off. Minutes for the break period before continued to the next cycle. Supernatant from lysis process was centrifuged at 10,000 g, 4^{o}C for 30 minutes. The supernatant was transferred into another microtube and used as soluble fraction. The pellet was added 70 µl urea 8 M then as heated at 95^{o}C for 15 minutes and was centrifuged at 10,000 g , 4^{o}C for 10 minutes. The supernatant was transferred into another microtube as insoluble fraction.

Periplasm extraction

Preipalsm extraction was performed by adding 100 µL Tris-Cl Sukrosa-EDTA buffer (TSE buffer) into cell pellet in microtube than was resuspended and was incubated in ice bath for 30 minutes. It was centrifuged at 20,000 g, 4^{o}C for 30 minutes. Supernatant was transferred into another microtube as periplasma protein extract (Quan et al., 2013).

hEGF characterization by tricine SDS-PAGE

Expression sample 15 µL was mixed with 5 µL sample reduction buffer. The mixture was incubated at 37^{o}C for 15 minutes. 20 µL of the mixture was transferred into gel well. 7 µL biorad® protein marker was used as protein size markers. SDS-PAGE was performed at 30 V for 30 minutes until the marker and the sample run over the stacking gel, then the voltage was increased up to 100 V for 2 hours. Subsequently, gel was put in staining gel solution and was incubated at 25^{o}C for 18 hours in dark condition. Gel was put in destining solution at 25^{o}C for 6 hours.

hEGF measurement wiith ELISA

Standard EGF Preparation

Standard EGF standard (Abcam®) 130 ng was dissolved in 1 mL Assay Diluent B, then cascade dilution was made by diluting standard EGF standard stock (130 ng/mL) with Assay Diluent B to generate 8 EGF standards (200 pg/mL;80 pg/mL;32 pg/mL;12,8 pg/mL;5,12 pg/mL;2,05 pg/mL;0,82 pg/mL, dan 0 pg/mL).

Sample Preparation

Supernatant 1 mL was separated from BL21 (DE3) [pD881-PelB] culture on the 20th hour after induction. 10 µL of the supernatant was added into 990 µL Assay diluent B (100 fold dilution), then 10 µL of 100 fol d culture dilution was added by 990 µL Assay diluent B to generate 10,000 fold dilution. 10 µL of 10,000 fold culture dilution was added 990 µL Assay diluent B to generate 1,000,000 fold dilution. 500 µL of 1,000,000 fold culture dilution was added 500 µL Assay diluent B to generate 2,000,000 fold dilution (Abcam, 2014).

Measurement of hEGF concentration using ELISA

hEGF standard/sample 100 µL was transferred onto ELISA plate well than was incubated for 16-18 hours at 4^{o}C with low rpm shaking. Solution in the wall was discarded and each well was washed with 300 µL Wash Buffer. Washing solution was discarded, then well was dried by placing the plate on absorbent paper. Washing was performed 4 times. 100 µL Biotinylated EGF Detection Antibody was added into each well then was incubated for 1 hour at 25^{o}C with low rpm shaking. Solution in the wall was discarded and the wall was washed 4 times with Wash Buffer. 100 µL HRP-Streptavidin solution was added into each well then was incubated for 45 minutes at 25^{o}C with low rpm shaking. Solution in the well was discarded, then the well was washed 4 times with Wash Buffer. 100 µL TMB One-Step Substrate reagent was added into each well and was incubated for 30 minutes at 25^{o}C with low rpm shaking. 50 µL Stop Solution was added into each well dan its absorbance at wavelength 450 nm was measured (Abcam, 2014).

RESULTS

hEGF gene optimization and plasmid construction

ene optimization and plasmid construction The chance of getting high level expression of heterologous protein is related to Codon Adaption Index(CAI). The ideal CAI is 1, nevertheless CAI>0,8 is considered good enough for desired expression level from a host (Poigbo et al., 2007). The CAI from this study was 1. The ideal percentage of the GC content range is 30%-70%. %GC beyond the range will affect the efficiency of transcription and translation process. Average %GC in this study was 50,93, hence the optimum expression result is possible to generate. Codon distribution percentage shows the quality of the expressed codon group. Codon value of 100% is the frequency of the amino acid usage in the expression. Codon value below 30% shows speculative expression efficiency. In this study, codon value of 100% was made, therefore efficient expression was expected. Plasmid construction was made after hEGF gen optimization by inserting PelB signal peptide which is available in DNA 2.0 as shown in fig 1.

