

The Effect of Single Co-expression of The DnaK-DnaJ-GrpE and GroEL/ES Chaperones and Their Combination on Expression Intein-pretrombin-2 in *Escherichia coli* ER2566

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Abstract

The use of recombinant thrombin in the manufacture of fibrin glue allows diseases contamination to be avoided. However, the expression of recombinant protein in *E. coli* still has a disadvantage of the formation of inclusion bodies, so it needs to be minimized by co-expression of chaperones. Therefore, the aim of this study was to determine the effect of single DnaK-DnaJ-GrpE and GroEL/ES chaperone co-expression and their combination on the expression of intein-pretrombin-2_{Ti,pH} on *E. coli* ER2566. The method started with isolation of pTWIN1-prethrombin-2_{Ti,pH} and pG-KJE8 from *E. coli* TOP10F' and DH5a respectively, the co-transformation of the expression host *E. coli* ER2566 using pG-KJE8 and pTWIN1-prethrombin-2_{Ti,pH} vectors, the chaperone co-expression was induced using L-Arabinosa before IPTG induction and cell culture growth was incubated at 22 °C. The expression products were characterized by using Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The results of the co-expression of chaperone showed that the number of soluble fraction was higher than the one without co-expression of chaperone. In addition, the co-expression of chaperone using pG-KJE8 in intein-prothrombin-2_{Ti,pH} expression was sufficient using tetracycline as an inducer.

Keywords: Intein-pretrombin-2_{Ti,pH}, chaperone co-expression of DnaK-DnaJ-GrpE and GroEL/ES, *E. coli* ER2566

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1. INTRODUCTION

Autologous fibrin glue (AFG) is an adhesive biomaterial, which consist of thrombin, fibrinogen and factor XIII, that can be applied as substitute to surgical suture (Enus *et al.*, 2006). Providing fibrinogen and Factor XIII from patient themselves are rather quick and simple however, preparation of autologous thrombin takes long time and requires trained personnel. Therefore, recombinant human thrombin were suggested to overcome the problem, while application of recombinant human thrombin in AFG also prevent contamination from infectious disease contaminants.

In this research, recombinant human prethrombin-2 (*hPT-2*) was expressed as thrombin precursor to AFG component. Expression were conducted under *E. coli* expression system, using intein as purification

tag which later purified using thiol induction (intein-pretrombin-2_{Ti}) and pH induction (intein-pretrombin-2_{pH}). This is based on previous research on recombinant thrombin that has been successfully produced in *E. coli* (Soejima *et al.*, 2001). The target protein, *hPT-2*, was fused with intein tags and chitin binding domain (CBD) that allows purification through affinity column, which is then continued by IMPACT system (Silaban *et al.*, 2014). IMPACT is a purification system developed by New England Biolabs which utilize self-cleavage properties of intein, analogous protein to intron, in its splicing components. The cleavage is induced whether by thiol reagent or pH gradient (Silaban *et al.*, 2016).

Expressing recombinant protein in *E. coli* offers advantages such as rapid growth, simple culture medium, vast strain are available, simple procedure, and relatively

inexpensive (Maksum *et al.*, 2017a; Maksum *et al.*, 2017b). Although, high expression of heterologous protein often lead to improper protein folding due to inadequate number of chaperone, which may induce formation of insoluble protein aggregate known as inclusion bodies (Choi and Lee, 2004). Recent studies showed that *in vivo* folding can be assisted by molecular chaperone. Chaperone induce appropriate isomerization of target protein through hydrophobic interaction with protein target (Baneyx, 1999).

