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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

High-Level Production of MMLV Reverse Transcriptase Enzyme in *Escherichia coli*

Escherichia coli'de MMLV Ters Transkriptaz Enziminin Yüksek Düzeyde Üretimi

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Abstract
Reverse transcriptase (RT) of *Moloney murine leukemia virus* (MMLV) is the most widely used enzyme for cDNA synthesis and RNA amplification. In this study, we aimed to produce MMLV RT enzyme recombinantly due to its importance in molecular studies. In this context, the DNA fragment encoding the MMLV RT enzyme was cloned into pTOLT plasmid and expressed in *E. coli BL21 (DE3) pLysE* cells. Since the high-level expression of the protein caused the protein molecules to aggregate in the inclusion bodies, co-expression of MMLV RT and chaperone plasmids (pG-KJE8, pGro7, pKJE7, pGTf2, pTf16) was performed to obtain the MMLV RT protein in soluble form. Contrary to our expectations, because it could not be obtained in soluble form, the protein was recovered from the inclusion bodies using refolding process. Finally, the protein was purified by affinity chromatography and the activity of the protein was checked using RT-PCR technique.
Keywords: Inclusion bodies, Moloney murine leukemia virus, Reverse transcriptase, cDNA synthesis.

Öz
Moloney murin lösemi virüs (MMLV) ters transkriptazı (RT), cDNA sentezi ve RNA amplifikasyonu için en yaygın kullanılan enzimdir. Bu çalışmada moleküler çalışmalardaki önemi nedeniyle MMLV RT enziminin rekombinant olarak üretilmesi amaçlanmıştır. Bu bağlamda, MMLV RT enzimini kodlayan DNA fragmanı pTOLT plazmidine klonlanmış ve *E. coli BL21 (DE3) pLysE* hücrelerinde eksprese edilmiştir. Proteinin yüksek düzeyde ekspresyonu, protein moleküllerinin inklüzyon cisimciklerinde toplanmasına neden olduğundan MMLV RT proteininin çözünür formda elde edilebilmesi için MMLV RT ve şaperon plazmidlerin (pG-KJE8, pGro7, pKJE7, pGTf2, pTf16) birlikte ekspresyonu gerçekleştirilmiştir. Beklentilerimizin aksine, protein çözünür formda elde edilemediği için, yeniden katlama prosesi kullanılarak inklüzyon cisimciklerinden geri kazanılmıştır. Son olarak protein, afinite kromatografisiyle saflaştırılmış ve proteinin aktivitesi RT-PCR tekniği kullanılarak kontrol edilmiştir.
Anahtar Kelimeler: İnklüzyon cisimcikleri, Moloney murin lösemi virüsü, Ters transkriptaz, cDNA sentezi.

I. INTRODUCTION

Reverse transcriptase (RT) enzyme is an enzyme used in molecular studies to create complementary DNA from the RNA template. RTs are widely used for RT Polymerase Chain Reaction (RT-PCR), microarray analysis, cDNA library construction and RNA amplification. Due to their high catalytic activities and reliabilities, avian myeloblastosis virus (AMV) and Moloney murine leukaemia virus (MMLV) reverse transcriptases are the most commonly used enzymes in cDNA synthesis [1]. The MMLV reverse transcriptase enzyme is encoded by the pol gene and synthesized as part of the 200 kDa gag-pol precursor [2]. The MMLV reverse transcriptase is a 75 kDa monomer, while the AMV reverse transcriptase is a heterodimer consisting of 63 kDa and 95 kDa subunits [3]. The pol gene of MMLV encodes reverse transcriptase which has no DNA endonuclease activity and contains lower RNase H activity than AMV/MAV RT. MMLV RT RNase H activity is 4 times less effective and 4 times less stable than AMV/MAV RT. Therefore, when comparing the cDNA synthesis efficiency, the cDNA efficiency of MMLV RT is 6-8 times higher than AMV/MAV RT. Also, MMLV RT is able to generate longer transcripts [4].

