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Modification of recombinant human epidermal growth factor (rh-EGF) expression vector by site-directed mutagenesis for therapeutic protein production

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ABSTRACT Recombinant human epidermal growth factor (rh-EGF) has high value in therapies for h-EGF deficiency-related diseases. The expression of the h-EGF gene was designed by using the pET21b(+) vector and *Escherichia coli* BL21(DE3) as the expression host. In a previous study, the sequence of a 6xHis tag without any restriction sites was fused to the h-EGF gene, yet it was not possible to obtain a purified and single rh-EGF by this approach. In this study, we modified the rh-EGF expression vector using site-directed mutagenesis (SDM) to remove the sequence of the 6xHis tag. The vector modification was carried out by inserting a stop codon and the *Eco*RI restriction site, along with deleting the 6xHis tag sequence. The results of PCR showed non-specific bands, while 2-step cycles PCR produced one non-specific band, and 3-step cycles PCR produced two non-specific bands. After purification of the PCR products, the SDM-recombinant plasmids treated for template plasmid-free product were transformed into *E. coli* DH5α. Even though the transformation efficiency was low, the planned gene mutations including the deletion of the 6xHis tag and insertion of the stop codon and *Eco*RI restriction site in plasmid pET21b(+) were successfully carried out. When using this modified vector in expression studies, rh-EGF of a similar size to that of the rh-EGF standard and approximately 1 kDa smaller than the rh-EGF-6xHis of the previous study was obtained.

KEYWORDS *Escherichia coli* BL21(DE3); pET21b(+); recombinant human epidermal growth factor (rh-EGF); site-directed mutagenesis (SDM)

1. Introduction

Human epidermal growth factor (h-EGF) is a small protein to stimulate cell proliferation, differentiation, viability in various cell types such as epithelial cells, fibroblast cells, endothelial cells including tumor cells with complex regulatory mechanisms (Citri and Yarden 2006; Su et al. 2006; Xian 2007; Higashiyama et al. 2008; Zeng and Harris 2014). It is functional protein from derivate large precursor gene, this large precursor gene includes encoding membrane receptor, EGF-like repeats, and EGF or growth factor family (Bell et al. 1986; Zeng and Harris 2014). This protein can be detected in saliva, milk, intestinal fluid, and others (Fisher and Lakshmanan 1990; Xian 2007; Dvorak 2010). h-EGF protein is composed of 53 amino acids with three disulfide bonds with conserved be cysteines from C1 to C6 specified in glycine and arginine. This mature protein has molecule mass about 6.2 kDa (Savage et al. 1972, 1973; Zeng and Harris 2014). However, the rh-EGF used in this study has been added a methionine residue at the N-terminal for intracellular expression in *E. coli*. In our sequence, disulfide bonds were formed by binding of C7-C21; C14-C31, and C33-C43. In an in vivo study with pig and mice, it is showed that EGF can prevent and treat necrotizing enterocolitis and also be important in gastrointestinal repair (Nair et al. 2008). EGF suppresses fibrosis in mice liver induced by thioacetamide (TAA) associated with inhibiting hepatic stellate cells (Huang et al. 2012). EGF is important in remodeling the cytoskeleton for endocytosis and cell proliferation (Kharchenko et al. 2007). Growth factor family such as EGF contributed to wound healing in various tissues and their possibility play roles in therapy based on stem cells (Fu et al. 2002; Pikuła et al. 2015).

h-EGF has been synthesized by several recombinant protein techniques due to its importance in treatments from h-EGF lack-related diseases. The recombinant h-EGF (rh-EGF) can be used for therapies in kidney destruction diseases such as glomerular disease (Flamant et al. 2012; Klein et al. 2016). Moreover, rh-EGF for patients with diabetic ulcers with level 1–4 showed significant repair of injury (Putri and Sriwidodo 2016) and rh-EGF spray also significantly useful for reduced mucositis oral induced by radiotherapy (Wu et al. 2009).

In our previous study, rh-EGF was successfully inserted into the expression vector pET21b(+) and transformed to *Escherichia coli* DH5α (Nurmalasari 2010). In the recombinant plasmid, the gene of h-EGF was fused with a gene of 6xHis tag, but without any cleavage sites between them. Therefore, it is not possible to obtain single and purified h-EGF. In this study, the recombinant plasmid was modified by using PCR based-SDM to insert stop codon and delete the 6xHis tag sequences. Thus, it is expected that purified h-EGF could be obtained by other purification methods than affinity chromatography.

