Factors Affecting Expression Level of Recombinant Human Epidermal Growth Factor in *Escherichia coli* BL21(DE3) and Size Exclusion Purification Thereof

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Abstract

Recombinant human epidermal growth factor (rhEGF) has been developed to provide the protein for therapeutic uses. Isopropyl β-d-1-thiogalactopyranoside (IPTG)-induced expression in *Escherichia coli* BL21(DE3) has shown the most effective system among other inducers systems in a similar host. However, suitable conditions related to IPTG concentration, incubation time, and incubation temperature are different depending on the amino acid content of the recombinant protein. This study aimed to evaluate the effects of various IPTG concentration, incubation time, and incubation temperature on rhEGF concentration. According to each analysis of those factors, induction with 0.05 mM IPTG for 2 h at 23°C was the most appropriate condition to obtain the highest concentration of rhEGF. The rhEGF was positively confirmed with a monoclonal anti-hEGF antibody and purified in high purity reaching 95.2%, yet recovery was low (1.44%) due to loss in fractions containing endogenous proteins. Therefore, further studies related to type of matrix, column length, and sample concentration in applying size exclusion chromatography are requested for higher recovery.

Keywords: IPTG concentration, incubation time, incubation temperature, recombinant human epidermal growth factor, *Escherichia coli* BL21(DE3)

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Introduction

Human epidermal growth factor (hEGF) is a 6.2 kD polypeptide containing 53 amino acid residues and three disulphide bonds. It plays an important role in regenerating epithelial and endothelial cells and stimulating tissue recovery (Eissazadeh *et al.*, 2017). Conventionally, hEGF was purified from animal urine, but it yields low efficiency and needs complex processes. Therefore, Wong *et al.* (2018) reported developing of protein by genetic engineering as a recombinant hEGF (rhEGF).

Escherichia coli BL21(DE3) strain is commonly used as a host for recombinant protein production. FDA has approved the BL21(DE3) strain for therapeutic purposes. The chromosome of DE3 prophase in BL21(DE3) expresses T7 polymerase RNA under the control of the lacUV5 promotor. This strain lacks of Lon and OmpT protease leading to stabilize expression of recombinant protein (Joseph *et al.*, 2015).

In the other studies, rhEGF has been expressed in *E. coli* using solubilizing tagging, such as GB1, SUMO, and thioredoxine (Trx) that cost expensive proteases to purify the rhEGF with the tagging proteins (Zheng *et al.*, 2016; Ma *et al.*, 2016; Shams *et al.*, 2019). In this study, the rhEGF was designed without any solubilizing tagging, which generates challenges in protein purification and refolding process.

Expression of rhEGF in this study was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG has been known to offer the highest yield compared

with other inducers, such as L-rhamnose and L-arabinose (Gellisen, 2005). IPTG works under lac operon control (Mühlmann *et al.*, 2017). However, several optimization-related to IPTG concentration, incubation time, and incubation temperature are requested to obtain an optimized result with IPTG induction.

Numbers of protein purification methods are available. Even so, for the rhEGF, only size exclusion chromatography is applicable, because it had been designed without any tagging and contaminated with massive endogenous proteins. This leads to other methods like affinity and ion exchange chromatography to be unsuitable (Figure 1). Moreover, size exclusion chromatography is considered the most efficient because no incubation is needed and relatively faster than other methods (Hagel & Haneskog, 2010).

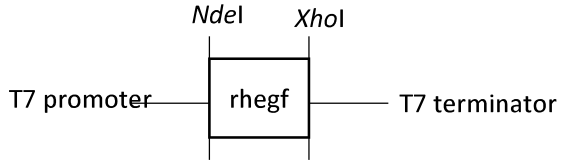

Figure 1. rhegf construction in vector pET21. The gene was inserted at multiple cloning sites using *Nde*I and *Xho*I restriction enzymes (Rodiansyah *et al.,* 2019).

This study aimed to investigate induction conditions for the highest result and to purify rhEGF using size exclusion chromatography.

Materials and Methods

Expression of Recombinant Human Epidermal Growth Factor (rhEGF) in *E. coli* BL21(DE3) with Variation of IPTG Concentration and Incubation Conditions (Temperature and Time). A single colony of BL21(DE3) harboring recombinant plasmid pET21-rhEGF was obtained from the previous study on site-directed mutagenesis of rhEGF (Rodiansyah *et al.*, 2019). The cells were precultured in 1 mL Luria Bertani (LB) (Caisson) medium added with 1 µg/mL ampicillin (Sigma), and then incubated at 37°C, 200 rpm overnight.