There is a link between activity and stability in various enzymes. It has been reported in various studies that mutating hydrophobic amino-acids increases protein solubility while decreasing enzyme activity and protein stability [5-9]. In general, protein stability is adjusted by generating artificial genetic mutations which will change the polypeptide to be synthesized and when protein stabilization increases, a decrease in enzyme activity is observed [6]. Das and Georgiadis [7] introduced some mutations that affect the amino-acid sequence of the protein to increase the solubility of the MMLV protein and decrease the inclusion body formation. They also reported that

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removing the 24-amino acid portion in the N-terminal region resulting in increased protein solubility while the protein itself becoming resistant to proteolytic activities. In addition, an increase in solubility of the protein was observed due to the amino-acid leucine (L435K), a hydrophobic amino-acid located in the connection domain. While this mutation did not significantly affect the stability of the protein, it showed 78% activity compared to the wild form of the protein. Fei et al. [8] aimed to increase the solubility of MMLV reverse transcriptase protein, achieved higher solubility compared to previous studies. In their study, the solubility of the protein was increased by changing five hydrophobic amino-acids (L432K, V433K, I434K, L435K, A436K) in the protein's connection domain. They also concluded that the histidine amino-acids used in purification in the N-terminal did not affect the activity and stability of the protein.

Recombinant proteins often tends to misfold and form inclusion bodies, especially when they are over-synthesized [9]. Co-production of large amounts of chaperone proteins with the target proteins in the host cell may increase protein folding *in vivo* [10]. It is also prefered to increase the solubility of the proteins in *E. coli* by co-expression of proteins with molecular chaperones is a frequently used method. In *E. coli*, chaperone proteins such as DnaK, DnaJ, GrpE, GroEL, GroES and trigger factors are widely used alone or in combination [11-15]. GroEL-GroES and DnaK-DnaJ-GrpE, the most efficient chaperone combinations, have been reported to achieve protein refolding [16-18]. The GroEL-GroES chaperones prevent peptides form inclusion bodies, while DnaK-DnaJ-GrpE combination helps unfolded proteins to be secreted from inclusion [19]. Trigger factor (TF) binds to ribosomes near the peptide exit site (E site on ribosome) and interacts with short chains [20]. In addition, the TF improves GroEL-substrate binding to facilitate protein folding by interacting with GroEL [21]. It was reported by Chen et al. that 95% of His6-MMLV RT was obtained in soluble form when co-expressed at low temperature (28°C) with pG-Tf2 plasmid expressing TF and GroEL-GroES chaperone proteins [22].

In this study, we produced the MMLV RT protein as a fusion with the TolAIII peptide for its high expression. We investigated the co-expression of TolAIII-MMLV RT and chaperone proteins on reducing inclusion body formation. TolAIII-MMLV RT protein was recovered from the inclusion body and its activity was tested by RT-PCR method.

II. MATERIALS AND METHODS

2.1. Cloning of the MMLV RT gene into the pTOLT vector

The required MMLV RT gene sequence was taken from NCBI (National Center for Biotechnology Information) (Accession number: NC001501). Our patented vector pTOLT vector was used for cloning [23]. *Ncol*, *BamHI*, *XhoI* and *KpnI* restriction sites introduced to our vector prior to cloning. The designed gene sequence was synthesized by Biomatic company and the plasmid (pBSKMMLV) containing the MMLV reverse transcriptase gene synthesized by the company was digested with restriction enzymes *BamHI* and *KpnI*. The resulting MMLV gene separated in 1% agarose gel at first, then purified in accordance with the protocol with the “Biobasic Gel and PCR Clean-Up" kit from the gel. It was ligated with the pTOLT vector digested with the same restriction enzymes before. The ligation mixture was transferred to *E. coli* DH5α cells by heat shock. The obtained colonies were grown at 37°C overnight. Plasmid DNA from cells was purified using the "Biobasic Plasmid DNA Purification" kit. Diagnostic restriction digestion was performed on the obtained plasmids with the enzymes *KpnI* and *BamHI*.

2.2. Co-expression of TolAIII-MMLV RT with chaperone proteins

The pTOLTMMLV plasmid was transferred into *E. coli BL21 (DE3) pLysE* cells. *E. coli BL21 (DE3) pLysE* cells were inoculated into LB (Luria-Bertani) medium containing chloramphenicol (20 µg/ml) and ampicillin (100 µg/ml). When the culture reaches an OD600: 0.7, protein production was induced by adding 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) to the medium. Total cell lysate before and after induction was analyzed in 12% SDS-PAGE.