Site-directed mutagenesis (SDM) or site-specific mutagenesis is a technique in genetic engineering to introduce defined mutations into target DNA. The mutation is needed to improve the expression of a recombinant gene and in protein/enzymes studies (Xu et al. 2003; Antikainen and Martin 2005; Walquist and El-Gewely 2001). In genetic and protein engineering, the SDM is beneficial to generate DNA sequence with mutated codons including insertion or deletion by using polymerase chain reaction (PCR) (Edelheit et al. 2009; Walquist and El-Gewely 2001). PCR is a common amplification technique to obtain extremely high copies of target DNA (Bhatia and Dahiya 2015). PCR-based SDM method requires a pair of forward and reverse primers to amplify full-length plasmid containing a sequence of interest, 5'phosphorylation of the blunt-ended amplification product, degradation of PCR template with *Dpn*I enzyme, ligation, and transformation (Rabhi et al. 2004; Walquist and El-Gewely 2001). The SDM technique has been applied in a number of studies, for instances to study interaction of type III secretion (T3S) components in model pathogenic bacterium of *Yersinia* (Francis et al. 2017), PCR-free CRISPR/Cas9 mutagenesis (She et al. 2018), and SDM in *Arabidopsis* with zinc finger nuclease (Osakabe et al. 2010).

According to the reported studies, PCR-based SDM offers a lot of advantages, such as almost 100% successful targeted mutation (Wan et al. 2012) and > 60% of subclones contain the desired mutation (Xu et al. 2003). Moreover, the PCR-based SDM is able to be designed with specific restriction sites which simplify the mutant screening and increase mutation reliability and fidelity (Edelheit et al. 2009; Zhang et al. 2009; Walquist and El-Gewely 2001).

2. Materials and methods

2.1. Preparation of SDM-recombinant plasmid

A template plasmid, namely pET21b(+)_*egf.syn_6xHis tag* was isolated from the recombinant subcloning host *E. coli* DH5α_ pET21b(+)_*egf.syn_6xHis tag* using the alkaline lysis method (Ausubel et al. 2002). The obtained recombinant plasmid was purified with Geneaid^{TM} Gel/PCR DNA Frgments Extraction kit and then used as a template in PCR for SDM with KOD-Plus-Mutagenesis kit. Primers used for SDM are reverse primer: 5'-TTA TCA ACG AAG TTC CCA CCA TTT CAG ATC ACG ATA CTG GCA-3' and forward primer: 5'-GAA TTC GAT CCG GCT GCT AAC AAA GCC CGA AAG-3' (purchased from Integrated DNA Technologies-IDT®, Singapore). The PCR mixture was prepared in accordance with the standard protocol for the KOD-Plus-Mutagenesis kit and KOD-Plus-Neo kit. Subsequently, the 2-step cycles PCR was performed as follows: pre-denaturation: 2 min at 94°C, denaturation: 10s at 98°C, annealing: 30s at 68°C, extension: 2 min 45 s at 68°C, hold at 4°C, and for annealing temperature screening use 3-step cycles PCR was performed as follows: pre-denaturation: 2 min at 94°C, denaturation: 10 s at 98°C, annealing: 30 s at [56.3–64.2]°C, extension: 2 min 45 s at 68°C, hold at 4°C. Afterward, the obtained PCR product was treated using *Dpn*I enzyme mixture to digest the template plasmid which contaminated the PCR product at 37°C 1 h. The template plasmid free-PCR product was purified with a Geneaid^{TM} Gel/PCR DNA Fragments Extraction kit. The target template plasmid free-PCR product was then ligated with a ligation enzyme in a KOD-Plus-Mutagenesis kit. The product of the ligation was subsequently named as the SDM-recombinant plasmid.

2.2. Transformation of SDM-recombinant plasmid to E. coli DH5α and transformants stability test

Transformation of product the SDM-recombinant plasmid using calcium chloride ($CaCl_2$) heat-shock treatment (Ausubel et al. 2002). The competent cells were prepared by harvesting subculture of *E. coli* DH5α at OD 0.25–0.3 and centrifugation at 6000 rpm, 10 min at 4°C. The pellets were resuspended with $CaCl_2$ then incubated in ice water for 30 min. The $CaCl_2$ treated pellet was centrifuged again at 6000 rpm, 10 min at 4°C and resuspended with $CaCl_2$ + 15% glycerol. The competent cells can be stored in the freezer −80°C. Prior to transformation, 7.5 µL of SDM-recombinant plasmid was added to 50 µL competent cells and incubated on ice for 20 min. Heat-shock treatment was conducted by placing the transformation mixture in 42°C water for 30 s then keeping it in ice water for 5 min. After heat-shock treatment, 450 µL LB medium was added to the transformation mixture and incubated at 37°C, 200 rpm, 1 h. A number 100 µL and 150 µL of the transformation mixture was spread on LB agar plate with ampicillin (AMP) 10 µg/mL and incubated at 37°C overnight. The transformants stability test was performed by regeneration the single colonies of the transformants on LB agar + AMP 10 µg/mL plate.

