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Construction, Expression and Purification of DNA Pol I ITB_1 and its Deletion Mutants

Hira Helwati, Rukman Hertadi, Fida Madayanti and Akhmaloka*

Biochemistry Research Group, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jln Ganesha 10, Bandung. Indonesia.

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A gene encoding DNA polymerase I, namely DNA Pol I ITB_1, was cloned from *Geobacillus thermoleovorans*. Heterologous expression of DNA Pol I ITB_1 in *Escherichia coli* was carried out under expression vector pET30a(+). The vector contains his-tagged in the upstream of the gene, thus the expressed protein carried poly his on the N-terminal. Recombinant plasmid containing DNA Pol I ITB_1 gene was constructed by inserting *Nco*I – *Bam*HI gene fragment of $pITB_8$ containing the whole coding region of DNA PolI ITB_1 into pET30a(+) resulting $pITB_{20}$ plasmid. The deletion mutants of the genes were constructed through *in frame* deletion of the gene by using *Eco*RI, and *Xho*I restriction enzymes resulting $pITB_{21}$ and $pITB_{22}$ plasmids respectively. The expression of the wild type and the deletion mutants were carried out in *Escherichia coli* BL21-DE3. High expression levels of the genes were shown on SDS-PAGE. The proteins were purified by immobilized metal-ion affinity chromatography (IMAC) using Ni-NTA resin and single band protein products were shown on the gel following SDS PAGE analysis.

Key words: DNA Polymerase I, Deletion mutant, Heterologous expression, His-tag.

DNA polymerases are present in all organisms and involve in genomic replication and repair. DNA Pol I is the most abundant DNA polymerase found in *E. coli* and also the first polymerase characterized[1]. DNA Pol I consists three discrete structural domains, namely 5'→3' exonuclease, 3'→5' exonuclease and 5'→3' polimerized domains. The 5'→, 3' exonuclease degrades duplex DNA from its 5' end. It enables DNA polymerase I to remove RNA primers of Okazaki fragment and fill with DNA sequences. The 5'→3' exonuclease is also capable of excising DNA lesion, such as thimin dimers, during DNA repair. The 3'→5 exonuclease degrades single or double stranded DNA from their 3'end. The ability to remove mismatched nucleotides provides proofreading activity of DNA replication[1].

More than 100 DNA polymerase genes have been cloned and studied from various organisms, including thermophiles and archaea[2]. As expected, these enzymes have been shown an elevation temperature activity and poses thermal stability compared to the mesophilic enzymes. Thermostable DNA Pol I isolated from

* To whom all correspondence should be addressed.
Tel.: +62-22-2515032, Fax: +62-22-2502360
E-mail: loka@chem.itb.ac.id

thermophilic microorganisms play an important role in molecular biology through their used in Polymerase Chain Reaction (PCR), particularly. PCR has become powerful method for the identification and amplification of genes, direct sequencing and clinical diagnosis. At the early development of PCR, the experiment used thermolabile Klenow Fragment, which required addition of the enzyme for every cycle. The introduction of a thermostable DNA polymerase allowed the automation of the process[3,4].

Thermostable DNA Pol has been studied from a wide range of bacteria. DNA polymerase from *T. aquaticus* (Taq polymerase) was the fist cloned and expressed in *E. coli*[5]. A number of archaebacterial DNA polymerases have also been isolated and purified[3]. Thereafter, many of DNA Pol I from *Thermu*s strains have been studied. *Tfl*, *Tth*, *Tfi* and *Top* Polymerase have been applied to PCR[3,6,7,8]. However, their base-insertion fidelity is low since some DNA Pols have no 3'→5' exonuclese activity. The high fidelity of DNA Pol, which has 3'→ 5' exonuclease activity dependent proofreading activity, should be required for error correction during the polymerization. Several thermostable DNA polymerases with proofreading activity (*Pfu*, *Vent*, *Deep Vent*, and *Pwo*) have also been studied and introduce from high-fidelity PCR amplification[3]. A few moderately thermostable DNA polimerases have been isolated and purified from thermophilic *Bacillus* species[9,10]. Bst DNA polymerase was isolated from *B. stearothermophilus*[11,12,13]. Bca DNA polymerase was isolated and cloned from *B. caldotenax*[13,14]. Bst DNA polymerase has been used for DNA sequencing.

Most of the native thermostable enzymes are synthesized at very low levels by the thermophilic bacteria and therefore cumbersome to purify[15]. All of thermostable DNA Polymerases were produced in a biologically active form in *E. coli* expression vector [16,17], however the commercial available DNA Pols have some advantages and disadvantages properties, therefore a new and improved thermostable DNA Pol are still needed.

