Sys Rev Pharm 2020;11(7):15-17
A multifaceted review journal in the field of pharmacy

Expression of LM TK 1 and evaluation of its ability to activate nucleoside analogues in *E. coli*

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ABSTRACT

Thymidine kinase 1 is a central enzyme in synthesis of DNA precursors. In this study, *Listeria monocytogenes* thymidine kinase 1(LM TK1) gene was cloned into *E. coli* to determine the optimum conditions for expression and purification of LMTK1 enzyme. In addition, the enzyme's ability to activate some nucleoside analogues in bacteria and its effects on these bacteria were also assessed. *E. coli* BL21 (DE3) were transformed with pET expression vector carrying LM Tk1 gene. Expressed TK1 enzyme was purified using affinity chromatography with Ni-sepharose IMAC resin. The ability of the TK1 gene product for increasing susceptibility of Tk1-deficient *E. coli* toward nucleoside analogues was investigated. LM Tk1 gene cloning and expression in *E. coli* was successful. Results showed that the optimal induction time was at 4 hours. with specific activity of 307.668 u/mg. TK1 enzyme was readily purified and its molecular weight was 25 KDa. Tk1-deficient *E. coli* host showed variable sensitivity toward various nucleoside analogues.

Keywords: *Listeria monocytogenes*; Thymidine kinase 1; Cloning; Expression; Nucleoside analogues.

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Introduction

Listeria monocytogenes is a small gram-positive rod bacterium. It is a major cause of food-borne listeriosis in humans. All strains of *L. monocytogenes* possess various pathogenic potential, as some strains are very virulent, whereas others are non-infectious agents. [1,2] Accordingly, regarding food safety and public health, evaluating the pathogenic potential of *L. monocytogenes* is of great importance.[3]

Thymidine kinase is a member of the deoxyribonucleoside kinases family. During the S phase of the cell cycle, thymidine kinase is up-regulated and its presence in cells indicates strong cell proliferation and is therefore considered to be an important marker of tumour proliferation.[4,5] Gram negative bacteria (GNB) possess only one TK1, while GPB have many TK1. On the other hand, four essential dNKs are found in mammals.[6] Identification of virulent strains can be achieved through Thymidine kinases (TKs) that is found in prokaryotes, eukaryotes and different viruses. It can be found in nearly all living organisms as it plays a crucial role in thymidine salvage pathway. [7]"TK" catalyzes the irreversible phosphorylation of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP).[8] *E. coli*'s thymidine kinase was the first bacterial TK to be purified and characterized. *E. coli*-TK plays an important role in phosphorylation of deoxythymidine (dThd), deoxyuridine (dUrd) and some 5'-halogenated dUrd analogues. ATP and dGTP were classified as phosphate donors whereas dTTP were considered as feedback-inhibitor.[9] There are several probable factors that could be attributed to the variation between classes and orders regarding the presence of the gene. Such factors may include genetic distance, host and environmental factors, and nucleotide metabolism. Additional studies are required to expand our understanding of the reasons behind the presence or absence of TK1 gene in bacteria. [10] Stimulation of nucleoside analogues are activated by bacterial-BDRK in a species-specific manner. [11]

The objectives of the present work are to produce LM TK1 in large quantity via cloning LM TK1 gene in *E. coli*, purification of TK1 enzyme using affinity chromatography and to study the activation and lethal effect of some nucleoside analogues on bacteria.

MATERIALS AND METHODS

Extraction of genomic DNA from *L. monocytogenes*

Genomic DNA from the growth cultures was extracted and purified by using the manufacturer's protocol (Geneaid "Presto", Korea). The integrity of genomic DNA was confirmed by running DNA samples on 1% agarose gel for one hour at 70 volts.

Amplification of *tk*1 gene by PCR

The nucleotide sequence of *L. monocytogenes tk*1 gene was obtained from the NCBI website. The primers F: 5-TACCGTGTCAATAAAAAGGTT-3 and R:5-AATAAAATAACCACCTAACCGT were designed to amplify full length 576 bp of *tk*1 gene.

