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Stress-Governed Expression and Purification of Human Type II Hexokinase in *Escherichia coli*

JEONG, EUN-JU[1], KYOUNGSOOK PARK[1], SO YEON YI[1], HYO-JIN KANG[1], SANG J. CHUNG[1], CHANG-SOO LEE[1], JIN WOONG CHUNG[2], DAI-WU SEOL[3], BONG HYUN CHUNG[1], AND MOONIL KIM[1]*

[1]*BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-600, Korea*
[2]*Laboratory of Immunology, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-600, Korea*
[3]*Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, U.S.A.*

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Abstract The full encoding sequence for human type II hexokinase (HXK II) was cloned into the *E. coli* expression vector pET 21b and expressed as a C-terminally hexahistidine-tagged protein in the BL21 (DE3) strain. The IPTG-induced HXK II approximately accounted for 17% of the total *E. coli* proteins, and 81% of HXK $II_{6\times His}$ existed in inclusion bodies. To improve the production of soluble recombinant HXK II protein, in the functionally active form, we used low temperature, and the osmotic stress expression method. When expressed at 18°C, about 83% of HXK $II_{6\times His}$ existed in the soluble fraction, which amounted to a 4.1-fold yield over that expressed at 37°C. The soluble form of HXK $II_{6\times His}$ was also highly produced in the presence of 1 M sorbitol under the standard condition (37°C), which indicated that temperature downshift and low water potentials were required to improve the yield of active recombinant HXK II protein. The expressed protein was purified by metal chelate affinity chromatography performed in an IDA Excellose column charged with Ni^{2+} ions, resulting in about 40 mg recombinant HXK II protein obtained with purity over 89% from 5 l of *E. coli* culture. The identity of HXK $II_{6\times His}$ was confirmed by Western blotting analysis. Taken together, using the stress-governed expression described in this study, human active HXK II can be purified in sufficient amounts for biochemical and biomedical studies.

Keywords: Human type II hexokinase, expression, purification, low temperature, osmotic stress

Glycolytic metabolism of glucose is a major pathway for the generation of energy (ATP) in all organisms from the microbial to mammalian. In bacteria, yeast, animals, and plants, hexokinases (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) not only catalyze sugar phosphorylation as the first step of hexose metabolism but also sense glucose levels and transmit the sugar signal to the nucleus [18, 19, 21]. Recent studies indicate that the mitochondria-associated hexokinase plays an important role in the control of apoptosis in mammals [2, 3, 9]. The mitochondrial pathway of apoptosis is initiated through release of mitochondrial cytochrome *c* into the cytosol through the permeability transition pore (PTP) in response to cellular stresses [5, 10, 11].

There are four different enzymes distinguishable from each other by its kinetic properties and different tissue distribution, designated as types I, II, III, and IV in humans [6, 15, 24]. The deduced amino acid sequences of the hexokinase have shown high similarity between those from a variety of organisms, indicating evolutionary conservation of hexokinase across species. Types I, II, and II isozymes show molecular weight of about 100 kDa, whereas type IV hexokinase (commonly named as glucokinase) has a molecular mass about 50 kDa [4, 12, 25]. Given the amino acid similarity of types I, II, and III isozymes between the N- and C-terminal regions, the evolutionary hypothesis of mammalian hexokinases that the types I-II isozymes resulted from duplication and fusion of a gene encoding an ancestral 50-kDa type IV hexokinase has been suggested. For example, the type II hexokinase is a 100-kDa protein with both N- and C-terminal halves possessing the catalytic function [1, 23].

Studies have been demonstrated that the type II hexokinase is highly expressed in many tumors and bound to the outer mitochondrial membrane [13, 20]. The type II hexokinase contains hydrophobic N-terminal sequences (15 amino

*Corresponding author
Phone: 82-42-879-8447; Fax: 82-42-879-8594;
E-mail: kimm@kribb.re.kr

acids) necessary and sufficient for its binding to the voltage-dependent anion channel (VDAC) [22], enabling the hexokinase to target mitochondria. Recent studies have demonstrated that mitochondrial bound hexokinase prevents Bax-induced cytochrome *c* release, whereas dissociation of the mitochondrial type II hexokinase promotes cytochrome *c* release and induction of apoptosis [14]. Although more studies and substantial investigations are needed to confirm the role of the type II hexokinase in cancer biology, much attention has been paid to the link between glucose metabolism and apoptosis.

