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Research Article

COMPARISON OF EXTRACELLULAR SECRETION OF RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR USING TORA AND PELB SIGNAL PEPTIDES IN *ESCHERICHIA COLI* BL21 (DE3)

RIMA MELATI[1], ANNISA INDRIYANI[1], SHABARNI GAFFAR[1], SRIWIDODO[2], IMAN PERMANA MAKSUM[1]*

[1]Biomolecular Laboratory of Health and Food, Department of Chemistry, Faculty of Mathematics and Natural Science, Padjadjaran University, Indonesia. [2]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Padjadjaran University, Indonesia. Email: iman.permana@unpad.ac.id

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ABSTRACT

Objective: The objective of this study was to evaluate two signal peptides (TorA and PelB), representing the most common secretion pathways in *Escherichia coli*, for their ability to secrete recombinant human epidermal growth factor (rhEGF) protein in the extracellular expression.

Methods: *E. coli* BL21 (DE3) as the host cell to be transformed using recombinant plasmid pD881-TorA the consensus already containing hEGF gene and the signal peptide TorA or PelB, then expressed by L-rhamnose induction. rhEGF purified by heat treatment and ion-exchange chromatography. The hEGF protein was characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and ELISA.

Results: The result showed that PelB was secreting more hEGF protein compared to TorA with protein expression results of 48.2 µg/L and purification results of 0.360 µg/L, with a purity level of 83%.

Conclusion: The results of this study explain in extracellular expression of hEGF protein in *E. coli*, PelB helps hEGF protein secretion to culture media better than TorA.

Keywords: Human epidermal growth factor, *Escherichia coli,* Extracellular expression, TorA, PelB.

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INTRODUCTION

Escherichia coli is classified as a rod-shaped, Gram-negative bacterium in the family *Enterobacteriaceae* [1]. *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 [2]. To be able to secrete recombinant protein into the membrane human epidermal growth factor (hEGF) periplasm or to the culture medium to be used the signal peptide which is fused with the target protein to be passing lane, sec twin-arginine translocation (TAT) or sec-pathway [3,4].

The general extracellular secretion route, termed Sec-pathway, catalyzes the transmembrane translocation of proteins in their unfolded conformations, whereupon they fold into their native structure at the transitions of the membrane. The twin-arginine translocation pathway, termed Tat pathway, catalyzes the translocation of secretory proteins in their folded state. Although the targeting signals that direct secretory proteins are to these pathways show a high degree of similarity, the translocations and translocations involved are vastly different [3,5].

EGF is a single chain, 53 amino acid residues polypeptide with molecular weight of 6.2 kDa [6]. Specifically, EGF interacts with its receptor across the entire epidermis and particularly in the basal layer, promoting epithelial growth through activation of several pathways [7]. Through binding to the EGF receptor in the cell membrane, EGF can induce its autophosphorylation and initiate signal transduction, thus regulating a wide range of biological functions. EGF is widely used in basic research and clinical medicine, which has created a great demand for it [8].

hEGF recombinant production was carried out in *E. coli*. The previous studies have succeeded in expressing rhEGF extracellular secretion pathways [9]. *E. coli* is also relatively easy for the recombinant plasmid which is inserted into it so that the gene hEGF can be easily inserted into the plasmid vectors of *E. coli* [10]. hEGF had potential as effective wound healing and had function as problems of premature aging, that is, why hEGF used not only for wound healing, corneal transplantation, and gastric ulcer treatment but also in the cosmetic industry [9]. Therefore, the method for producing large and high efficiency of hEGF needed[A]. This study was to evaluate two signal peptides (TorA and PelB), representing the most common secretion pathways in *E. coli*, for their ability to secrete hEGF protein in the extracellular expression.

MATERIALS AND METHODS

Reagent and chemicals

Material used in this study were Bacto agar Bacto (Oxoid), agarose (Sigma-Aldrich), ammonium persulfate (Bio Basic INC), Bromophenol blue (Merk), Coomassie Brilliant blue (SERVA), EDTA (1st Base), DNA 1 kb marker (Thermo Fisher Scientific), *E. coli* BL21 (DE3), Gel Red (Biotium), hEGF synthetic gene inserted in pD881-PelB plasmid, and pD881-TorA (synthesized by DNA 2.0, California, USA), glycerol (1st Base), Kanamycin Sulfate (Sigma-Aldrich), L-rhamnose (Sigma-Aldrich), protein marker (Biorad), sodium chloride (Merk), sodium dodecyl sulfate (SDS) (Merk), tetracycline, and TEMED (Sigma-Aldrich).