The chaperone plasmids used in this study were purchased from Takara Bio Inc. The chaperone plasmids used and their properties are given in Table 1. The pTOLTMMLV was transferred to *E. coli BL21 (DE3)* cells. For co-expression with chaperone plasmids, pTOLTMMLV was separately transferred to *E. coli BL21 (DE3)* cells with the plasmids in Table 1.

Table 1. Chaperone plasmids used in this study and their properties

Plasmid	Chaperones	Inducer
pG-KJE8	**dnaK**-70 kDa **dnaJ**-40 kDa **grpE**-22 kDa **groES**-10 kDa **groEL**-60kDa	L-arabinose (0.5 mg/ml), Tetracyclin (5 ng/ml)
pGro7	**groES**-10 kDa **groEL**-60 kDa	L-arabinose (0.5 mg/ml)
pKJE7	**dnaK**-70 kDa **dnaJ**-40 kDa **grpE**-22kDa	L-arabinose (0.5 mg/ml)
pG-Tf2	**groES**-10kDa **groEL**-60 kDa **tig**-56kDa	Tetracyclin (5 ng/ml)
pTf16	**tig**-56 kDa	L-arabinose (0.5 mg/ml)

Overnight culture was inoculated into 600 ml LB medium containing chloramphenicol (20 µg/ml) and ampicillin (100 µg/ml). The production of chaperone proteins was induced by adding L-arabinose (0.5 mg/ml) or/and Tetracyclin (5 ng/ml) to the cells grown at 240 rpm at 28°C when OD_{600}:0.5. After 20 minutes, 0.5 mM IPTG was added and the bacterial culture

incubated at 240 rpm for 5 hours at 28°C. The cells were collected by centrifugation and lysed with a sonicator. The disrupted cells were centrifuged at 12,000 rpm for 15 minutes and total, insoluble, and soluble fractions were analyzed in 12% SDS-PAGE.

2.3. Recovering of the TolAIII-MMLV RT from the inclusion body

The pTOLTMMLV plasmid was transferred to the *E. coli BL21 (DE3) pLysE* cells by heat shock. *E. coli* BL21 (DE3) pLysE cells was inoculated into 4 ml LB medium with antibiotics and incubated overnight at 37°C at 240 rpm. The cells were then inoculated into a 2-L sterile flask containing 600 ml LB medium. When the OD reaches 0.7, the cells was induced by adding 0.1 M IPTG. After 4 hours of incubation, the cells were harvested by centrifugation. The harvested cells were disrupted with a sonicator. After the sonicator process, centrifugation was carried out at 12,000 rpm for 15 minutes. The obtained pellet was suspended twice with resuspension buffer (50 mM Tris-HCl (pH: 8.0), 2.5% Triton X-100, 20% sucrose, (5ml/g)), and the pellet was collected by centrifugation at 12,000 rpm for 15 minutes. The washed pellet was dissolved by mixing gently with solubilizing buffer (50 mM Tris-HCl, 8 M urea (pH: 8.0) (5ml/g)) for 5 hours. Afterward, centrifugation was carried out at 12,000 rpm for 15 minutes. The cold refolding buffer (100 mM Tris-HCl, 10 mM DTT, 20% glycerol (pH: 8.0)) was added dropwise to the obtained supernatant so that the final protein concentration was less than 0.2 mg/ml [24]. The mixture was gently stirred at 4°C for 16 hours. Afterward, centrifugation was carried out at 12,000 rpm for 15 minutes. The supernatant was dialyzed at +4°C overnight (Spectra/por MWCO = 6-8,000), 20 mM Tris-HCl, 300 mM NaCl (pH: 8.0) solution was used as a dialysis solution. Then, the MMLV reverse transcriptase protein was purified using our previous protocol with minor modifications [25; 26]. In summary, 20 mM Tris-HCl, 300 mM NaCl (pH: 8.0) were used as purification buffers. 20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole (pH: 8.0) were used as wash buffers. Elution of the protein from the column was carried out using 20 mM Tris-HCl, 300 mM NaCl 300 mM imidazole (pH: 8.0) buffer. The obtained fractions were analyzed in 12% SDS-PAGE.