On the following day, the preculture was refreshed in 10 mL LB medium with 10 µg/mL ampicillin (Sigma) and incubated at 37°C, 200 rpm for 1.5-2 h or until reaching optical density (OD) 0.7-0.8. Afterward, the cultures were incubated at 23°C, 30°C, or 37°C, induced with IPTG (Thermo Scientific) (0.01, 0.5, and 1 mM), and re-incubated with varied incubation times (2 h, 4 h, and 18 h) (Table 1). Duplo experiment set was performed.

Table 1. Experimental design of the rhEGF expression with IPTG induction at various temperatures and incubation time.

Treatment		
Incubation temperature at 23°C	Incubation time for 2 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 4 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 18 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
Incubation temperature at 30°C	Incubation time for 2 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 4 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 18 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
Incubation temperature at 37°C	Incubation time for 2 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 4 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 18 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced

Note: The experiment was conducted in duplication in which each replication consisted of two batches. For the data analysis, all samples of uninduced groups were calculated as one group.

The induced cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C. Subsequently, the cell pellet was solubilized in 2 mL solubilization buffer containing 8 M urea, 80 mM β-mercaptoethanol, and 50 mM glycine (Biobasic and Merck), without pre-

isolation step that had been optimized in our previous study (unpublished data). For solubilization, the cell suspension was incubated for 3 days at cold temperature of 4-8°C. Solubilized protein and cell debris were separated by centrifugation at high speed (12,000 rpm) for 15 min at 4°C.

The solubilized protein in the supernatant was analyzed by tricine (Sigma) SDS PAGE. The tricine SDS PAGE samples were treated with 2×loading dye (Sigma) and boiled at 95-100°C for 5 min. A 15 µL boiled sample was used. Tricine SDS PAGE was conducted using 15% acrylamide-bisacrylamide gel (Biorad) at 70 V for 2.5-3 h.

Characterization of rhEGF with Western Blotting. The rhEGF was characterized using western blotting with a monoclonal anti-hEGF antibody (Santa Cruz) and visualized with alkaline phosphatase (Thermo Scientific). After transferring the protein into a nitrocellulose membrane (Biorad), the membrane was blocked with 10% skim milk in 1×TBS (Merck and Biobasic) for 2 h. Prior to applying the antibody, the membrane was washed with 0.01% Tween 20 (Merck) in 1×TBS (15 min, 15 min, and 5 min, respectively). In order to visualize the blotted band, the membrane was added with 1-2 mL alkaline phosphatase (Biorad). The band appeared less than 5 min after incubation in a dark room at room temperature.

Semiquantification of rhEGF Concentration Using ImageJ Software. The area of the rhGEF band was measured with ImageJ Software ver 1.5b (National Institute of Health, USA). The measured area was plotted to linear regression obtained from the BSA standard (2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.0313 mg/mL).

Data Analysis. Data were grouped according to each parameter (IPTG concentration, incubation time, and incubation temperature). The variance of groups was analyzed with One Way ANOVA ($\alpha = 0.05$) followed by post hoc test LSD method ($\alpha = 0.05$) using IBM SPSS Statistics 26 (IBM®).

Purification of rhEGF Using Size Exclusion Chromatography. Sephadex G-50 matrix (Sigma) was set up in a chromatography column with 1 cm in diameter and 30 cm in length. Before usage, the matrix was equilibrated with Tris-Cl pH 6.0 to remove the ethanol used during storage. Protein crude was diluted with Tris-Cl pH 6.0 (1:1 or 10 mL of total volume) and then applied to the column. Twenty-five fractions were collected. Each fraction (1 mL) was collected in a 1.5 mL microtube. Several representing fractions were analyzed with SDS PAGE and Western blotting.

Results

Influence of IPTG Concentration, Incubation time, and Incubation Temperature for Expression Level of rhEGF.

Experiment to investigate the effect of IPTG concentration, incubation time, and incubation temperature for the expression level of rhEGF was carried out simultaneously using a factorial design. Twenty-seven groups of treatment were conducted in duplicate. Figure 2 represents samples in one set experiment. Protein of interest was found at size approximately 6 kD, and no target band was observed from uninduced treatment on the stained SDS PAGE.