Methods

E. coli BL21 (DE3) (pD881-TorA-hEGF) growth and post-induction curve

Transformant was grown in LB medium 5 mL containing kanamycin about 16–18 jam at 37°C with shaking at 200 rpm. 1000 µL of culture was transferred into LB medium 100 mL in shake flask containing kanamycin for 12 h at 37°C with shaking at 200 rpm. OD_{600} was measured every hour. Starter culture 1000 µL was transferred into LB medium 100 mL containing kanamycin and was grown to OD_{600} reach 0.6. Inducer L-rhamnose 4 mM final concentration was added. The culture was incubated for 20 h at 37°C with shaking at 200 rpm. OD_{600}nm was measured every hour.

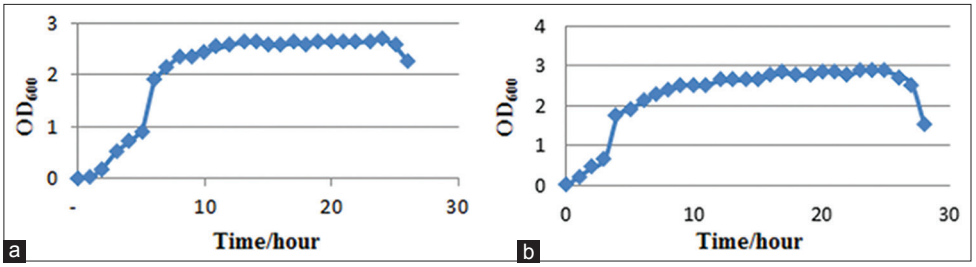

Fig. 1: Growth curve *Escherichia coli* BL21 (DE3) incubation at 37°C using LB shaking speed 200 rpm. (a) *E. coli* BL21 (DE3) (pD881-TorA-human epidermal growth factor (hEGF]). (b) *E. coli* BL21 (DE3) (pD881-PelB-hEGF)

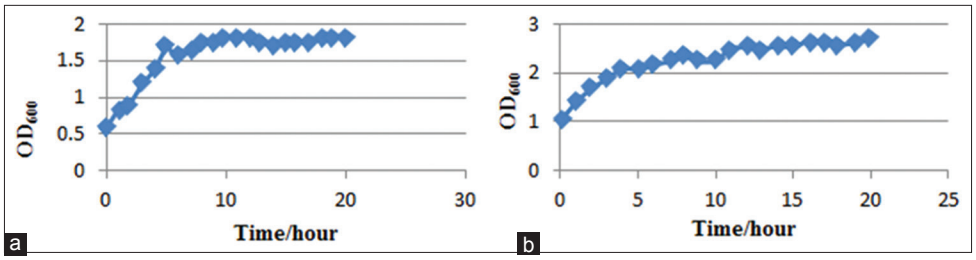

Fig. 2: Post-induction growth curve *Escherichia coli* BL21 (DE3) incubation at 37°C using LB media, shaking speed 200 rpm. (a) *E. coli* BL21 (DE3) (pD881-TorA-human epidermal growth factor [hEGF]). (b) *E. coli* BL21 (DE3) (pD881-PelB-hEGF)

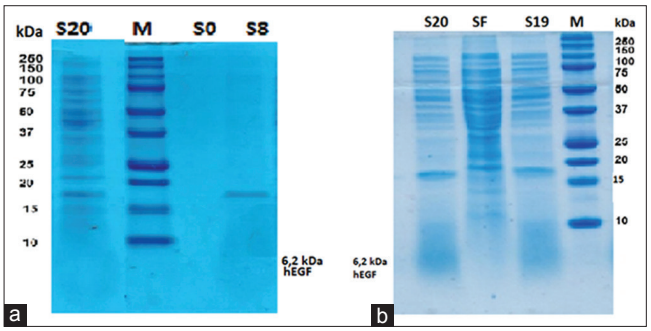

Fig. 3: Characterization of human epidermal growth factor (hEGF) protein expression results in *Escherichia coli* BL21 (DE3) using Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. M: Protein marker. Sn: Supernatant at n h post-induction (S20: Supernatant at the 20th h post-induction), SF: Soluble fraction. (a) Result of hEGF expression using: *E. coli* BL21 (DE3) (pD881-TorA-hEGF) and (b) using *E. coli* BL21 (DE3) (pD881-PelB-hEGF)