In general, inclusion bodies are improved via *in vitro* denaturation and renaturation processes; however, a large fraction of enzymes is lost during the processes [17]. In this study, we describe an efficient expression and purification system to obtain biologically active human type II hexokinase from inclusion bodies expressed in *E. coli*. In particular, a stress-governed expression strategy including low temperature, and an osmotic pressure expression were applied to improve the production of soluble hexokinase using immobilized metalion affinity chromatography (IMAC).

MATERIALS AND METHODS

Strains, Vectors, and Enzymes

E. coli strain DH5α was used as the host for subcloning and *E. coli* BL21 (DE3) (Novagen, WI, U.S.A.) for gene expression. The *E. coli* strain was grown in LB medium at 37°C and 50 mg/ml ampicillin was added for plasmid-containing strains. pBluescript SK+ (Stratagene, CA, U.S.A.) was used as the vector for subcloning and amplification of the full-length gene, and pET 21b (Novagen, WI, U.S.A.) for expression. Restriction enzymes and modifying enzymes were purchased from Boehringer-Mannheim (Germany) and used according to the recommendations of the supplier. A preparation of vector DNA was carried out using an QIAEX II gel extraction kit (Qiagen, Germany).

Expression of Recombinant Protein

The construct of pHXK $II_{6\times His}$ was transformed into *E. coli* DH5α. Ampicillin-resistant colonies were screened by DNA isolation of 3-ml overnight cultures followed by restriction mapping. Plasmid DNA was prepared and purified by a QIAprep spin miniprep kit (Qiagen, Germany). Recombinant protein expression was performed as described previously [16]. Briefly, plasmids were transferred to expression host, *E. coli* BL21 (DE3) (Stratagen, CA, U.S.A.), and plated on LB plates. A single colony from a fresh plate was picked and grown at 37°C in 3 ml of Luria-Bertani broth, containing 100 mg/ml ampicillin until OD_{600}=0.6. They were inoculated in 100 ml of LB with ampicillin. Cells were grown at 37°C with shaking until OD_{600}=0.6. Cells were induced with 1 mM isopropyl-2-D-thiogalactopyranoside (IPTG) (GibcoBRL, NY, U.S.A.) and grown for 4 h. Cells were then harvested by centrifugation at 6,000 ×*g* at 4°C for 10 min. To compare the solubility of HXK $II_{6\times His}$ under different expression conditions, cells were subjected to the by low temperature (18°C) and osmotic stress (1 M sorbitol) expression method. Cells were harvested, and adjusted to the same OD value at 600 nm. Harvested cells were resuspended in 50 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The crude cell lysates were separated into total, soluble, and insoluble fractions, which were analyzed by 10% SDS-PAGE. The solubility was determined as soluble protein/total expressed protein ×100%.

Affinity Purification of Recombinant Protein

The transformants were harvested from 5-l culture medium, resuspended in 50 mM Tris-HCl buffer (pH 8.0), and lysed by sonication. IDA Excellose (particle size: 125–210 μm, Bioprogen Co., Korea) was poured onto a chromatographic column, and the column was saturated with nickel ions. A 50 mM solution of metal was applied to the column in the form of sulfate salt to remove unbound metal ions. The column was washed with distilled water and finally with 50 mM Tris-HCl/0.5 M NaCl/0.5 M imidazole buffer (pH 8.0). The solution containing HXK $II_{6\times His}$ was loaded onto the column. For the elution of proteins, the column was washed with 50 mM Tris-HCl/0.5 M NaCl buffer (pH 8.0) and a continuous imidazole gradient was applied by the gradual mixing of 50 mM Tris-HCl/0.5 M NaCl buffer (pH 8.0) containing 0.5 M imidazole with 50 mM Tris-HCl/0.5 M NaCl buffer (pH 8.0). After elution, the yield of purified HXK $II_{6\times His}$ was quantified by the Bradford method [7] and the identity of HXK $II_{6\times His}$ was confirmed by Western blotting using the polyclonal antibody of HXK II.

