Research Article

The Role of Exogenous GroEL/GroES Chaperon System in Improving the Expression of Auto-Activated Thrombin Precursor Genes in *Escherichia Coli* ER2566

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Received: 10-07-2018; **Revised:** 30-07-2018; **Accepted:** 10-08-2018.

ABSTRACT

Despite the advances of current biotechnology techniques, the activation of recombinant thrombin, as a component of fibrin glue, still requires exogenous activator such as ecarin. Ecarin is a metalloprotease derived from snake venom, which could transmit the pathogen to the end-product. Prethrombin-2, the smallest precursor of thrombin, is a mutant at positions of E14e, D14l, E18, and G14m (EDGE) that automatically converted to prothrombin-2. This study aims to express the prethrombin-2 (pt2) EDGE mutant gene in *Escherichia coli* ER2566. The result showed that pt2 (EDGE) gene was successfully expressed in *E. coli* ER2566. Moreover, the co-expression with pG-KJE8 increased the solubility of PT2 (EDGE). This result was evidenced by the decreasing of inclusion bodies, as confirmed in the SDS electropherogram in the form of the CBD-Ssp fusion protein of DNAB-PT2 (EDGE), with a molecular weight of ± 63 kDa.

Keywords: Autoactivation, GroEL/GroES, E. coli ER2566, co-expression, pG-KJE8.

INTRODUCTION

Thrombin is synthesized in the form of zymogen and turned into a mature enzyme during an activation process by a specific enzyme, usually with the help of a cofactor. In the blood, prothrombin and pretrombin-2 (PT2) zymogens require protrombinase to convert them into thrombin as an active enzyme. To change PT2, which is the smallest precursor of thrombin to thrombin that is actively used, it needs a specific enzyme, which is ecarin, that has high activity. Ecarin is a metalloprotease of the snake Echis- carinatus[1].

The use of ecarin for the activation of PT2 may result in transmission of disease, thus making necessary the purification of the pathogenic component and removal of the ecarin present in the thrombin after the activation process. These increases production costs in the development of fibrin glue, which is used as an alternative to stitching replacement procedures, especially in ophthalmologic procedures. Therefore, it is necessary to develop a PT2 automated product which has the function and structure equivalent to the thrombin found from wild animals. Autoactivation is one of the innovative strategy employed in the production process of therapeutic proteins without the need for exogenous enzymes (ecarin) for activation. This PT2 autoactivation product is obtained from the expression of pt2 gene, which the four amino acid residues (E14eA / D14lA / G14mP / E18A) have mutated. The replacement of E14e, D141 and E18 acid residues into Ala in the activation domain causes R15 to escape from the anion cage and its side chains are directly exposed to the surface for proteolysis, while the replacement of G14m residue to Pro allows the urgent side chain from R15 to rise to the surface, allowing the zinc The EDGE mutants to undergo immediate self-activation after refolding[2].

This automation product is obtained through cloning techniques and heterologous protein expression. The mutant pt2gene EDGE (pJ211-pt2 plasmid (EDGE) has been express using E.coli ArcticExpress DE3 as expression hosts that have endogenous Cpn10 and Cpn60 endemic chaperones which help the protein folding process[3]. However, when using another *E. coli* strain as an expression host, it is often involving the protein produced in an insoluble state, which is in the form of inclusion bodies due to the unequal amount of endogenous chaperones to perform their tasks in protein folding with heterologous protein expression[4]. Therefore, it is necessary to increase the amount of chaperones to overcome the inclusion bodies, to increase the solubility of PT2 mutants, which can be achieved by the co-expression of chaperones.

Co-expression of chaperones can increase the solubility of expression and purification results from iron-regulatory protein 1 (IRP1) protein in *E.* coli[5]. Increased dissolved fraction of expression results also increases the effectiveness of protein purification. The results of IRP1 purification increase when expressed with GroEL / ES compared with the result without co-expression, i.e. increases to 87% of the eluting protein of 10%. Co-expression uses pG-KJE8, which carries the DnaJ / K-GrpE chaperon gene and GroEL / ES. DnaJ / K-GrpE co-expression, and GroEL / ES increased non-mutant PT2 solubility. However, co-expression using GroEL / ES gave the best result of improved expression of pt2 non mutant gene, seen from the absorption data of dissolved fraction at 280 nm wavelength[6]. Chaperon GroEL helps protein

folding through a functional cycle that begins with the binding of ATP and GroES after the capture of polypeptide substrate, then through hydrolysis of ATP and protein folding, followed by the release of GroES and folded proteins and the new polypeptide folds [7].

The purpose of this study is to investigate the effect of exogenous chaperone GroEL/GroES system in enhancing the expression of auto-activated thrombin precursor genes in E. coli strains ER2566.