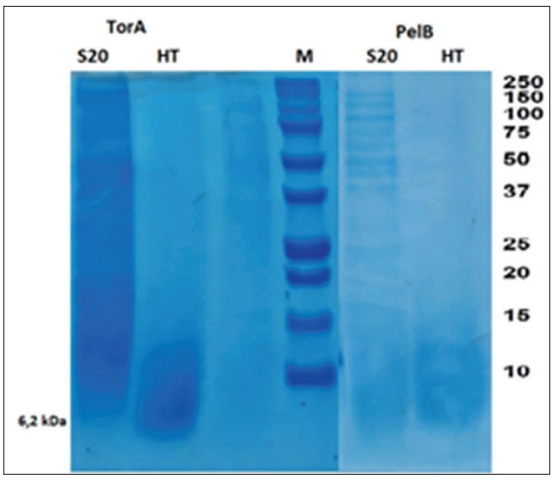

Fig. 4: Human epidermal growth factor protein characterization after heat treatment using Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. M: Protein marker; S20: Supernatant at the 20th h post-induction. HT: Supernatant fraction resulting from heat treatment

Expression of hEGF by *E. coli* BL21 (DE3) (pD881-TorA-hEGF) and *E. coli* BL21 (DE3) (pD881-PelB-hEGF)

E. coli BL21 (DE3) (pD881-TorA-hEGF) and (pD881-PelB-hEGF) that was characterized, grown in 5 mL of liquid Luria Bertani (LB) media which contained kanamycin (25 µL/mL) for about 16–18 h, in temperature 37°C and 200 rpm shaking speed. Each cell culture 1000 µL was added into different Erlenmeyer flask containing 100 mL liquid LB and kanamycin (25 µL/mL). Then, all cultures in the Erlenmeyer flask incubated at 37°C and shaking speed 200 rpm until OD_{600} reached at 0.6. Then, 1 mL of each culture taken as t_0 (before induction of L-rhamnose) and collected into a 1.5 mL microtube, then cell pellet and it's supernatant separated using centrifugation with velocity 3000 g, 4°C for 20 min. Then, the cultures in Erlenmeyer flasks added by L-rhamnose total concentration of 4 mM in culture, then incubated at 37°C, shaking speed 200 rpm for 20 h. For about 2 mL, each culture was taken as t_i (after induction of L-rhamnose) 1 mL, separated the cell pellet and its supernatant by centrifugation with a velocity of 3000 g, 4°C for 20 min. Pellets are used for cell lysis and extraction processes periplasmic membrane and media can be analyzed using SDS-polyacrylamide gel electrophoresis (PAGE).

Purification of hEGF protein using heat treatment and anion exchange chromatography

Cultures are harvested 20 h after induction, separated the cell pellet and its supernatant by centrifugation with a velocity of 3000 g, 4°C for 20 min. Supernatant (soluble fraction) heated at 80°C for 20 min, then centrifuged at a speed of 20,000 g, for 30 min.

Supernatant was taken and hEGF contained in it was then purified by anion exchange chromatography with DEAE matrix Sephadex A-25, eluen I (citrate-phosphate buffer pH 5.6), and eluen II (citrate-phosphate buffer pH 5.6 with sodium chloride 2 M), which is integrated in the AKTA START. The heat-treated 5 mL sample was filtered using 0.22 mm pore diameter filter units and loaded onto the DEAE sepharose fast flow

column with a flow rate of 2 mL/min. The rhEGF protein detected by UV absorbance at 280 nm.

