

Enhanced Expression and Purification Strategy for Recombinant Bacterially-Expressed Human Hexokinase II

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ABSTRACT. Dengue virus hijacks the host cellular mechanism to propagate and survive during viral infection, in which the central carbon mechanism plays a crucial role to upregulate DENV infection through the increase of human hexokinase II (HKII) activity. Since the enzyme governs the glycolytic pathway, it has potentials as a target for anti-dengue (DENV) drug development. In this study, the production of human hexokinase II protein has been enhanced by using bacterial system for anti-dengue therapeutic purpose. The HKII gene was cloned into pET28b vector and transformed into the *E. coli* strain BL21 (DE3) for HKII expression. In order to obtain soluble recombinant HKII in an active form, we optimized protein expression under specific conditions at 18 °C for 19 hours using Terrific Broth media, in the presence of 0.5 mM isopropyl-2-D-thiogalactopyranoside (IPTG). The pET28b-HKII construct expressed in BL21 (DE3) system exhibited adequate protein expression, thus, this construct was subsequently proceeded to purification process. The expressed protein was purified to homogeneity by a combination of Immobilized Metal Ion Affinity Chromatography (IMAC) and size exclusion chromatography (SEC), resulting in pure, active bacterially-expressed HKII with a specific activity of 80.90 U.mg⁻¹. The amount of HKII obtained from 2 L culture is 80 mg, with a yield percentage of 10.50%. Hence in this study, human HKII has successfully been cloned and expressed as a soluble protein that can be utilized for further therapeutic studies.

Keywords: Gene cloning, glycolysis, human Hexokinase II protein expression, protein purification.

INTRODUCTION

Hexokinase is a crucial enzyme that governs the glycolytic pathway of all organisms, ranging from bacteria, yeast, and plants to humans and other vertebrates. Hexokinase is the controller of the first rate-limiting step of the intracellular glucose metabolic pathway, which catalyzes the conversion of hexose to hexose-6-phosphate using MgATP as a phosphoryl donor, thereby permitting glucose entry and its utilization in all major metabolic pathways of cells (Schaffingen, 2020). In addition, HK is an important regulator of cell death (Roberts, & Miyamoto, 2015). The four mammalian HKs (ATP: D-hexose-6-phosphotransferase; EC 2.7.1.1) are designated as I, II, III, and IV. These enzymes, although highly conserved in amino acid sequences, differ in molecular mass, tissue distribution, regulation, and catalytic properties. HKI-HKIII have molecular masses of ~100 kDa and a relatively high affinity for glucose, and are subject to feedback regulation by physiological levels of glucose-6-phosphate (G-6-P) (Schaffingen, 2020). HKIV, more commonly referred to as glucokinase (GCK), has a molecular mass of 50 kDa, is primarily located in the liver and pancreatic β -cells, has a lower affinity for glucose and is not subject to feedback regulation by physiological levels of G-6-

P (Lynedjian, 2013). The difference in molecular mass between GCK and HKI-HKIII led to the suggestion that the latter arose from gene duplication and tandem ligation of a GCK-like precursor (Berg, 2012;). The amino acid sequences of hexokinases I-III are 70% identical, and structural analyses have shown that the N and C-terminal halves of isoforms I-III possess similar amino acid sequences, probably as a result of gene duplication and tandem fusion (Ruzzo, Andreoni, & Magnani, 2008). Furthermore, another study revealed that among all mammalian hexokinase isoenzymes, HKII most closely resembled the ancestral 100 kDa, which contained 60% similar amino acid sequence in both N and C terminals, compared to the similarity percentage of type I and III hexokinases (Postic, Shiota, & Magnuson, 2010; Nawaz, Ferreria, & Rabeh, 2018). It has been noted that both halves of this isoenzyme contain a catalytic site and an open reading frame of 2751 bp, encoding a protein of 917 amino acids, with highly similar N (1474) and C (475-971) terminal domains (Ruzzo et al., 2008; Nawaz et al., 2018). HKs are also characterized by significant differences in kinetic features, regulatory properties, intracellular distribution, and patterns of expression that purportedly suit individual isoforms for distinct metabolic roles within cells (Shinohara et al., 2007).

