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Cloning and Induction of *E. coli* Pyruvate Kinase by IPTG

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Abstract: Pyruvate Kinase is a key enzyme in the glycolytic pathway. In almost every cell type it controls the flux through the pathway, together with the phosphofructokinase-1 and hexokinase. *E.coli* strain DH5ά was grown in liquid broth (LB) medium at 37°C. pET-26b (Novagen) was propagated in DH5ά in 200 ml LB medium supplemented with appropriate antibiotic, either 80 µg/ml ampicillin and 25- µg/ml Kanamycin, respectively. Strain BL21 (DE3) which contain T7 RNA polymerase gene integrated in its chromosomal was used for expression of recombinant protein. PCR amplification of Pyk gene was carried out with chromosomal DNA isolated from *E.coli* DH5ά as a template. The kinetic of induction of the expression of pyruvate kinase by IPTG or Lactose was followed after induction at various times from 1-5 hours. The level of pyk kinase induced was determined on 12%SDS_PAGE electrophoresis. Purity of the enzyme was examined by 12% sodium dodecyl sulfate gel electrophoresis (SDS-Page). Results of the present work indicate that pyruvate kinase (pyk) gene of *E.coli* can be used for the induction and purification of pyruvate kinase enzyme. The present study has provided a new insight into a new method for cloning and induction of *E. coli* pyruvate kinase by Isopropyl-1-thio-ß-D-galactopyranoside (IPTG).

Key words: Pyruvate kinase · *E. coli* · Induction and purification-Isopropyl-1 · Thio-ß-D-galactopyranoside (IPTG)

INTRODUCTION

The polymerase Chain Reaction (PCR) method of in vitro DNA amplification was invented by Saiki *et al.* [1], and was developed by Mullis and Faloona [2]. The technique is based on the heat stability of some DNA polymerases like the *taq* polymerase of *Thermus aquaticus* or *pfu* of *Pyrococcus furiosus*, microorganisms livening in thermal vents in the ocean. These polymerases are stable up to more than 100°C. With recent advanced in PCR technology, deoxyribonucleoside triphosphates (dNTPs) have become idispensable reagents used in polymerase chain reaction for cloning and diagnostic purposes in addition to Tag DNA polymerase [3]. 2-Deoxynucleoside triphosphates (dNTPs) are important reagents used in polymerase chain reaction. To enzymatically synthesis dNTP from deoxynucleoside monophosphate (dNMP), three purified enzymes, namely cytidine Monophosphatekinase (CMK), nucleoside diphosphate kinase (NDK) and pyruvate Kinase (Pk) from *Escherichia coli* are used for enzymatic synthesis of deoxycytidine triphosphate (dCTP), using deoxycytidine monophosphate (dCMP) as a substrate and adenosine triphosphate (ATP) as a phosphoryl donor [4]. Pyruvate Kinase is a key enzyme in the glycolytic pathway. In almost every cell type it controls the flux through the pathway, together with the phosphofructokinase-1 (PFK) and hexokinase [5,6,7]. This enzyme catalyzes the essentially irreversible transphosphorylation from phosphoenolpyruvate (PEP) and ADP to pyurvate and ATP [8, 9, 10]. Almost all organisms at least have one pyruvate kinase gene and one enzyme. However the Arabidopsis genome initiative recently indicate that *A.thaliana* contains at least seven genes that encode different polypeptides [11,12].The two most studied allosteric pyk isoenzymes are those of *E.coli* [13] and *S.typhimurium* [14] type I (pykF) and typeII (pykA). PEP/puryvate kinase is the most efficient system for the generation of ATP from ADP. The phosphorlating agent PEP can be prepared in a mole scale [15, 16]. The aim of this work is the induction and purification of pyk enzyme using pyk gene of *E.coli.*

MATERIALS AND METHODS

***E.coli* Strains, Plasmids and Culture Media:** *E.coli* strain DH5ά was grown in liquid broth (LB) medium at 37°C. pET-26b (Novagen) was propagated in DH5ά in

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200 ml LB medium supplemented with appropriate antibiotic, either 80 μg/ml ampicillin and 25 μg/ml Kanamycin, respectively. Strain BL21 (DE3) which contain T7 RNA polymerase gene integrated in its chromosomal was used for expression of recombinant protein.