2.4. Activity Determination of TolAIII-MMLV RT Protein

We used 'Thermo Scientific Revert Aid First Strand cDNA Synthesis kit' to test the activity of the cloning product of MMLV RT enzyme against its commercial form. In the experiment we use six different concentrations of both cloning product of TolAIII-MMLV RT protein and the commercial form. The obtained cDNAs were amplified using "Thermo Scientific DreamTaq PCR Master Mix", GAPDH primer (reverse) and GAPDH primer (sense).

The experimental steps of this study are briefly illustrated in Figure 1.

III. RESULTS AND DISCUSSION

3.1. Cloning of the MMLV RT gene into the pTOLT vector

MMLV gene and the pTOLT vector were digested at first and then ligated. The *E. coli* DH5α strain was transformed with ligation products and colonies were inoculated into 4 ml LB medium. Subsequently, plasmid DNA was isolated from the cultures and the obtained plasmids were digested with the same restriction enzymes used in cloning. After two hours of digestion, the digest products were analyzed in 1% agarose gel electrophoresis (Figure 2). As a result, the pTOLTMMLV plasmid was obtained (Figure 3).

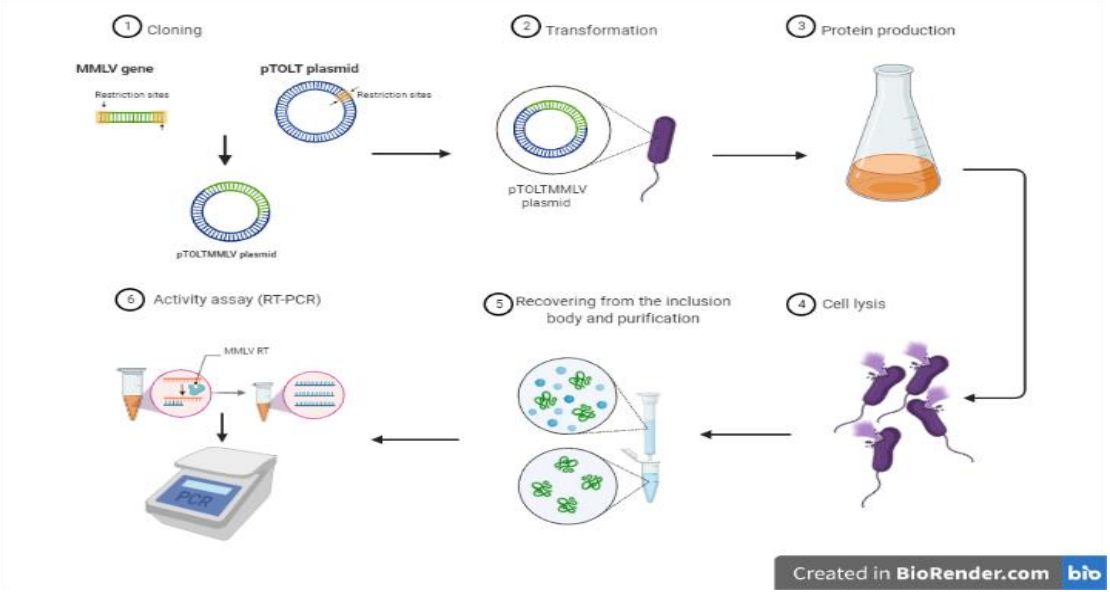

Figure 1. Experimental steps for recombinant production of TolAIII-MMLV RT protein (Created in BioRender.com)

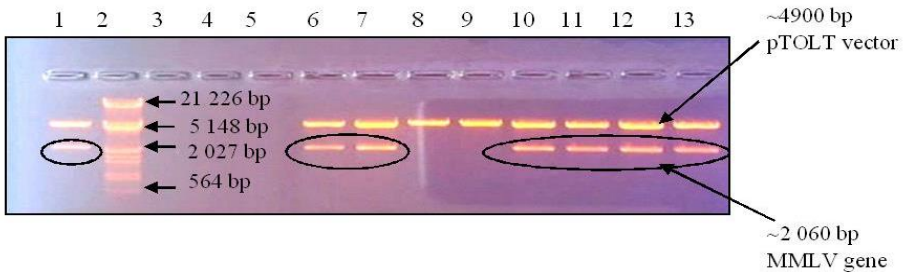

Figure 2. Analysis of diagnostic restriction digest of pTOLT-MMLV plasmid DNA samples in 1% agarose gel. 1, 6, 7, 10, 11, 12, 13 show plasmids containing the MMLV gene of 2060 bp, 2. DNA marker (Lambda HindIII/EcoRI).

