Recombinant Expression and Purification of Serine Protease Enzyme of *Haloarcula* sp. TG1 in *Escherichia coli* BL21(*DE3*)

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Introduction

Microorganism-derived enzymes are generally used in the industry because of having high catalytic activity, absence of unwanted by-products, being more stable and being able to produce more at low cost (Illanes vd, 2012; Singh et al., 2016). Hydrolases constitute 65% of the enzyme market worldwide and serine proteases constitute 40% of the produced hydrolases (Mechri et al., 2017). The main parameters that make industrial applications of enzymes difficult are stability, catalytic activity and specificity factors. Many industrial applications take place in extreme environments that negatively affect these parameters. Various approaches have been tried to overcome such deficiencies, including screening of different enzymes from natural sources, random mutations, immobilization (Elleuche et al., 2014; Choi et al., 2015). Halophilic microorganisms have active and stable enzymes at high salt and low water concentration. Haloenzymes are of industrial interest due to their optimum activity over a wide temperature and pH range, and the flexibility of the sterilization step of archaea (Abanoz et al., 2017).

Materials and Methods

In this study, the extreme halophilic *Haloarcula* sp. TG1 strain with high protease activity, isolated from Lake Tuz by Abanoz et al. (2017) was used as the gene source. Cells were collected by taking a 5 ml sample from the *Haloarcula* sp TG1 liquid culture and centrifuged at 7.500 rpm for 10 minutes. Genomic DNA isolation from the cell pellet was performed using Qiagen DNA Extraction Mini Kit. The serine protease gene (rTG1) belonging to *Haloarcula* sp. TG1 strain was cloned with the oligonucleotides (Figure 1) designed targeting the serine protease gene (*WP_044952818.1*) of *Haloarcula hispanica* ATCC 33960 using PCR technique.

Figure 1. Nucleotide sequences of the primer pair used in cloning the rTG1 gene

The Primer3Plus web application at *https://primer3plus.com* was used in design of the primers, and these primers were verified with the online BLAST web application of the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov*). The rTG1 gene cloned with the PCR was first transferred into the pGEM-T® Easy (*Promega*) intermediate cloning vector, followed by the pET-28a (+) (*Novagen*) expression plasmid for heterologous expression (Figure 2).

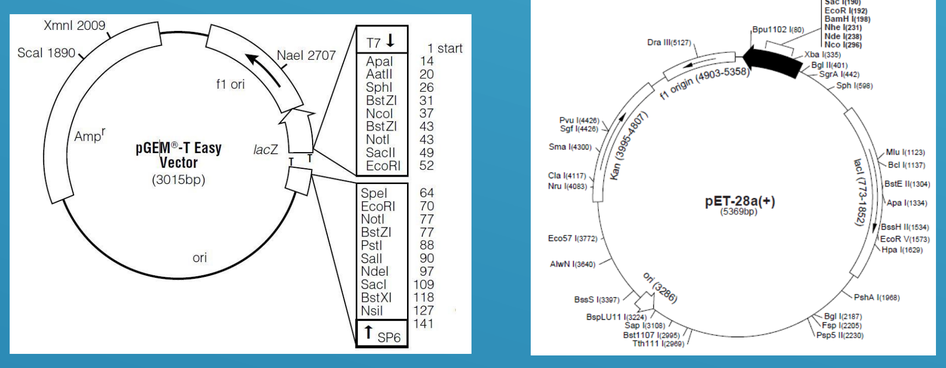

Figure 2. Vector maps of plasmids used in cloning and expression of the rTG1 gene

The cloned gene was amplified in *Escherichia coli* DH5α cells with the intermediate cloning vector. In order to generate the recombinant expression vector, ligation reaction was performed by using the pET-28a (+) plasmid linearized with *BamHI-HindIII* enzymes and the rTG1 gene rescueded from recombinant pGEM-T easy vector cut with the same enzymes. The ligation product was then transformed into *E. coli* DH5α and the resulting recombinant pET-28a(+) carrying rTG1 (pET-TG1) was introduced into *Escherichia coli* BL21 (*DE3*) cells. Mid log culture of this recombinant strain was induced with 1 mM IPTG for heterologous expression. Total cell protein contents obtained from IPTG-induced and non-induced cultures were compared by SDS-PAGE analysis. The recombinant serine protease enzyme (rTG1) verified by SDS-PAGE analysis was purified using Protino Ni-TED (Macherey-Nagel) affinity columns under denatured conditions (*8 M urea*) .

Results

The rTG1 PCR amplicon obtained using *Haloarcula* sp. TG1 genomic DNA as template is given in Figure 3.

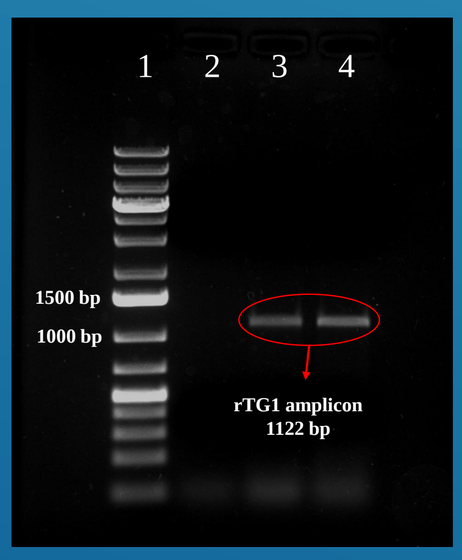

Figure 3. PCR amplicon of rTG1 gene from *Haloarcula* sp. TG1 genomic DNA (3-4). 1: Molecular marker (O'GeneRuler™ 1 kb), 2: Negative control reaction using water instead of template DNA

The rTG1 fragment cloned with PCR was first cloned into pGEM®-T Easy and transferred to *E. coli* DH5α cells by transformation. Sequence accuracy of rTG1 in plasmids isolated from recombinant cells was verified by sequencing reaction. The gene was separated from the vector with appropriate cutting enzymes and cloned into the pET-28a (+) expression vector cut with the same enzymes and transformed into *E. coli* DH5α cells. The accuracy of recombination was achieved with PCR and enzyme digestion reactions (Figure 4).