PCR reaction was carried out to amplify the gene by mixing 10 µl 10x thermopol buffer, 5µl of 2µM primer (forward), 5µl of 2µM primer (reverse), 1µl of 10 mM dNTP, 1µl template ,0.5 U vent polymerase and 27.5 µl of DNase-RNAse free deionized distilled water. The reaction mixture was cycled through the following temperature profile 94 ˚C 5min, 35 cycles of (94 ˚C 45 sec. , 58 ˚C 45 sec., 72 ˚C 1min.,) and 72 ˚C 10min. PCR product was analysed by applying sample to 1.5 % agarose gel and run at 70 V for one hour in an 1X TAE buffer and subsequently gel was documented with gel doc. PCR-product was purified on a spin column using IIIustra GFX PCR DNA and gel band purification kit from GE.Healthcare.

Cloning of *tk*1 gene

The purified PCR-product (*TK1* gene) was cloned using Champion PET Directional TOPO Expression Kit. *E. coli* Top10 competent cells were transformed with TOPO cloning reaction following standard molecular biology procedures.[12]

Competent cells were plated onto nutrient agar containing 0.1 mg/ml ampicillin and incubated overnight at 37 ˚C. Positive clones were identified using colony PCR with primers annealing to the TOPO vectors cloning site. Single colonies were incubated overnight in LB media containing 0.1 mg/ml ampicillin. Plasmid preparation was made using Fermentas GeneJet Plasmid miniprep kit according to the manufacturer's protocol. Plasmid samples were sequenced by Eurofins MWG operons, Germany.

Expression of TK 1 in *E. coli*

The plasmid containing the *TK1* gene from *L. monocytogenes* and a C-terminal 6X histidine tag sequence was introduced to *E. coli*-BL21 star (DE3) cells (from Invitrogen) via transformation. *E. coli* BL21 star (DE3) cells were incubated to grow to an OD600=0.5–0.7 at 37˚C and then inducted for 0 – 6 h at 25˚C with 100 mM IPTG. Cells were harvested by centrifugation and the pellets were frozen at -80˚C. The cell pellet was re-suspended in lysis buffer ((Tris/HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100 and 10% glycerol) with EDTA-free complete inhibitor cocktail (Roche diagnostics, Denmark) and disrupted by running the suspension twice through a French Press (1000 psi using a SLM Aminco French press from SLM Instrument). The lysate was centrifugated at 13000 rpm using rotor JA-25.50 at 4 ˚C for thirty minutes and the supernatant was filtered by using 0.45µm Millipore filter. Purification was carried out using affinity chromatography on ÄKTA Explorer 100 systems (GE Healthcare) with column consisting of Ni sepharose high nickel charged IMAC resin. Purity of protein was estimated with SDS-polyacrylamide gel electrophoresis whereas total protein concentration was determined by Bradford method using (BSA) as a standard.[13]

Estimation of enzyme activity

The activity of the purified Tk1 enzyme was estimated according to Stedt *et. al*. [14]

Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration was determined using nucleoside analogue – containing media and a kinase deficient *E. coli* cells transformed with pET expression vectors with and without insert of LM *tdk* 1 gene as described previously [14].

Results and Discussion

*L. monocytogenes tk*1 genes were amplified from genomic DNA successfully and cloned into pET expression vectors. This was confirmed by Colony PCR. The recombinant pET vector containing LM *tdk*1 gene was successfully transformed into *E. coli* BL21. After transformation, the cells were grown in ampicillin containing LB agar medium to confirm transformation.

The LM TK1 gene was over expressed to produce tdk1 protein by adding IPTG as an inducer for different periods 0–6 h. Results showed that the optimal induction time was at 4h with specific activity of 307.668 u/mg, while induction at (0, 1, 2, 3, 5, 6) were (37.0, 18.56, 258.67, 147.98, 245. 30, 274.66 u/ mg) respectively.