Tricine-SDS-PAGE analysis

Secreted proteins were separated on a 15% Tricine SDS-polyacrylamide gel. 10 µL of each sample was boiled in a 5 µL sample buffer (312 mM Tris-HCl pH 6.8, glycerol 50% (v/v), bromophenol blue 0.05% (w/v), and dH2) for 10 min before the sample mixture was being loaded onto the gel using Haider *et al*. protocol [11].

Measurement of rhEGF using ELISA

Method measurement of hEGF concentration used the ELISA method with protocols and kit from Quiescence.

Measurement of total protein by Lowry method

Method measurement of total protein concentration by Lowry method with protocol used folin phenol reagent [12].

RESULTS AND DISCUSSION

Growth curve of *E. coli* BL21 (DE3) (pD881-TorA-hEGF)

Growth in prokaryotic organisms such as *E. coli* is defined as an increase in cell volume and size and also an increase in cell numbers. The growth curve is made to determine the log phase or exponential. The log or exponential phase is a phase where cells in an active state divide at a constant speed. Induction carried out in this phase will increase the desired target protein yield.

Based on Fig. 1, the growth curve of *E. coli* BL21 (DE3) (pD881-TorA-hEGF), it can be seen that at the 1st–2nd h a lag phase after that at the 3rd–8th h, the bacteria undergo an exponential phase. Then, from the 8th until 26th h, the bacteria had the stationary phase and when the bacterial growth hours reached the 26th began to decline in OD_{600} values this indicates that the bacteria had begun to reach death phase. While *E. coli* BL21 (DE3) (pD881-PelB-hEGF) growth curve 1st–2nd h, it was a lag phase, induction in this phase will produce a low recombinant protein. At the 3rd to the 8th h is an exponential phase. The purpose of making a growth curve is to determine the optimum time of induction used for recombinant protein expression. Induction is carried out when the bacteria reach the exponential phase, if there is an induction when the stationary phase the number of living cells is equal to the number of dead cells so the production of recombinant proteins will be low. Hence, expression using PelB signal peptide was induced at the 3rd–4th h and TorA signal peptide at the 4th–5th h.

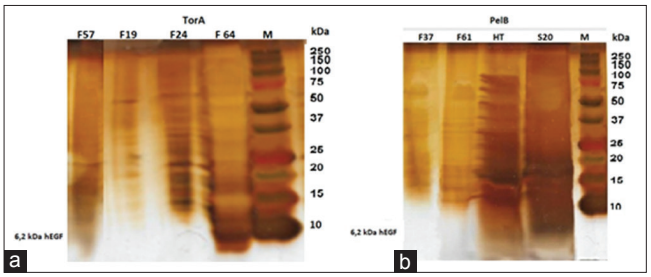

Fig. 5: (a and b)The human epidermal growth factor (hEGF) characterization in anion exchange chromatography fraction using Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. F57: 57th fraction, F19: 19th fraction, F24: 24th fraction, F64: 64th fraction, F61: 61th fraction, F37: 37th fraction M: Protein marker. S20: Supernatant at the 20th h post-induction. HT: Supernatant fraction from heat treatment. hEGF molecular weight is 6.2 kDa

Post-induction curve of *E. coli* BL21 (DE3) (pD881-TorA-hEGF)

The post-induction growth curve in Fig. 2 showed that the estimated time of cell harvest for both occurs at the 20th h post-induction because it has a high cell density based on OD_{600} values. $OD6_{00}$ values were measured using the turbidimetry method, which is a measurement of the number of bacteria based on turbidity where OD is comparable to the number of cells. The OD_{600} value states the large number of cells that are in the culture medium. It is assumed that the greater the OD_{600} value the more rhEGF proteins that have been successfully secreted.

Expression of hEGF protein in *E. coli* BL21 (DE3)

Characterization of hEGF in Fig. 3, it can be seen that at the 19th h post-induction in the culture medium, there is already a fairly thick band on the post-induction culture medium; then, at the 20th post-induction, the intensity increases in the protein markers area below 10 kDa. hEGF protein was successfully secreted into the culture medium, the optimum harvest time is at the 20th post-induction time. From Fig. 3, it can be seen in the soluble fraction strip that at the 20th h post-induction, there is no band on the protein size below the 10 kDa marker, meaning that at that hour all the proteins have been successfully secreted to the culture medium (supernatant). The soluble fraction is a collection of dissolved proteins that are still present in the cytoplasm of *E. coli* which are not excreted in the culture medium.