MATERIALS AND METHODS

Material

The materials used in this study are pJ211-pt2 synthetic genes (EDGE) pH 2 ng / µL (synthesized in Dna 2.0, California, USA), E. coli TOP10F ', expression vectors pTWIN1 2 ng / µL, E. coli ER2566 , plasmid insulation kits (High-Speed Plasmid Mini Kit GeneaidTM), DNA purification kits from gel (Tiangel Midi DNA Purification Kit), TxE (Tris Acetate EDTA) 1x, GelRed (Biotium) buffer, 1 kb ladder (Thermo) marker, enzyme restriction of SapI & BamHI (NEB), buffer cut smart (NEB), loading dye (Thermo), tetracycline antibiotics, kanamycin, chloramphenicol, and ampicillin, SOC, IPTG, SDS 10%, urea 8M, TEMED, tris-HCl pH 6 8, Tris-HCl pH 8.8, APS 10%, Commasie Briliant Blue R-250 staining solution, destaining solution, 5x SDS sample buffer, 40% acrylamide (Biorad), DNA marker 1 kpb (Thermo), unstained protein mark (Thermo).

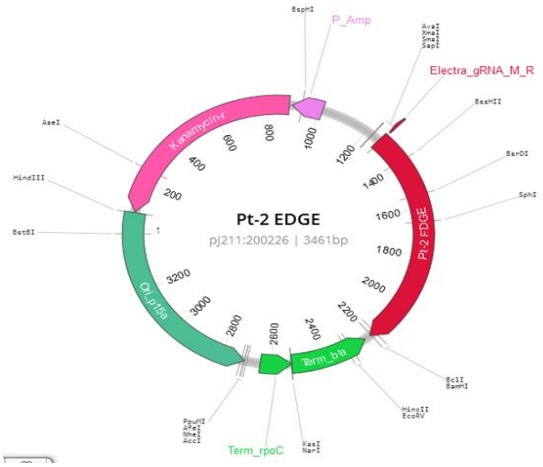

Figure 1: Map plasmid pJ211-pt2 (EDGE) pH -Plasmid Carrier, synthesized in Dna 2.0, California, USA.

Transformation of E. coli TOP10F'using Plasmid Carrier and Expression Vector pTWIN1

The single colon of E. coli TOP10F' was grown in 5 mL of liquid LB medium containing 5 µL tetracycline antibiotic (10 mg / mL) at 37 ° C for 16-18 hours at a rate of 150 rpm. A total of 1 mL cells were grown into 100 mL of LB liquid medium without NaCl, then incubated at 37° C at 250 rpm to OD600 nm at 0.8-1.0. Cells were incubated in ice bath for 10-15 minutes. The cells were then transferred into a sterile falcon tube, and then centrifuged at 5000 rpm, 4° C for 5 minutes. The obtained cell pellets were resuspended with cold sterile aquabidest of a number of cells inserted into the falcon tube, then centrifuged at 5000 rpm, 4 °C for 5 minutes. Then the cell pellets were collected and resuspended with 50 mL cold sterile akuabides. The obtained cell pellets were resuspended with 2 mL cold sterile aquabidest then centrifuged at 5000 rpm, 4 ° C for 5 minutes. Then the obtained cell pellets were diluted with 200 µL-300 µL cold sterile aquabidest for instantly used transformation. If not directly used for transformation of cell pellets dissolved with 200 µL-300 µL 10% glycerol then stored at -20 °C.

Transformation of E. coli using expression vector pTWIN1

The transformation of pJ21-pt2(EDGE) to pTWIN1 used electroporation method. A 2 µL plasmid of pJ21-pt2(EDGE) synthesis gene (1 ng/µL) and 2 µ Expression expression pTWIN1 (2 ng/µL) were each added to a micro tube containing 50 µL competent cells before being piped up and down. A micro tube containing a mixture of plasmids and competent cells was fed into a cuvette that had been incubated in an ice bath for 10 minutes. The cuvette was then cleaned underneath and inserted into a 1500 mV voltage-regulated electrophorator device. The ms and voltage values used were then recorded. As a positive control of transformation, a TOP10F transformation of E. coli was performed using quantifiable pPICZαB plasmids. Then, 1 mL of SOC liquid medium was added to the cuvette, resuspended, and fed into the test tube, before being incubated for 1 hour at 37 ° C at 250 rpm. A total of 100 µL transformed cultures were grown on an LB solid medium containing antibiotics suitable for selection at 37 ° C for 16-18 hours. Transformances were taken from a single colony and replicated.

Isolation of Plasmid Carrier Cloning Results and Expression Vectors

Isolation of cloning results using plasmid isolation kits GenAid High Speed Plasmid Mini Kit. The TOP10F E. coli transformant containing pTWIN-pt2 (EDGE) plasmid was grown in 5 mL of liquid LB medium which had been added with 5 µL tetracycline (10 mg / mL) and 5 µL ampicillin (100 µg / mL) for 16-18 h, at the temperature 37oC, with a speed of 150 rpm. The TOP10F E. coli cell culture was inserted into a 1.5 mL microcentrifuge tube and centrifuged at 12,000 rpm for 1 minute to collect pellets. Cell pellet was added 200 µL buffer PD1 (added RNase) and dissolved with vortex before 200 µL buffer PD2 was added, and the microspiration tube was inverted 10 times. A 300 µL PD3 buffer was then added to the tube and then inverted 10 times. The tube was centrifuged at 13,400 rpm for 5 min. The supernatant was transferred into the PD column and centrifuged at 13,400 rpm for 1 minute before being removed. A 400 µL W1 buffer was added to the PD column and centrifuged at 13,400 rpm for 1 minute and then its eluate was discharged. Then, as much as 600 µL washing buffer (added ethanol) was added to the PD column and centrifuged at 13,400 rpm for 1 minute and then its eluate was discharged. After