Materials: Restriction enzymes *N*deI, *X*hoI, T4 DNA ligase, Ni-NTA resin was obtained from Qiagen. Tag DNA polymerase was obtained from Dragon Egg Biolab. Isopropyl-1-thio-ß-D-galactopyranoside (IPTG), PEP, B-NADH and other chemicals were purchased from Sigma. The designed primers were purchased from ku-vector.

Isolation of the Chromosomal Dna and Plasmids from *E.coli*: Chromosomal DNA from *E.coli* strain DH5ά was isolated by Triton-Prep method. Briefly, after growing 50 ml of cell culture in LB medium overnight, cells were harvested by centrifugation at 4,000 rpm and resuspended in 9 ml STET buffer (8% sucrose, 5% Triton X-100 and 50 mM Tris-HCl [pH 8.0]. After addition of lysozyme (1 mg) and RNase (10 μg), the reaction mixture was boiled for 1 min and centrifuged at 10.000 rpm for 15 minute. The supernatant was extracted with equal volume of STET-saturated phenol. To the aqueous layer 0.1 volume of 4 M lithium chloride was added and placed in ice for 5 minute. After centrifugation at 10.000 rpm for 10 min, equal volume of isopropanol was added to supernatant. Chromosomal DNA was recovered by centrifugation at 10.000 rpm for 5 min and dissolved in TE buffer. The plasmid pET-26b was isolated from DH5 ά by the standard alkali method and purified as described by Sambrook *et al.* [17].

PCR Amplification and Cloning of pyk Gene: PCR amplification of Pyk gene was carried out with chromosomal DNA isolated from *E.coli* DH5ά as a template. Based on the nucleotide sequence of the pyk gene, the forward primer (pyk-1: 5-CCC<u>GAATTCCATATG</u>TCCAGAAGGCTTCGCAGA-3, where the underline indicate the added *E*coRI and *N*deI linker) and reverse primer (pyk-2: 5-CCC<u>CTCGAG</u>CTCTACCGTTAAAATACGCGT-3, where the underline indicate the added *X*hoI linker). In 10 μl of reaction mixture were contained DNA template 0.5 μl; forward and reward primers 0.25 μl; dNTP 0.8 μl; taq polymerase 1 μl; 10x polymerase buffer 1 μl and H_2O 6.2 μl. The reaction mixture initiated by denatureing at 94 C°for 5 min and subjected to 30 cycles of 1^{-min} denaturation at 94°C, 45 seconds annealing at 60°C and 2 min extension at 72°C. After 30 cycles, the reaction mixture was incubated for final extension at 72°C for 7min then cooled to 4°C. The amplified product was analyzed on 1% agarose gel and purified with PCR purification kit (Qiagen). The purified PCR product, corresponding to the Pyk gene, was double digested with *Nde*I and *Xho*I. The digested PCR product was ligated into pGEM-T, which had been previously double digested with *Nde*I and *Xho*I, using T4 DNA ligase. The ligation DNA product was transformed into competent *E.coli* DH5ά cell prepared by calcium Iodide protocol Colonies allowed to grow on LB agar plates contain 25μg/ml Kanamycin overnight at 37°C. To determine the transformed colonies contain the recombinant pGEM-pyk, the plasmid was purified with the alkali lysis method. The pyk gene was obtained by double digestion of pGEM-pyk with *Nde*I and *Xho*I. This pyk gene was ligated to pET-26, which had previously double digested with *NdeI* and *XhoI*. Ligation mixture was transformed into competent BL21 which prepared by calcium chloride method. Transformed cells were growth on LB agar plate containing 25μg/ml Kanamycin.