2.3. Transformants verification by PCR, digestion by enzyme restriction, and DNA sequencing

Verification of the transformants was generated with three methods: PCR, *Eco*RI restriction enzyme digestion and DNA sequencing. First, single colonies obtained from the transformations (or named as transformants) were verified by PCR, using primers of targeted gene (*egf.syn*) (IDT®, Singapore) with set as follows: pre-denaturation: 5 min at 95°C, denaturation: 30 s at 95°C, annealing: 30 s at 55°C, extension: 30 s at 72°C, final extension 60 s at 72°C and hold at 4°C. Second, the SDM-recombinant plasmid was

also checked with *Eco*RI restriction enzyme which is specific for the SDM-recombinant plasmid. The restriction mixture reaction contained buffer orange 1 µL (Thermo Scientific, Lithuania), sample (SDM-recombinant plasmid) 1 µL, *Eco*RI enzyme (Thermo Scientific, Lithuania) 5 units 1 µL, and dH_2O 3 µL. The mixture was incubated at 37°C overnight and checked on agarose gel electrophoresis 0.7 percent. Final verification was DNA sequencing of the SDM-recombinant plasmid with primer sequencing T7 promoter and T7 terminator.

2.4. *Expression of SDM modified rh-EFG in* E. coli *BL21(DE3)*

Purified SDM-recombinant plasmid from *E. coli* DH5α was transformed to *E. coli* BL21(DE3) expression host. The transformants were then pre-cultured in 1 mL LB medium with AMP 10 µg/mL, incubated at 37°C; 180 rpm overnight. On the following day, 0.5 mL of preculture was added to 5 mL LB medium and incubated at 37°C; 180 rpm; approximately for 2 h or until reaching OD 0.6–0.8. Subsequently, the culture was added with isopropyl β-D-1-thiogalactopyranoside or IPTG (Thermo Scientific, Lithuania) (final concentration 0.1 mM) and incubated for 6 h at 37°C; 180 rpm. After IPTG induction, the cells (pellet) were collected by centrifugation at 8,000 rpm; 5 min; 4°C. The pellet was then solubilized with solubilization buffer (8 M urea; 50 mM glycine; 80 mM β-mercaptoethanol) and incubated at a cold temperature (8–10°C) for 72 h. The solubilized protein was obtained by centrifugation at 12,000 rpm; 15 min, and then analyzed with tricine SDS PAGE (acrylamide 15%; 70 volts), also verified with western blotting against monoclonal antibody EGF (Santa Cruz, USA). The SDM modified rh-EGF was compared with rh-EGF standard (Sigma) and rh-EGF with 6xHis tag.

3. Results and discussion

3.1. *Preparation of SDM-recombinant plasmid*

The result of plasmid purification from gel extraction contained two bands (Figure 1). Based on the theory, plasmids are separated on gel electrophoresis according to its topologies (De Mattos et al. 2004; Cebrián et al. 2014), which are supercoiled (SC), open-circle (OC) or linear (L)

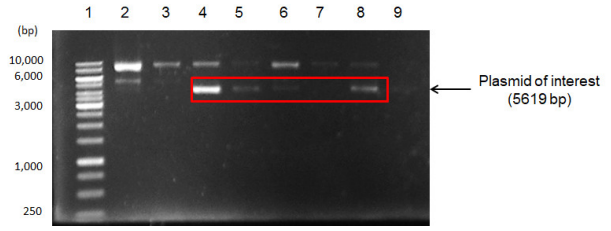

FIGURE 1 Electroforegram of plasmid purification. 1 = Marker (1 kb), 2 = $3B_1$ 1st elute, 3 = $3B_1$ 2nd elute, 4 = $3B_2$ 1st elute, 5 = $3B_2$ 2nd elute, 6 = $3D_1$ 1st elute, 7 = $3D_2$ 2nd elute, 8 = $3D_2$ 1st elute, 9 = $3D_2$ 2st elute. Sample $3B_2$ 1st elute is used as a template PCR for SDM. Code of samples "B" and "D" was a single colony from the previous study.

(Balagurumoorthy et al. 2008; Carbone et al. 2012). The plasmid yield was varied significantly depending on the elution. The first elution produced a thicker band than second elution did. The purified plasmid in line 4 was used as a PCR template because it showed the thickest band or in other word had the highest concentration among the other purified plasmids.

The result of PCR products can be seen in Figure 2. Electroforegram of amplicon in 0.7% electrophoresis gel showed that two non-specific bands were found after 3-step cycles PCR (Figure 2a) and one non-specific band was from 2-step cycles PCR (Figure 2b).