that, the PD column was centrifuged at 13,400 rpm for 5 minutes, then the column was transferred to a new microcentrifuge tube. The TE buffer was added to a 30 µL CP3 column then incubated in a 55 ° C water bath for 3 min and centrifuged at 13,400 rpm for 2 min. Next, the eluate was re-added into the PD column then incubated in water bath with the temperature of 55 °C for 3 minutes and centrifuged at 13,400 rpm for 2 minutes.

Characterization of carrier plasmid isolation results and expression vectors

The isolation results were characterized by the cutting of one and two restriction enzymes. A total of 5 µL isolates were inserted into the microtube and added 0.5 µL SapI enzyme and 0.5 µL of the BamHI enzyme for restriction by the addition of 1 µL buffer cut smart and 3 µL nuclease-free water, then incubated for 2-3 hours at 37 °C. To cut the restriction enzymes, only the BamHI enzyme was used. The results of the restriction were characterized using 0.4% (w/v) agarose electrophoresis. Markers and mixtures containing isolated plasmids were made for characterization. The markers contained a mixture of 4 µL nuclease free water, 1 µL loading dye, 1 µL gel red, and 1 µL ladder DNA of 1 kb. Then, 1 µL of loading dye, 1 µL gel red, and 5 µL of plasmid isolation without cutting while for cutting plasmid were included in all the volumes of the restriction reaction results before being introduced into the agarose gel well 0.8% (w/v). Electrophoresis was carried out with a voltage of 80 volts for ± 40 minutes and TAE 1x buffer was used as a current delivery medium. DNA bands were witnessed with the help of ultraviolet light at λ312 nm.

Recombinant plasmid construction pTWIN1-PT2 (EDGE) and cloning in E. coli TOP10F'

Isolates pJ211-pt2 (EDGE) and pTWIN1 were cut with restriction enzymes BamHI and SAP with a total reaction volume of 20 µL and the remaining isolates were concentrated to a volume of 0 µL and then added 2 µL buffer cut smart, 1 µL SapI enzyme, 1 µL of BamHI enzyme, and 16 µL of nuclease free water and then incubated for 2-3 hours at 37°C. The restriction DNA fragment was analyzed with 0.4% (w/v) agarose electrophoresis. The SapI-pt2-BamHI fragments were cut from the agarose gel and purified using a Tiangel Midi DNA Purification Kit purification kit. In the same way, plasmid pTWIN1 was also cut from agarose gel and purified.

The agarose gel slices (fragment SapI-pt2-BamHI and pTWIN1) were fed into a microcentrifuges tube and then added with 400 µL PN buffer and incubated in 50°C water bath while inverted until dissolved for 10 min. The microcentrifuges tube was cooled, and allowed to stand for 2 minutes, both at room temperature. The solution of the DNA fragment was transferred to a spin column that had been previously added with 500 µL buffer BL and centrifuged at 12,000-13,400 rpm for 1 minute and then the eluate was discharged. The spin column containing the DNA fragment solution was centrifuged at a rate of 12,000-13,400 rpm for 1 minute and the eluat was discharged. Then, as much as 600 µL of PW buffer (added ethanol) was added to the spin column and centrifuged at 12,000-13,400 rpm for 1 minute and then removed. Then, the column was allowed to stand for 2-5 minutes at room temperature.

The addition of PW buffer was repeated 2 times. Thereafter, the spin column was centrifuged at the rate of 12,000-13,400 rpm for 2 minutes and to dry the spin column, the column was open and left for a few minutes at room temperature, before being transferred to a new micro centrifuge tube. EB buffer was added to the 40 µL spin column and then incubated in a water bath at 55°C for 3 minutes and centrifuged at 12,000-13,400 rpm for 2 minutes, before being added back to the 30 µL spin column and incubated in the water bath temperature with the temperature of 55°C for 3 minutes and centrifuged at 12,000-13,400 rpm for 2 minutes. The ligation reaction was carried out with a 1:8 molar ratio between the pTWIN1 vector and the SapI-pt2-BamHI insert genes. The ligation reaction was carried out using T4 DNA ligase enzyme with a total ligation reaction volume of 10 µL at 160C for 16-18 hours.