Most of prethrombin-2 (PT-2) produced in *E. coli* has minor activity, due to formation of inclusion bodies (Soejima *et al.*, 2001). Therefore, simple strategies to overcome inclusion bodies formation were needed, such as: 1) Intracellular expression with refolding of fusion protein using *E. coli* Artic express (Maksum *et al.*, 2019; S Silaban *et al.*, 2019a; Silaban *et al.*, 2019b) or co-expression using chaperone (Gupta, 2002; Silaban *et al.*, 2014; Rizkia *et al.*, 2015; Silaban *et al.*, 2019c), 2) Extracellular expression using signal peptide and phospholipase co-expression (Sriwidodo *et al.*, 2017; Maksum *et al.*, 2017c; Melati *et al.*, 2019; Indriyani *et al.*, 2019; Indriyani *et al.*, 2020; Latifah *et al.*, 2020).

Co-expression of chaperone was conducted in this research with the aim to overcome inclusion bodies formation. Chaperone molecules are classified in three functional subclasses based on its mechanism that is, folding chaperone, stabilizing chaperone, and disaggregating chaperone. Folding chaperone (such as GroEL-ES) depends on ATP which promote conformational change to mediate substrate refolding (Baneyx and Mujacic, 2004).

According to Shen *et al.*, 2011, chaperone co-expression also improves the solubility of target protein while also reduce aggregation of recombinant protein lipase LIP-948 in *E. coli*. LIP-948 were efficiently expressed while co-expressed with chaperone combination, GroEL/ES and DnaK-DnaJ-GrpE compared with no co-expression added. This strategy produces 41.8% soluble fraction from total LIP-948 with respective specific activity 108.77 U/mg, while without co-expression only generate 4.5% soluble fraction with specific activity of 66.51 U/mg (Yan *et al.*, 2012).

Other studies (Juozas and Neubauer, 2011) also conduct chaperone co-expression in ribonuclease inhibitor (RI) expression. Co-expression of GroEL/GroES in RI expression showed activity enhancement compared without co-expression (Siurkus, 2013). Both of these studies confirm that chaperone co-expression can improve protein solubility, which later enhance its activity.

Nevertheless, further studies about chaperone-mediated folding are required since each chaperone has different mechanism and its influence in various target protein. Several proteins may have preference to certain chaperone system, LIP-948 were efficiently expressed along with GroEL/ES and DnaK-DnaJ-GrpE (from pG-KJE8) co-expression while human basic fibroblast growth factor (hbFGF) expression is best with trigger factor from pTf16 (Alibolandi *et al.*, 2010). Therefore, expression of intein-prethrombin-2_{Ti,pH} using pG-KJE as chaperone-containing plasmid, will also observe influence of both each chaperone GroEL/ES, DnaK-DnaJ-GrpE and combination of both chaperone.

2. MATERIALS AND METHODS

Materials

Sample used in this research were *E. coli* TOP10F' [pTWIN1-*pretrombin-2_{Ti}*], TOP10F' [pTWIN1-*pretrombin-2_{pH}*], DH5 α [pG-KJE8], dan untransformed ER2566. Agarose, tris base and glycerol [Genetika Science], bacto agar [Pronadisa], acrylamid [BioRad], aquabidest, ampicillin, APS, acetic acid, *comasie brilliant blue* R-250 *staining solution*, EDTA, hydrochloric acid, n-butanol, yeast extract [Titan Biotech LTD], restriction enzyme *Nde*I, *Bam*HI & *Xho*I [NEB], absolute ethanol, ethanol 70%, etidium bromide, glycine dan urea [Merck], IPTG [Thermo Scientific], plasmid isolation kit (*High-Speed Plasmid Mini Kit GeneaidTM*), chloramphenicol, calcium chloride, L-arabinose, *loading dye*, DNA marker, protein marker, sodium hydroxide, sodium chloride, SDS [Merck], TEMED, tetracycline, tryptone [Becton].

pTWIN1-*pretrombin-2_{Ti,pH}* dan pG-KJE8 Plasmid Isolation from Its Respective *E. coli* TOP10F'

