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Recombinant production of *Thermus aquaticus* single-strand binding protein for usage as PCR enhancer

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ABSTRACT

Single-stranded DNA-binding (SSB) proteins play an important role in DNA metabolism involving DNA replication, recombination, and repair in all living beings. In molecular biology, SSB proteins are used as enhancers to increase the efficiency and specificity of PCR. Thermostable SSB protein eliminates secondary structure or dimer formation and significantly increase the effectiveness of amplification of DNA fragments. In this study, it was ensured that the SSB gene of thermophilic bacteria *Thermus aquaticus (T. aquaticus)* was cloned into the pET28b vector and expressed in *E. coli BL21 (DE3) PLysE* cells. Then, the purification of the SSB protein produced in *E. coli BL21 (DE3) PLysE* cells was performed. 20 mg SSB protein was obtained from 1L bacterial culture, and its purity was more than 90%. It was shown by the PCR experiment that the SSB protein produced in this study could increase the amplification efficiency.

1. Introduction

PCR (polymerase chain reaction) technique is one of the most basic techniques of molecular biology. PCR protocols are used in a wide range such as routine diagnosis, genomic, and transcriptomic analysis, and these protocols need to be developed [1]. Due to the intrinsic properties of template DNA used in PCR such as high GC content and tendency to form secondary structures, PCR products do not occur under standard reaction conditions. This is a factor that limits the routine use of PCR. Strategies that can provide low-cost and reliable reaction conditions are needed for large scale PCR experiments [2]. In general, template DNAs contain long homopolymer regions, high GC content, and tandem repeats; therefore, it is difficult to amplify the template DNA by PCR. DNA templates with more than 65% GC content give very weak signals when observed under standard PCR conditions, and non-specific product formation is observed [3]. PCR can be improved by making some changes in reaction conditions. For example, “Touch-Down PCR”, performed by decreasing the annealing temperature step by step in each cycle, and “Hot Start PCR” using modified DNA polymerases cause serious improvement in PCR results [2]. In addition, when various enhancers such as tetramethylammonium chloride (TMAC), dimethyl sulfoxide (DMSO), Betaine, Glycerol, Formamide, non-ionic detergents, and their combinations are added in PCR, these enhancers increase efficiency, specificity, and reproducibility of the PCR amplification. It is particularly effective in ensuring the specificity of formamide and DMSO PCR products. In particular, formamide and DMSO are effective in ensuring the specificity of PCR products. Betaine can reduce the Tm value of DNA and it is effective in DNA amplification with a long and high GC content [4]. It also increases PCR's product efficiency and detection sensitivity. Often, 2 or more PCR enhancers are used together to make a PCR reaction work better. In addition

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to the known classic effect enhancers, new chemicals and substances are also being investigated. Trehalose, homoectoine, Zn^{2+}-1,7-bis(4-quinolylmethyl)-1,4,7,10-tetraazacyclododecane (Zn^{2+}-Q2-cyclen), and some nanoparticles are among the investigated chemicals. For example, trehalose, which displays a function like betaine, can facilitate PCR of GC-rich DNA by reducing the Tm value and DNA polymerase. Thanks to trehalose, the long PCR process can be also improved [5]. Homoectoine, which is a derivative of 1-ectoine, is more effective than betaine. Homoectoine reduces the Tm value and can increase the specificity of PCR at concentrations lower than betaine [6]. Zn^{2+}-Q2-cyclen, which can be specifically bound to deoxythymidine (dT), disrupts the hydrogen bond between adenine and thymine, decreases Tm value, and is effective in increasing PCR specificity [7]. However, despite their potential to greatly increase PCR effectiveness, commercial enhancers have significant disadvantages, such as the cost and unknown composition [2].