Plasmid transformation

The transformation was successfully performed on E. coli BL21 (DE3) electro-competent cell with plasmid [pD881-PelB]. Electroporation transformation uses electricity power to disrupt the membrane host cell stability so the plasmid can be inserted into the cytoplasm. Successful transformation can be seen by the growth of host cell on agar medium containing antibiotics (Fig 2). Positive control of E. coli BL21(DE3) electro-competent cell was seen to grow in LB medium containing kanamycin while the negative control of E. coli BL21(DE3) electro-competent cell did not grow on LB medium containing kanamycin due to the absence of nptII gene which encode the neomycin phosphotransferase II (NPT II/Neo) that cause the resistance of kanamycin (Ghanem, 2011).

E. coli BL21(DE3) pPICZαB plasmid was used as trans formant control that was grown in LB medium. E. coli BL21(DE3) [pPICZα B] grow in the medium containing skin if the transformation was successfully performed due to the presence of the ble gene in pPICZαB plasmid that cause resistance to zeocin (Invitrogen, 2010). E. coli BL21(DE3) transformant grow in medium containing kanamycin showed the transformation was successfully performed due to the presence of nptII gen in pD881-PelB expression plasmid.

Growth curve and production curve of E. coli BL21

As an organism, E. coli grows and multiplies in volume, size and number. Every phase in its growth can be observed by turbidimetry, a method to measure the microbial number which its OD is directly proportional with the cell number (Kaitu et al., 2013). The bacterial turbidity can be measured at OD600nm and the absorbance can be used to generate bacterial growth curve to determine the optimum condition to do L-rhamnosa induction. Inducer was added at the exponential phase of bacterial growth where the cell is active in proliferation with a constant rate. Induction performed at this point would increase the desired protein product.

Based on E. coli BL21(DE3)[pD881-PelB] growth curve, induction was set. Exponential phase started on OD600nm 0.883 at the 3rd hour and ended on OD600nm 2.24 at 4th hour. Optimum condition for induction was in the middle of exponential phase where the median of the log phase in the E. coli BL21(DE3)[pD881-PelB] growth curve was on OD600nm 0,7. (fig 3)

Based on the production curve of E.coli BL21(DE3)[pD881-PelB] the highest OD reach at the 5th hour after the first induction at OD 0.7, thus harvesting culture can be done at 5th hour or after because the higher the OD the more cell in the culture, the more desired protein generated. (fig. 4)

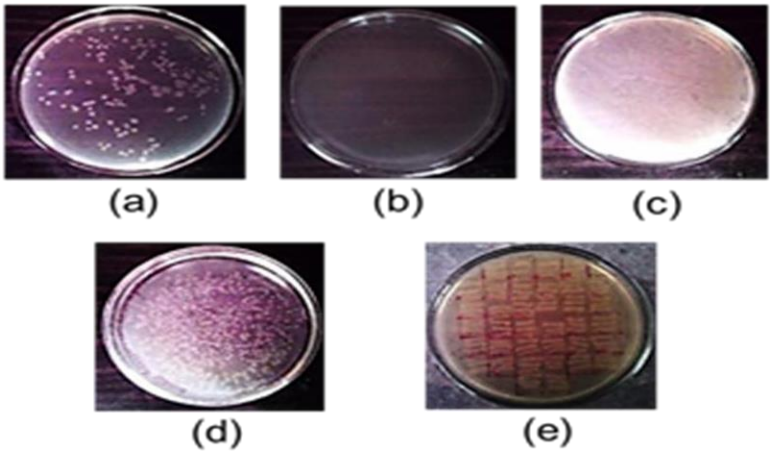

Figure 2: E. coli BL21(DE3) transformation: E. coli BL21(DE3) [pD881-PelB] transformant (a). Negative control of E. coli TOP10F' (b). Positive control of E. coli TOP10F' (c). Positive control of of E. coli BL21(DE3) [pPICZαB] transformant (d). E. coli BL21(DE3)