A number of thermophilic microorganisms have been isolated from few hot spring around Indonesia[18,19,20]. DNA pol I gene from one of the isolated has been cloned and sequenced[2], namely DNA Pol I ITB_1. Thermal stability of the protein have been studied through Molecular Dynamic Simulation[21,22]. However, structure function of the protein need to be elucidated. In order to further probe to structure function of the protein, in this report we would like to present over-expression and purification of DNA Pol I ITB_1 and its deletion mutants in *E. coli*.

MATERIAL AND METHODS

Bacterial Strains and Plasmids

The strains used for cloning was *E. coli* DH5a (supE44 lacU169 (Q90 LacZM1) hsdR17 recA1 endA1 gyrA96 thi-1 reiA1). *E. coli* BL21(DE3)(F^- ompT hsdSb (rB^-mB^-) gal dcm(DE3) was used for heterologous expression of the gene. The $pITB_8$ that contains DNA Pol I ITB_1 gene was used for construction of DNA Pol I ITB_1 deletion mutants, while pET30a(+) (Novagen,UK) was used as expression system.

Growth Condition and DNA Manipulation

E. coli strains were grown aerobically at 37°C in Luria Bertani (LB) broth or plated on LB containing appropriate antibiotics when required. *E. coli* BL21 (DE3) was grown at 37°C on LB medium supplemented with 34 µg/ml kanamycin. All routine DNA isolation and manipulation were performed as described by Sambrook *et al*[23]. Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, USA). Small-scale preparation of plasmid DNA from *E. coli* cells was performed using plasmid miniprep kit (Qiagen, Hilden, Germany).

Construction of Recombinant Vector

Recombinant vector ($pITB_{20}$), carrying DNA PolI wild type gene was constructed by inserting *Nco*I-*Bam*HI fragment of DNA PolI gene fragment from $pITB_8$ plasmid into pET30a(+) (Fig. 1A). The ligation process was carried out by standard method[23]. Meanwhile recombinant vectors ($pITB_{21}$ and $pITB_{22}$), carrying *Eco*RI and *Xho*I deletion fragments respectively, were constructed from $pITB_8$ plasmid. *Eco*RI or *Xho*I fragment of $pITB_8$ was removed by restriction of *Eco*RI or *Xho*I restriction enzyme. The rest of the vectors were religated resulting $pITB_8$-A and $pITB_8$-B plasmids respectively (Fig 1B, C). Furthermore, DNA PolI gene fragment from

pITB$_8$-A and pITB$_8$-B were removed by *Nco*I and *Bam*HI restriction digest. The fragments were inserted into linearized pET30a(+) vector with the same (*Nco*I and *Bam*HI) enzymes (Fig. 1B,C).

Expression and Purification of Recombinant Proteins

E. coli strain BL21(DE3) transformed by pITB$_{20}$, pITB$_{21}$ and pITB$_{22}$ were grown at 37°C in 1000 ml LB containing 34 mg/ml kanamycin to an optical density (OD_{660}) = 0.5 reached. IPTG was then added to the final concentration of 1 mM. The cells were harvested after 4 h by centrifugation (9 g of cell mass) and the pellet was resuspended in 20 ml of B buffer (50 mM posphat, pH 7.9, 500 mM NaCl, 1 mM PMSF and 1 mg/ml lysozime). The cells were disrupted by sonication, and the insoluble debris was removed by centrifugation. The cleared lysate was heated at 70°C in water bath for 10 minute, cooled on ice for 20 min, and then centrifuged at 16,000*g,* 4°C for 20 min. The supernatant was then applied directly onto Ni^{2+}-NTA agarose column pre-equilibrated with 4 vol of B buffer. After loading, the column was washed several times with 10 ml of the same buffer until the UV absorption returned to the baseline. His6-tagged DNA Pol I ITB-1 was eluted by B buffer containing 200 mM imidazole. Protein fractions resulting from Ni-NTA chromatography were analyzed following SDS-PAGE and comassie brilliant bule staining. The eluted fractions were pooled and dialyzed against a buffer containing 50 mM fosfate, pH 7.9 and 50 mM NaCl[23].

RESULTS

Construction of the Recombinant Vectors

pET system (Novagen, Madison, USA) is one of the useful and powerful recombinant protein expression systems in *E. coli*. The pET vector has a strong T7 promoter and could be harbored in combination with pLysS to provide additional stringency[15,24,25]. The pITB$_{20}$, pITB$_{21}$ and pITB$_{22}$ have been successfully constructed by ligation of the *Nco*I-*Bam*HI fragments of the genes as illustrated in Fig.1A, B, and C. The parent plasmid, pITB$_8$ is a recombinant pGEMT plasmid carrying *Kpn*I-*Bam*HI PCR fragment of DNA polymerase I ITB$_1$[26].