Comparison of the Level Expression of Pyruvate Kinase Induced by iPTG and Lactose: *E. coli* BL21 carrying pET-pyk was incubated into 50 ml of LB broth supplemented with 25-μg/ml Kanamycin. Cells were allowed to grow and reach OD_{600} of 0.5 in two separated flasks, the expression of recombinant pyruvate kinase was induced by addition of either IPTG at 1mM or Lactose 1- mM. After induction one milliliter of cells suspension were harvested by centrifugation at 4,000 rpm for 5 minutes. The kinetic of induction of the expression of pyruvate kinase by IPTG or Lactose was followed after induction at various times from 1-5 hours. The level of pyk kinase induced was determined on 12%SDS_PAGE electrophoresis.

Over Expression of the Recombinant Pyruvate Kinase: *E.coli* strain BL21 (DE3) harboring pET-pyk was grown at 37°C in 2L of LB containing 25-μg/ml Kanamycin. Overexpression was induced by addition of lactose to final concentration of 1- mM at OD_{600nm} of 0.5. After 3 hours of induction, cells were harvested by centrifugation at 5,000 rpm. The cells pellet was store at-70°C until use.

Purification of the Recombinant Pyurvate Kinase: The cell pellet was resuspended in lysis buffer (20mM Tris-HCL, pH 8.0; 50 mM KCL; 1mM EDTA; 50%Tween20; 1mM PMSF) and disrupted by sonication. Crude extract was clarified by centrifugation at 10.000 rpm for 20 minutes; pyurvate kinase consisted of 10% of total protein in cell free extract.

To cell free extract, ammonium sulfate (0-80% saturation) was added slowly. After 30 minutes of stirring, Protein was precipitated from cell free extract by centrifugation at 10.000 rpm at 4°C for 20 minutes. The pellet of Ammonium sulfate saturation was dissolved in 6 ml lysis buffer and dialyzed four times against 1L of 50-mM potassium phosphate buffer, pH 7.5 contained 0.1 mM of EDTA and then loaded onto 2 ml Ni^{+2}-NTA affinity column which had been equilibrated with buffer B (20 mM Tris HCL [pH 7.9], 5 mM imidazole, 500 mM NaCl and 0.1 %Triton x-100). The column was washed with five bed volumes of buffer B. Pyruvate kinase was subsequently eluted with three volumes of buffer B containing 20, 40, 60 and 100 mM imidazole respectively. The elute fraction were pooled and concentrated by ultrafiltration, using Amicon pressure cell. Purity of the enzyme was examined by 12% sodium dodecyl sulfate gel electrophoresis (SDS-Page).

Assay Activity of Pyruvate Kinase: The recombinant pyruvate kinase activity was determined using coupled spectrophotometric assays absorbance at 340 nm. The reaction mixture (0.5 ml) contained 10 mM Tris HCl [pH 7.5]; 50 mM KCl; 2 mM $MgCl_2$; 2 mM ADP; 2 mM phosphoenolpyruvate; 0.2 mM NADH and 5 U of partially purified pyruvate kinase and lactatedehydrogenase. The reaction was started by addition of crude cell extract or pure protein and the decrease in A_{340} was measured for 5 minutes.

RESULTS AND DISCUSSION

E.coli strain DH5ά was allowed to grow in 50 ml LB at 37°C. Cells were harvested by centrifugation at 4.000 rpm and resuspended in 9 ml STET buffer. After addition of lysozyme and RNaseA, the Chromosomal DNA was recovered as mentioned in the material and method and dissolved in 400 µl TE buffer. Atypical yield of DNA obtained was approximately 40 µg per 50 ml of cell culture. Jonathan *et al.* [18] studied the reconstitution and analysis of the multienzyme *Escherichia coli* RNA Degradosome. Analysis of size of chromosomal DNA isolated on 1% agarose gel electrophoresis indicated that they are high molecular weight (Fig. 1).