DNA-binding proteins of bacteriophage T4, such as gp32 T4 and SSB protein from *Escherichia coli* (EcoSSB), significantly increase the effectiveness of amplification of DNA fragments [8]. SSBs bind to single-stranded DNA and protect it from the digestion of nuclease. It ensures that ssDNA remains in a suitable conformation in DNA replication, repair, and recombination processes. In addition, SSB protein can physically interact with some proteins that play a role in this DNA metabolism. Therefore, it can be said that SSBs also play an important role in DNA metabolism [9, 10]. Since thermostable SSB proteins bind without denaturing the primers, they eliminate secondary structure or dimer formation. Thermostable SSB proteins are also highly effective in increasing the effectiveness of PCR when PCR conditions are considered [11]. In particular, SSB protein prevents primer dimers in multiplex PCR studies carried out with primers that have different annealing temperatures. In the studies conducted so far, the SSB protein of many thermophilic bacteria has been produced and their roles in increasing the effectiveness of PCR have been revealed [12-20].

The thermostable SSB of all bacteria belong to the *Deinococcus-Thermus* phylum except for SSB from *Thermoanaerobacter tengcongensis* [17]. They have been found in *T. thermophilus* [12], *T. aquaticus* [12], *D. geothermalis* [13], *D. murrayi* [14], *D. radiopugnans* [15], *D. radiodurans* [18], *D. grandis*, and *D. proteolyticus* [19].

Dabrowski et al. showed that the SSB protein of *T. aquaticus*is highly effective in providing amplification of weakly amplified regions by conducting experiments with a wide variety of DNA templates [12]. The SSB protein of *T. aquaticus*, which is a thermophilic bacteria, contains 266 amino acids and its molecular weight is 30 kDa [21, 22].

E. coli, which is frequently used in recombinant protein production, has advantages such as low cost and rapid production of recombinant proteins. Many proteins are produced by the recombinant DNA technology using *E. coli* strains [23].

In this study, *T. aquaticus* SSB gene used as a PCR enhancer was cloned into the pET28b vector. Expression of *T. aquaticus* SSB (*Taq*SSB) protein was performed in *E. coli BL21 (DE3) PlysE* cells. Thereafter, *Taq*SSB protein was produced and purified. It was shown that the purified *Taq*SSB protein could be used as a PCR enhancer.

2. Material and Method

*2.1. Cloning of Taq*SSB *gene into the pET28b vector*

The SSB gene sequence of *T. aquaticus* was amplified by PCR using the primers SBBBamHISense 5'TTTTGGATCCAATGGCTCGAGGCCTGAAC3', SSBHindIIIReverse 5'TTTTTAAGCTTTCAAAACGGC AAATCCTCCTC 3'. Primers are designed using the *Taq*SSB gene nucleotide sequence (AF276705) in NCBI (National Center for Biotechnology Information). Sense primer has BamHI restriction cutting site and reverse primer has HindIII cutting site.

PCR was performed using 50 ng template DNA, 10 mM dNTP mix, 10mM sense primer and reverse primer, 10 X Pfu Polymerase PCR buffer, and 1 U Pfu DNA polymerase that had a final volume of 50 µl. The program of the PCR device was set as follows: first, 2 min 1 cycle at 95°C; then, a total of 31 cycles, including 1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension; and 5 min at 72°C for the final extension. The obtained PCR products were purified with the PCR products cleaning kit and digested with BamHI and HindIII restriction enzymes. The pET28b plasmid to be used for cloning was also cut with the same restriction enzymes. *Taq*SSB gene and pET28b plasmid digestion by restriction enzymes were ligated with T4 DNA ligase enzyme at room temperature for 16 hours after purification performed with PCR products cleaning kit. The ligation products were transferred to *E. coli* DH5α cells and spread on LB medium containing kanamycin (50 mg/ml). Plasmid DNA isolation was done from the colonies, and diagnostic restriction digest and diagnostic PCR were performed on plasmids. The obtained products were analyzed in 1% agarose gel (Figure 1 and Figure 2, respectively).