All of the recombinant plasmids have been confirmed by restriction digest analysis using appropriate restriction enzymes. The size of pITB$_{20}$ is around 8 kb, meanwhile pITB$_{21}$ and pITB$_{22}$ are 7.5 and 7.0 kb respectively. The size of pITB$_{20}$ was confirmed by double digest of the plasmid using *Bam*HI and *Nco*I. Following agarose gel electrophoresis, the result showed that two bands represents fragment DNA with the sizes of 5.4 kb from pET30a(+) vector and 2.6 kb from DNA PolI insert respectively (Fig 2A). pITB$_{21}$ and pITB$_{22}$ carries deletion fragment of *Eco*RI with the size of 0.6 kb and *Xho*I with the size of 1.1 kb respectively. Agarose gel electrophoresis following double digest by *Bam*HI and *Nco*I showed that two fragments were appeared from both plasmids with the respected sizes (Fig. 2 B, and C). Further analysis to confirm the constructed plasmid was carried out by sequencing of the fragments. Sequence analysis showed that all recombinant plasmids carried ATG start codon originally from pET30a(+) vector, six histidine codons and in-frame junction on *Nco*I and *Bam*HI sites (data not shown). No other mutations were found within the coding sequence of the genes.

Expression and purification of DNA Pol I and Its Mutant Proteins

The whole coding region of DNA Pol I ITB and its deletion mutant genes were successfully cloned into plasmid pET30a(+) through *Nco*I and *Bam*HI restriction sites resulting on pITB$_{20}$, pITB$_{21}$, and pITB$_{22}$ respectively. In these plasmids the gene are controlled under promotor of bacteriphage T7. The plasmids were used to transform *E. coli* BL21(DE3) which is suitable for the expression of plasmid since this strain carrying T7 RNA polymerase from bacteriophage DE3. The gene under T7 promotor remaining transcriptionally silent until the expression a chromosomal copy of T7 RNA Polymerase was induced. The expression of DNA Pol I ITB1 and its deletion mutant proteins were carried out under the induction of IPTG when the culture cells reached on the logarithmic phase. The results showed that the proteins were over-expressed up to 15% of the total protein following densitometric measurement on the gel (data not shown). The size of the over-expressed proteins were expected, 100 kDa for the wild type, 75 kDa for the *Eco*RI