Transformation of E. coli TOP10F 'using pTWIN1-pt2 (EDGE) - Recombinant Plasmid

A total of 10 µL recombinant plasmid pTWIN1-pt2 (EDGE) were added to a microcentrifuges tube containing 50 µL competent cells, then piped up and down. A microcentrifuges tube containing a mixture of plasmids and competent cells was fed into a cuvette that had been incubated for 10 minutes in an ice bath. The cuvette was cleaned underneath and then inserted into a 1500 V voltage regulated electrophorator device. The ms and voltage values used were recorded. As a positive control of the transformation, a transformation of TOP10F E. coli was performed using quantized plasmids of pTYB21. Then, 1 mL of SOC liquid medium was added to the cuvette, resuspended, and fed into the test tube, then incubated for 1 hour at 37°C at 250 rpm. A total of 100 µL transformed cultures were grown on an LB solid medium containing antibiotics suitable for selection at 37°C for 16-18 hours. Transformances were taken from a single colony and replicated.

The TOP10F E. coli transformant containing pTWIN1-pt2 (EDGE) plasmid pH was grown in 5 mL of liquid LB medium which was added with 5 µL tetracycline (10 mg / mL) and 5 µL ampicillin (100 µg / mL) for 16-18 hours at 37°C and a speed of 150 rpm. The TOP10F E. coli cell culture was inserted into a 1.5 mL micros centrifugation tube and centrifuged at 12,000 rpm for 1 minute to collect pellets, which DNA were isolated using plasmid insulation kit of GenAid High Speed Plasmid Mini Kit. The isolation results were characterized by the cutting of two restriction enzymes. A total of 5 µL isolates were inserted into the micro tube and added with 0.5 µL SapI and 0.5 µL of the BamHI enzymes for restriction by addition of 1 µL

buffer that had been cut and 3 µL nuclease free water before being incubated for 2-3 hours at 37°C. Isolates without restriction enzyme cutting and the restriction results were characterized using 0.4% (w/v) agarose electrophoresis.

Expression PT2 (EDGE) and co-expression of GroEL / ES in E. coli ER2566

Single colonies of E. coli ER2566 were grown in 5 mL of liquid LB medium at 37°C for 16-18 hours at a rate of 150 rpm. A total of 200 µL cells were grown into 20 mL LB liquid medium, then incubated at 37°C at a rate of 150 rpm until OD600 nm reached 0.2-0.4. The cell culture of E. coli ER2566 was inserted into a 1.5 mL microscaping tube and centrifuged at 5000 rpm, at 4°C, for 5 minutes to collect pellets. Then, the cell pellets were added with 1 mL CaCl2 0.1 M cold and resuspended. The microcentrifuge tube was incubated in an ice bath for 10 minutes before being centrifuged at 5000 rpm, at 4°C for 10 minutes and the supernatant was discarded. The addition of 0.1 M cold CaCl2 was done twice. After that, the cell pellets were diluted with 300 µL CaCl2 0.1 M cold and stored in an ice bath for 2-24 hours, at 4°C. Competent cells were then ready for transformation.

The transformation of pTWIN-pt2 (EDGE) and pG-KJE8 was using heat shock method. A total of 3 µL pTWIN1-pt2 (EDGE) pH and 2 µL pG-KJE8 were added to a microcentrifuges tube containing 50 µL of competent cells, then pipetted up and down. A microcentrifuge tube containing a mixture of plasmids and competent cells was incubated for 30 minutes at 4°C, then heat shock was performed at 42°C for 90 seconds. Then, the transformation was repeated using only the pTWIN1-pt2(EDGE). The microcentrifuge tube was incubated in ice for 10 min, then 900 µL liquid LB medium for pT-pG-KJE8 and pT2-pt2 (EDGE) plasmid was added. The microcentrifuge tube was incubated at 37°C for 1 hour at a rate of 250 rpm, and then centrifuged at 5000 rpm for 30s at 4°C. As much as ± 900 µL supernatant was discarded, leaving the cell pellet to resuspend with the remaining 100µL supernatant, then grown on LB solid medium containing antibiotics suitable for selection at 37°C for 16-18 hours. Transformances were taken from a single colony and replicated.

The E. coli ER2566 transformant containing pTWIN1-pt2(EDGE) + pG-KJE8 plasmisd was grown in 5 mL of liquid LB medium which had been added with 5 µL ampicillin (100 µg/mL), and 5 µL chloramphenicol (50 mg/mL) for 16-18 hours, temperature 37oC, with a shake rate of 150 rpm. Also grown E. coli ER2566 transformant containing pTWIN1-pt2 (EDGE) plasmid in 5 mL of liquid LB medium which has been added 5 µL ampicillin (100 µg / mL) for 16-18 hours, at 37°C, at the rate of 150 rpm. Culture cells E. coli ER2566 were inserted into a 1.5 mL microcentrifuge tube and centrifuged at 12,000 rpm for 1 minute to collect pellets which were then isolated using plasmid isolation kits GenAid High Speed Plasmid Mini Kit. The isolation result was characterized by the cutting of two restriction enzymes. A total of 5 µL isolates were inserted into the microtube and added 0.5 µL SapI enzymes and 0.5 µL of the BamHI enzyme for restriction by addition of 1 µL buffer cut and 3 µL nuclease free water then incubated for 2-3 hours at 37°C. The isolates without the restriction enzyme cutting and the restriction results were characterized by 0.4% (w/v) agarose electrophoresis.