Wan et al. (2012) and Edelheit et al. (2009) mentioned that several factors in primer design might cause non-specific bands such as complicated mutation, high content of G-C, complex secondary structure, tandem and inverted sequence, and insertion of long primers. In our study, the

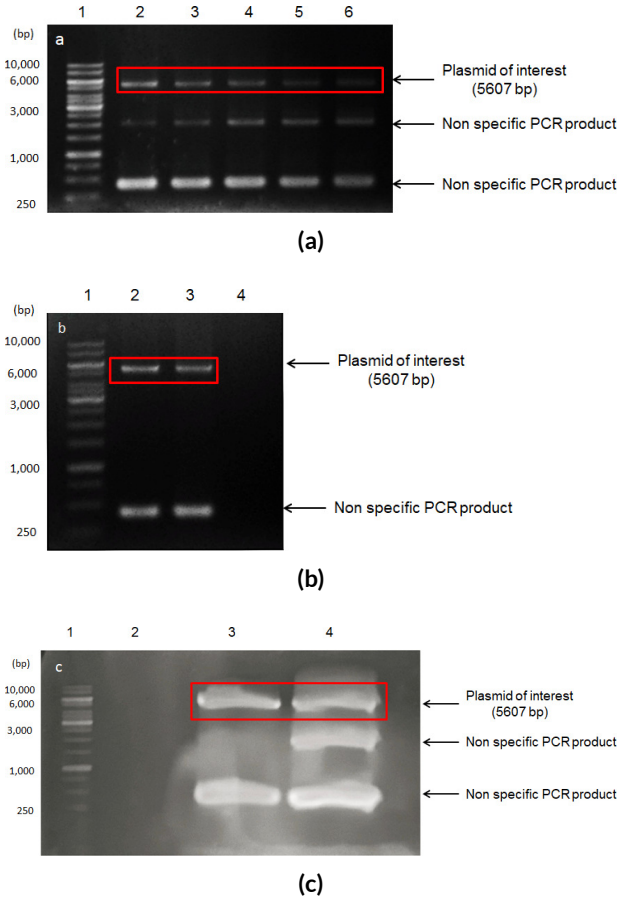

FIGURE 2 Electroforegram of amplicon for SDM with various annealing temperature, a) Electroforegram of 3-step cycles PCR. 1 = Marker (1 kb), 2 = 64.2°C, 3 = 62.2°C, 4 = 60.2°C, 5 = 58.1°C, 6 = 56.3°C. PCR product for SDM showed 2 nonspecific products, b) Electroforegram of 2-step cycles PCR (Tm = 68°C). 1 = Marker (1 kb), 2 = once dilution template, 3 = without dilution template, 4 = control (dH_2O). PCR product for SDM showed 1 band nonspecific product. c) Purification of template free-PCR product after treated with *Dpn*I enzyme, 1 = Marker. 2 = template plasmid free-PCR product from KOD-Plus-Mutagenesis kit, 3 = template plasmid free-PCR product from KOD-Plus-Neo kit 2-step cycles PCR, 4 = template plasmid free-PCR product from KOD-Plus-Neo kit 3-step cycles PCR.

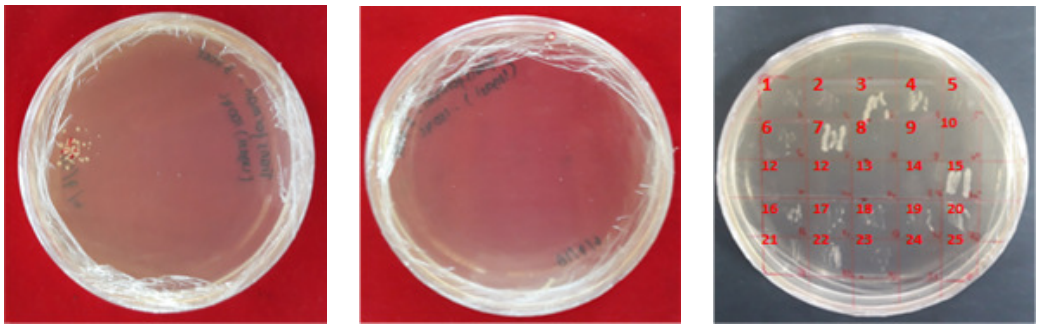

FIGURE 3 a) Plate100 µL of transformation culture, b) Plate150 µL of transformation culture, c) Replica plating of transformant single colonies from plate a (8–25) and transformation culture plate b (1–7).

