Cloning and Characterization of D-xylulose Kinase from *Kocuria gwangalliensis* Strain SJ2

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D-Xylulose is phosphorylated to D-xylulose-5-phosphate by D-xylulose kinase before it enters glycolysis via the nonoxidative pentose phosphate pathway. A gene encoding a novel D-xylulose kinase (XK) from *K. gwangalliensis* strain SJ2 was sequenced and expressed in *E. coli*. The sequence of the isolated XK gene was 1,419 bp, encoding 472 amino acids. The XK protein was more closely related to the *Arthrobacter phenanthrenivorans* XK than to the *Bifidobacterium catenulatum* one, as reflected in the sequence identity (54.9% vs. 38.7%). The XK gene was subcloned into the pCold-II expression vector. The resulting plasmid was transformed into *E. coli* strain BL21 (DE3) cells and the expression of the recombinant XK protein was induced by the addition of IPTG. The resulting protein was expressed as a fusion protein of approximately 48 kDa containing a N-terminal six-histidine extension that was derived from the expression vector. The expressed protein was homogenized by affinity chromatography and showed enzymatic activity corresponding to D-xylulose kinase. XK enzyme kinetic studies with D-xylulose and ATP showed a *Km* of 250±20 μM and 1,300±50 μM, respectively. The results obtained from this study will provide a wider knowledge base for the characterization of D-xylulose kinase at the molecular level.

**Key words**: Characterization, D-xylulose kinase, enzyme kinetic, gene cloning, *Kocuria gwangalliensis*

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**Introduction**

In nature, D-xylose occurs mainly in the polysaccharide form as xylan, arabinobiose, gluconoarabinoxyran, xyloglucan and xylogalacturan. Mixed linkage D-xylans are also found in certain seaweed species and a similar polysaccharide is thought to make up the backbone of psyllium gum. Free D-xylose is found in guava, pears, blackberries, loganberries, raspberries, aloe vera gel, kelp, echinacea, bosophiella, broccoli, spinach, eggplant, peas, green beans, okra, cabbage and corn. It is the second-most common saccharide in the natural world [1, 2, 4, 13]. D-xylose undergoes different pathways in prokaryotes and eukaryotes to become D-xylulose and then convert to D-xylulose-5-phosphate. In prokaryotes, D-xylose isomerase transforms D-xylulose into D-xylulose; however, D-xylose converts into D-xylose via a two-stage oxidation-reduction reaction in yeast or fungi.

D-xylose is deoxidized to D-xylitol by D-xylose reductase, and then D-xylitol is oxidized by dehydrogenase to D-xylulose. D-xylulose is phosphorylated to D-xylulose-5-phosphate by D-xylulose kinase and ATP. This phosphorylated intermediate is then metabolized by the pentose phosphate and Embden-Meyerhof-Parnas pathway [6, 7, 8].

The bacterial gene for D-xylulose kinase (xylB) was first cloned from *Escherichia coli* in 1984 [5, 9]. In 1989 Ho and Chang [3] reported cloning the *Saccharomyces cerevisiae* gene for D-xylulose kinase by complementing an *E. coli* xylB deficiency.

This research isolated the XK gene coding for D-xylulose kinase (KM986628) from *Kocuria gwangalliensis* strain SJ2, a marine bacterium that generates a pink pigment. The gene was cloned into the pCold-II expression vector and the recombinant protein was expressed in *E. coli* BL21 (DE3) cell. We then examined the purification and characterization of the expressed D-xylulose kinase protein.

**Materials and Methods**

**Bacterial strains and growth conditions**

A pink-orange pigmented bacterial strain, *K. gwangalliensis* strain SJ2 [11], was isolated from seawater collected on the Gwangalli coast of Busan. This strain was isolated on...
nutrient agar medium (Difco, USA) and maintained on PIPPE-II medium containing (L-1) 1 g Bacto-tryptone (Difco, USA), 1 g Bacto-Soytone (Difco, USA), 1 g Bacto-yeast extract (Difco, USA), 0.01 g ferric citrate, 2 g poly peptone (Difco, USA), and 30 g NaCl (Junsei, JPN) by serial inoculation.

Escherichia coli strain XL-l-blue [F’; Tn10 proA+ B- lacIq Δ (LacZ) M15/reaA1 endA1 gyrA96 thi hsdR17 (rK- mK+) supE44 relA1 lac] was used for the gene cloning procedures, and BL21 (DE3) cell [F- ompT hsdSB (rB-mB) dcm gal (DE3) tonA] was used for the expression of the recombinant protein.