PCR Amplification of pyk Gene: According Mullis and Faloona [2] a prerequisite for the PCR is the knowledge of short stretches of DNA (RNA) which flank the regions of interest. In the case of the 16S rRNA, there are highly conserved sequences at the start (5' end) and at the end (3' end). These short sequences are chemically synthesized and used as primers that bind to the target sequence. PCR ampilification of pyk gene was carried out with chromosomal DNA of *E.coli* strain DH5 ά as a template. Based on the nucleotide sequence of the pyk gene, the forward primer pyk-1 and reverse primer pyk-2 were used, in 10 µl of the reaction mixture contained DNA template 0.5 µl; Forward and reward primers 0.25 µl; dNTP 0.8 µl; Taq polymerase 1 µl; 10x polymerase buffer 1 µl and H_2O 6.2 µl. The reaction mixture initiated by denatureing at 94°C for 5 min and subjected to 30 cycles of 1min denaturation at 94°C, 45 seconds annealing at 60°C and 2 min extension at 72°C. After 30 cycles the reaction mixture was incubated for final extension at 72°C for 7-min then cooled to 4°C. The amplified product was analyzed on 1% agarose gel and purified with PCR purification kit (Qiagen). Kenichiro *et al.* [12] could obtain the crystal structure of Pyruvate Kinase from *Geobacillus stearothermophilus*. In our study, approximately 4 µg of pyk gene was obtained (Fig. 2).

Construction of the Recombinant Vector Producing His_6-tagged Pyruvate Kinase: The purified PCR product, corresponding to pyk gene (1.450 bp), was ligated into the cloning vector pGEM-T. The ligation mixture was transformed into *E.coli* DH5á. One colony harboring recombinant plasmid pGEM-pyk was detected by restriction enzyme *Nde*1and *Xho*1 (Fig. 3). The data indicated that pyk gene had been successfully inserted into the multiple cloning site of pGEM-T. The isolated recombinant plasmid, pGEM-pyk was used after digestion with *Nde*1 and *Xho*1 restriction enzyme as a source of pyk fragment to be clone into the pET-26b *Nde*1-*Xho*1 sites giving the recombinant pET-pyk plasmid. Transformation of the ligation mixture into the competent *E.coli* BL21 that allowed growing on LB agar plate was supplemented with 25 µg/ml kanamycin. Plasmid from these colonies was isolated by the alkali lysis miniprep method. Moreover, pyk gene is detected by the double digestion. Our findings are agreed with those reported by Jonathan *et al.* [18] who studied the reconstitution and analysis of the multienzyme *Escherichia coli* RNA Degradosome.

Comparison of the Level Expression of Pyruvate Kinase Induced by IPTG and Lactose: *E.coli* BL21 carrying pET-pyk was incubated into 50 ml of LB broth supplemented with 25 µg/ml kanamycin. Cells were

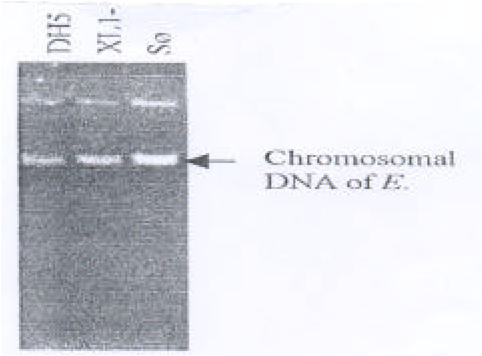

Fig. 1: Analysis of the size of chromosomal DNA isolated from *E.coli* strain DH5 α′

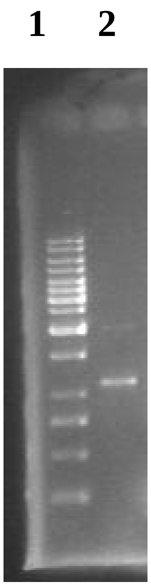

Fig. 2: Analysis of PCR product from amplification of Pyk gene from *E.Coli* chromosomal DNA Lane 1 DNA marker Lane 2 PCR product (pyk gene).1450 bp