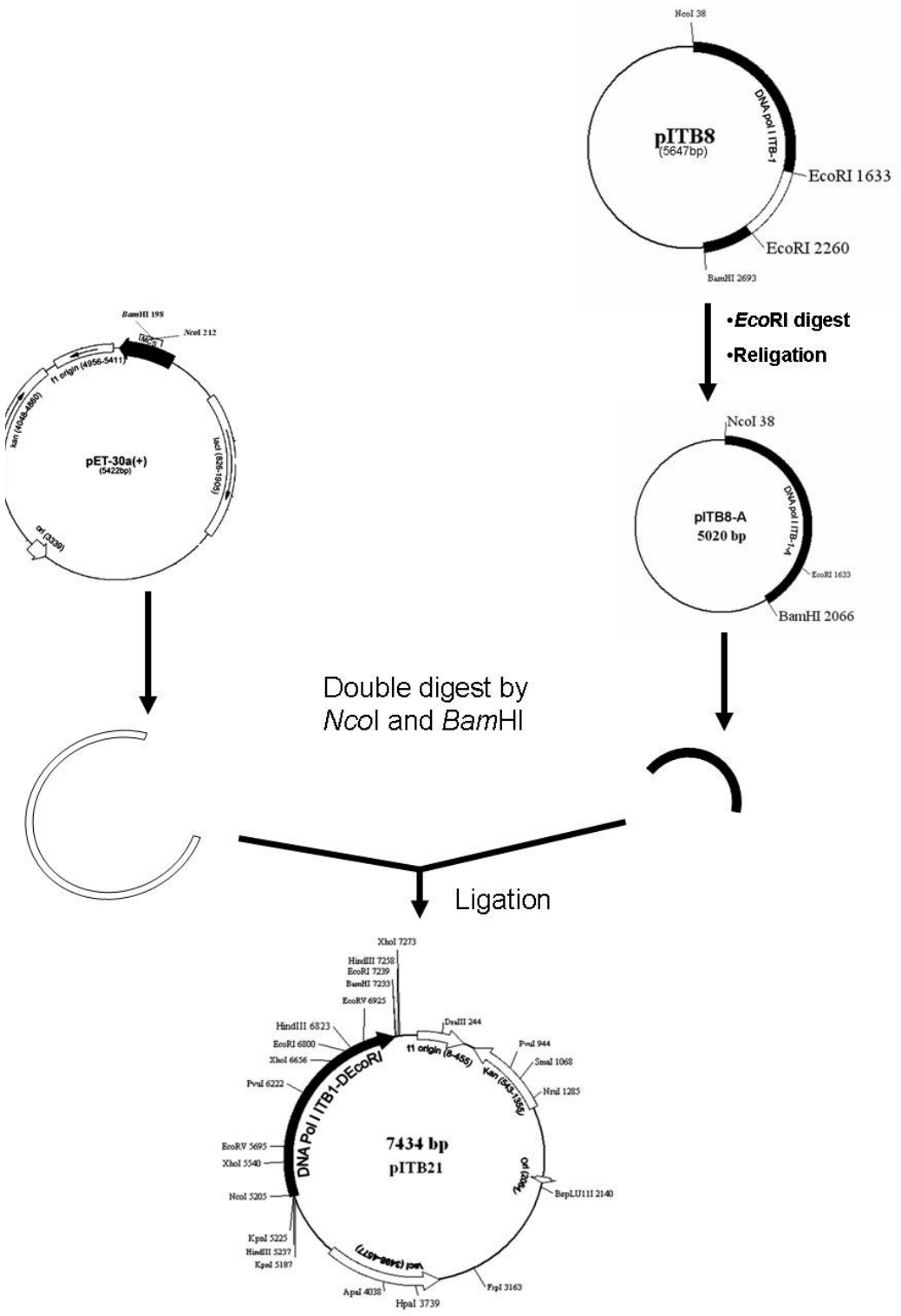

Fig. 1A. Construction Strategy of pITB$_{20}$. *Nco*I and *Bam*HI fragment of DNA PolI gene from pITB$_8$ was inserted into linearized pET30a(+) digested by *Nco*I and *Bam*HI restriction enzymes