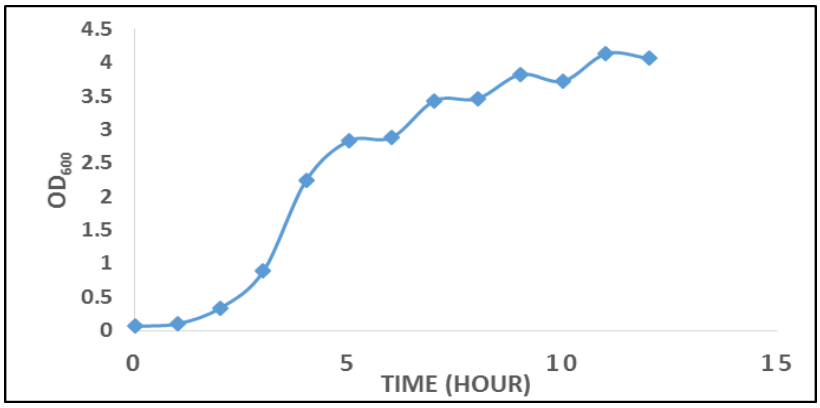

Figure 3: Growth curve of E. coli BL21(DE3) [pD881-PelB]

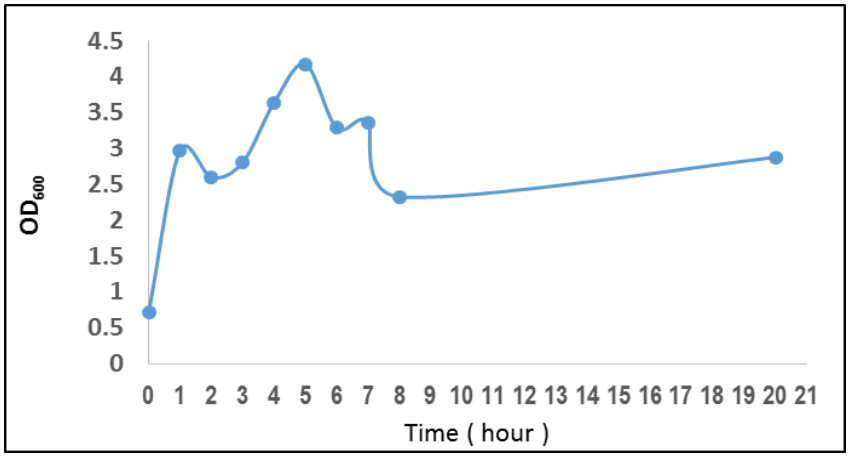

Figure 4: Production curve of E.coli BL21(DE3)[pD881-PelB]

L-rhamnosa induction optimization

L-rhamnosa has important role in the rhamnosa operon system beacuse rhaPBAD promoter controls the expreddion of the gene in pD881-PelB plasmid that uses L-rhamnosa as carbon and energy source (Wickstrum et al., 2005).

Based on SDS-PAGE electroforegram of L-rhamnosa concentration optimization in (fig. 5), it can be concluded that the best concentration of L-rhamnosa induction was 4 mM because it produces a more desired protein either insoluble fraction in medium or in the form of inclusion bodies at t(20) because not all rhEGF was translocated to periplasma to be secreted to medium.

hEGF secretion

rhEGF encoding gene in pD881-PelB plasmid could generate pelcate lyase Bsignal peptide - rhEGF based on the secreted rhEGF in medium. Pelcate lyase B is a