plasmid template has a size of 5,619 bp, thus the possibility that several bases are compatible with the primers is high. The primers could bound to a region that was not in the target primer-template binding. Non-specific bands can be seen in Figure 2a and 2b. Other reasons for non-specific bands are the PCR condition. We used either 2-step cycles PCR or 3-step cycles PCR in our experiment. A 2-step PCR cycling includes denaturation and annealing/extension, while in 3-step PCR extension step after annealing is added (Lorenz 2012). Too low denaturing temperature (Roux 1995) and not optimal annealing temperature (Vestheim and Jarman 2008; Kalendar et al. 2017) might also cause non-specific bands. Primers melting temperature (Tm primer) is the most important factor in the success of PCR. In addition, optimized cycling condition and reagent concentration also contribute to producing amplicons with the expected size. Changing a parameter can influence other parameters, hence the production of the amplicon is affected as well (Lorenz 2012). The result showed the best annealing temperature was at 68°C in 2-step cycles PCR with an indicator such as forming one non-specific band produced, while in annealing temperature below 68°C in 3-step cycles PCR (64.2–54.3°C) program were formed two non-specific PCR products. The optimum PCR reactions ensure the amplification of the DNA target and increase the quality of amplicons target (Joko et al. 2011). Therefore, differences in temperature used in annealing with 3-step cycles and 2-step cycles PCR program produce a number of different bands.

All PCR products either from 3-step cycles PCR or 2-step cycles PCR with KOD-Plus-Neo and KOD-Plus-Mutagenesis were treated with *Dpn*I enzyme and purified on gel electrophoresis (Figure 2c). *Dpn*I enzyme is specific to digest PCR templates, particularly at the methylated adenine base of GATC sequence in parental strand (Johnston et al. 2013), but not in the PCR product (Walquist and El-Gewely 2001). DNA methylation is often described as a parental epigenetic sign and can be inherited through cell division, this mechanism is caused by DNA binding proteins that bind DNA (Jones 2012; Moore et al. 2013) so this site is found repeatedly in parental strand (Sánchez-Romero et al. 2015). The repetition of this site makes the template degradation more effective so that pure PCR products are easy to be obtained. The template plasmid free-PCR product was then purified and ligated with ligation enzyme. Ligation enzyme catalyzes the formation phosphodiester bonds between 3' hydroxyls (OH) and 5' phosphates (PO_4) in nucleic acid residues (Lohman et al. 2013) from this formation the circular plasmid can be formed.

3.2. *Transformation of SDM-recombinant plasmid to* E. coli *DH5α and transformants verification by PCR*

Successful transformation is characterized by the growth of colonies on selective medium (LB + Amp 10 µg/mL). The successful transformation was only obtained from the SDM-recombinant plasmid which was ligated from 3-step cycles PCR product (Figure 3). On the plate with 100 µL transformation culture (Figure 3a), 16 single colonies were found while on the other plates, seven single colonies grew (Figure 3b).

The successive rate of this transformation method is determined by Ca^{2+} and heat shock temperatures (Rahimzadeh et al. 2016). A pulse of 30 s duration at 42°C heat shock temperature followed by a 10 min ice incubation exhibited maximum efficiency, with a transformation efficiency 3×10^8 cfu/µg in DH5α (Singh et al. 2010). Transformation efficiency in this study is low, so it is necessary to repeat the transformation procedure. Few or no transformants present might be caused by cells which are

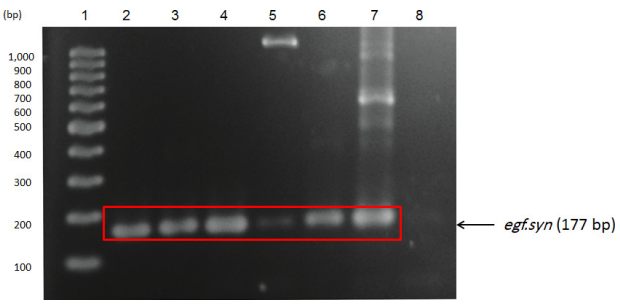

FIGURE 4 Verification SDM-recombinant plasmid with target gene (*egf.syn*). 1 = Marker (100 bp), 2 = colony 3, 3 = colony 7, 4 = colony 15, 5 = colony 22, 6 = positive control (1st elute from miniprep), 7 = positive control (ligation plasmid), 8 = negative control (dH_2O).