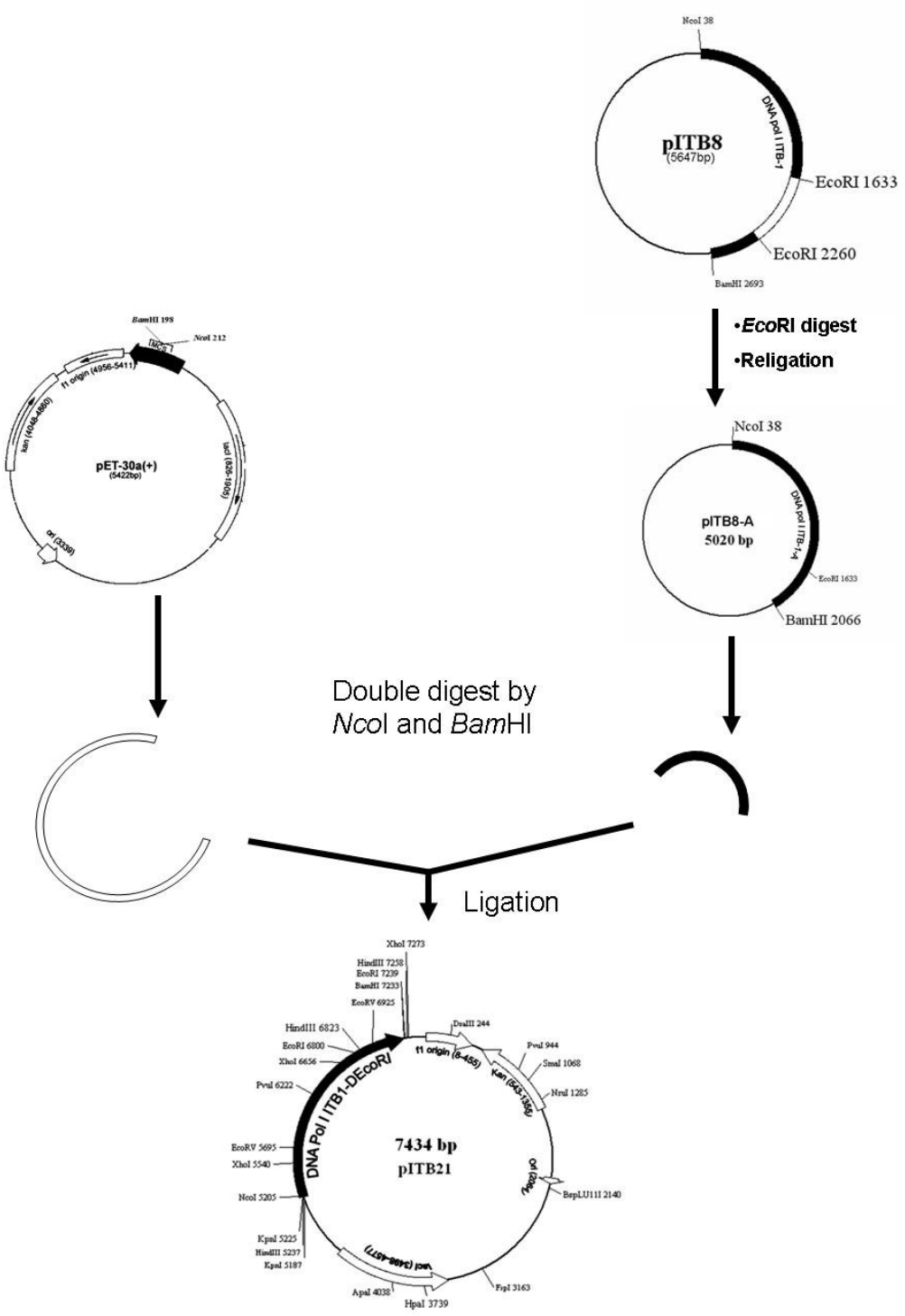

Fig. 1B. Construction Strategy of pITB$_{21}$. *Eco*RI fragment of DNA PolI gene was removed from pITB$_8$, then the vector was religated resulting pITB$_8$-A. *Nco*I and *Bam*HI fragment of DNA PolI gene from pITB$_8$-A was inserted into linearized pET30a(+) digested by *Nco*I and *Bam*HI restriction enzymes