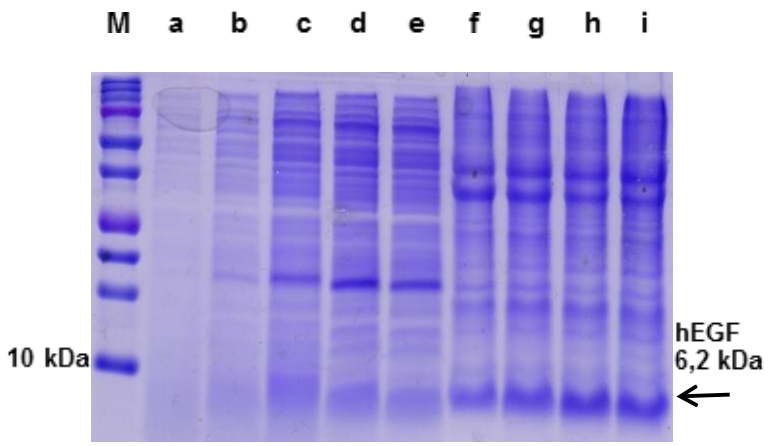

Figure 5: Electroforegram of L-rhamnosa induction optimization SDS-PAGE: Protein marker (M). Medium t(20) of 40 µM L-rhamnosa induction (a). Medium t(20) of 1 mM L-rhamnosa induction (b). Medium t(20) of 2 mM L-rhamnosa induction (c). Medium t(20) of 4 mM L-rhamnosa induction (d). Medium t(20) of 6 mM L-rhamnosa induction (e). Inclusion bodies t(20) of 1 mM L-rhamnosa induction (f). Inclusion bodies t(20) of 2 mM L-rhamnosa induction (g). Inclusion bodies t(20) of mM L-rhamnosa induction (h). Inclusion bodies t(20) of 6 mM L-rhamnosa induction (i).

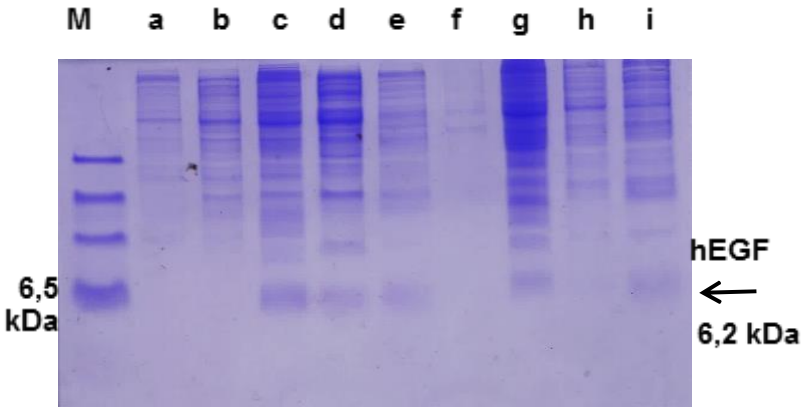

Figure 6: Elektroforegram of protein translocation SDS-PAGES: protein marker (M). Soluble protein fraction before induction (a). Medium protein fraction before induction (b). Soluble protein fraction after 8 hours of induction (c). preiplasm protein fraction after 8 hours of induction (d). medium protein fraction after 8 hours of induction (e). Insoluble protein fraction after 8 hours of induction (f). Soluble protein fraction after 20 hours of induction (g). Periplasm protein fraction after 20 hours of induction (h). Medium protein fraction after 20 hours of induction (i).

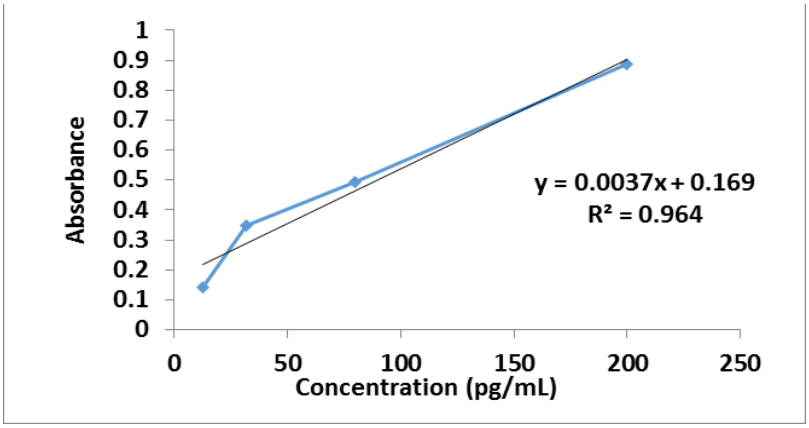

Figure 7: Standard curve of human EGF. Based on the calculation, concentration of rhEGF in medium t(20) was 310,81 µg/mL after multiplying with dilution factor

