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Cloning and Expression of the Alkaline Phosphatase Gene from the Persian Type Culture Collection *Escherichia coli* K-12

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Abstract: The structural gene for alkaline phosphatase (phoA) of *E. coli* K-12 strain obtained from the Persian type culture collection (PTCC 1268) was cloned into pTZ57R plasmid as cloning vector and pGEM-3Z plasmid as expression vector, respectively. The recombinant plasmids were confirmed by different restriction enzymes and determination of the nucleotide sequence. Protein expression was induced by isopropyl β-D thiogalactopyranoside (IPTG) and was analyzed using polyacrylamide gel electrophoresis (PAGE). The obtained results demonstrate a complete homology of the DNA sequence between the cloned alkaline phosphatase gene with the sequence present in the gene banks.

Key words: Alkaline phosphatase, *E. coli* K-12, plasmid, cloning, expression

INTRODUCTION

Alkaline phosphatase is a homodimeric enzyme with a molecular weight of 56 kDa[1]. It is a metalloenzyme, binding two zinc atoms and one magnesium ion per monomer[2-5].

The enzyme catalyzes the hydrolysis of a wide variety of phosphomonoesters and also catalyze a transphosphorylation reaction with the transfer of the phosphoryl group to the alcohol in the presence of certain phosphate acceptors.

In *Escherichia coli*, alkaline phosphatase is encoded by the phoA gene, which is located between 8 and 9 min on the *E. coli* map[6]. The synthesis of this enzyme is regulated by a two-component regulatory system composed of the products of the phoB and phoR genes[7-10].

The enzyme is synthesized as a precursor monomer with a signal peptide at the amino-terminal end. After translocation into the periplasmic space, the signal peptide is removed and two of the resulting mature monomers dimerize, in the presence of four Zn^{2+} ions and two Mg^{2+} ions, to produce an active enzyme[11].

Escherichia coli alkaline phosphatase is a valuable reagent for removal of terminal monoesterified phosphate from both ribo- and deoxyribo-oligonucleotides[12,13]. Also Highly active bacterial alkaline phosphatase variants can be used as reporter enzymes, which are fused to foreign proteins via their amino or carbohydrate groups. These are particularly convenient novel tools that can be used in a wide range of applications, including expression, epitope mapping, histochemistry, immunoblatting, mutant analysis and competition or sandwich ELISAs[14]. Therefore, because of its widespread use we decided to clone and express the alkaline phosphatase gene from the available resources in Iran. This study would open the way for future investigations on structure and function of this enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids: The bacterial strains used in this study were *E. coli* K-12 strain (PTCC 1268 from Persian type culture collection), *E. coli* Hb101 and XL1-blue strains (from Cinagen Co).

The pTZ57R cloning vector and the pGEM-3Z expression vector were obtained from Fermentas Co.

Media and chemicals: Luria Bertani (LB) media was prepared according to Sambrook *et al.*[15]. Antibiotic screening was performed on LB Agar plates using ampicillin at 100 μg mL^{-1} which was obtained from Sigma Co. 5-bromo -4-chloro -3-indolyl-β-D-galactopyranoside (Xgal) and 0.1 M isopropyl β-D thiogalactopyranoside (IPTG) were used for blue/white colony screening. 0.1 mM and 0.5 mM IPTG were also used for induction of phoA gene. These reagents were purchased from Frementas Co.

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All of the restriction enzymes were purchased from Fermentas and Roche companies.

Cloning of phoA gene: The genomic DNA from *E. coli* K-12 (to be used as template for PCR amplification) was isolated by high pure PCR template preparation kit, which was purchased from Roche Co. Specific primers were designed by Wdnasis software and were synthesized by Faza pajooh Co, for the PCR amplification of phoA gene (APF 5′- cgtggaaagcttaatgtcacggccgag - 3′ and APF 5′- cgtggatccttatttcagccccaga - 3′). These primers were designed to have Hind III and BamHI sites at the 5′ and 3′ ends of the PCR product, respectively. Additionally, the sequence of the forward primer was designed in such a way that it was in frame with the lac Z protein of the pGEM-3Z plasmid (which its expression is under the control of lac promoter). The amplified DNA was analyzed by agarose (0.7%) gel electrophoresis to check for purity and proper size of the amplified product. PCR product was isolated by QIAquick Gel Extraction Kit and then it was directly ligated into pTZ57R plasmid using InsT/A clone PCR product cloning kit (from Fermentas Co) and was transformed into XL1Blue competent cells. The appropriate colonies were screened for the presence of the insert. Subsequently pTZ57R vector having the insert (phoA/pTZ57R) and also pGEM-3Z vector were cut by HindIII and XbaI enzymes. The insert and digested pGEM-3Z plasmid were isolated from agarose gel and then ligation was performed with T4 DNA Ligase (from Fermentas Co) at 16°C overnight in water bath. Before ligation, the vector was dephosphorylated by treatment with calf intestinal alkaline phosphatase (from Roche Co) to remove 5′-terminal phosphate and to prevent recircularization of the plasmid. The ligated DNA then was transformed into HB101 competent cells using $CaCl_2$ method[15].