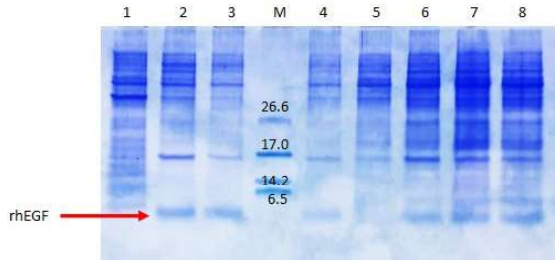

Figure 2. SDS PAGE of rhEGF expressed at 37ºC, various IPTG concentrations, and various incubation times. Lane 1: uninduced, 2 h; 2: 0.01 mM IPTG, 2 h; 3: 0.05 mM IPTG, 2 h; M: ultra-low protein marker; 4: 1 mM IPTG, 2 h; 5: uninduced, 4 h; 6: 0.01 mM IPTG, 4 h; 7: 0.05 mM IPTG, 4 h; 8: 1 mM IPTG, 4 h.

Protein concentration was measured semiquantitatively by identifying the area of the rhEGF band using ImageJ software. The band area was plotted to a linear equation of the BSA standard (Figure 3).

Protein concentration was calculated per optical density value. Figures 4, 5, and 6 depict the effect of each parameter. IPTG induction at 0.05 mM resulted in the highest protein concentration, yet the other two concentrations

allow statistically similar protein expression levels. All IPTG induction proved a significant effect compared with the uninduced group (Figure 4). In uninduced group, a small area under curve (AUC) was detected and calculated as value of protein concentration. However, this area could be ignored and considered as noise in the densitometry using ImageJ software. Moreover, this finding is strongly supported by the statistical analysis.

IPTG is known to be toxic to the bacteria; however, since used in low concentration (below 0.1 mM), no negative outcome was found in bacterial growth. Either induced or uninduced group showed almost similar optical density.

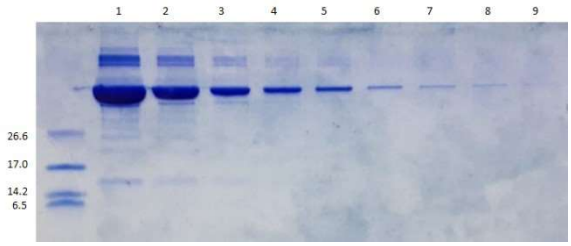

Figure 3. SDS PAGE of a serial concentration of BSA standard.

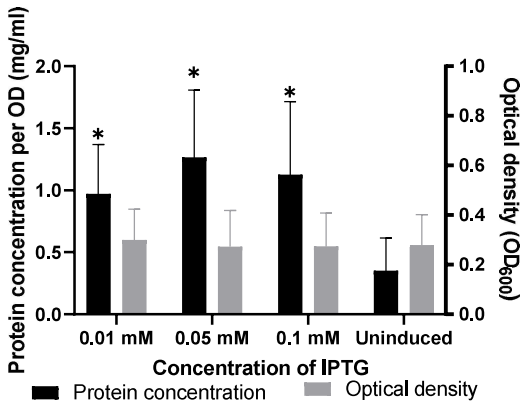

Figure 4. The effect of concentration of IPTG to protein concentration and optical density at all temperatures and incubation times (* shows a significant difference with α = 0.05).

Incubation time revealed a contrary influence on protein concentration and optical density (the longer incubation, the higher optical density). However, the highest protein concentration was found in the group incubated for 2 h after IPTG induction (Figure 5).

Figure 6 illustrates the influence of incubation temperature on protein concentration and optical density. Surprisingly, the incubation time did not show linear trend related to both parameters. The highest protein concentration was obtained by incubation at 23ºC, then followed by 37ºC and 30ºC. Meanwhile, the lowest to the highest optical density was found in group that incubated at 37ºC, 23ºC, and 30ºC.

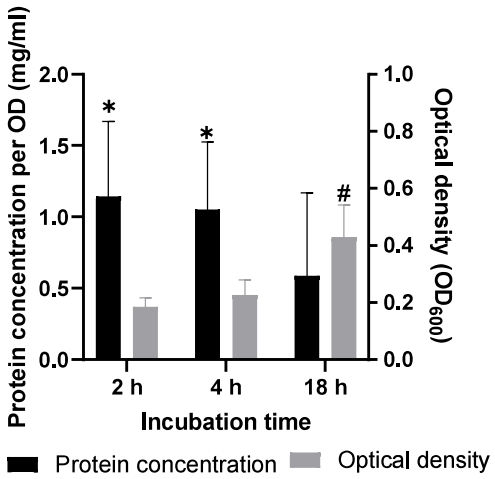

Figure 5. The effect of incubation time on protein concentration and optical density at all IPTG concentrations and temperatures. (*shows a significant difference of protein concentration groups with α = 0.05; # a significant difference of OD groups with α = 0.05).