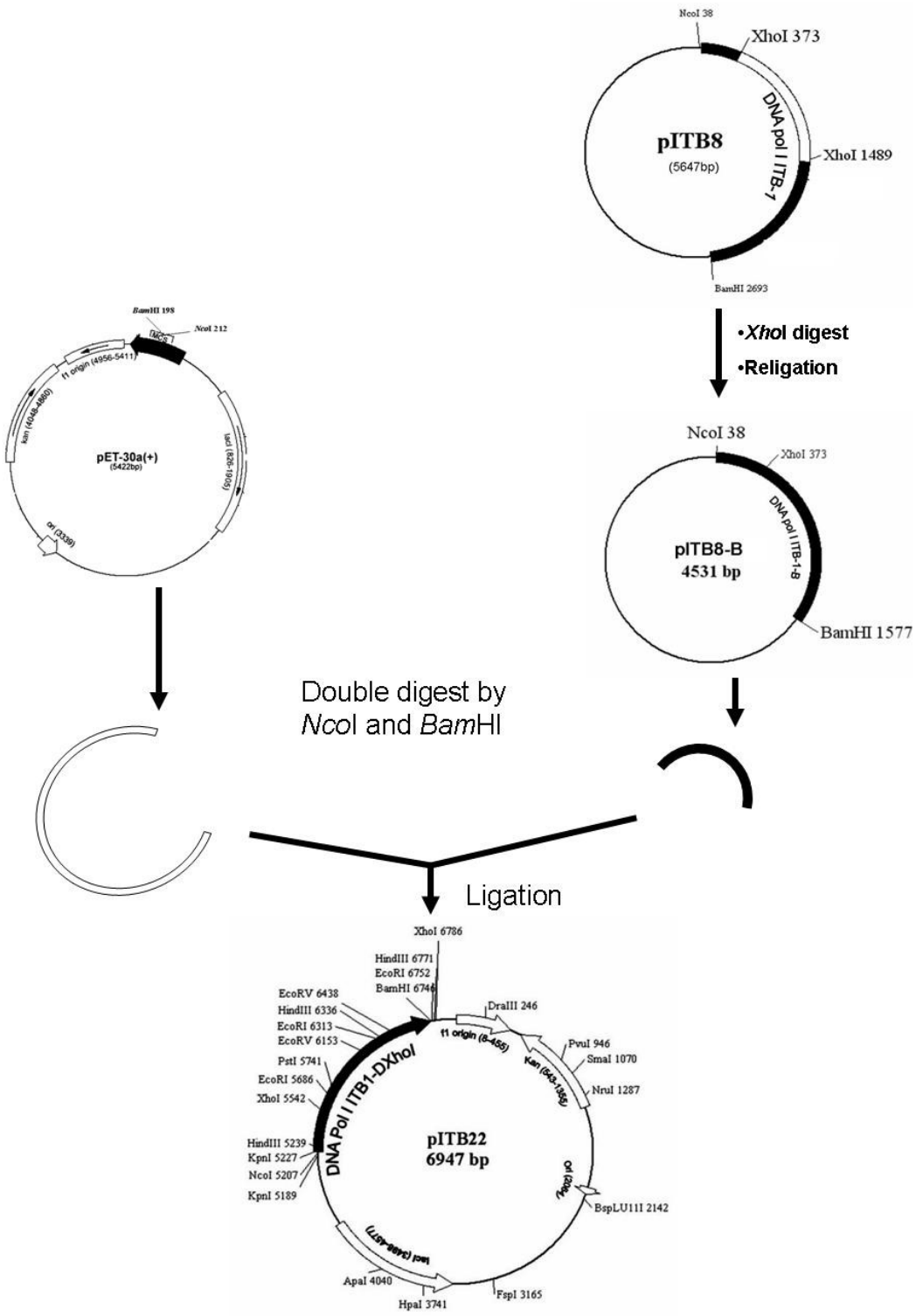

Fig. 1C. Construction Strategy of pITB$_{21}$. *Xho*I fragment of DNA PolI gene was removed from pITB$_8$, then the vector was religated resulting pITB$_8$-B. *Nco*I and *Bam*HI fragment of DNA PolI gene from pITB$_8$-B was inserted into linearized pET30a(+) digested by *Nco*I and *Bam*HI restriction enzymes

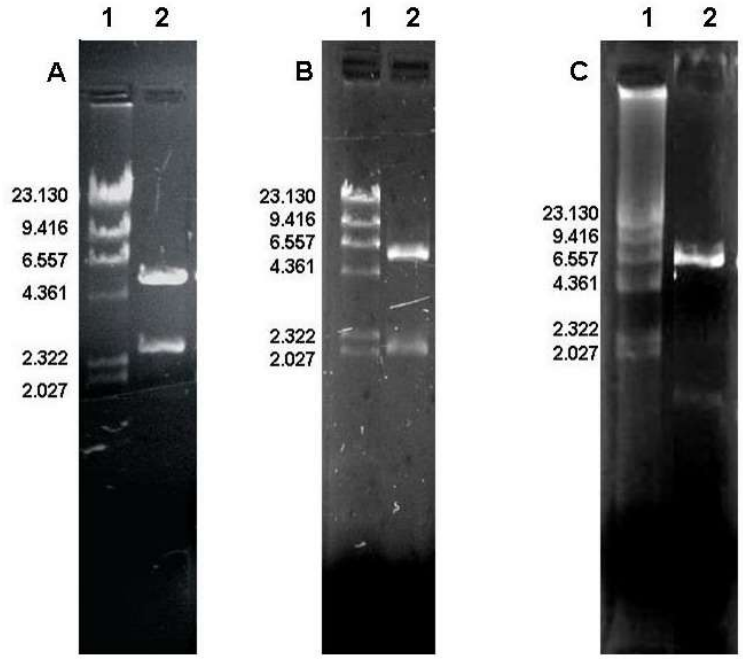

Fig. 2. Electrophoregram of Agarose Gel Electrophoresis. (A) pITB$_{20}$, (B) pITB$_{21}$, (C) pITB$_{22}$ digested with *Nco*I and *Bam*HI. Lane 1 (A,B,C) Lamda DNA digested by *Hind*III. Lane 2 (A, B, C) Double digest fragment with *Nco*I and *Bam*HI

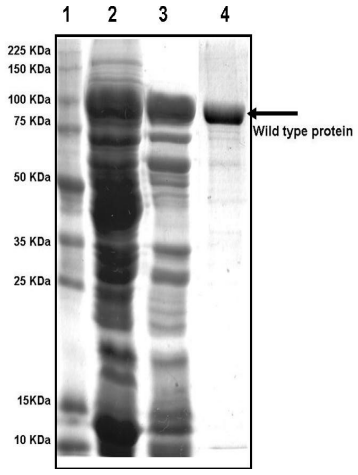

Fig. 3A. Electrophoregram of SDS-PAGE from DNA PolI ITB$_1$ Wild Type. (1) marker protein, (2) crude extract, (3) crude extract after heated at 60oC for 10 min, (4) purified protein following IMAC purification

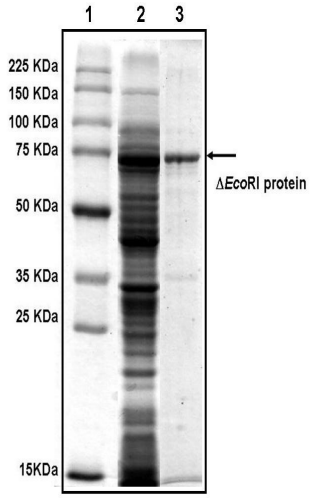

Fig. 3B. Electrophoregram of SDS-PAGE from DNA PolI ITB$_1$ *Eco*RI Deletion Mutant. (1) marker protein, (2) crude extract, (3) purified protein following IMAC purification