The obtained colonies were used for plasmid preparation using High Pure Plasmid Isolation Kit (from Roche Co) and a portion of the plasmids DNA were digested with the restriction enzymes such as HindIII, BamHI, EcoRI and XbaI and loaded onto a 0.7% agarose gel to verify the presence of the phoA insert. Integrity of the insert DNA was verified by automated DNA sequencing in Faza pajooh Co.

Expression of phoA gene: A culture of phoA/pGEM-3Z containing bacteria was grown in 5 mL LB/ampicillin (100 μg mL^{-1}) overnight and 100 μL of the overnight culture was used to inoculate 5 μL of prewarmed LB/ampicillin. The culture was grown in a shaking incubator at 37°C until OD_{550} reached approx.0.5-1. Protein expression was induced by the addition of IPTG with final concentrations of 0.5 and 0.1 mM and the culture was grown for an additional 2 or 3 h. At these time points, 1.5 mL of each culture was transferred to a microfuge tube and cells were harvested by centrifugation at 13000×g for 1 min at room temperature and after removing of supernatant, the bacterial pellets were resuspended in 15 μL of 6×SDS gel-loading buffer. The samples were heated to 100°C for 3 min. The tubes were centrifuged at 13000×g for 1 min at room temperature and were stored on ice until loading of the samples into a gel. Protein expression was analyzed using sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE). For this purpose, a 12% polyacrylamide gel was utilized (100 V). Proteins were stained with coomassie blue for 20 min and individual proteins were visualized by destaining with methanol:acetic acid solution[15].

RESULTS

Construction of recombinant plasmids: To clone the phoA gene of *E. coli* K-12, at first the phoA gene was PCR amplified using specific primers. A 0.7% agarose gel was run to verify the presence of the proper size of PCR product and only a single band was present with an estimated size of approx. 1500 base pairs. To verify this band, a portion of the PCR product was digested by EcoRI and RsaI restriction enzymes which produced two (~900 and ~300 bp) and three bands (900, 300 and 160 bp), respectively (Fig. 1).

Then, PCR product was directly ligated into pTZ57R vector by InsT/A clone PCR product cloning kit. Because the PCR product could be attached in two different directions, the vector was digested by HindIII enzyme. This restriction site is present not only in the PCR product but also in the vector. Therefore the use of HindIII enzyme can demonstrate the proper orientation of the insert. The presence of the insert within the plasmid was also verified by double digestion of HindIII and XbaI restriction enzymes (Fig. 2) and single digestion of EcoRI restriction enzyme. Sequence integrity was determined by automated DNA sequencing (Fig. 3). The obtained phoA gene sequence completely matches the one deposited in NCBI database (NC_000913). We changed the "t" in the beginning of the coding sequence to "a" so instead of "ttg" we could have "atg". This may help to make the obtained gene easier to express.

For expression of phoA gene it was necessary to transfer the insert from phoA/pTZ57R into pGEM-3Z plasmid. Therefore, pGEM-3Z vector and phoA/pTZ57R were digested by HindIII and XbaI enzymes and then the insert and pGEM-3Z vector were ligated together at a molar ratio of 3:1. After transformation and plasmid

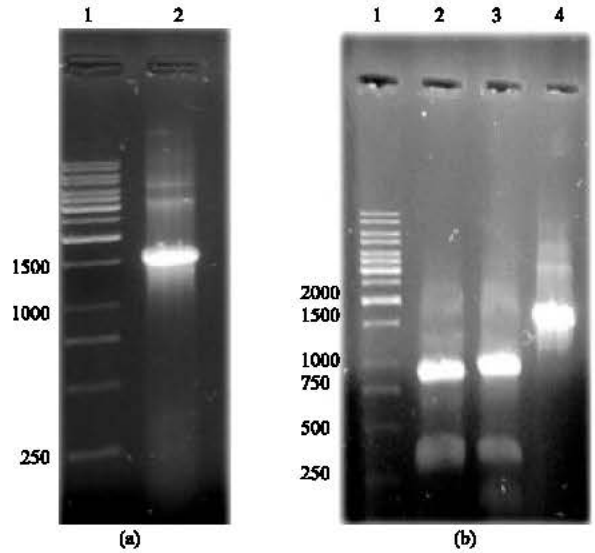

Fig.1. Agarose gel electrophoresis of a) the PCR product of *E. coli* K-12 phoA gene and b) digestion of the PCR product by EcoRI and RsaI restriction enzymes. a) lane 1: ladder, lane 2: the PCR product. b) lane 1: ladder, lane 2: the digested PCR product by EcoRI enzyme, lane 3: the digested PCR by RsaI enzyme, lane 4: the undigested PCR product