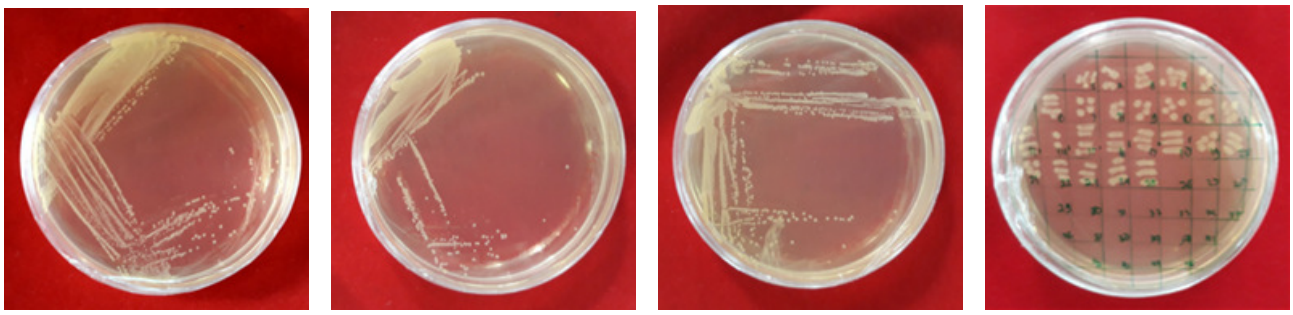

FIGURE 5 a) streak colony 3 , b) streak colony 7, c) streak colony 15, d) replica plating for streak colony 3 (1–10), streak colony 7 (11–20) and streak colony 15 (21–25).

not competent and incorrect heat-shock protocol. Most cases of transformation failure due to heat shock temperature is inaccurate (Chang et al. 2017). The temperature is important because the heat-shock step is a facilitator for competent cells to intake DNA or plasmid (Das and Dash 2015; Chang et al. 2017). This problem can be fixed by preparing a new batch of competent cells by improvement in heat-shock and incubation temperatures and duration and use a water bath for accuracy temperature in heat-shock step.

Subsequently, each colony was regenerated in a replica plating. Replica plating is the technique to inoculate each colony/clone into multiple plates that used to select specific hybridization, reduce the risk of environmental microbial contamination and cross-contamination between colonies (Carson et al. 2019). The best growth was found in replica colonies number 3 and 7 from a plate with 100 µL transformation culture and replica colonies number 15 and 22 from a plate with 150 µL transformation culture (Figure 3a). The single colonies number 3, 7 and 15, 22 from replica plating were used for PCR colonies for screening *egf.syn* sequence. Visualization of PCR colonies using target gene (*egf.syn*) primer on gel electrophoresis 0.7%. The result PCR colony showed that replica colonies number 3, 7 and 15 positively had *egf.syn* sequence, while the colony 22 showed a thin band of *egf.syn* and a contaminant band. Positive control showed band of *egf.syn*, but they had non-specific PCR product and also no band was found from negative control (Figure 4). From this result, colonies number 3, 7 and 15 were streak again to regenerated on a new plate.

Figure 5 shows the results of colony streak regeneration from colonies number 3, 7 and 15, whereas colony number 22 was not regenerated because suspected to be a contaminant product. From the colony number 3, 7 and 15, a number of re-single colonies were obtained, i.e 55 colonies (Figure 5a), 26 colonies (Figure 5b) and 15 colonies (Figure 5c), respectively. Subsequently, from the regenerated single colony, 10 of 55; 10 of 26 and 5 of 15 colonies were randomly reselected to be replicated on a new plate (Figure 5d). Three sub-single colonies from each colony on the plate in Figure 5d have isolated the plasmid and then used in SDM verification with PCR colonies to screening *egf.syn* sequence.

Visualization of the PCR sub-colonies using primer target gene (*egf.syn*) for second replica cultures from streak colonies 3, 7 and 15 are shown on Figure 6. All samples were found to have a *egf.syn* sequence. Based on these results, sub-colonies 3.2, 7.2 and 15.2 were selected to be checked with the *Eco*RI restriction enzyme.

3.3. *Transformants verification by digestion with enzyme restriction, and DNA sequencing.*

3.3.1. *Digestion by* EcoRI *restriction enzyme*

The *Eco*RI restriction enzyme was used to check specific sequence (G/AATTC) in SDM-recombinant plasmid

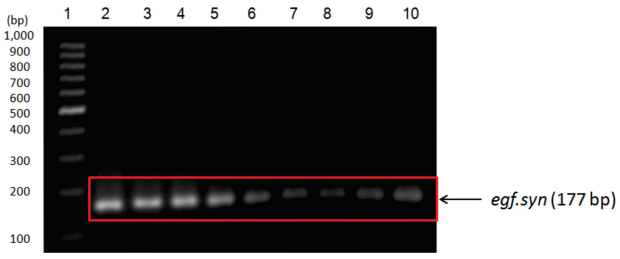

FIGURE 6 Verification SDM-recombinant plasmid target gene (*egf.syn*), 1 = Marker (100 bp), 2 = colony 3.1, 3 = colony 3.2, 4 = colony 3.3, 5 = colony 7.1, 6 = colony 7.2, 7 = colony 7.3, 8 = colony 15.1, 9 = colony 15.2, 10 = colony 15.3.