Genomic DNA extraction and construction of cosmid library

Bacterial genomic DNA was extracted from K. gwangalliensis strain SJ2 with an AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea) and the following procedure. Bacterial cells (1-2 ml, OD600 = 0.8-1.0) were harvested by centrifugation at 13,000 rpm for 1 min. After centrifugation, the supernatant was removed and pellet was completely resuspended in 200 μl of cell lysis buffer. Proteinase K (20 μl) was added to the tube and the mixture was inverted several times until mixed well. After incubating the reaction at 56 °C for 15 min, 100 μl of isopropanol was added and completely mixed by pipetting. Cell lysates were loaded on a binding column attached to a collection tube and centrifuged at 13,000 rpm for 1 min. In addition, the lysate, 500 μl of washing buffer was added and centrifuged at 8,000 rpm for 1 min. Genomic DNA was eluted with 150 μl of elution buffer by centrifugation at 13,000 rpm for 1 min. In addition, the manufacture of the cosmid genomic library modified the part to construct the genomic library.

Cloning of D-xylulose kinase gene from K. gwangalliensis

To screen the D-xylulose kinase (XK) gene from the K. gwangalliensis strain SJ2 cosmid genomic library, primers were designed from conserved sequences of previously identified XK genes obtained from the National Center for Biotechnology Information (NCBI) nucleotide and protein sequence database. Degenerative oligonucleotides designed based on the conserved sequences and synthesized by GenoTech (Korea) were used to screen for the XK gene. The oligonucleotide sequences used in this study are shown in Table 1.

The XK gene was amplified by PCR using each upstream (XK-F1, XK-F2) and downstream (XK-R1, XK-R2) primer. Pfu DNA Polymerase was used for primer extension reactions. The amplified gene was subcloned into the vector pGEM-T (Promega) and its nucleotide sequence was confirmed by DNA sequencing.

Comparative sequence analysis of D-xylulose kinase gene

To examine the molecular evolution of D-xylulose kinase from K. gwangalliensis, the following sequences were imported from the GeneBank database. The nucleotide sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). A multiple sequence alignment was performed using Clustal W2 (http://www.ebi.ac.uk/clustalw2), and sequence identities were calculated using GeneDoc (http://www.pcs.edu/biomed/gendoc). A phylogenetic tree was constructed from the amino acid sequences encoded by the XK gene according to the neighbor-joining method with the program Treecon [12].

D-xylulose kinase overexpression

To overexpress the cloned D-xylulose kinase gene in E. coli, the coding region of the cloned DNA was amplified by PCR using a pair of oligonucleotides containing the restriction sites NdeI and HindIII (Table 1). After enzyme digestion with NdeI and HindIII, the pCold-XK recombination DNA was constructed from the PCR product and the pCold

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Table 1. Oligonucleotide primers used for this study

| Primer | Sequence | Remark |
|--------|----------|--------|
| XK-F1  | 5'-GCTCGCATCGTGAGCGAGCA-3' | Target gene cloning forward primer |
| XK-F2  | 5'-ATGTCGGCAGGTGTTGGG-3' | |
| XK-R1  | 5'-ACGGAATCTGCGAAGCGGCGG-3' | Target gene cloning reverse primer |
| XK-R2  | 5'-TGTCGCTAGCTGTTTTC-3' | |
| XKFN  | 5'-CATATGTCGCCAAGCGGTT-3' | XK expression (NdeI) |
| XKRH  | 5'-AAGCTTGGCTAGCTGTTTTC-3' | XK expression (HindIII) |
vector and transformed into BL21 (DE3) cells. The transformed cells were cultured in LB medium containing 50 μg/ml of ampicillin at 37°C until OD600 = 0.5, and then the recombinant protein was overexpressed by shaking the culture at 16°C after adding 0.1 mM of IPTG (isopropyl-β-thiogalactopyranoside). The protein was ran on a with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for analysis.

**Refinement of D-xylulose kinase through His-tag affinity chromatography**

To purify the D-xylulose kinase, the overexpressed, transformed cell culture was centrifuged at 3,000 rpm for 30 min at 4°C, and the pellet was completely resuspended in 20 ml of buffer A (20 mM Tris-HCl, 0.5 M NaCl, 1 mM NaN3, pH 7.9). The mixture was sonicated three times for 30 s each using an Ultra sonicator under 90~100% power and then centrifuged at 13,000 rpm for 10 min. The supernatant was filtered through 0.22 μM filter [11].

The D-xylulose kinase was then purified via affinity chromatography and a His-Trap chelating HP-column (GE Healthcare, USA). First, the column was equilibrated with buffer A and then the extracted protein sample was injected into the column. After injection, the other proteins were separated from D-xylulose kinase with Buffer B (Buffer A containing 40 mM Imidazole). Then D-xylulose kinase was extracted from the column with Elution buffer (Buffer A containing 100 mM Imidazole).