Measurement of Hexokinase Activity

Hexokinase activity was measured by the spectrophotometric assay, in which G-6-P formation is coupled to NADPH production, and monitored at 340 nm in the presence of excess G-6-P dehydrogenase, as described previously [26]. The assays were conducted in 1 ml of reaction buffer [40 mM Tris-HCl, pH 8.5, 6.7 mM $MgCl_2$, 6.6 mM ATP, 3.3 mM glucose, 10 mM 1-monothioglycerol, 0.5 mg/ml NADP, and 1 unit of NADP-dependent glucose-6-phosphate dehydrogenase (Sigma, MO, U.S.A.)].

SDS-PAGE and Western Blotting

E. coli cells were harvested and suspended in 50 mM Tris-HCl buffer (pH 8.0). After cell disruption by sonication, soluble and insoluble fractions were separated by centrifugation at 10,000 ×*g* for 10 min. The total cell lysate, and soluble and insoluble fractions were resolved on a 10% SDS gel

and transferred onto the nitrocellulose membrane. The gel was stained with Coomassiae Brilliant Blue R250. Quantitative protein determination was made using a densitometer (Bio-Rad Model GS-700, CA, U.S.A.) to read band intensities of Coomassie stained gels that were scanned in at a grayscale pixel density of 300 dpi on a scanner (HP ScanJet 6100C, CA, U.S.A.). Protein concentrations were determined by the Bradford method with bovine serum albumin as a standard. Western blotting analysis was performed as described previously [8] using antibody for human type II HXK (Santa Cruz, CA, U.S.A.)

Results and Discussion

Construction of the Expression Vector pET 21b-HXK II

For the construction of the pET 21b-HXK II expression vector, the full-length gene encoding human type II hexokinase (2,754 bp, 100 kDa) was first amplified with the 5 primer (CGG AAT TCA TGA TTG CCT CGC ATC TGC) and the 3 primer (ACT AAG CTT TCG CTG TCC AGC CTC ACG) using a polymerase chain reaction (PCR) with the synthesized cDNA as a template. The 5' and 3' termini were designed to contain EcoRI and HindIII restriction enzyme cleavage sites, respectively. The PCR products were purified using a DNA purification kit (Qiagen, Germany) and digested with the indicated restriction enzymes. The resulting DNA fragments were ligated with a pET 21b vector using a ligation kit (Takara, Japan) (pHXK $II_{6\times His}$), and cloned into the pET 21b vector using EcoRI and HindIII sites to generate in-frame fusion of pET 21b-HXK II, in which the hexahistidine peptide consisting of six histidine residues genetically fused to the C-terminal end of the recombinant protein. This modification facilitates purification of the target protein by immobilized metalion affinity chromatography (IMAC). After cloning of the pET 21b-HXK II, the HXK $II_{6\times His}$ fusion gene was confirmed by DNA sequencing. The map of the recombinant pET 21b-HXK II expression vector is presented in Fig. 1A.

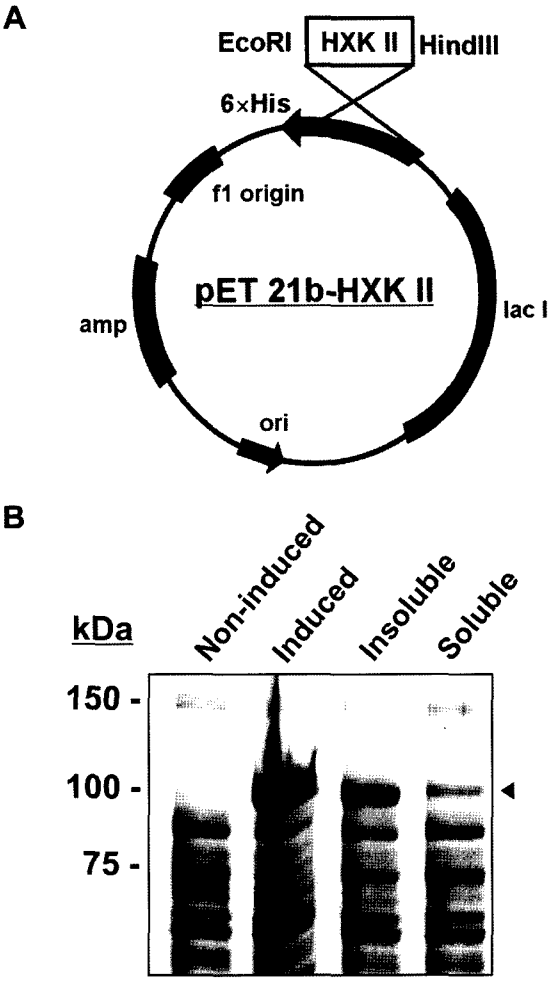

Fig. 1. Construction of the pET 21b-HXK II vector and expression of HXK $II_{6\times His}$.