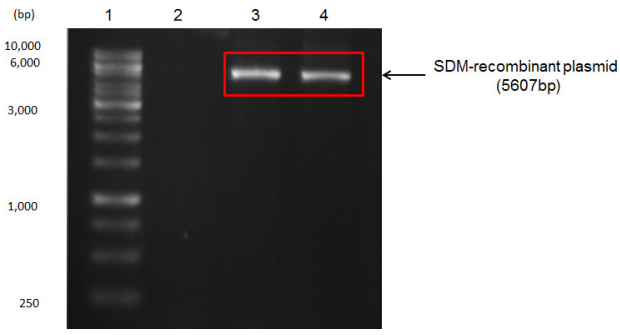

FIGURE 7 Verification SDM-recombinant plasmid with *Eco*RI restriction, 1 = subclone 3.2, 2 = subclone 7.2, 3 = subclone 15.2, 4 = marker 1 kb.

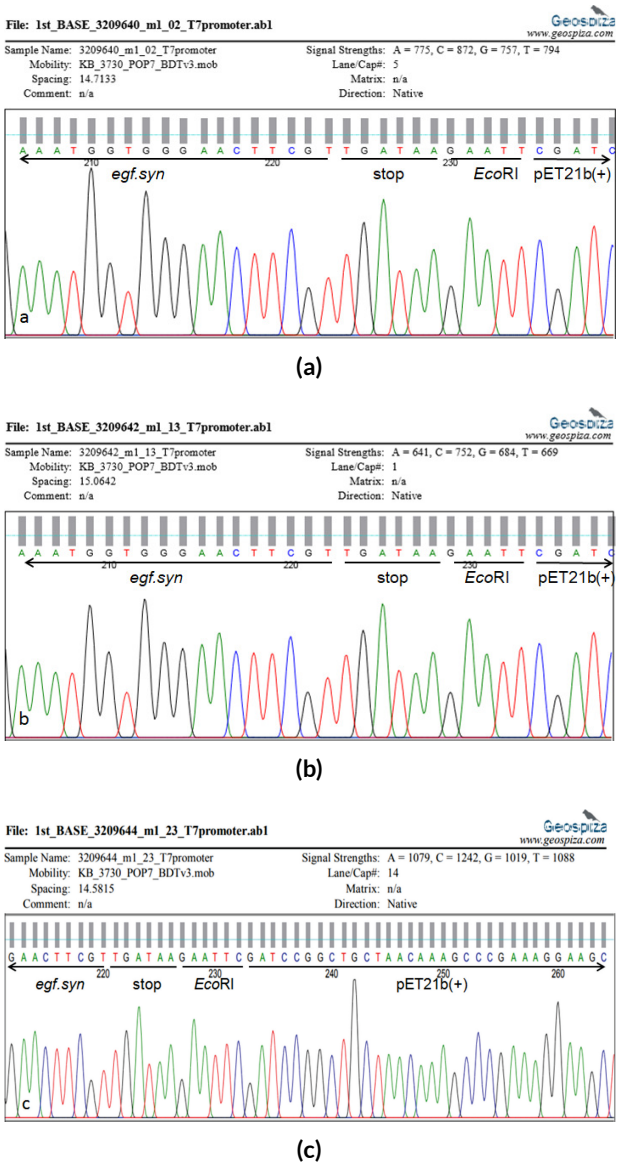

FIGURE 8 Final verification SDM-recombinant plasmid with DNA sequencing. a) DNA sequencing result of sub-colonies 3.2, b) DNA sequencing result of sub-colonies 7.2, c) DNA sequencing result of sub-colonies 15.2.

(Shivanand and Noopur 2010). The restriction *Eco*RI sequence has been encoded on the SDM forward primer. Design restriction site in mutagenesis primers to ensure efficient mutant screening (Zhang et al. 2009). Visualization of successful *Eco*RI restriction can be seen on electrophoresis gel 0.7% (Figure 7). All samples showed similar bands with the same band location with an estimated size of 5,607 bp. Line number 3 did not show a band because no template added in the mixture for *Eco*RI restriction treatment.

3.3.2. *DNA sequencing*

Three transformants (sub-colonies number 3.2, 7.2 and 15.2) were confirmed by DNA sequencing using 1st BASE (Malaysia). The universal primers used for DNA sequencing were the T7 promoter primer and T7 terminator primer. All of the samples had the expected mutations, conserved reading frame and no mutations were observed in the target gene (*egf.syn*). In addition, they were successfully added with two stop codons and *Eco*RI restriction sites also succeeded in deleting 6xHis tag sequences (Figures 8a, 8b and 8c). Sequence alignment with software *clustalX* and *Bioedit* of sequence from DNA sequencing results shows more clearly the deletion 6xHis tag sequence and insertion *Eco*RI restriction sequence and stop codons (Figure 9).