signal peptide that has MKYLLPTAAAGLLLLAAQ PAMA sequence which takes rhEGF into periplasm. MKY fragment in pectate lyase B is an N-terminal that is recognized on sec-dependent B pathway so the fusion protein fusi can be moved into periplasm. PAMA fragment in pectate lyase B is a C-terminal that is recognized by peptidase signal as alanin box due to its sequence consist of Ala-X-Ala which would be cut by peptidase signal (Choi & Lee, 2004).

Fig. 6 shows the hEGR production in the cytoplasm which then was transferred into the periplasm and finally into culture medium is a time consuming process. Optimum concentration of hEGF was found in the periplasm at t(8) after induction and in medium at t(20) after induction yet rhEGF concentration at t(20) was decreased

hEGF measurement by ELISA

rhEGF that has successfully expressed and purified was measured by commercial ELISA kit. The kit used Sandwich ELISA method where in each well contains specific primary antibodies that would bind the human hEGF hence when washing with wash buffer only hEGF bound to the primary antibody will remain. Biotinlated secondary antibody was added and bound to streptavidin HRP enzyme that will convert TMB substrate that turns the solution become blue then yellow if stop solution was added. The absorbance of the solution on ʎ450nm was measured (Abcam, 2014).

Standard EGF equation obtained was y=0.0037x + 0.169, with R^2 = 0.964. (fig. 7) This equation was used to determine the measured hEGF sample from medium t(20) that was 0.782 then the result was multiplied by dilution factor 2,000,000. The equation also used to determine rhEGF sample from purification by cation exchange chromatography that was 0.467 then the result was multiplied by dilution factor 10,000.

The hEGF measurement by ELISA kit showed rhEGF in the medium after 20 hours was 310,81 µg/mL. This showed the expression of rhEGF gene with Pectate Lyase B signal peptide in pD881-PelB plasmid is potentially scaled-up to produce rhEGF. Some studies that constructed and expressed rhEGF gene showed lower results compare to the result of this study. Chen et al, (2005) produced 31,2 µg/mL of rhEGF, Kwong and Wong (2013) produced 103 µg/mL of rhEGF, and Zhang et al., (2015) produced 248 µg/mL of rhEGF.

DISSCUSION

hEGF was successfully secreted from E. coli BL21 (DE3) facilitated by pectate lyase B (PelB) signal peptide from type II Sec Dependent. hEGF expression in cytosol can be identified by the presence of band in SDS-PAGE gel showing the soluble fraction at 6,2 kDa. hEGF expressed in cytosol was showed to be trans located into periplasma by PelB signal peptide based on the presence of the same size band (6,2kDa) on SDS-PAGE gel after 8 hours of induction. hEGF secretion into the culture medium reaches its optimum condition at the 20th hour after induction. It was shown by the decrease of band thickness at 6,2 kDa on the soluble fraction lane from periplasm extraction and the increase of band thickness from the medium. Optimization of hEGF synthetic gene with CAI=1, %GC 50,93% and 4mM L-rhamnosa induction time at the 3rd-4th hour has generated hEF production up to 310,81 µg/mL which was harvested from medium at 20th hour. The determination of hEGF concentration by ELISA indicated the expressed hEGF was active.

CONCLUSION

This study showed that the secretion of expressing hEGF using E. coli BL21(DE3) system with optimized synthetic gene and production process, include induction time and L-rhamnose concentration, was effective in producing hEGF without the needs of cell lysis and periplasm extraction.

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