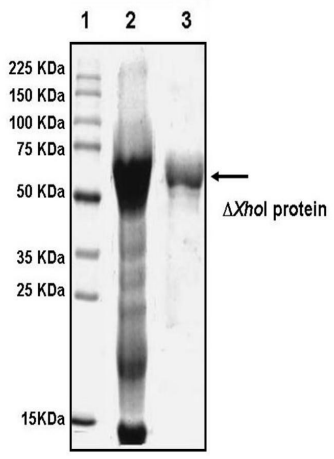

Fig. 3B. Electrophoregram of SDS-PAGE from DNA PolI ITB$_1$ *Xho*I Deletion Mutant. (1) marker protein, (2) crude extract, (3) purified protein following IMAC purification

deletion, and 60 kDa for *Xho*I deletion respectively (Fig 3A, B, and C).

Purification of recombinant proteins were performed by Ion Metal Affinity Chromatography (IMAC) system since the genes under pET30a(+) containing His-tag (6 histidine residues) in the N-terminal of the proteins. The purification using IMAC system was based on the specific interaction between transitional metal ion (Co^{2+} or Ni^{2+}) with a donor electron from amino acid residues[27,28]. In the case of DNA Pol I ITB_1, the histidine was used as a donor electron meanwhile Ni^{2+} was the acceptor. The soluble crude extracts of the proteins were directly subject to Ni-NTA affinity chromatography. The proteins were eluted by addition of immidazoles. Fig 3A, B, and C (lane 3) showed the elctrophoregram of purified proteins for the wild type, *Eco*RI and *Xho*I deletion mutants respectively. It showed that DNA PolI ITB_1 wild type and its deletion mutants have been well purified and ready for further used.

CONCLUSIONS

DNA Pol I ITB_1, D*Eco*RI and D*Xho*I genes were successfully cloned into pET30a(+) expressions vector resulting $pITB_{20}$, $pITB_{21}$ and $pITB_{22}$ recombinant plasmids. Restriction and sequence analysis of all of recombinant plasmids confirmed that the DNA Pol I ITB-1 genes were clone in right and correct positions. The genes from $pITB_{20}$, $pITB_{21}$, and $pITB_{22}$ have been successfully expressed in *E. coli* BL21 (DE3) and purified using Ni-NTA resin.

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REFERENCES

1. Kornberg, A., Minireview : DNA replication. *J. Biol. Chem.*, 1988; **236**: 1-4.
2. Akhmaloka, Pramono, H., Ambarsari, L., Susanti, E., Nurbaiti, S., Madayanti, F., Cloning, homological analisis, and expression of DNA Pol I from *Geobacillus thermoleoforans*. *IJIB*, 2007; **1**: 206-215.
3. Kim,D.J., Jang, H.J., Pyun, Y.R. and Kim, Y.S., Cloning, Expression, and Characterization of Thermostable DNA Polymerase from *Thermoanaerobacter yonseiensis*, *Journal of Biochemistry and Molecular Biology*, 2002; **35**: 320-329.
4. Choi, J.J., Jung S.E., Kim, H.K., and Kwon, S.T., Purification and properties of *Thermus filiformis* DNA polymerase expressed in *Escherichia coli*, *Biotechnol. Appl. Biochem*., 1999; **30**: 19-25.
5. Chien, A., Edgar D.B., and Trela, J.M., Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J.Bacteriol.* 1976; **127**: 1550-1557.
6. Burrows, J.A. and Gowaard ,C.R., Purification and properties of DNA polymerase from *Bacillus caldotenax*. *Biochem. J.,* 1992; **287**: 971-977.
7. Kaledin, A.S., Sliusarenko, A.G., and Gorodetskii, S.I., Isolation and Propertis of DNA polymerase from the extreme thermophilic bacterium *Thermus Falvus*. *Biokimiia*., 1981; **46**: 1576-1584.
8. Ruttiman, C., Cotoras, M., Zaldivar, J., and Vicuna, R. DNA polymerase from the extremely thermophilic bacterium *Thermus thrmophilus* HB. *Eur: J.Biolchem*. 1985; **149**: 41-46.
9. Akhmaloka, Tika, I.N., Nurbaiti, S., Sindumarta, M., and Madayanti, F., Isolation and Characterisation of A Novel Thermostable DNA Polymerase from *Bacillus thermophilus sp1*. *Indonesian Journal for Microbiology*, 2006: **11**(2): 82-86
10. Perler, F.B., Kumar S., Kong, H. Thermostable DNA polymerases. *Adv. Protein Chem,* 1996; **48**: 377-435.
11. Stenesh, J. and Roe R.A., DNA Polymerase from mesophilic and thermophilic bacteria. I. Purification and properties of DNA Polymerase from *Bacillus licheneformis* and *Bacillus stearothermophilus*, *Biochem. Biophys. Acta*, 1972; **272**: 156-166.
12. Kaboev, O.K., Luckia L.K, Akhmedov, A. T., and Bekker, M. L., Purification and properties of deoxybucleic acid polymerase from *Bacillus stearothermophilus*, *Biokhimia*. 1981; **45**: 664-651.
13. Sellman, E., Scroder K. L, Knoblich, I.M, and Westermann, P., Purification and