FIGURE 9 Alignment DNA sequencing of SDM-recombinant plasmid with control sequence (recombinant plasmid before SDM).

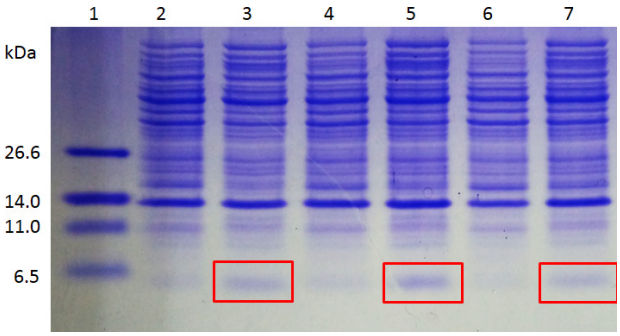

FIGURE 10 SDS PAGE profile of solubilized cells pellet from rh-EGF expression in *E. coli* BL21(DE3). Lane 2; 4; 6: uninduced condition; lane 3; 5; 7: IPTG induced (0.1 mM). Lane 2; 3: BL21(DE3)_pET21b(+)_rh-EGF_no.3.2; Lane 4; 5: BL21(DE3)_pET21b(+)_rh-EGF_no.7.2; 6;7: BL21(DE3)_pET21b(+)_rh-EGF_no.15.2. The rh-EGF is clearly expressed with IPTG induction, but slighly appear in uninduced condition. Lane 1: low protein marker.

3.4. *Expression of SDM modified rh-EFG in* E. coli *BL21(DE3)*

The SDM-modified rh-EGF vector has been used to express the rh-EGF without 6xHis tag. The rh-EGF was expressed by IPTG induction at 0.1 mM and found at less than 6.5 kDa in size (Figure 10). Some samples from uninduced treatment also shows rh-EGF expression, but not significant. It was expressed in uninduced condition due to leaky of the expression system having a strong promoter such as T7 promoter Tegel et al. (2011).

In Figure 11, it was proven that after removing the 6xHis tag, the rh-EGF was obtained at similar size with the standard, while the rh-EGF_6xHis tag has bigger size, approximately 1 kDa, the theoretical size of 6xHis tag (Zhu and Qian 2012). In regard to the removing 6xHis tag, affinity chromatography, particularly Ni-NTA chromatography, is not suitable to purify the rh-EGF from other *E. coli* endogenous proteins (Hochuli et al. 1988). Other purification methods, like size exclusion and/or ion exchange chromatography, might be applied (Suortti 1997).

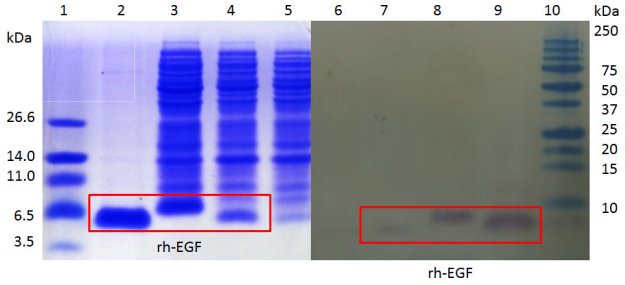

FIGURE 11 SDS PAGE (1–5) and western blot (6–10) profile of rh-EGF after SDM modification compared with that of with 6xHis tag and rh-EGF standard. Lane 2 & 9: rh-EGF standard; Lane 3 & 8: rh-EGF_6xHis tag; Lane 4 & 7: IPTG induced rh-EGF; Lane 5 & 6: uninduced sample. The rh-EGF standard has a similar size with rh-EGF after SDM modification which is less than 6.5 kDa. The rh-EGF_6xHis tag has app. 1 kDa size bigger than the rh-EGF.

4. Conclusions

The rh-EGF vector has been successfully modified by SDM. *Eco*RI restriction site sequence, stop codons had been inserted, and 6xHis tag had been successfully deleted from plasmid expression vector pET21b(+) recombinant. Therefore, the modified vector expressed rh-EGF without 6xHis tag and had a similar size with rh-EGF standard. In this study, it was proven that the PCR based SDM was an effective, precise, simple, and low-cost technique to improve or modify a recombinant plasmid.

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Authors' contributions

AMF and RDP designed the study; AR and SZ carried out the laboratory work; AR and RDP analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that no competing interests of this work.

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