A. The map of recombinant plasmid pET 21b-HXK II for expression of HXK $II_{6\times His}$. amp, ampicilline resistant gene; ori, origin of replication. **B**. SDS-PAGE analysis of recombinant HXK $II_{6\times His}$ protein. Lane 1, total cell lysates of non-induced BL21 (DE3); lane 2, total cell lysates of IPTG-induced BL21 (DE3); lane 3, insoluble fraction; lane 4, soluble fraction. About 50 μg of protein was loaded per lane. The positions and molecular masses of protein marker are indicated. The arrow indicates the expressed HXK $II_{6\times His}$ protein.

Expression of the Recombinant HXK II

After IPTG induction, there was an obvious additional band around the molecular weight of 100 kDa, which is consistent with the expected molecular weight of HXK $II_{6\times His}$, when compared with the *E. coli* strain containing the control plasmid pET 21b (Fig. 1B). The expression of recombinant HXK $II_{6\times His}$ accounted for about 17% of total cellular proteins; however, about 81% of HXK $II_{6\times His}$ existed in inclusion bodies (Fig. 1B). In this study, to improve the production of soluble, biologically active recombinant HXK II, low temperature cultivation and osmotic stress expression were employed. As shown in Fig. 2, when recombinant HXK $II_{6\times His}$ was expressed at 18°C, the soluble HXK $II_{6\times His}$ increased about 4.1-fold compared with that at 37°C. When recombinant HXK $II_{6\times His}$ was expressed at 37°C with 1 M sorbitol for osmotic stress, 78% of HXK $II_{6\times His}$ appeared in the soluble fraction, as determined by soluble protein/total expressed protein ×100%, which resulted in about 3.8-fold more yield than that expressed in the absence of sorbitol under the standard condition (37°C), indicating that stress conditions such as low temperature expression and low water potentials were required to improve the yield of soluble recombinant HXK $II_{6\times His}$ protein. However, when HXK $II_{6\times His}$ was expressed at 18°C with 1 M sorbitol, the solubility was similar to that of the low temperature alone (data not shown), indicating