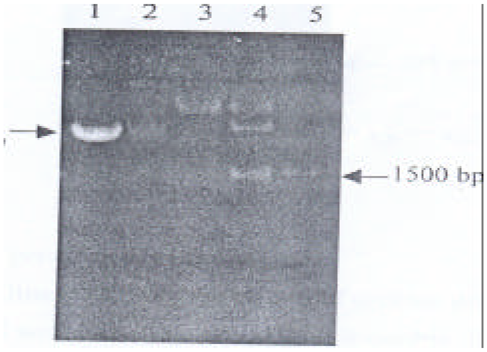

Fig. 3: Detection of pyk gene insert in pGEM-pyk with double digestion. Lane 1 pGEM-T linear (3.0 kb); lane 2, PGEM-pyk no digestion, lane 3 pGEM-pyk digested with X*h*o1, Lane 4 pGEM-pyk double digestion with N*de*1 and X*h*o1, Lane 5 1500 DNA fragment

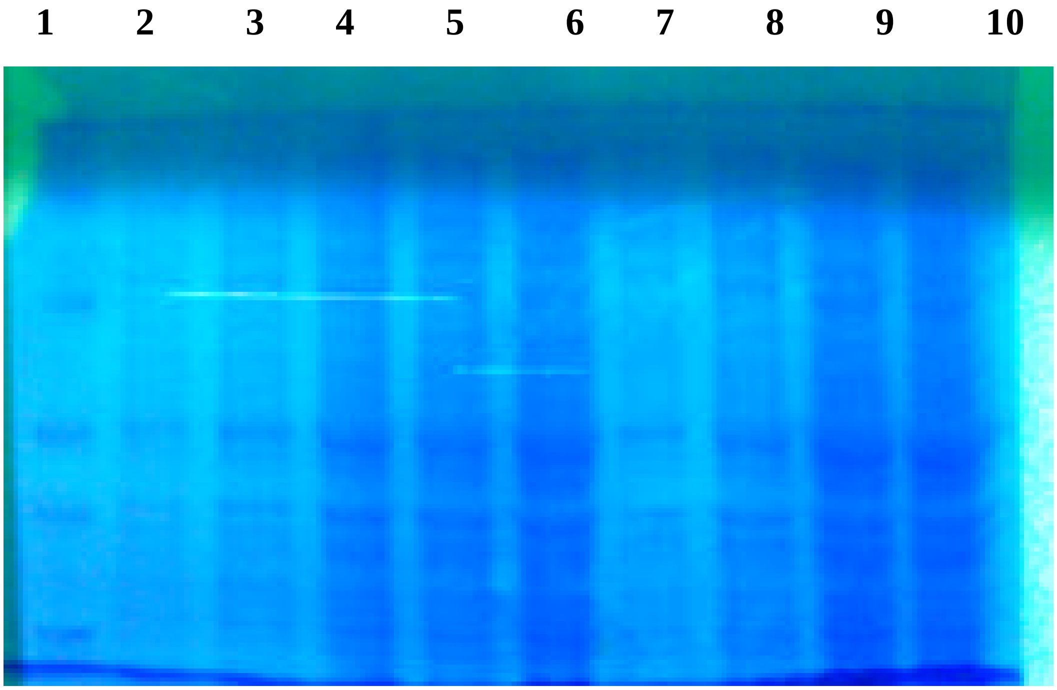

Fig. 4: Comparison of effectiveness of induction of the expression of the pyk gene byIPTG and lactose.

Lanes, 1, 2 controls; lanes, 3, 4, 5 IPTG; Lanes, 6, 7, 8,9,10 Lactose lanes

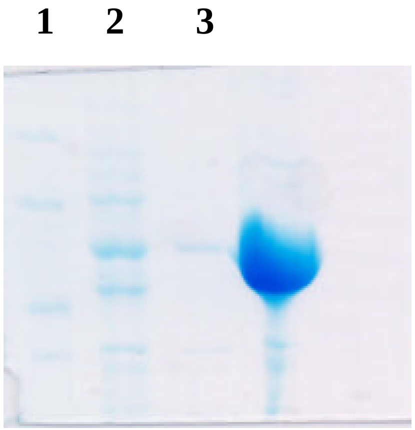

Fig. 5: Recombinant pyruvate kinase purified using NI-NTA affinity column

Lane 1 crude extract, lane 2 washing buffer, lane 3 recombinant pyk

Table 1: Summery of the purification of pyruvate kinase from *E. coli*. One unit of enzyme is the amount that catalyzes the phosphorlyation of 1 μmole of ADP per minute at 37°C