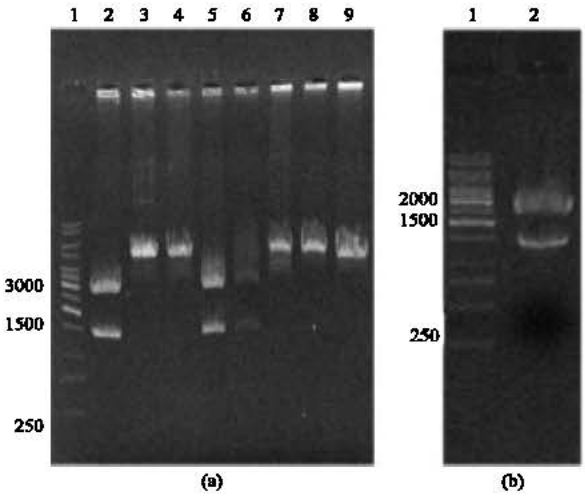

Fig. 2: Agarose gel electrophoresis of the pTZ57R vector with the insert by a) HindIII restriction enzyme and by b) HindIII and XbaI restriction enzymes. a) lane 1: ladder, lanes 2, 5 and 6: incorrect orientation, lanes 3, 4, 7, 8 and 9: correct orientation b) lane 1: ladder, lane 2: the digested pTZ57R vector with the insert by HindIII and XbaI restriction enzymes

preparation form the obtained colonies, a portion of the plasmid DNA was digested by HindIII and XbaI restriction enzymes to extract the insert with approximate size of 1500 bp. The correct orientation of the insert was verified by EcoRI restriction enzyme when a band of 300 bp was obtained (Fig. 4).

Expression analysis: Protein expression was analyzed using SDS-PAGE. The expressed protein should have a small portion of the Lac Z protein at its N-terminal since the phoA gene was framed with lac Z gene to be able to use the lac promoter of the vector for the expression of our protein. Electrophoresis was performed according to Sambrook *et al.*[15]. About 10 µL of each sample was loaded in wells and together with protein molecular weight marker were run on gel. After staining of proteins by coomassie blue and destaining, in addition to other bands, a weak band was appeared with an estimated size of ~55 kDa on lane of the induced sample (Fig. 5).

DISCUSSION

One of the main purposes of this laboratory is to produce and enhance the function of commercial enzymes. Since alkaline phosphatase is an enzyme vastly used in research and industry, we decided to focus our study on producing and improving the function of this enzyme. The first step was to clone the DNA encoding alkaline phosphatase from the available resources in Iran. The vectors used and the experimental design was also unique. Therefore by designing primers specific for phoA, we obtained a PCR band corresponding to this enzyme. However we could not insert this PCR product into our pGEM-3Z vector. Upon investigation we noticed that one of the primers had a wrong base at its restriction site sequence therefore not allowing the enzyme to cut the obtained product. One solution was to put the PCR product into a cloning system that didn't require restriction digestion. Thus we used pTZ57R vector for this purpose. The insert was extracted using the vector restriction sites (Hind III and XbaI sites) and it was then ligated into pGEM-3Z vector. Due to having one wrong base in 3′ end of the PCR product, there was no framshift of the insert and could be expressed using the lac prometer of pGEM-Z vector. Upon expression of protein using phoA/pGEM-3Z plasmid a weak band in an expected position was observed. We didn't succeed to express this gene in larger quantities that may be due to the fact that the expression of the phoA gene is induced under phosphate-limited conditions[16]. About 80% of the phoA gene product fails to be secreted and forms insoluble inclusion body in the cytoplasm[17,18]. Therefore

TCGTCCCCCAACCGAGCTTCCGCTCGCTCGCAGTCGACGGGCGGGATCCGATTGGAAAGCTTA
ATGTCACGGCCGAGACTTATAGCTCGTCTTTTGTTTTTATTTTTTAATGTATTTGTACATGGAG
AAAATAAAGCGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCCTGTGA
CAAAAGCCCGGACACCGGAAATGCCTGTTCTGGAAAACCGGGCTGCTCAGGGCGATATTACT
GCACCCGGCGGTGCTCGCCGTTTAACGGGTGATCAGACTGCCGCTCTGCGTGATTCTCTTAGC
GATAAACCTGCAAAAAATATTATTTTGCTGATTGGCGATGGGATGGGGGACTCGGAAATTACT
GCCGCACGTAATTATGCCGAAGGTGCGGGCGGCTTTTTTAAAGGTATAGATGCCTTACCGCTT
ACCGGGCAATACACTCACTATGCGCTGAATAAAAAAACCGGCAAACCGGACTACGTCACCGA
CTCGGCTGCATCAGCAACCGCCTGGTCAACCGGTGTCAAAACCTATAACGGCGCGCTGGGCGT
CGATATTCACGAAAAAGATCACCCAACGATTCTGGAAATGGCAAAAGCCGCAGGTCTGGCGA
CCGGTAACGTTTCTACCGCAGAGTTGCAGGGATGCCACGCCCGCTGCGCTGGTGGCACATGTG
ACCTCGCGCAAATGCTACGGTCCGAGCGCGACCAGTGAAAAATGTCCGGGTTAACGCTCTGG
AAAAAGGCGGAAAAGGATCGATTACCGAACAGCTGCTTAACGCTCGTGCCGA…