*2.2. Production and Purification of Taq*SSB *protein*

For the expression of N terminal hexahistidine-tagged (6xHis) *Taq*SSB protein, plasmid DNAs from positive

clones (pET28bSSB) were transferred to *E. coli BL21 (DE3) PLysE* cells. Transformed cells were inoculated into 50 ml LB medium containing kanamycin (50mg/ml) and chloramphenicol (34 mg/ml) and induced by IPTG when OD_{600}: 0.6. Before and after induction performed with IPTG, the total cellular protein was analyzed in 12% SDS-PAGE (Figure 3).

E. coli BL21 (DE3) PLysE cells producing *Taq*SSB protein were incubated for 3 hours at 240 rpm at 37°C after the induction with IPTG. Then, the cells were collected by centrifugation at 8000 rpm for 5 minutes. The cells were dissolved in lysis buffer (100 mM sodium phosphate, 100 mM NaCl, and pH 7.8); then, PMSF (100 mM) and Benzamidine (100 mM) were added and lysed on ice by a sonicator. The cell lysate was kept at 95 °C for 20 minutes and then centrifuged at high speed for 60 minutes at 30 000 rpm. Purification of the SSB protein in the supernatant was carried out with the Ni-NTA column thanks to His-tag added to the protein. 100 mM sodium phosphate, 100 mM NaCl, and pH 7.8 were used as purification buffers. Elution of the protein from the column was carried out using 100 mM sodium phosphate, 100 mM NaCl, 300 mM imidazole, and pH 7.8 buffer [24]. The obtained protein was analyzed in 12% SDS-PAGE (Figure 4) and its amount was determined by the Bradford method.

*2.3. Usage of Taq*SSB *protein for PCR amplification*

Different concentrations of the purified SSB protein (50 ng/µl, 100 ng/µl, 250 ng/µl,) were added to the PCR mixture. Plasmid DNA containing the proteinase K (proK) gene was used as a template in PCR. The PCR was carried out using proK specific primers (ProteinazKNDEIsense TTTTCATATGGCTGCGCAG ACCAACGCTCCTT and ProteinazKHINDIIIreverse TTTTTAAGCTTTCAAGCCTGGTAGTTGTTGTA).

The program of the PCR device was set as follows: first, 2 min 1 cycle at 94°C; then, a total of 34 cycles, including 30 seconds at 94°C for denaturation, 1 min at 60°C for annealing, and 30 seconds at 72°C for extension; and 5 min at 72°C for final extension. PCR products were analyzed in 1% agarose gel electrophoresis (Figure 5).

3. Results and Discussion

3.1. Cloning of *Taq*SSB gene into the pET28b vector

*Taq*SSB gene was cloned into the pET28b vector. Confirmation of cloning was first performed by validation restriction cut using BamHI and HindIII restriction enzymes. As seen in Figure 1, it is revealed with the presence of the SSB gene region around 800 bp and the pET28b vector around 5300 bp.

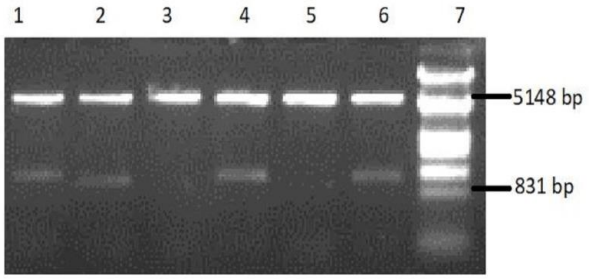

Figure 1. Analysis of the validation restriction cut result of the SSB gene cloned into the Pet28b vector in 1% agarose gel. 1, 2, 4, 6 are pET28b vector containing the SSB gene (positive clones pET28bSSB plasmids). 3, 5 are pET28b vector without the SSB gene and 7 is λDNA/EcoRI/HindIII Marker