Steps	Total volume (ml)	Total Protein (mg)	Total unit (Unit)	Specific activity (U/mg)	Fold purification
Cell free extract	28	260	62	0.24	1.0
0-80% NH_3SO_4	24	280	380	1.36	5.7
Ni-NTA affinity	5	20	830	41.50	173.0

allowed to grow and reach OD600 of 0.5 in two separated flasks, the expression of recombinant pyruvate kinase was induced by the addition of 1mM IPTG or lactose. Seonghun Kim and Sun Bok Lee [19] reported that the soluble expression of archaeal proteins in *Escherichia coli* could be obtained by using fusion-partners. The result showed that kinetic of induction of pyk by IPTG occurred rapidly after one hour of induction. However, the kinetic of induction of pyk by lactose occurred at much slower rate (Fig. 4). Moreover, after three hours of induction the amount of pyk reached almost at high level as induced by IPTG. Thus, these result showed that lactose at 1mM can be used as a substitute induce for IPTG for effective induction of pyruvate kinase.

Purification of the Recombinant Pyruvate Kinase: *E.coli* BL21 harboring pET-pyk was grown at 37°C in 2 L LB containing 25 μg/ml kanamycin. Over expression of pyruvate kinase was induced by the addition of lactose 1 mM at OD600 of 0.5. After three hour of induction, cells were harvested by centrifugation at 10.000 rpm. and 3 grams of cells were obtained. The cell pellet was resuspended in 20 ml lysis buffer and disrupted by sonication. Crude extract was clarified by centrifugation at 10.000 rpm for 20 minutes. To crude extract, ammonium sulfate (0-80% saturation) was added slowly. After 30 minutes of stirring, protein was precipitated from the cell free extract by centrifugation at 10.000 rpm at 4°C for 30 minutes. The pellet of 0-80% ammonium sulfate saturation was dissolved in 6 ml of lysis buffer and dialyzed four times against 1L of 50 mM potassium phosphate buffer, pH 7.5 contained 0.1 mM EDTA and then loaded onto 2 ml Ni-NTA affinity column which had been equilibrated with the lysis buffer. The column was washed with five volumes of the lysis buffer. Pyruvate kinase was subsequently eluted with three volumes of lysis buffer containing 20, 40, 60 and 100 mM imidazole. Alejandro Yevenes and Perry A. Frey [20] studied the cloning, expression, purification, cofactor requirements, and steady state kinetics of phosphoketolase-2 from *Lactobacillus plantarum*.

The data obtained showed that the enzymes began to elute at 20 mM imidazole and consisted of major band migrated at 55 kDa the recombinant pyruvate kinase purified was analyzed for purity on 15 %SDS-page (Fig. 5).

Recombinant pyruvate kinase activity was determined spectrophotometrically at wavelength 340 nm using couple enzyme assays. The reaction mixture (0.5 ml) contained 10 mM Tris HCl [pH 7.5]; 50 mM KCl; 2 mM mgCl2; 2 mM ADP; 2 mMphosphoenolpyruvate; 0.2 mM NADH and 5 U of partially purified pyruvate kinase of crude cell extract and decrease in A340 was measured for 5 minutes.

Results of the present work indicate that pyruvate kinase (pyk) gene of *E.coli* can be used for the induction and purification of pyruvate kinase enzyme.

In conclusion, the present study has provided a new insight into a new method for cloning and induction of *E. coli* pyruvate kinase by Isopropyl-1-thio-ß-D-galactopyranoside (IPTG).

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