5 mL cell culture were collected into microtube and centrifuged in 14000 g for 1 minute to collect cell pellet. Next, 200 μ L PD1

buffer with RNase were added and resuspended using vortex machine. Cell then lysed by adding 200 μ L PD2 buffer and mixed by 10X inversion. Mixture then incubated for 2 minutes before neutralized by 300 μ L PD3 buffer, then supernatant collected through centrifugation in 14000 g for 3 minutes. PD column were placed on the collection tube, then added by the supernatant. Later, pellets were collected through centrifugation at 14000 g for 30 seconds. PD column then placed back in the collection tube and centrifuged for 3 minutes with same speed. Next step, 600 μ L washing buffer containing ethanol were loaded into PD column then centrifuged in 14000 g for 3 minutes to dry out column matrix. Later, 50 μ L elution buffer or TE loaded into center of matrix column and allowed to stand for 2 minutes in order to allow absorption. Last, plasmid was collected by centrifugation at 14000 g for 2 minutes.

***E. coli* ER2566 Transformation with pG-KJE8 and pTWIN1-pretrombin-2_{Ti,pH}**

0.5 μ L plasmids pG-KJE8 and pTWIN1-*prethrombin-2_{Ti,pH}* were loaded into microtube containing 50 μ L competent cell for the chaperone combination method. While for single chaperone method, each *E. coli* were added by 50 μ L of its respective plasmids before incubation in 4 °C for 30 minutes. Transformation were conducted through heat shock in 42 °C for 90 seconds, then rapidly cooled for 10 seconds in ice bath. Mixtures then added by 900 μ L LB media and incubated with 150 rpm, 37 °C for 2 hours. After incubation, culture was centrifuged at 13000 g for 30 seconds and 850 μ L supernatant was discarded while the remaining 150 μ L will be grown in solid LB media, containing 50 μ g/mL chloramphenicol dan 100 μ g/mL ampicillin. Culture then incubated in 37 °C for 16-18

hours. Transformation of *E. coli* ER2566 by sole pTWIN1-*prethrombin-2_{Ti,pH}* plasmid also conducted as control without chaperone co-expression.

Chaperone Co-expression in *E. coli* ER2566 [pG-KJE8 + pTWIN1-pretrombin-2_{Ti,pH}] with Single and Combined Chaperone

Transformants *E. coli* ER2566[pG-KJE8+pTWIN1-*prethrombin-2_{Ti,pH}*] were grown in 5 mL liquid LB containing 50 μ g/mL chloramphenicol dan 100 μ g/mL ampicillin for 16 hours. Next, 10 μ L of culture were loaded into Erlenmeyer flask with 50 mL LB media with 1 mg/mL L-arabinose (DnaK-DnaJ-GrpE inducer) and/or 10 ng/mL tetracycline (GroEL/ES inducer) and antibiotics (50 μ g/mL chloramphenicol dan 100 μ g/mL ampicillin) then incubated in 150 rpm, 37 °C for 3-4 hours. After incubation, pellet was collected from 1 mL through centrifugation at 15000 g for 10 minutes and added by 30 μ L glycine buffer (as t_0 before induction by IPTG). The remaining culture then induced by IPTG with total concentration 75 μ M in media, and incubated in 22 °C for 6 hours. Later, pellet was collected from 1 mL of culture through centrifugation 15000 g for 10 minutes and added by 60 μ L glycine buffer (as t_1 : after IPTG induction). The remaining induced culture then harvested and pellet was collected in microtube using centrifugation at the same speed for 10 minutes. The pellets collected then added by 500 μ L glycine buffer, sonicated, and centrifuged in 15000 g, 4 °C for 20 minutes. Later supernatant was separated into fresh microtube as soluble fraction or crude extract. While pellet, was added by 50 μ L 8 M urea, heated in 95 °C for 15 minutes, and centrifuged at 15000 g in 4 °C for 10 minutes. The supernatant then collected as insoluble fraction.