E. coli ER2566 pTWIN1-pt2 (EDGE) that has been characterized, was grown in 5 mL of liquid LB medium which had been added with 5 µL ampicillin (100 µg/mL) for 16-18 hours, at 37°C, at the rate of 150 rpm. The E. coli ER2566 cell culture was fed as much as 20 µL into an erlenmeyer flask containing 20 mL of liquid LB and an appropriate antibiotic for selection. E. coli ER2566 [pTWIN1-pt2 (EDGE) pH + pG-KJE8] that had been characterized, grown in 5 mL of liquid LB medium which had been added with 5 µL ampicillin (100 µg/mL) and 5 µL chloramphenicol (50 mg/mL) for 16-18 hours, at the temperature of 37oC, with a rate of shake of 150 rpm. The E. coli ER2566 cell culture was incorporated with as much as 20 µL into an erlenmeyer flask containing 20 mL of liquid LB and an appropriate antibiotic for selection and 20 µL tetracycline (10 mg/mL) as a GroEL / ES chaperon inducer. An empty E. coli ER2566 cell was also grown and the culture was inserted with as much as 20 µL into an erlenmeyer flask containing 20 mL liquid LB. Then, all the cultures within the erlenmeyer flask were incubated at 37°C, with a matching rate of 150 rpm to 0.4-0.8 OD600 nm. A total of 1 mL of culture was taken to (before the induction of IPTG) and inserted into microsentrifugation tube, then the pellet was collected by centrifugation at 8000 g, 4°C for 10 min. Then, the culture in the erlenmeyer flask was added with IPTG 0.1 mM / mL culture volume, then incubated at 22oC, with a shuffle rate of 150 rpm for 5-6 hours to OD600 nm 1.0-1.5. A total of 1 mL of culture was taken as ti (after induction of IPTG) and inserted into a microsentrifugation tube, then pellet was collected by centrifugation at 8000 g, 4°C for 10 min. Then, the collected pellets in the remaining cultures were collected by centrifugation at the speed of 8000 g 4°C for 10 minutes. Then, as much as 500 µL of glycine buffer was added. The dilisis was done using a sonicator, and centrifuged at 10,000 g at 4°C for 30 minutes. The supernatant was transferred to a new microscaping tube as a soluble fraction. The pellets were added 100 µl of urea 8 M, then heated to 95°C for 15 min and centrifuged at 8000 g at 4°C for 10 min. The supernatant was fed into a new microtube as an insoluble fraction. For to and ti, they were dissolved with 40 µL buffer glycine.

As much as 40 µL of the samples of expression results were taken and mixed with 5 µL SDS sample buffer 5x in micro centrifugation tube. The mixture was then heated at 95°C for 15 minutes at the waterbath, then centrifuged at 8000 g for 1 minute at 4°C to lower the vapor. Each sample of 10 µL and a protein marker of 5 µL were fed into the well. Electrode cable was paired with

electrophoresis device, then the gel was electrophoresed at 100 volts for ± 120 minutes. The gel was then immersed in a staining solution (Commasie Brilliant Blue R-250 0.25%, 45% methanol, 10% acetic acid) for 1 hour with slow shaking or during overnight, then stored in the destaining solution until the excess gel dye was lost. The positive results of SDS-PAGE were characterized by the emergence of the ± 63 kDa recombinant protein band of intein-pretrombin-2.

RESULTS AND DISCUSSION

Transformation of plasmid carrier and expression vector

The process of plasmid transformation was successful on E. coli TOP 10F ' using electroporation method or electric shock. The principle of this method is to disrupt the stability of cell membranes using high electric current, to enable the plasmids to enter the cells through the pores formed. The electric voltage used in this transformation was 1500 mV which is the optimum electric voltage with one electric current through the cuvette with a slit size of 0.4 cm (transformed in Figure 2). The result of this transformation was obtained transformant E. coli TOP 10F 'containing pJ211-pt2 (EDGE) plasmid and pTWIN1 expression vector. Transformation of E. coli TOP10F '[pJ211-pt2 (EDGE) pH] was grown on a dense LB medium that had been given tetracycline antibiotics and kanamycin, while transformant E. coli TOP10F '[pTWIN1] was grown on a dense LB medium which had been given tetracycline antibiotics and ampicillin. Meanwhile, the positive control, which was pPICZαB, was grown in 20 mL of solid medium LSLB (Low Salt Luria Bertani) with zeosine and tetracycline antibiotics. Single columns of transformants were rejuvenated and replicated in a new solid LB medium with appropriate antibiotics (Figure 2).