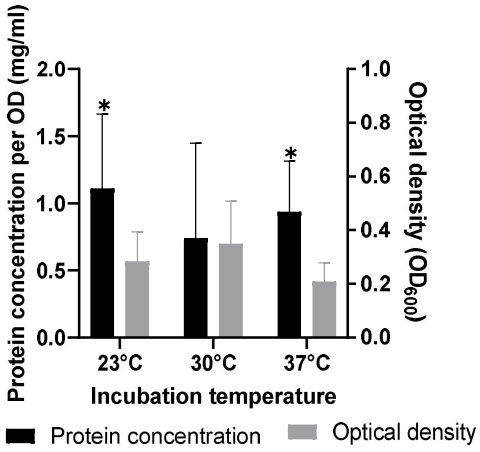

Figure 6. Effect of incubation temperature to protein concentration and optical density at all IPTG concentrations and incubation times (* shows a significant difference with α = 0.05).

Purification of rhEGF Using Size Exclusion Chromatography and Protein Confirmation with Western Blotting.

Figure 7 shows fractions collected in the purification process. Proteins started to appear in the fifth fraction (Figure 7 lane 6).

Unfortunately, the protein of interest was also released in early elutions, which also contained host endogenous proteins (lanes 6-10). Starting from lanes 11-13, the contaminant protein significantly decreased. Purified rhEGF was obtained in lanes 14-15. In lanes 16-18, all proteins have been released from the column. Recovery of purified protein was 1.44% with purity 95.2%. Protein confirmation was carried out using a monoclonal anti-hEGF antibody (Figure 8). A single band at ~6 kD was blotted in crude protein and elution fractions containing rhEGF.

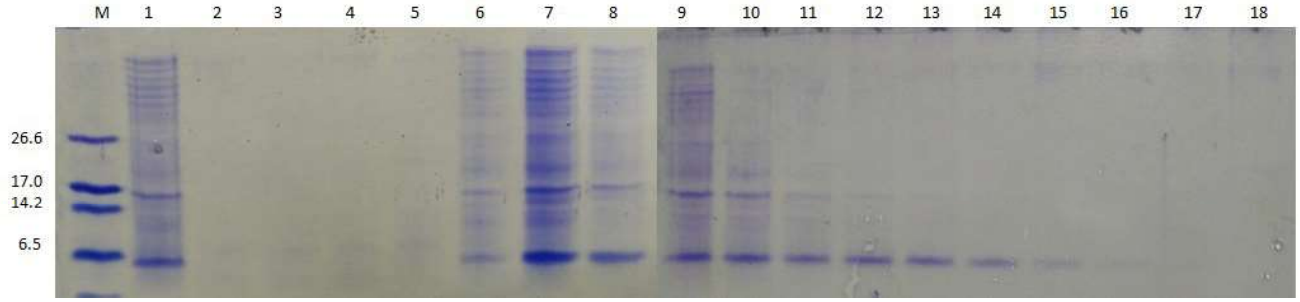

Figure 7. SDS PAGE of protein purification using size exclusion chromatography. Lane M: protein marker, 1: crude protein, 2-18: elution fractions

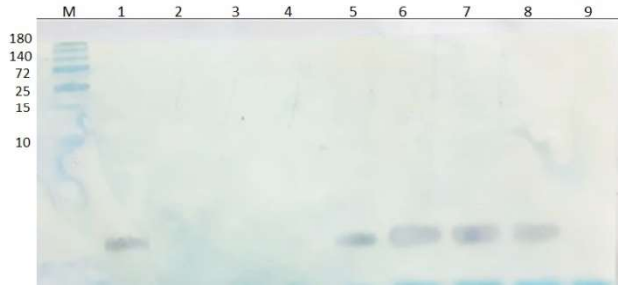

Figure 8. Western blotting using a monoclonal anti-hEGF antibody. Lane M: protein marker, 1: crude protein, 2-4: elution fraction No. 2-4, 5-6: elution fractions No. 6-7, 7-8: elution fractions No. 14-15, 9: elution fraction No. 18