Table 1. Variation of induction treatment on chaperone co-expression in *E. coli* ER2566 [pG-KJE8 + pTWIN1-*prethrombin-2_{Ti,pH}*] singly and in combination

Induction type	Variations in Induction Treatment		
	Chaperone co-expression		Without chaperone co-expression
	Single	Combination	
Tetracycline	√	-	√
L - Arabinose	-	√	√

Protein Characterization using SDS-PAGE and UV-Vis Spectrophotometer

The protein expressed was characterized using SDS-PAGE. Gel was prepared by casting resolving gel and stacking gel. Resolving gel was loaded in glass platesheet up to 2/3 volume, then n-butanol was added to help flatten the gel surface and let stands for 30 minutes. After gel hardened, n-butanol was discarded then, stacking gel and comb were loaded and allowed to stand for another 30 minutes. Later, gel was assembled in electrophoresis tank filled with 1X running buffer. Samples were prepared by mixing 10 μL of each samples with 5 μL 1X loading dye, then incubated in 95 °C using water bath for 15 minutes before centrifuged at 15000 g for 1 minutes. Later, each samples were loaded 20 μL into well and 5 μL protein marker was also loaded. Electrophoresis then run at 120 V for 90-120 minutes. After electrophoresis, gels were stained using *coomassie brilliant blue R-250 staining solution* for 5 hours and destained for 2 hours, then rinsed in aquadest before dried and analyzed.

Protein wavelength absorption from 2 expression system (pTWIN1-*prethrombin-2_{Ti,pH}* dan pTWIN1-*prethrombin-2_{pH}*) with/without chaperone co-expression (both single or combination method) were measured at $\lambda 280$ nm using UV-vis spectrophotometer [Ultrospec 3000 Pro].

3. RESULTS AND DISCUSSION

Without Chaperone Co-expression

Ekspresi of ER2566 [pTWIN1-*prethrombin-2_{Ti,pH}*] without chaperone co-expression were conducted as control comparison. Protein expression induced by IPTG with total concentration 75 μM and incubated in 22 °C for 6 hours. The results were shown in Fig. 1A, *prethrombin-2_{Ti,pH}* fused with intein gives protein band in 63 kDa which appeared in insoluble fraction. High-rate expression and inadequate folding are reasons of this result. Therefore, chaperone co-expression was conducted to resolve this problem.

Intein-*prethrombin-2_{Ti,pH}* Expression with Combined GroEL/ES + DnaK-DnaJ-GrpE Chaperone Co-expression

Both chaperones inducer, L-arabinose and tetracycline for DnaK-DnaJ-GrpE and GroEL/ES respectively, were added into media

before transformant was first cultured in 37 °C at 150 rpm. While cultured grown, both chaperones will be expressed first since both were induced first. This corresponds to Table 1, which both chaperone absorption was greater with prethrombin as a result of early chaperone induction compared to protein target. Early chaperone induction allowed cells to provide chaperones, so when protein target expression was induced chaperones were ready to help protein folding. It is shown that intein-*prethrombin-2_{Ti,pH}* expression under GroEL/ES co-expression has better protein yield in soluble fraction compared to DnaK-DnaJ-GrpE solely and in combination of chaperones (Figure 1). This is caused by GroEL role which help protein folding by providing folding space, so that intein-*prethrombin-2_{Ti,pH}* folding undergoes without surrounding disturbance. It is shown that co-expression induced by L-arabinose has less soluble fraction, it is likely when DnaK-DnaJ-GrpE is combined with GroEL/ES, it will reduce the soluble protein from intein-*prethrombin-2_{Ti,pH}* due to competition for binding of protein substrates (intein-*prethrombin-2_{Ti,pH}*) between GroEL and DnaK, which when intein-*prethrombin-2_{Ti,pH}* expressed with DnaK is no better than when expressed with GroEL. Apart from that, tetracycline seems has optimum effect than L-arabinose, since host and its vector do not contain any tetracycline-resistant gene.