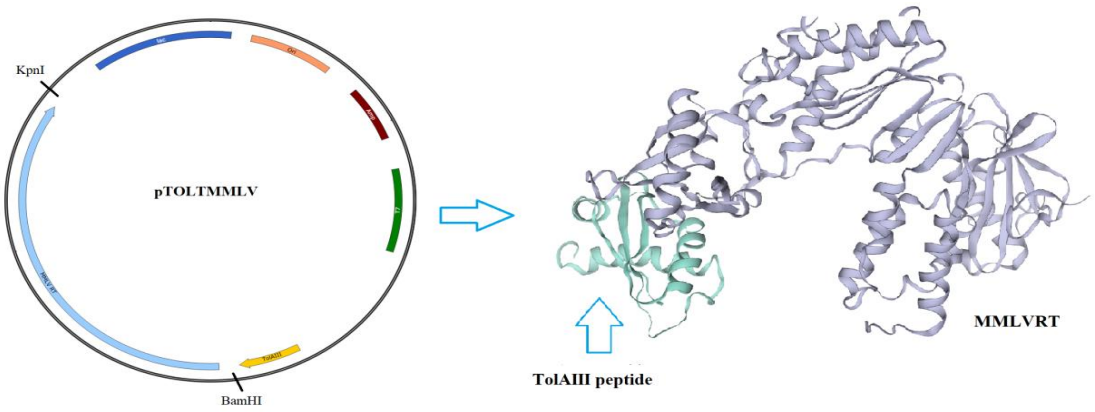

Figure 3. pTOLTMMLV construct used in order to produce TolAIII-MMLV fusion

3.2. Co-expression of TolAIII-MMLV RT with chaperone proteins

As expected, induction of the gene expression in *E. coli BL21 (DE3) pLysE* cells with IPTG resulting in an approximately 84 kDa TolAIII-MMLV RT protein in Figure 4. TolAIII-MMLV RT protein constitutes 32% of the total cell lysate (Analyzed with ImageJ program.).

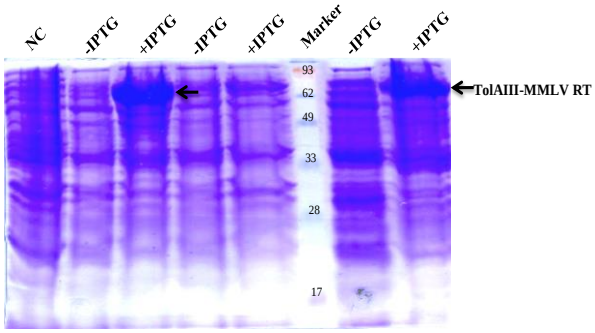

Figure 4. Analysis of recombinant production of TolAIII-MMLV RT protein in 12% SDS-PAGE. **NC** : negative control (plasmid-free *E. coli BL21 (DE3) pLysE* cells), **-IPTG**: *E. coli BL21 (DE3) pLysE* cells before inducing with IPTG, **+IPTG**: *E. coli BL21 (DE3) pLysE* cells after inducing with IPTG.

Co-expression with chaperone plasmids caused a decrease in expression of the TolAIII-MMLV RT protein. At the same time, TolAIII-MMLV RT could not be expressed in soluble form. (Figure 5 and Figure 6). Therefore, it is ensured that the highly expressed TolAIII-MMLV RT protein was recovered from the inclusion bodies.