The protein was purified readily by using affinity chromatography containing nickel column. SDS–PAGE technique was effective for assessing both the purity and the molecular weight of the proteins. The molecular weight of purified recombinant protein was 25 KDa. Unlike previous studies that used P-GEX -2T expression vector to produce recombinant TK 1, in this study LM TK1 was cloned as his-tagged fusion protein into a pET vector which was then used to transform *E. coli* -TK- deficient hosts aiming to produce considerable amount of high purity protein appropriate for crystallization. The reason behind using pET vector for expressing the TK1 gene is that the expression is under the control of bacteriophage T7 RNA polymerase promoter which is a strong promoter that produces high level of transcription in *E. coli* [14]. According to the previous studies, bacterial TK1 expression by using P-GEX, which contain the weak *tac* promoter, resulted in lower amounts of recombinant protein compared to that of pET[15, 16]. Although *tk*1 gene expression by using pET vectors gives high target proteins amount, the recombinant protein produced was less soluble and had a greater tendency to precipitate. A previous study attempted expressing human TK1 using different pET vectors in several *E. coli* hosts had resulted in obtaining only insoluble protein with poor yield and activity [17]. On the other hand, in this study LMtk1 was purified successfully with reasonable level of purity. There are several benefits for selecting affinity chromatography for protein purification over other techniques. Affinity chromatography consists of a single step purification with high selectivity and binding capacity for the desired proteins. Furthermore, the purification process is robust and easy to conduct [18]. Several earlier studies have been performed to purify GST-tagged bacterial TK1 using affinity chromatography.

The LM *tdk*1 gene has been introduced into the TdK1-defficient *E. coli* strain then the host cells were subjected to a variety of nucleoside analogues (2,2-difluoro-deoxycytidine (gemcitabine,dFdC), 5-fluoro-deoxyuridine (5F-dU) and 3-azido-thymidine (AZT)) to test their effect . The sensitivity of bacteria toward these analogues was expressed as LD 100 which defined as the lowest concentration of the tested analogue that completely inhibits the bacterial growth. The MIC values in *E. coli* that has been transformed with pET vector that does not have the *tdk*1 gene (as a control) were very low on dFdC 2 µM, while transformed bacteria with pET-*tk*1 gene showed LD100 at 0.8 µM. Treatment with 5F-dU resulted in 16 µM of LD 100 in control and 2 µM. Finally, the LD 100 of (AZT) exposure was more than 100 µM in control and at 0.5 µM in transformed bacteria. Nucleoside analogues operate as antimetabolites due to their structural similarities to nucleosides. In which the analogues are phosphorylated by the same enzymes involved in salvage pathways of nucleosides and then incorporated as building blocks of the newly synthesized DNA strands [19,20]. Clinically, nucleoside analogues are very important compounds that can be used as antiviral and anticancer drugs. In fact, the nucleoside analogues mechanism of action that are activated have been described according to cancer research [21]. Initially, nucleoside analogues enter plasma membrane of cells by nucleoside transporter. After entering the cell, the nucleoside analogues go through multiple phosphorylation steps (diphosphorylated or triphosphorylated performed by NMPKs and NDPKs, respectively, that of cellular of viral origin) transforming into their active form [21]. The activated nucleoside analogues toxicity is executed by binding to the elongating DNA chain during DNA replication leading to the termination of the replication process. In addition, activated mono-, di- or triphosphorylated nucleosides may

act as inhibitors of human or viral enzymes such as DNA or RNA polymerases [22].

Acknowledgments
Many thanks to Dr. A. R. Clausen for their support, help and evaluation of cloning.

Conflict of interest.
I have no conflicts of interest to disclose.