Result of combined chaperone co-expression shown in Fig 1B, exhibit intein-*prethrombin-2_{Ti,pH}* band in soluble fraction. This indicate that chaperones assist intein-*prethrombin-2_{Ti,pH}* folding which may result to improving soluble fraction of target protein. Furthermore, availability of folding space provided by GroEL allowed efficient folding of intein-*prethrombin-2_{Ti,pH}*, although insoluble fraction also exhibit notable intein-*prethrombin-2_{Ti,pH}* band.

Single DnaK-DnaJ-GrpE Chaperone Co-expression in Intein-*prethrombin-2_{Ti,pH}* Expression

DnaK-DnaJ-GrpE was expressed after L-arabinose induction. Figure 1C exhibit intein-*prethrombin-2_{Ti,pH}* band, yet it has lower band thickness compared to Fig 1A. The reduction of soluble fraction from DnaK-DnaJ-GrpE co-expression might be caused by absence of folding space as well as provided in

GroEL system, this may have allowed environmental disturbance to disturb folding. It is also caused by Hsp70 including DnaK, which help folding and degradation of unstable protein (Henderson and Pockley, 2010), execute intein-pretrombin-2_{Ti,pH} degradation due to protein crowd which occupy limited space of cytoplasm, to prevent stability disturbance of surrounding proteins. Other possibility, L-arabinose used as inducer may have been metabolized by cells as carbon source so it does not utilize as inducer optimally (Yan *et al.*, 2012). Furthermore, there were no mutation found on *E. coli* ER2566 which may disrupt L-arabinose metabolism. While this problem has already countermeasured by increasing L-arabinose concentration, it appears that optimization of L-arabinose concentration still need to be studied to achieve high-yield protein target.

Single GroEL/ES Chaperone co-expression in intein-pretrombin-2_{Ti,pH} expression

GroEL/ES were expressed after tetracycline induction. Fig. 1D shows that intein-pretrombin-2_{Ti,pH} in soluble fraction has more protein than soluble fraction from sole DnaK-DnaJ-GrpE and combined chaperones system. It is strongly estimated caused by folding space provided by GroEL which help folding and reduce environmental disturbance. As discussed in previous section, co-expression induced by L-arabinose yields lower soluble fraction of intein-pretrombin-

2_{Ti,pH}, which may correspond to reduced yield in DnaK-DnaJ-GrpE + GroEL/ES combination system. It is also due to GroEL and DnaK binding competition for protein substrate that gives no better result as previously described. While tetracycline also may play role as it might have optimal role as inducer solely compared to L-arabinose, due to absence of tetracycline-resistant gene.

Although chaperone help intein-pretrombin-2_{Ti,pH} folding which lead to increasing its soluble fraction, but total protein produced using chaperone co-expression was decreased. This was measured using spectrophotometer at 280 nm from respective soluble fraction samples, as shown in Table 2. It is appropriate that co-expression of chaperons can inhibit the growth of *E. coli* and reduce the level of expressed proteins (Garcia-carbonero *et al.*, 2013). Competition between inducers seem to play a role since they were in same system, whether IPTG, L-arabinose, and tetracycline, thus interfere each induction process and resulting in lower protein production. However, chaperone co-expression using pG-KJE8 induced by tetracycline exhibit highest value among other chaperone co-expression system. Although without chaperone co-expression, especially [ER2566+pTWIN1-pretrombin-2_{Ti}], exhibit greater absorbance, it has no intein-pretrombin-2_{Ti} appeared in soluble fraction as shown in Fig. 1A.