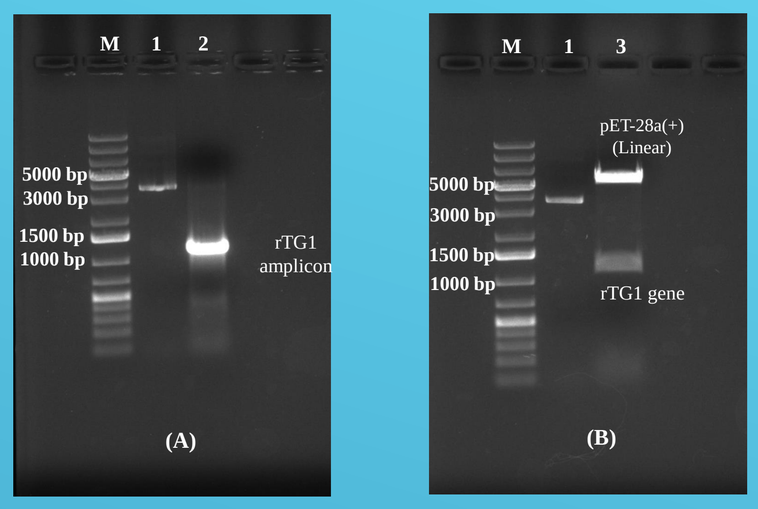

Figure 4. Verification of pET-TG1 in *E. coli* DH5α by PCR (A) and double digestion with *BamHI-HindIII* enzymes (B). M: Molecular marker (O'GeneRuler™ 1 kb), 1: Circular pET-TG1, 2: PCR amplicon of rTG1 obtained using pET-TG1 as template DNA, 3: rTG1 rescued from pET-TG1 after *BamHI-HindIII* digestion

Bioinfomatic analysis by using Expasy web tool (*https://web.expasy.org*) demonstrated that the rTG1 serine protease enzyme is consisted of 373 amino acids, theoretically pI value at 4.84. Its aliphatic index and molecular weight are 79.8 and 39.8 kDa, respectively. BLAST analysis of its amino acid sequence showed that it has 99.46% sequence identity to *H. hispanica* ATCC 33960 serine protease. After induction of cultures with 1 mM IPTG the cells were disrupted by sonication and the cell free lysate was run on SDS-PAGE to confirm heterologous expression of rTG1 expression in *E. coli* BL21 under the control of strong T7 promoter (Figure 5). Then, the rTG1 enzyme was purified using Protino Ni-TED affinity columns (Figure 6).

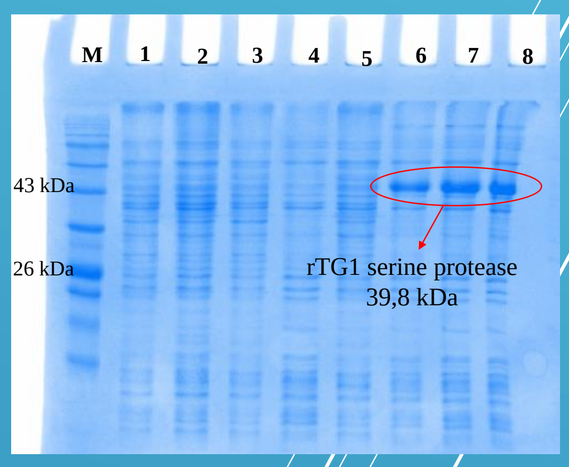

Figure 5. Induction of heterologous rTG1 expression in 1 mM IPTG induced *E. coli* BL21 cells containing pET-TG1. M: Molecular marker (BioLabs, P7719), 1: Total protein from IPTG non-induced BL21 cultures, 2: Total protein from IPTG induced BL21 cultures, 3: Total protein from IPTG non-induced BL21 carrying pET-28a(+), 4 : Total protein from IPTG-induced BL21 carrying pET-28a (+), 5: Total protein from IPTG non-induced BL21 carrying pET-TG1, 6-7-8: Total protein and rTG1 expression in IPTG induced BL21 cells carrying pET-TG1

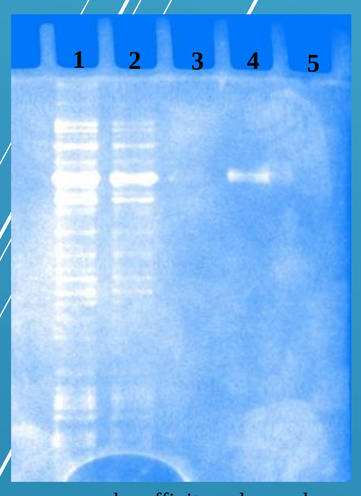

Figure 6. Purification of rTG1 serine protease enzyme by affinity column chromatography. 1: Flow through, 2: Wash 1 fraction, 3: Wash 2 fraction, 4: Elution 1 fraction, 5: Elution 2 fraction

Conclusion

The gene belonging to the rTG1 serine protease enzyme was amplified and the related enzyme was successfully expressed heterologously in *E. coli* BL21 cells. In subsequent applications, the conditions under which the purified rTG1 enzyme exhibits optimum activity will be determined. Later, this enzyme will be arrested in polyhydroxybutyrate nanoparticles and the enzyme activity and stability will be evaluated.

Acknowledgement

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