characterization of DNA Polymerase from bacillus species, *J. Bacteriol*. 1992; **174**: 4350-4355.

14. Uemori, T., Ishino Y., Fujita, F., Asada, K. and Kato, I., Cloning of the DNA polymerase gene of *Bacillus caldothenax* and characterization of the Gene Product. *J. Biochem*, 1993; **113**: 401-410.
15. Dabrowski, S., and Kur, J., Cloning and Expression in *Escherichia coli* of the Recombinant His-Tagged DNA Polymerases from *Pyrococcus furiosus* and *Pyrococcus woesei* protein Expression and Purification, 1998; **14**: 131-138.
16. Kim, Y.J., Lee, H.S., Bae, S.S., Jeon, J.H., Lim, J.K., Cho, Y, Nam, K.H., Kang, S.G., Kim, S.J., Kwon, S.T., and Lee, J.H. Cloning, purification and characterization of new DNA polymerase from a hyperthermophilic archeon, *Thermococcus* sp. NA1. *J. Microbiol. Biotechnol.* 2007; **17**: 1090-1097.
17. Shin, H-J., Lee S. K., Choi, J.J., Koh, S., Lee, J.H., Kim, S.J., and Kwon, S.T. Cloning, expression, and characterization of a family B-type DNA polymerase from the hyperthermophilic crenarcheon *Pyrobaculum arsenaticum* and its aplication to PCR. *J. Microbiol. Biotecnol*, 2005: **15**: 1359-1367.
18. Yohandini, H., Madayanti F., Aditiawati, P., Akhmaloka, Diversity of microbial thermophiles in a neutral hot spring (Kawah Hujan A) of Kamojang Geothermal Field, Indonesia. *J Pure Appl Microbiol*. 2008; **2**: 283–294.
19. Aminin, A.L.N., Warganegara, F.M, Aditiawati, P., Akhmaloka, Simple enrichment and independent culture to expand bacterial community analysis from Gedongsongo hot spring. *J. Biosci. Bioeng.* 2008; **106**: 211-214.
20. Aditiawati, P., Yohandini, H., Madayanti, F., and Akhmaloka, Microbial Diversity of Acidic Hot Spring (Kawah Hujan B) in Geothermal Field of Kamojang Area, West Java-Indonesia, *The Open Microbiology Journal*. 2009; **3**: 58-66.
21. Hertadi, R., Nurbaiti, S., and Akhmaloka, Molecular Dynamic Analysis of Thermostable DNA PoI ITB-1. *Microbiology Indonesia*. 2007; **1**(3): 101-104.
22. Nurbaiti, S., Hertadi, H., Martoprawiro, M.A., Akhmaloka Interface domain interaction on early stage unfolding of DNA polymerase I Klenow fragment, *IJIB*, 2009; **6**(1): 46-52
23. Sambrook dan Russell, *Molecular cloning: a laboratory manual*, third *ed.*, 2001: New York.
24. Schlicke, M., and Brakman, S., Expression and puriWcation of histidine-tagged bacteriophage T7DNA polymerase, *Protein Expression and Purification*, 2005; **39**: 247-253.
25. Studier, F.W., and Moffatt, B.A., Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes, *J. Mol. Biol.* 1986; **189:** 113-130.
26. Akhmaloka, Pramono, H., Tika, I.N., Ambarsari, L., Moeis, M.R. and Madayanti, F. Cloning and Expression of DNA Pol I Gene from *Bacillus thermoleovorans* in *Escherichia coli*. The FEBS Journal, 2005; **272**: 509.
27. Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R., and Stüber, D., Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *BioTechnology*, 1988; **6:** 1321-1325
28. Van Reeth, T, Dreze, P.L, Szpirer, J., Szpirer, C., Gabant, P., Positive selection vectors to generate fused genes for the expression of his-tagged proteins. *Biotechniques*, 1998; **25**: 898-904.