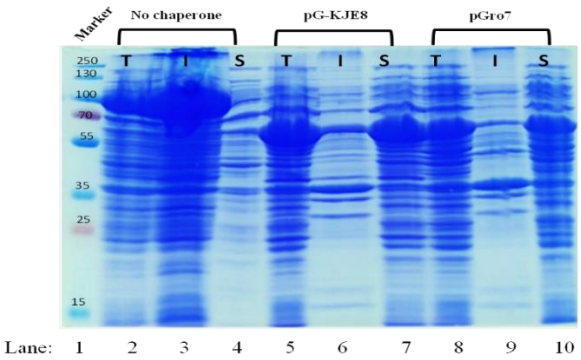

Figure 5. Expression analysis of TolAIII-MMLV RT with pG-KJE8 and pGro7 chaperone plasmids in 12% SDS-PAGE. 1.Protein marker (Page Ruler Plus Prestained Protein Ladder, 2, 3, 4. lanes are total, insoluble and soluble fractions of *E. coli* cells, respectively, containing the plasmid pTOLTMMLV. 5, 6, 7. lanes are total, insoluble and soluble fractions of *E. coli* cells, respectively, containing the plasmid pTOLTMMLV and pG-KJE8. 8, 9, 10. lanes are total, insoluble and soluble fractions of *E. coli* cells, respectively, containing the plasmid pTOLTMMLV and pGro7. **T**, **I** and **S** represent total, insoluble and soluble fractions, respectively.

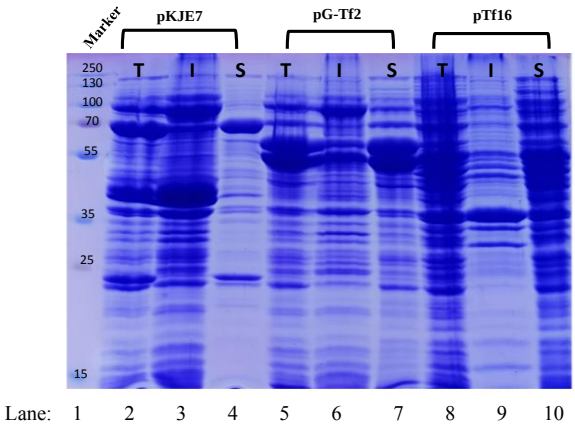

Figure 6. Expression analysis of TolAIII-MMLV RT with pKJE7, pG-Tf2, and pTf16 chaperone plasmids in 12% SDS-PAGE. 1. Protein marker (Page Ruler Plus Prestained Protein Ladder). 2, 3, 4. lanes are total, insoluble, and soluble fractions of *E. coli* cells, respectively, containing the plasmid pTOLTMMLV and pKJE7. 5, 6, 7. lanes are total, insoluble, and soluble fractions of *E. coli* cells, respectively, containing the plasmid pTOLTMMLV and pG-Tf2. 8, 9, 10. lanes are total, insoluble and soluble fractions of *E. coli* cells, respectively, containing the plasmid pTOLTMMLV and pTf16. **T**, **S** and **I** represent total, soluble and insoluble fractions, respectively.

3.3. Recovering of the TolAIII-MMLV RT from the inclusion body

E. coli BL21 (DE3) pLysE cells were induced with 0.1 mM IPTG when the culture's OD reaches 0.7. The induced cells collected 4 hours after induction. The collected cells were disrupted with a sonicator. The refolding procedure was applied to the collected pellet after centrifugation and refolded proteins purified by affinity chromatography. The samples obtained were analyzed in 12% SDS-PAGE (Figure 7). Protein concentration was determined as 5.4 mg/ml via Bradford method.