Isolation and characterization of plasmid carrier and expression vector

The transformant colonies obtained from the replica were then isolated and characterized to ensure a growing TOP10F' E. coli transformant, containing pJ211-pt2 (EDGE) plasmid and pTWIN1 expression vector. Bacterial culture of E. coli TOP10F' [pJ211- pt2 (EDGE) pH], E. coli TOP10F' [pTWIN1], and E. coli TOP10F' [pPICZαB] used liquid LB media. Then, the bacterial culture was inserted into microcentrifuges tube to collect pellets which was then entered into plasmid isolation stage, using the High-Speed Plasmid Mini Kit GeneaidTM kit. After isolation, pJ211-pt2 (EDGE) plasmid isolate, pTWIN1 vector, and positive control of pPICZαB vector transformation were characterized using agarose gel electrophoresis (Fig. 3). The presence of multiple bands on a single line indicates that the isolation results contain the same plasmid DNA but have different conformations such as supercoiled, single stranded circular, and nicked circular/relaxed circle.

Then, the plasmids were analyzed using two restriction enzymes (double digest). Restriction analysis using these two enzymes ensured the size of the two plasmids, as if they are circular plasmids, they can adopt conformations that cannot be matched with markers which are also sequences of linear nucleotides. Thus, they need to be first made linear by cutting using the corresponding restriction enzymes. In accordance with the synthetic gene design pt2 (EDGE), the enzymes used were SapI and BamHI as they work optimally on two different buffers.

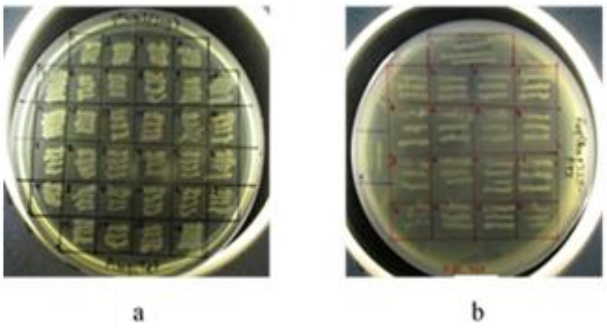

Figure 2: Replication of transformers resulting from subcloning plasmid pJ211-pt 2 (EDGE) pH) and pTWIN1. (b) E. coli TOP10F '[pTWIN1] (tet, amp).

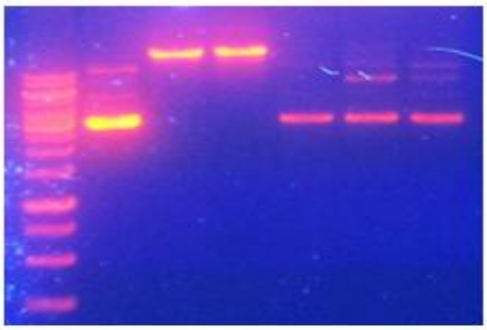

Figure 3: 0.17% agarose electrophoregram (b / v) of pJ211-pt2 plasmid isolation (EDGE) pH, pTWIN1 and pPICZαB. (M) DNA marker 1 kb; (1) plasmid pPICZαB; (2,3) pTWIN1 colonies 1 and 2; (4,5,6) pJ211- pt2 (EDGE) pH colonies 1, 2, and 3.

The SapI restriction enzyme had a 100% activity on the NEB CutSmart buffer, and could be incubated at 37°C without initiating star activity, while the restriction enzyme BamHI had the same activity as Sap but in NEB buffer, the restriction analysis using SapI and BamHI enzymes was performed using CutSmart buffer with incubation 37oC and not overnight. From the characterization of circular plasmid pJ211-pt2 (EDGE) and pTWIN1, the 2nd colonies of each plasmid were chosen because of the brightest bands, which indicated considerable concentration. Restriction was carried out at 37oC for 2-3 hours. The following is the result of characterization of two pJ211-pt2 (EDGE) and pTWIN1 colonies 2 with 0.17% agarose gel electrophoresis (b/v) (Fig. 4).

Purification of pt2 (EDGE) gene and pTWIN1 expression vector of agarose gel

Purification of pt2 gene (EDGE) and pTWIN1 used TIANgel Midi DNA Purification Kit (TIANgen). Purification of pH2 gene (EDGE) and pTWIN1 vector of other fragments was

done by cutting agarose gels on the gene band (EDGE) and pTWIN1 expression vectors. The purified agarose gel piece was re-characterized using 0.4% (w/v) agarose electrophoresis.

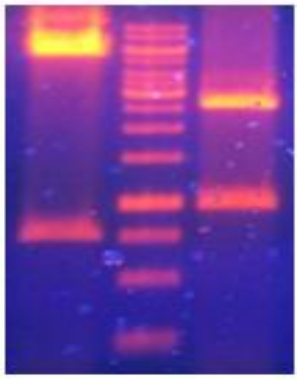

Figure 4: Electroforegram resulted from the characterization of two restriction enzymes SapI-BamHI plasmid pJ211-pt2 (EDGE) and pTWIN1. (M) DNA marker 1 kb; (1) pTWIN1 colony 2; (2) pJ211- pt2 (EDGE) colony pH 2.