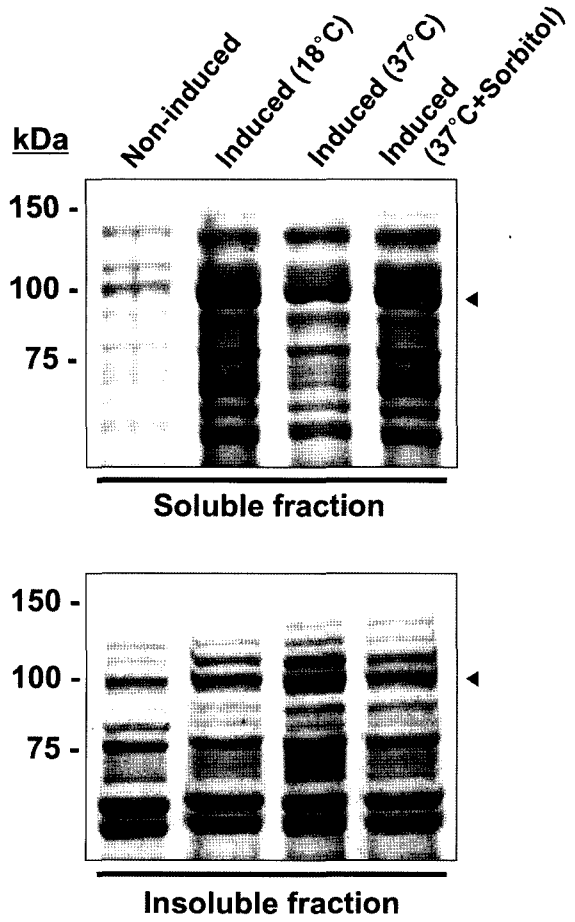

Fig. 2. SDS-PAGE analysis of recombinant HXK $II_{6\times His}$ protein under the different expression conditions.
Lane 1, cell lysates of non-induced BL21 (DE3); lane 2, cell lysates of IPTG-induced BL21 (DE3) at 18°C; lane 3, cell lysates of IPTG-induced BL21 (DE3) at 37°C; lane 4, cell lysates of IPTG-induced BL21 (DE3) at 37°C with 1 M sorbitol. About 50 μg of protein was loaded per lane. The positions and molecular masses of protein marker are indicated. Soluble fractions (upper panel), insoluble fractions (lower panel). The arrows indicate the expressed HXK $II_{6\times His}$ protein.

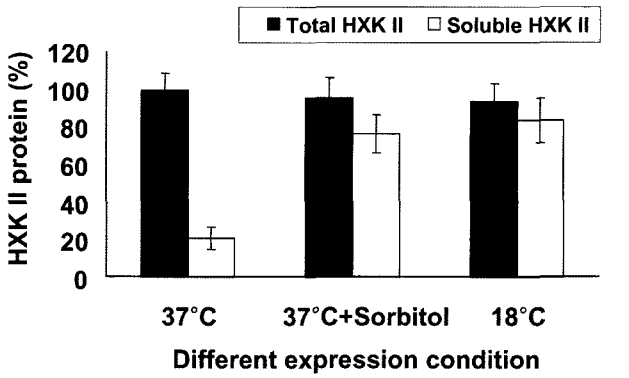

Fig. 3. Analysis of solubility and yield of HXK $II_{6\times His}$ under the different expression conditions.
The solubility and yield of HXK $II_{6\times His}$ protein were determined by densitometric analysis of SDS-PAGE. The estimates were averaged among triplicate independent induction samples.

that this increase of soluble yield at low temperature with osmotic pressure is not synergistic, which may be due to the saturation of the protein translation system. These results were validated by the densitometric quantification analysis of SDS-PAGE (Fig. 3). Since lowering the expression temperature facilitates the correct folding of HXK $II_{6\times His}$, and improves the production of soluble HXK $II_{6\times His}$, in the functionally active form, it is thought that low temperature contributed to the solubility of HXK $II_{6\times His}$. During the environmental or physiological stress conditions such as temperature downshift or osmotic pressure, translations slow, which may provide more chance for the target protein and its refolding enzymes to proceed with protein folding. Moreover, upon the low growth conditions, the *E. coli* translational apparatus undergoes modifications, allowing the selective translation of the transcripts of cold shock-induced genes, while bulk protein synthesis is drastically reduced.

Purification of the Recombinant HXK II

The recombinant HXK $II_{6\times His}$ protein was purified using metal chelate affinity chromatography performed in an IDA Excellose column charged with Ni^{2+} ions. Purification of the recombinant HXK $II_{6\times His}$ protein was carried out according to the manufacturer's instructions. Five l of the cell lysate was loaded onto an IDA Excellose affinity column (Bioprogen Co., Korea), and the column was washed three times with 100 ml of equilibration buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 8.0). The recombinant protein was eluted with 200 ml of 0.5 M imidazole in the equilibration buffer, and dialyzed in dialysis buffer (50 mM Tris-HCl, pH 8.0) for 48 h at 4°C. The recombinant HXK $II_{6\times His}$ fraction collected was concentrated using ultrafiltration (MW cutoff: 10,000) to the final concentration of 0.5 mg/ml and kept in an ice bath. After the recombinant HXK $II_{6\times His}$ protein expressed in *E. coli* was purified to homogeneity, the catalytic activity of the homogeneous recombinant HXK II was obtained by the spectrophotometric assay in which G-6-P formation is coupled to NADPH production, monitored at 340 nm. The purification results are summarized in Table 1. As a result, the final purity and the recovery yield were 89% and 19% respectively. The specific activity of the purified recombinant HXK $II_{6\times His}$ displayed approximately 20 times higher than the glucose-phosphorylation activity of the control sample without recombinant HXK $II_{6\times His}$, measured by a spectrophotometric assay in which G-6-P formation is coupled to NADPH production, indicating that the structural modification of the hexahistidine tags on the C-terminal end of the protein did not contribute to the functional properties of human type II hexokinase. Fig. 4A shows the chromatographic profile of the recombinant HXK $II_{6\times His}$ protein by immobilized