**Identification of D-xylulose kinase through western blot analysis**

All the protein in the cell lysates, including D-xylulose kinase, was separated on a 12% SDS-PAGE, and the gel was transferred to a nitrocellulose membrane by a semi-dry method. The membrane was placed on a plate and soaked in a solution of PBS buffer and 5% skim milk. After blocking the membrane in a rotator for 1 hr, the skim milk was discarded and the membrane was washed with PBS buffer three times for 10 min each. Subsequently, a polyclonal antibody against mouse anti-6-Histidine (diluted 1:500) was added to the membrane and incubated for 1h at room temperature. After three washes with PBS, the membrane was incubated with anti-mouse antibody conjugated with alkaline phosphatase (Sigma; diluted 1:2,000 in PBS, USA) at room temperature for 30 min. A solution of 20 ml alkaline phosphate buffer with 200 μl each of NBT and BCIP was added to the membrane. The plate was then covered with foil and allowed to react for 30 min.

**In vitro enzymatic assay**

Cells were grown in synthetic complete medium on 20 g L⁻¹ glucose and extracted with glass beads. D-xylulose kinase activity was assayed in a medium containing 50 mM Pipes K pH 7.0, 100 mM KCl, 5 mM MgCl₂, 5 mM ATP, 0.2 mM NADH, 1 mM phosphoenol pyruvate, and pyruvate kinase 5 U ml⁻¹ (Boehringer. GER). The reaction was started by the addition of D-xylulose (Sigma, USA) to a final concentration of 10 mM. The concentration was calculated from the substrate absorbance change at 340 nm when the reaction was completed after 15 min.

The Michaelis-Menten constants were measured using the partially purified enzyme, where no background ATPase activity could be detected. The constants for D-xylulose and ATP were measured in a medium containing 50 mM Pipes K pH 6.5, 100 mM KCl, 5 mM MgCl₂, 0.2 mM NADH, 1 mM phosphoenol pyruvate, pyruvate kinase 5 U ml⁻¹ and 1 mM D-xylulose. The reaction was started by addition of D-xylulose. The assay was performed with different concentrations of purified enzyme at 30°C for 6 hr under constant shaking in the dark.

**Results and Discussion**

D-Xylulose kinase gene cloning from K. gwangalliensis strain SJ2

The genomic DNA extracted from K. gwangalliensis strain SJ2 was verified that higher than 10⁸ titer. The extracted DNA was used to clone the XK gene through PCR with degenerate primers targeting a relatively conserved domain after comparing the homogeny of the gene base sequence of D-xylulose kinase previously revealed in bacteria (Table 1). The ORF (open reading frame) of the cloned XK gene was 1,419 bp, and the codon TGA sequence was positioned at 1,417-1,419 bp. The expected molecular weight (MW) and isoelectric point of XK gene were 48.1 kDa and 8.64, respectively, and XK gene expressed a D-xylulose kinase composed of 472 amino acids (NCBI accession No. KM986628).

Several programs are available to predict the secondary structure. We selected the SABLE website and TMpred (Prediction of Transmembrane Regions and Orientation) to predict the secondary structure. Fig. 1 shows the XK protein secondary structure prediction. It includes the location of α-
helices, β-sheets, and random coils.

**Amino acid sequence analysis of D-xylulose kinase**

The nucleotide and deduced amino acid sequences of the XK gene from *K. gwangalliensis* were analyzed. Amino acid sequence identity was determined using the GeneDoc program. The deduced amino acid sequence for the XK gene showed homology and a high degree of sequence conservation with previously identified XK enzymes from other species (Fig. 2). Molecular phylogenetic analysis revealed the evolutionary relationship among the XK genes of various organisms (Fig. 3). The bacterial D-xylulose kinase whose homology with XK is highest was D-xylulose kinase from *Arthrobacter phenanthrenivorans* (NCBI accession No. YP004240341), which showed 54.9% of homology. The lowest homology came from D-xylulose kinase from *Bifidobacterium catenulatum* (NCBI accession No. ZP03324559), which showed 38.7% homology.

**Expression of D-xylulose kinase in E. coli**

To identify the expression of the XK gene and optimized expression time in *E. coli*, BL21 (DE3) cells transformed with pCold-XK were allowed to grow until OD₆₀₀ = 0.5, and then overexpression was induced with IPTG. The overexpression
Fig. 2. Multiple alignment of deduced amino acid sequences of XK and other bacteria. The amino acid sequences are obtained from GenBank: Arthrobacter phenanthrenivorans (YP004240341), Candidatus Aquiluna (ZP09962252), Synoeca cyanus (ZP09745127), Saccharothrix xinjiangensis (ZP09984153), Amycolatopsis mediterranei (YP0003762961), Intrasporangium calcum (YP004099328), Nocardiopsis dassonvillieri (YP003679324), Thermobispora bispora (YP003650776), Solanum roseum (YP00336129), Thermobifida fusca (YP289682), Gordonia terrae (ZP09798456), Bifidobacterium catenulatum (ZP03324559), Clavibacter michiganensis (YP001710782), Kocuria gwangalliensis (KM986628). The amino acids are shaded in different colors of grey, which indicate the degree of consensus between the different sequences. "-" non-conserved amino acids.