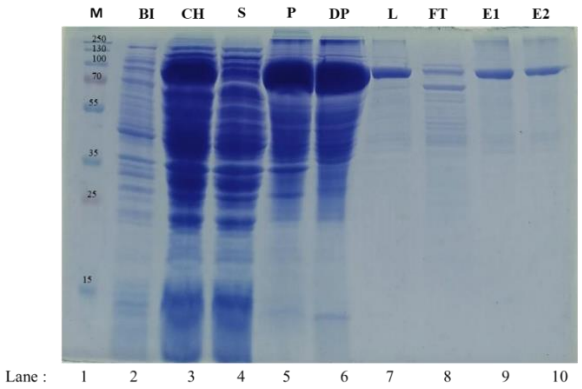

Figure 7. Analysis of the refolding process of TolAIII-MMLV RT in 12% SDS-PAGE. 1. Protein marker (Page Ruler Plus Prestained Protein Ladder). 2. *E. coli* cells containing the pTOLTMMLV plasmid before IPTG induction. 3. *E. coli* cells lysed by sonicator 4. The supernatant after centrifugation. 5. The pellet after centrifugation (dissolved with resuspension buffer). 6. The pellet dissolved in solubilizing buffer. 7. The sample loaded onto the column after dialysis. 8. The sample flowing down from the sample loaded onto the column containing the His-Link affinity resin after dialysis. 9-10. The sample obtained by passing the elution buffer containing 300 mM imidazole. M: protein marker, BI: before induction CH: Crude homogenates, S: supernatant, P: pellet, DP: dissolved pellet, L: Load, FT: Flow-through, E1: Elution 1, E2: Elution 2

3.4. Activity determination of TolAIII-MMLV RT Protein

The activity of recombinantly synthesized MMLV reverse transcriptase enzyme was analyzed in two steps. Firstly, human GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) RNA was converted to cDNA, then PCR reaction was performed using the obtained cDNAs as templates. The resulting PCR products was analyzed in 1% agarose gel electrophoresis (Figure 8). Approx. 496 bp amplicons are observed in the gel. The obtained gel images proved that our cloned, refolded and purified enzyme, MMLV RT, showed enzymatic activity like its commercial form.

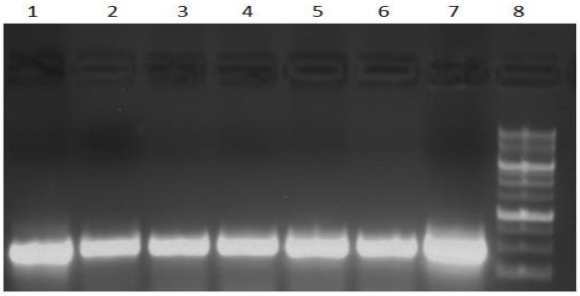

Figure 8. MMLV RT enzyme activity analysis result in 1% agarose gel. In the cDNA synthesis reaction, different amounts of MMLV reverse transcriptase enzyme (0.025 µg/µl, 0.05 µg/µl, 0.075 µg/µl, 0.1 µg/µl, 0.15 µg/µl, 0.25 µg/µl) and 1 µl of commercial MMLV reverse transcriptase enzyme (200U/µl) (7) were used. 8. DNA Marker (Hyper Ladder 1 kb)

IV. CONCLUSION

The MMLV RT protein was highly expressed when it fused with the TolAIII peptide. TolAIII is a small domain (92 amino-acids), expressed in high yields as a soluble protein in the cytoplasm of *E. coli*. The greatest advantage of expression in combination with TolAIII is that, it provides high efficiency protein production in *E. coli* [23]. As a matter of fact, about 32% of the total cellular protein is TolAIII-MMLV RT protein (Figure 4). Chen et al. reported that approximately 15% of the total cellular protein is MMLV RT [22]. The TolAIII peptide significantly increased the production of the protein. The expression with chaperone proteins at low temperatures was provided to obtain the protein in soluble form. 94% of His6-MMLV RT was obtained in

soluble form when co-expressed at low temperature (28°C) with pG-Tf2 plasmid by Chen at al [22]. However, contrary to their study, the protein was not obtained in soluble form and there was a decrease in the expression of the TolAIII-MMLV RT protein in our study. In the literature, it has been revealed that the expression of proteins with chaperones may show undesirable side effects related to protein yield and quality [13, 27-30]. This may be caused by the TolAIII peptide that we added for high level expression. Fei et al. [8] were reported that the histidine amino-acids used in purification in the N-terminal did not affect the activity and stability of the protein. In our study, the histidine amino-acid and TolAIII peptide that we added to the N-terminal of the protein did not affect the activity and stability of the protein.

Insoluble TolAIII-MMLV RT protein was converted to efficiently soluble form using refolding process. Then, it was purified by affinity chromatography and it was revealed that the purified enzyme has similar reverse transcriptase activity when we compare it against commercial enzyme through the activity test. 18 mg MMLV RT was obtained from 1 L of bacterial culture. As a result, obtained MMLV reverse transcriptase enzyme quality might be used commercially and produced enzyme has suitable properties for the development of PCR based diagnostic assay kits.

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