Table 1. Purification of human type II hexokinase expressed in *E. coli.*

	Total protein (mg/l)	HXK II (mg/l)	Purity (%)	Recovery (%)
Total extract	245	42	17	100
Affinity chromatography	9	8	89	19

metal ion affinity chromatography (IMAC). For the elution of HXK $II_{6\times His}$, a continuous imidazole gradient was applied by the gradual mixing of 50 mM Tris-HCl/0.5 M NaCl buffer (pH 8.0) containing 0.5 M imidazole with 50 mM Tris-HCl/0.5 M NaCl buffer (pH 8.0). After elution, the identity of HXK $II_{6\times His}$ was confirmed by SDS-PAGE (Fig. 4B) and Western blotting (Fig. 4C) using polyclonal antibody of HXK II. As shown in Figs. 4B and 4C, an apparent molecular mass of HXK $II_{6\times His}$ is approximately 100 kDa, which corresponded to that of the expected molecular mass of human type II hexokinase estimated using the Peptide Mass program (ExPASy Proteomics tools, http://www.expasy.org). Finally, the densitometric quantification analysis of SDS-PAGE was also used to validate the purity of the purified recombinant HXK $II_{6\times His}$ proteins. Using the low temperature (18°C) cultivation and expression method, the final yield of functionally active HXK $II_{6\times His}$ protein reached around 40 mg from 5 l of *E. coli* culture, with purity of over 89% (Table 1).

In conclusion, we used the low temperature culture and expression method to improve the solubility of HXK $II_{6\times His}$, as 81% of recombinant HXK $II_{6\times His}$ protein deposited as inclusion body in *E. coli.* When expressed at 18°C, and treated with 1 M sorbitol under the standard condition, the solubility of HXK $II_{6\times His}$ was significantly increased to 83% and 79%, respectively. The effect of the significantly reduced metabolic rate on the folding of recombinant protein can offer an explanation for the yield increase of soluble proteins by the stress-governed expression method. Taken together, using the method described in this study, human HXK II can be purified in sufficient amounts for biochemical and biomedical studies.

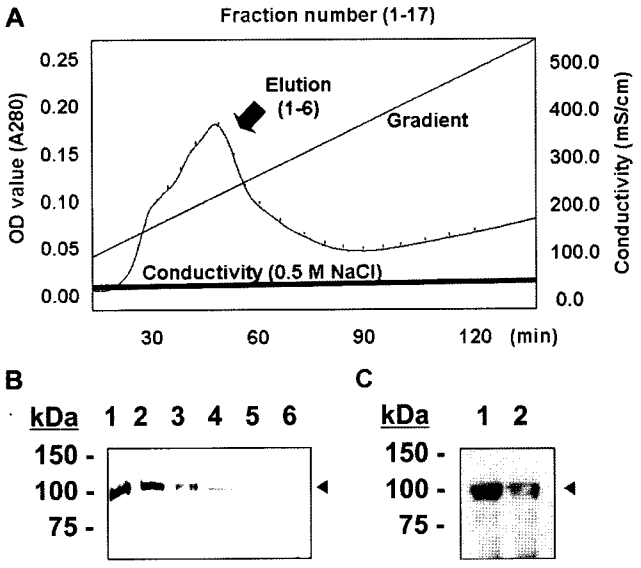

Fig. 4. Purification of recombinant HXK $II_{6\times His}$ protein.
A. Chromatographic profile of the recombinant HXK $II_{6\times His}$ protein by immobilized metal ion affinity chromatography (IMAC) using hexahistidine tags at the C-terminal end. The arrow indicates the HXK II-containing fractions. The detailed procedure is described under Materials and Methods. **B.** Purified HXK $II_{6\times His}$ was analyzed on 10% SDS-PAGE. Purified protein was visualized by Coomassie staining (fractions 1–6). **C.** This identity was confirmed by Western blotting using polyclonal antibody of HXK II. Lane 1, 2 μg of purified HXK $II_{6\times His}$; lane 2, 1 μg of purified HXK $II_{6\times His}$. The arrows indicate the expressed HXK $II_{6\times His}$ protein.

Acknowledgments

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