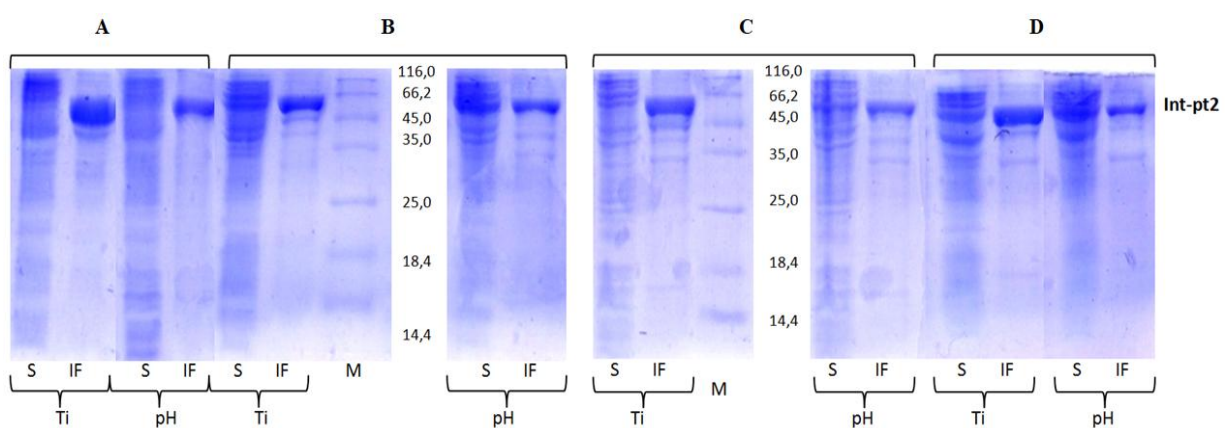


Figure 1. SDS-PAGE Electrophoregram of intein-pretrombin-2_{Ti,pH} expression with and without chaperone co-expression. A) without co-expression; B) GroEL/ES + DnaK-DnaJ-GrpE co-expression; C) DnaK-DnaJ-GrpE co-expression; and D) GroEL/ES co-expression. M, marker protein; S, soluble; IF, insoluble fraction; Ti, pTWIN1-pretrombin-2_{Ti}; pH, pTWIN1-pretrombin-2_{pH}; int-pt2 : intein-pretrombin-2_{Ti,pH}. Expression were conducted in 37 °C for 3 hours (before induction) and 22 °C for 6 hours (after induction).

Table 2. Soluble Fraction absorbance at $\lambda 280$ nm from two expression systems. (pTWIN1-*prethrombin-2_{Ti}*; pTWIN1-*prethrombin-2_{pH}*)

Expression System	Absorbance	
	Ti	pH
<i>E. coli</i> ER2566	0.757	0.551
<i>E. coli</i> ER2566 + pG-KJE8 combined chaperones (induced by L-arabinose and tetracycline)	0.467	0.420
<i>E. coli</i> ER2566 + pG-KJE8 single chaperone (induced by L-arabinose)	0.491	0.488
<i>E. coli</i> ER2566 + pG-KJE8 single chaperone (induced by tetracycline)	0.720	0.639

Note: Ti: pretrombin-2_{Ti} absorbance; pH: pretrombin-2_{pH} absorbance

In addition, since SDS-PAGE only give qualitative and semi-quantitative profile about protein of interest, it is better to comprehend SDS-PAGE data and have more precise result to help analysis. SDS-PAGE data may have come to bias in distinction our protein target with other protein that has similar molecular weight, in example, GroEL molecular weight were 57 kDa which close to our protein target 63 kDa and may give overlapping band. Therefore, to determine and assure our protein target, western blot analysis will be helpful while, accurate measurement of prethrombin-2 level requires further purification steps. However, one unique characteristic of expression using the T7 RNA polymerase regulated by the T7 promoter in the pTWIN1 vector is its high level of expression, and shows the thickest protein band among the others.

4. CONCLUSION

From the results of the research that has been done, it can be concluded that according to SDS-PAGE analysis and absorption analysis at $\lambda 280$ nm, chaperone co-expression of the GroEL/ES, DnaK-DnaJ-GrpE, and a combination of GroEL/ES and DnaK-DnaJ-GrpE in the expression of intein-pretrombin-2_{Ti,pH}, in *E. coli* ER2566 shows an increase in the soluble fraction compared without co-expression, especially when co-expressed with GroEL/ES solely.

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