Discussion

IPTG induces recombinant protein to be overexpressed under lac operon control to distinguish the protein of interest from endogenous protein (Mühlmann *et al.*, 2017). Unlike other inducers such as lactose and galactose, IPTG is unmetabolized in the cell; thus, the concentration remains constant during incubation. However, the IPTG concentration needs to be optimized to avoid extreme over-expression which eventually leads to inclusion bodies (Rizkia *et al.*, 2015). The amount of inducer depends on promotor potency, availability of repressor, cellular expression location of recombinant protein, the solubility of recombinant protein, and characteristics of the recombinant protein (Babeipour *et al.*, 2013). Ranjbari *et al.* (2015) mentioned that IPTG concentration is determined not only based on the characteristics of recombinant protein, but also expression of host and incubation conditions. Insufficient IPTG concentration causes ineffective recombinant protein expression; while excessive IPTG concentration is toxic to the expression host (Huang *et al.*, 2017; Ashengroph *et al.*, 2013).

In correlation to those other studies, rhEGF concentration obtained with 0.01 mM IPTG was the lowest among other IPTG-treated groups. This finding proves that 0.01 mM IPTG is deficient for rhEGF expression in the current expression system. The highest concentration of rhEGF was resulted after induced with 0.05 mM IPTG; meanwhile, the rhEGF concentration slightly decreased with the higher IPTG concentration (0.1 mM). However, up to 0.1 mM, no toxic effect was indicated because all groups showed similar optical density (Figure 4). All IPTG-induced groups were significantly different with uninduced groups (Figure 4). Figure 2 revealed that no rhEGF was expressed without IPTG induction.

Incubation time is related to bacterial growth. Figure 5 shows a significant increasing of optical density found after incubation for 18 h, yet the rhEGF concentration per optical density was notably the lowest among the others. Overall, incubation time and optical density are inversely correlated to rhEGF concentration.

Incubation temperature corresponds to *E. coli* growth. It has minimum, maximum, and optimum growth temperature of *E. coli* (Knob *et al.*, 2008). *E. coli* optimized growth temperature is at 37°C (Sulistiyoningrum *et al.*, 2013). Nevertheless, regarding recombinant protein expression induced with IPTG, the optimum temperature and other conditions differed for each recombinant protein depending on amino acid content (Gutiérrez-González *et al.*, 2019). Cysteines in the hEGF sequence showed positive correlation with IPTG, meanwhile lysine and arginine which influence solubility are not found in the hEGF amino acid sequence (Gutiérrez-González *et al.*, 2019).

High temperature tends to form inclusion bodies. On the other hand, low temperature causes ineffective growth of *E. coli*. In this study, among 23°C, 30°C, and 37°C, the incubation temperature at 30°C showed the highest optical density, yet the lowest rhEGF concentration. No significant difference was found in terms of optical density caused by variation of incubation temperature. Interestingly, rhEGF concentration was significantly higher in group 23°C and 37°C compared with that of in group 30°C. The highest rhEGF concentration was observed by incubation at 23°C resulting the second highest of optical density (Figure 6).

The rhEGF was designed without any tagging to facilitate purification to obtain a recombinant protein as similar to the native one. Besides, it is known difficult to separate a small protein such hEGF from its tagging protein after being cleaved with proteases. Therefore, the suitable method to purify the protein of interest is by using size exclusion chromatography. This method separates proteins based on hydrodynamic size in which the proteins with large hydrodynamic size are eluted first from chromatography matrix (Brusotti *et al.*, 2017). The rhEGF was the smallest protein in the protein crude, so this protein was eluted after all endogenous contaminants were completely released.

In our preliminary study, ion exchange and hydrophobic interaction chromatography has been applied to purify the rhEGF. However, the rhEGF purity was low due to abundant endogenous protein having similar pI and hydrophobicity-related physical characteristics (Agustina, 2019).

Using the size exclusion chromatography, the rhEGF was obtained in high purity, but percentage of recovery was low. The rhEGF was also released in the several first elutions along with other endogenous contaminant proteins. For further study, selecting other types of matrices, longer columns, and optimizing the sample concentration loaded into the matrix of size exclusion chromatography are needed to avoid preliminary release of rhEGF in the earlier fractions which eventually increase the purification efficiency.

Conclusion

Vary of IPTG concentration, incubation time, and incubation temperature have been evaluated for rhEGF expression. The rhEGF was purified using size exclusion chromatography. High purity was obtained, yet further studies are requested for higher percentage of recovery. The rhEGF was confirmed with a monoclonal anti-hEGF antibody.

Acknowledgements

The research project was supported by Indonesia Toray Science, Research Grant 2017.

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