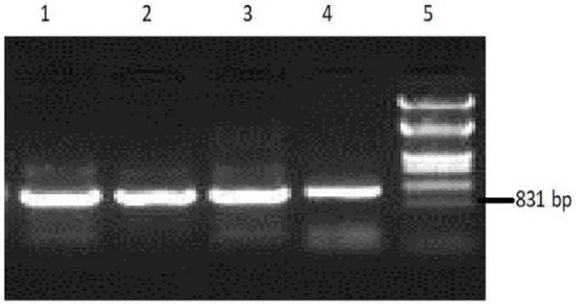

Figure 2. Analysis of the validation PCR result of the SSB gene cloned into the Pet28b vector in 1% agarose gel. 1, 2, 3, 4 are PCR products using pET28b plasmids (pET28bSSB) containing the SSB gene as a template in Figure 1. 5 is λDNA/EcoRI/HindIII Marker

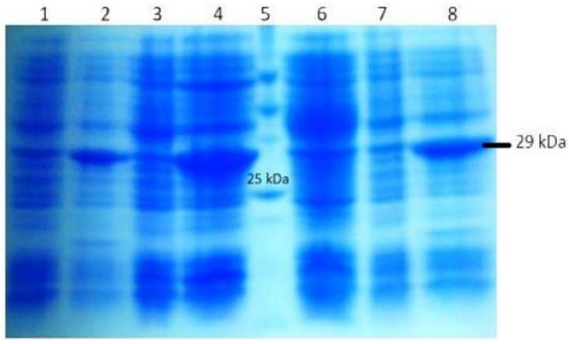

Figure 3. Analysis of the expression of *Taq*SSB protein in E. *coli* BL21 (DE3) PLysE cells in 12% SDS-PAGE. 1, 3, 7 are *E. coli BL21 (DE3) PLysE* cells containing the pET28bSSB plasmid before induction with IPTG. 2, 4, 8 are *E. coli* BL21 (DE3) PLysE cells containing the pET28bSSB plasmid after the IPTG induction. 6 shows *E. coli BL21 (DE3) PLysE* cells without pT28bSSB plasmid after the induction with IPTG. 5 is BioRad dual color precision plus protein marker

Diagnostic PCR was also performed by using plasmids that were found to be positive as a result of the diagnostic restriction digestion. As expected, the band belonging to the SSB gene, which appeared around 800 bp, demonstrated the verification of cloning (Figure 2).

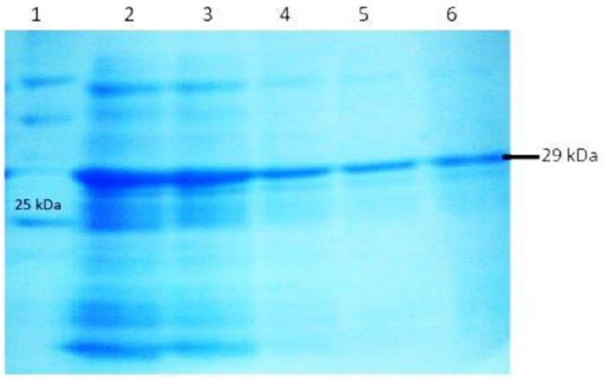

Figure 4. Analysis of the purified *Taq*SSB protein in 12% SDS-PAGE. 1: BioRad dual color precision plus protein marker, 2-6: Fractions of the purified *Taq*SSB protein