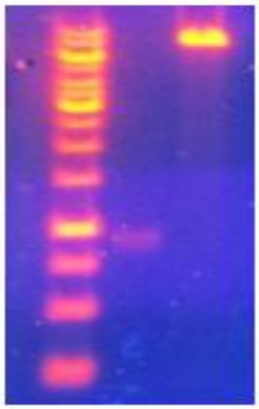

Figure 5: Electrophoregram pt2 gene (EDGE) pH and pTWIN1 expression vectors purified from agarose gel. (M) DNA marker 1 kb; (1) pt2 gene (EDGE) pH pure 938 pb; (2) pTWIN1 pure vector 6540 bp.

The result of characterization with 0.8% (w/v) agarose electrophoresis shows a ply band of pt2 gene (EDGE) of 938 bp and a 6540 bp pTWIN1 expression vector (Fig. 5 lanes 1 and 2), so it is certain that pt2 gene (EDGE) and pTWIN1 expression vectors had been pure.

pt2gene (EDGE) ligation with pTWIN1 expression vector

In the pt2 (EDGE) gene ligation process with pTWIN1 expression vector, it is important to know the gene concentration and the expression vector. The pt2 (EDGE) gene concentration of pt and expression vector TWIN1 can be determined by comparing pt2 gene (EDGE) thickness and pTWIN1 expression vectors with a 1 kb DNA marker concentration. The pt2 (EDGE) gene concentration and pTWIN1 expression vector was found to be 2.5 ng/µL and 12 ng/µL (the calculation was obtained from the ratio of gene band thickness to the DNA marker 250 pb multiplied by a co6centration of 250 pb DNA marker with gene volume). PH2 gene ligation (EDGE) pH with expression vector pTWIN1 is a sticky end ligation and performed with a molar ratio of pH2 gene (EDGE) pH: pTWIN1 expression vector is 8: 1, so the number of pTWIN1 expression vectors used is 50 ng, while pH2 gene (EDGE) pH was used 58 ng (calculation of molar ratio of ligation reaction found in Protocol NEB). The result of this ligation is a pH recombinant plasmid pH2 (EDGE) (Figure 5).

Recombinant plasmid cloning pTWIN1-pt2 (EDGE) in E. coli TOP10F'

Recombinant plasmid pTWIN1-pt2 (EDGE), which is the result of ligation was reproduced by cloning in E. coli TOP10F'. The TOP10F E. coli cells were transformed by electroporation method. Competent transformed cells used recombinant plasmid pT2 (EDGE), then grown in dense LB medium for 18 hours at 37°C . A single colony of transformation was replicated in a solid LB medium. The colonies were isolated and characterized using the SapI-BamHI restriction enzyme (Figure 7).

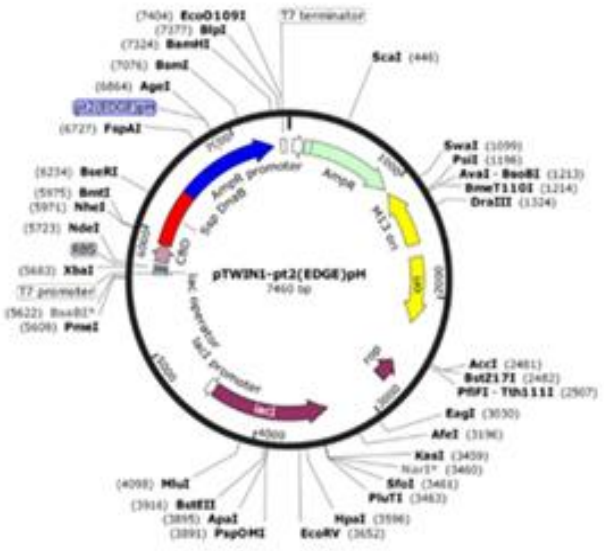

Figure 6: Map of recombinant plasmid pTWIN1-pt2 (EDGE).

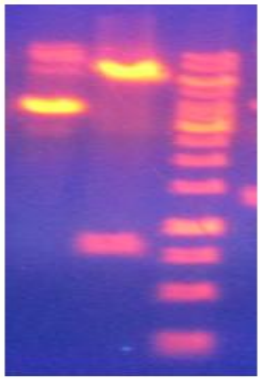

Figure 7: Electropherogram isolates and a transformant restriction analysis suspected to contain a pH recombinant pT2 (pGE) pT2 (EDGE) plasmid with two SapI-BamHI restriction enzymes. (M) DNA marker 1 kb; (1) isolate transformant (uncut); (2) isolate transformant colony 1 / SapI-BamHI.

Isolation and Characterization of Transformation E. coli ER2566 [pTWIN1-pt2 (EDGE)] and E. coli ER2566 [pTWIN1-pt2 (EDGE) + pG-KJE8]

A few colonies of transformant transformer E. coli ER2566 [pTWIN1-pt2 (EDGE)] and E. coli ER2566 [pTWIN1-pt2 (EDGE) + pG-KJE8] were isolated and then characterized by a reconstructive analysis of two SapI-BamHI enzymes and then the results recrystallization with 0.17% (w/v) agarose gel electrophoresis (Figure 10).