was induced at 0 time, 1 hr, 3 hr, 5 hr, 7 hr, 9 hr, and overnight, and the expression pattern was analyzed on a 12% SDS-PAGE. The optimized expression time of D-xylulose kinase from pCold-XK was about 5 hr after the IPTG addition. The molecular weight of the kinase samples averaged 48 kDa, as predicted by the amino acid sequence analysis, and six histidines were included in the N-terminal.

Purification of the D-xylulose kinase

The pCold-XK plasmid was overexpressed in BL21 (DE3) cells with IPTG. After a 5 hr induction period, the protein was extracted and affinity chromatography was conducted. The graph having passed the affinity column is shown in Fig. 4, and the extracted XK protein was analyzed on a 12% SDS-PAGE, as shown in Fig. 4(A). The western blot showed
the XK protein to be about 48 kDa (Fig. 4B).

**Enzyme activity of D-xylulose kinase**

The purified D-xylulose kinase was assayed with pyruvate kinase to measure the production rate of ADP. The $K_m$ for D-xylulose and ATP was 250±20 μM and 1,300±50 μM, respectively, (Fig. 5A, B). The apparent $V_{max}$ values of the reactions were about 540 nkat mg⁻¹ (540 with D-xylulose, 460 with ATP). $K_m$ and $V_{max}$ were obtained from Hanes-Woolf plots of the presented data. All the data was obtained under similar conditions at the pH 6.5.

In a preliminary study, the authors’ laboratory first reported the separation and identification of K. gwangalliensis, which produces pink-orange pigment, and conducted the cloning of the D-xylulose kinase gene from the organism. D-xylulose kinase is an enzyme that transforms D-xylulose
into D-xyulose-5-phosphate, and it is involved in the production of ethanol through the pentose phosphate and EMP pathways. *K. gwangalliensis*’s D-xyulose kinase gene has an ORF of 1,416 bp and codes for 472 amino acids. By analyzing the amino acid base sequence of the enzyme and the evolutionary flexibility of D-xyulose kinases identified in other species, homogenies of 54.9% with D-xyulose kinase from *A. phenanthrenivorans* and 38.7% with D-xyulose kinase from *B. catenulatum* were shown.

The current study showed an optimized expression time of 5 hr and a size of about 48 kDa for the recombinant protein. The enzyme kinetics studies showed a $K_m$ of 250±20 μM and a $V_{max}$ of 540 nkat mg$^{-1}$ with 5 mM ATP for D-xyulose and a $K_m$ of 1,300±50 μM and a $V_{max}$ 460 nkat mg$^{-1}$ with 1 mM D-xyulose for ATP. The results obtained from this study will provide a wider base of knowledge on the primary structure and characterization of the D-xyulose kinase at the molecular level.

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초록: *Kocuria gwangalliensis* strain SJ2에서 유래된 D-xylulose kinase 유전자와 특성

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D-Xylulose는 nonoxidative pentose phosphate 경로를 통해 glycolysis 과정으로 들어가기 전에 D-xylulose kinase에 의해서 D-xylulose-5-phosphate로 인산화 된다. *K. gwangalliensis* strain SJ2로부터 D-xylulose kinase (XK)를 암호화하는 유전자는 *E. coli*를 이용하여 서열분석 및 발현 하였으며, XK 유전자의 염기서열 1,419 bp로 구성되어 있으며 463개의 아미노산 잔기를 암호화하고 있다. 분석결과를 통해 XK 유전자가 전화과정 동안 잘 보존되었음을 보여 주었다. XK 유전자 발현을 위해 pCold-II 발현 벡터에 클로닝 하였으며 클로닝 된 플라스미드는 *E. coli* strain BL21 (DE3)에 형질전환 하여 IPTG를 이용해 발현을 유도하였다. 재조합 된 XK 단백질의 크기는 약 48 kDa이었다. 이 발현된 단백질은 affinity chromatography를 이용하여 정제하였으며 D-xylulose kinase에 따른 enzymatic activity를 분석하였다. D-xylulose와 ATP로 실험한 XK enzyme kinetic 연구는 각각 250±20 μM과 1,300±50 μM의 Km value를 보였다. 본 연구를 통해 얻어진 결과는 분자적 수준에서 D-xylulose kinase의 특성 연구의 보다 넓은 지식적 기초를 제공할 것으로 사료된다.

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