Purification of hEGF protein using heat treatment

EGF is a protein which heat resistance and not denatured or loses its biological activity at temperatures of 80°C for 30 min, EGF protein has an isoelectric point 4.2–4.6 [13-15]. The results of the Tricine-SDS-PAGE characterization showed that there was an hEGF protein in electrophoregrams with a molecular weight of ±6.25 kDa (Fig. 4). Other proteins that are not heat resistant were coagulated and precipitate so that hEGF can be separated from the impurity protein. The intensity of the color of the band from the S20 lane as a result of the heat treatment lane shows the presence of protein that is lost after purification. Heat-resistant proteins will remain soluble and stay in the supernatant. Increasing the temperature or concentration of hydrogen ions in solution causes the reaction speed accelerated so that a reaction between proteins and water that develops regularly causes the protein to coagulate.

Purification of hEGF protein using heat treatment and anion exchange chromatography

Purification is by ion-exchange chromatography method because the protein has a specific isoelectric point. Protein negatively charged at a pH consistent above the isoelectric point. Protein stability also influences the determination of the pH of the buffer used. Only proteins with negative charges bound to the matrix at anion exchange system.

Table 1: Human epidermal growth factor protein purification

Signal peptide	Sample	Volume (mL)	EGF* (µg/mL)	Total protein** (µg/mL)	Purify (%)
PelB	Supernatant	100	4.82	23.42	20.58
	Heat treatment	96	4.2	12.03	34.91
	IEC	96	0.035	0.043	81.40
TorA	Supernatant	100	3.8665	17.088	22.63
	Heat treatment	96	3.6163	10.759	33.61
	IEC	96	0.0360	0.045	80.00

The results of expression, after purification using the heat treatment method, and ion-exchange chromatography. *Data obtained from the ELISA method, **Data obtained from Lowry method. EGF: Epidermal growth factor, IEC: Intestinal epithelial cell

Proteins with a positive charge at pH 5.6 will be eluted in wash unbound phase. The hEGF protein is negatively charged because the buffer used has a pH above the hEGF isoelectric point. The hEGF isoelectric point is 4.6 while the pH buffer used is 5.6. The 19th and 24th fractions of the wash unbound phase did not appear band under the 10 kDa protein markers, indicating no hEGF eluted before salt addition. In the wash unbound phase, hEGF proteins are bound to the anion exchange matrix. The 64th fraction showed a band below the 10 kDa mark, indicating the presence of hEGF eluted during the elution and fractionation phases.

Data in Table 1 hEGF protein levels for expression results using TorA and PelB signal peptide obtained a total hEGF of 3.86 and 4.82 µg/mL. Table 1 shows that the hEGF protein was purer because there are fewer impurities that accompany the protein. Purification samples have also been characterized by Tricine-SDS-PAGE. Purification processes with heat treatment and ion-exchange chromatography are effective for the purification of hEGF because they produce EGF with a purity of more than 80%. However, it is necessary to optimize the purification conditions such as the volume of the matrix, pH, and concentration of salt used to obtain hEGF protein with higher levels of purification. The acquisition data in Table 1 show that extracellular hEGF expression using PelB signal peptide secretes more hEGF than TorA signal peptides.

CONCLUSION

The results of this study explain in extracellular expression of hEGF protein in *E. coli*. PelB helps hEGF protein secretion to culture media better than TorA. hEGF protein levels for expression results using TorA and PelB signal peptide obtained a total hEGF of 3.86 and 4.82 µg/mL. Purity of protein produced is 80 and 81.4% for hEGF using TorA and PelB signal peptide. Purification processes with heat treatment and ion-exchange chromatography are effective for the purification of hEGF because they produce EGF with a purity of more than 80%.

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AUTHORS' CONTRIBUTIONS

All authors declare that they have participated sufficiently in the work to take public responsibility for the content, including participation in the processed the experimental data, performed the analysis, designed the figures, calculations, manufactured the samples and characterized concept, design, analysis, writing, or revision of the manuscript.

CONFLICTS OF INTEREST

All authors declare that this material or similar material has not been and will not be submitted to or published in any other publication. There are no any potential conflicts of interest.

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