Expression test pt2 (EDGE) in E. coli ER2566

The expression began with the preparation of bacterial culture of E. coli ER2566 [pTWIN1-pt2 (EDGE) pH] from the stock of glycerol grown in liquid LB medium. In addition, E. coli ER2566 that does not carry any plasmid was grown and made the positive control of expression. The expression results with several fractions were characterized using SDS-PAGE electrophoresis. The result of characterization can be seen in Figure 8.

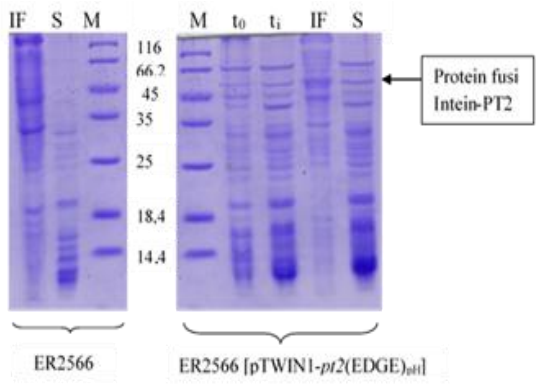

Figure 8: Result of characterization of 12% SDS-PAGE gel with IPTG induction of 0.1 mM expression of E. coli ER2566 [pTWIN1-pt2 (EDGE)] and cell E. coli ER2566 empty. (t0) fraction before induction of IPTG; (ti) fraction after induction of IPTG; (IF) undissolved fraction; (S) dissolved fraction.

The results as shown in Fig. 8 show pretrombin-2 combined with intein (Intein-PT2) yields 63 kDa molecular weight expressed at a soluble and soluble fraction. The possible cause was that the high-level expression of the protein is not matched by adequate folding by endogenous chaperones, resulting in much of the insoluble fraction (forming of inclusion bodies). Therefore, chaperon co-expression was performed to help maximize the folding of proteins especially Intein-PT2.

Co-expression pG-KJE8 on E. coli ER2566 [pTWIN1-pt2 (EDGE)]

For co-expression, bacterial culture of E. coli ER2566 [pTWIN1-pt2 (EDGE) + pG-KJE8] was prepared from the stock of glycerol grown in liquid LB medium. When the culture process was retained the GroEL/ES chaperon inducer was mixed with tetracycline in the hope that chaperones were expressed in advance so that they would be readily instrumental in helping to fold the protein when Intein-PT2pH was produced. The co-expression results using GroEL/ES chaperones were characterized using SDS-PAGE electrophoresis (Figure 9). pt2 gene (EDGE) could be expressed on E. coli ER2566 in dissolved and non-soluble fractions shown with SDS-PAGE analysis results of 63 kDa band. The formation of inclusion bodies in the non-soluble fractions could be reduced by the co-expression of the GroEL/ES chaperon using a pG-KJE8 plasmid on Intein-PT2 expression in E. coli ER2566, indicated by the increased Intein-PT2 dissoluble fraction compared without co-expression. This was because the GroEL/ES chaperon added to the E. coli ER2566 expression host as an exogenous chaperon could help the folding of Intein-PT2 eventually generated more dissolved fractions than without co-expression, since bigger number of GroEL / ES chaperones could offset the production of Intein-PT2pH in cells. The presence of a folding chamber provided by GroEL allowed Intein-PT2pH to run more efficient folding, thereby producing a native protein structure in the dissolved fraction.

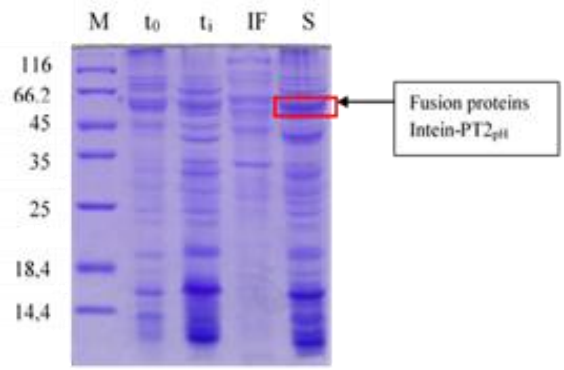

Figure 9: Electrophoregram of Grown / ES chaperon co-expression in E. coli ER2566. (t0) fraction before induction of IPTG; (ti) fraction after induction of IPTG; (IF) undissolved fraction; (S) dissolved fraction.

CONCLUSION

Exogenous chaperone co-expression of GroEL / ES can increase the expression of thrombin auto-activated gene pt2 (EDGE) in E. coli ER2566 which is resulting in more dissolved auto-acitivated thrombin fraction.

Acknowledgment: The researchers would like to thank the Ministry of Research, Technology and Higher Education Republic of Indonesia for supporting this research through National Competitive Grant - National Strategic of Superior Research (Pusnas) 2017.

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Source of Support: Nil, **Conflict of Interest:** None.