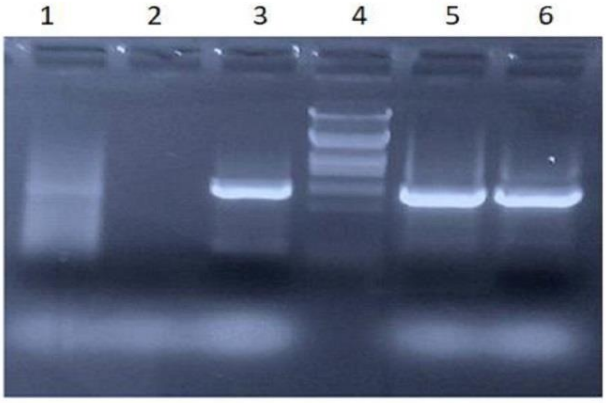

Figure 5. Analysis of the TaqSSB protein usage efficiency in PCR in 1% agarose gel. 1: Amplification of the proK gene without SSB protein. 3, 5, and 6: Amplification of the proK gene performed by adding various amounts of SSB protein; it contains SSB protein at concentrations of 50 ng/µl, 100 ng/µl, 250 ng/µl, respectively. 4: λDNA/EcoRI/HindIII Marker

3.2. Production and Purification of *Taq*SSB protein

The pET28b plasmid containing the SSB gene, which was confirmed to be cloned due to diagnostic restriction digest and diagnostic PCR, was transferred to *E. coli BL21 (DE3) PLysE* cells. For the analysis of protein expression, the total cell lysate before and after induction with IPTG was displayed in 12% SDS-PAGE. As seen in Figure 3, the expression of approximately 29 kDa size SSB protein was observed at the location where it was expected after induction with IPTG.

6xHis-*Taq*SSB protein was produced *in E. coli BL21 (DE3) PlySE cells* and purified by nickel affinity chromatography. The fractions of the purified *Taq*SSB protein analyzed in %12 SDS-PAGE (Figure 4), and its amount was determined by the Bradford method. As a result, 20 mg SSB protein was obtained from 1L bacterial culture and its purity was over 90%.

3.3. Usage of *Taq*SSB protein for PCR amplification

The *Taq*SSB protein in the 6th well shown in Figure 4 was taken in various volumes and added to the PCR reaction. PCR products were analyzed on 1% agarose gel. In Figure 5, it is shown that the *Taq*SSB protein, produced and purified recombinantly in this study, is very effective in increasing the effectiveness of PCR.

4. Conclusion

PCR is a powerful molecular biology technique. Various enhancers and their combinations are used to solve technical problems occurring in PCR. These enhancers increase the specificity, efficiency, and overall effectiveness of PCR. Traditionally used PCR enhancers (betaine, TMAC, formamide, DMSO) help solve complex secondary structure formation in GC-rich DNA templates. These enhancers can reduce the melting temperature of the primers as well as DNA templates [2]. SSB, an important protein for in vivo DNA replication, is effective in shortening PCR extension time and increasing PCR detection sensitivity [25]. SSB prevents primer dimer formation and increases PCR specificity [11]. Considering the temperature conditions in PCR, it can be said that SSBs isolated from thermophilic bacteria are very effective in PCR. Various studies have demonstrated the role of SSB proteins of thermophilic bacteria in increasing PCR activity [12-17]. *Taq*SSB protein provides highly effective amplification on a wide variety of weakly amplified DNA templates [12]. The use of SSB protein in the PCR technique have been routinely limited due to the difficulty of obtaining milligram amounts of purified protein. The cloning of the *ssb* gene into plasmids that cause increase in *ssb* gene expression made the purification of tens of milligrams of SSB protein a routine issue.

In this study, *Taq*SSB protein was cloned into the pET28b vector. Recombinant 6xHis-*Taq*SSB protein was produced in *E. coli BL21 (DE3) PlySE* cells and purified by nickel affinity chromatography. 20 mg of protein was obtained from 1L bacterial culture. It was shown that the *Taq*SSB protein produced and purified at high yield was highly effective in increasing the efficiency and specificity of PCR products. The production of *Taq*SSB protein in high amounts and purity recombinantly in this study may contribute its use as a tool to increase PCR efficiency and specificity.

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Declaration

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The authors also declared that this article is original, was prepared in accordance with international publication and research ethics, and ethical committee permission or any special permission is not required.

Author Contributions

All the authors have equally contributed.

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