Fig. 3: A part of the DNA sequence of phoA gene cloned

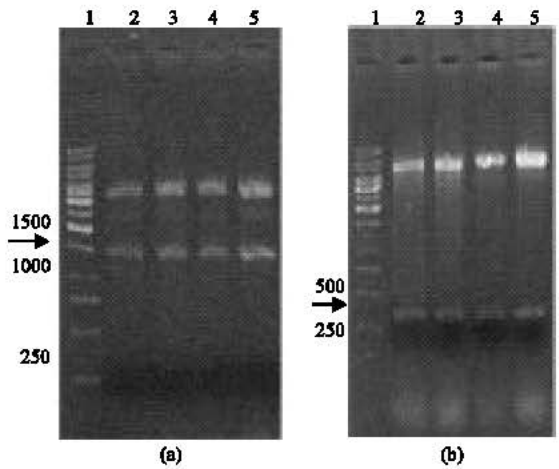

Fig. 4: Agarose gel electrophoresis of the pGEM-3Z vector with the insert by a) HindIII and XbaI restriction enzymes and by b) EcoRI restriction enzyme a) lane 1: ladder, lanes 2-5: HindIII digest of different colonies. Arrow head indicates position of the insert with an estimated size of ~1500 bp. b) lane 1: ladder, lanes 2-5: EcoRI digest of different colonies. Arrow head indicates position of the insert with an estimated size of 300 bp

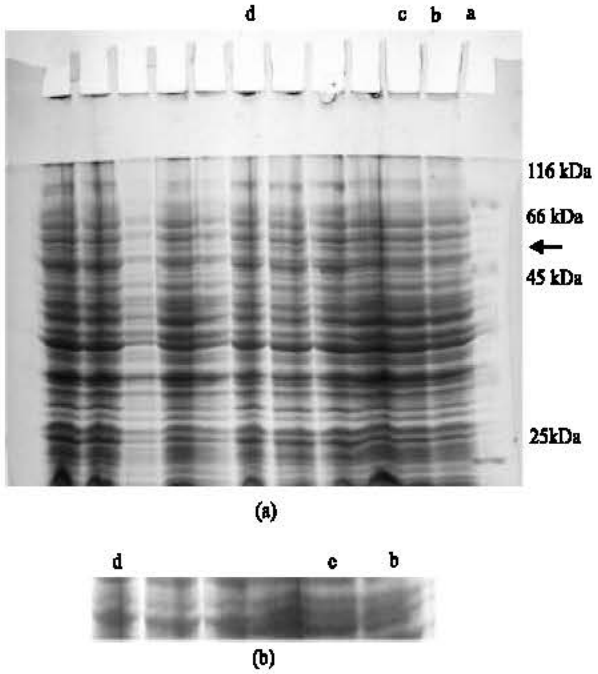

Fig. 5: SDS-PAGE analysis of the expressed phoA gene. Arrow head indicates position of a band with size of ~ 55 kDa related to the induced protein. lane a: protein molecular weight marker. lane b: protein band from the induced pGEM-3Z with the insert by addition of 0.1 mM IPTG. lane c: protein band from the induced pGEM-3Z with the insert by addition of 0.5 mM IPTG. lane d: protein band from the pGEM-3Z

we should test other expression methods and alter numerous parameters that are involved in protein expression system.

Regarding the DNA sequence of the cloned gene, we found a complete homology between this sequence and the ones present in the gene banks. Therefore it appears that the *E. coli* K-12 strain obtained from the Persian type culture collection is similar to the strain reported in the NCBI gene bank.

In conclusion we cloned the phoA gene and the plasmid with this gene can be used in protein expression, enzyme purification and mutagenesis to enhance catalytic activity of the enzyme. Since the phoA gene is ideal for

the construction of gene fusions for analysis of protein expression[19], a series of vector plasmids can be constructed suitable for fusion of secretory proteins to alkaline phosphatase[20].

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