REFERNCES

1. Liu D, Lawrence ML, Ainsworth AJ, Austin FW. (2007) Toward an improved laboratory definition of Listeria monocytogenes virulence. Int J Food Microbiol. 118(2):101–15. https://doi.org/10.1016/j.ijfoodmicro.2007.07.045
2. Liu D, Ainsworth AJ, Austin FW, Lawrence ML(2003) Characterization of virulent and avirulent Listeria monocytogenes strains by PCR amplification of putative transcriptional regulator and internalin genes. J Med Microbiol. 52(12):1065–70. https://doi.org/10.1099/jmm.0.05358-0
3. Zeinali T, Jamshidi A, Bassami M, Rad M (2016) Serogroup identification and Virulence gene characterization of Listeria monocytogenes isolated from chicken carcasses. Iran J Vet Sci Technol. 7(2):9–19. http://dx.doi.org/10.22067/veterinary.v7i2.43658
4. Jagarlamudi KK, Shaw M (2018) Thymidine kinase 1 as a tumor biomarker: technical advances offer new potential to an old biomarker. Biomark Med. 12(9):1035–48. https://doi.org/10.2217/bmm-2018-0157
5. Bi M-H, Han W, Liu J-J, Wang H-Y, Gao Z-Y, Tian W-L (2016) Clinical significance of serum thymidine kinase 1 (TK1) expression in patients with non-small cell lung cancer. Int J Clin Exp Med. 9(5):8536–42.
6. Sandrini MPB, Piškur J (2005) Deoxyribonucleoside kinases: two enzyme families catalyze the same reaction. Trends Biochem Sci. 30(5):225–8. https://doi.org/10.1016/j.tibs.2005.03.003
7. Sandrini MPB, Clausen AR, Munch-Petersen B, Piškur J (2006) Thymidine kinase diversity in bacteria. Nucleosides, Nucleotides and Nucleic Acids. 25(9–11):1153–8. https://doi.org/10.1080/15257770600894469
8. Okazaki R, Kornberg A (1964) Deoxythymidine kinase of Escherichia coli I. Purification and some properties of the enzyme. J Biol Chem. 239(1):269–74.
9. Carnrot C, Wehelie R, Eriksson S, Bölske G, Wang L (2003) Molecular characterization of thymidine kinase from Ureaplasma urealyticum: nucleoside analogues as potent inhibitors of mycoplasma growth. Mol Microbiol. 50(3):771–80. https://doi.org/10.1046/j.13652958.2003.03717.x
10. Sandrini MPB, Clausen AR, On SLW, Aarestrup FM, Munch-Petersen B, Piškur J (2007) Nucleoside analogues are activated by bacterial deoxyribonucleoside kinases in a species-specific manner. J Antimicrob Chemother. 60(3):510–20. https://doi.org/10.1093/jac/dkm240
11. Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM (1992) In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science (80-)256(5063):1550–2. https://doi.org/10.1126/science.1317968
12. Sambrook J, Russel DW (2001) Molecular Cloning: a laboratory manual. Cold Spring Harbor, New York
13. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72(1–2):248–54. https://doi.org/10.1016/00032697(76)90527-3
14. Stedt H, Samaranayake H, Kurkipuro J, Wirth G, Christiansen LS, Vuorio T, et al. (2015) Tomato thymidine kinase-based suicide gene therapy for malignant glioma—an alternative for Herpes Simplex virus-1 thymidine kinase. Cancer Gene Ther. 22(3):130. https://doi.org/10.1038/cgt.2014.76
15. Khan Z, Knecht W, Willer M, Rozpedowska E, Kristoffersen P, Clausen AR, et al. (2010) Plant thymidine kinase 1: a novel efficient suicide gene for malignant glioma therapy. Neuro Oncol. 12(6):549–58. https://doi.org/10.1093/neuonc/nop067
16. Christiansen L, Egeblad L, Munch-Petersen B, Piškur J, Knecht W (2015) New variants of tomato thymidine kinase 1 selected for increased sensitivity of E. coli KY895 towards Azidothymidine. Cancers (Basel). 7(2):966–80. https://doi.org/10.3390/cancers7020819
17. Birringer MS, Perozzo R, Kut E, Stillhart C, Surber W, Scapozza L, et al. (2006) High-level expression and purification of human thymidine kinase 1: quaternary structure, stability, and kinetics. Protein Expr Purif. 47(2):506–15. https://doi.org/10.1016/j.pep.2006.01.001
18. Schäfer F, Seip N, Maertens B, Block H, Kubicek J (2015) Purification of GST-tagged proteins. In: Methods in enzymology. Elsevier. p. 127–39. https://doi.org/10.1016/bs.mie.2014.11.005
19. Knecht W, Petersen GE, Munch-Petersen B, Piškur J (2002) Deoxyribonucleoside kinases belonging to the thymidine kinase 2 (TK2)-like group vary significantly in substrate specificity, kinetics and feed-back regulation. J Mol Biol. 315(4):529–40. https://doi.org/10.1006/jmbi.2001.5257
20. Clausen AR, Al Meani SAL, Piškur J (2010) Pasteurella multocida thymidine kinase 1 efficiently activates pyrimidine nucleoside analogs. Nucleosides, Nucleotides and Nucleic Acids. 29(4–6):359–62. https://doi.org/10.1080/15257771003729716
21. Young JD, Yao SYM, Baldwin JM, Cass CE, Baldwin SA (2013) The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. Mol Aspects Med. 34(2–3):529–47. https://doi.org/10.1016/j.mam.2012.05.007
22. Jordheim LP, Durantel D, Zoulim F, Dumontet C (2013) Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. Nat Rev Drug Discov. 12(6):447–64. https://doi.org/10.1038/nrd4010