Some specialized functions of individual hexokinase isoforms have already been identified (Stryer, 2008;), but the coexistence of multiple hexokinase isoforms within a single tissue, or within a given cell, remains incompletely defined (Shinohara et al., 2007). When examined, most mammalian tissues and cell types, including tumor cells, have been found to express multiple hexokinase isoforms (Postic et al., 2010; Coy et al., 2009; Shinohara et al., 2007). The high-affinity isoforms HKI, HKII, and HKIII, each have K_m values in the micromolar range and are characterized by sensitivity to feedback inhibition by their principal reaction product, glucose-6-phosphate. In contrast, HKIV (also referred to as glucokinase or GK) exhibits a relatively low affinity for glucose (K_m 6mM), is approximately half the size of its high-affinity counterparts, and represents the only mammalian hexokinase isoform that is not inhibited by glucose-6-phosphate (Stryer, 2008; Shinohara et al., 2007).

An important fundamental distinguishing feature of the mammalian HKI and HKII isoforms involves their unique capacity to directly interact with mitochondria, both physically and functionally. This property is not shared by the mammalian HKIII or GK (HKIV) isoforms or by yeast hexokinases. As such, HKI and HKII are often referred to as mitochondrial hexokinases. Hexokinase activity in both soluble and particulate fractions of mammalian tissue extracts has been recognized for over 50 years (Liberti, & Locasale, 2016), where the particulate fraction can be largely accounted for by specific HKI and HKII binding to the OMM at mitochondrial contact sites. This binding is both dynamic and regulated (Wilson, 2003) and involves specific interaction between the outer membrane of mitochondria OMM voltage-dependent anion channel (VDAC) and hydrophobic mitochondrial binding domains found in the amino-terminus of HKI and HKII, but not HKIII or GK (Azoulay, Israelson, Abu-hamad, & Shoshan, 2004; Hay, & Sonenberg 2014; Liu, Yang, Wang, & Tu, 2018). Mammalian VDAC directly participates in the specific binding of mitochondrial hexokinases to the OMM (Ritter, Wahl, Freund, Genzel, & Reichl, 2010; Fontaine, Sanchez, Camarda, & Lagunoff, 2015; Jin, Meihong, Dagang, & Yujin, 2016). Unlike their mammalian counterparts, yeast hexokinases do not bind mitochondria, and yeast mitochondria do not bind hexokinases (Shinohara et al., 2007).

There is considerable variation in the proportion of hexokinase activity bound to mitochondria in different cell types and tissues, with the majority found in the bound form in some tissues, such as the brain and kidney (Tanbin et al., 2019; Ritter et al., 2010). Mitochondrial hexokinase activity is also increased in many tumors, where it has been reported to correlate with *in vivo* tumor growth rates (Tanbin et al., 2019; Ritter et al., 2010). In the last decade, many seminal studies have emphasized the importance of metabolic re-programming in cancer biology. HK-II upregulation

has been suggested to be a major contributor to elevated glycolysis, even in the presence of oxygen, in cancer, as first reported by Otto Warburg in 1930 (Warburg effect). During tumor development in tissues normally expressing HK-IV, gene expression of HK-II is induced, whereas HK-IV is silenced (Stryer, 2008;). HK-II expression levels are closely associated with tumor grade and mortality in hepatocellular carcinoma (Fontaine et al., 2015). Although in normal brain and low-grade gliomas HK-I is the predominant isoform, HK-II is highly upregulated in human glioblastoma multiform (Jin et al., 2016), and poor prognosis is associated with upregulation of HK-II in human brain metastases of breast cancer (Claeysen, wally, Daniel, Morse, & Rivoal 2006) Thus HK-II upregulation is considered a consequence of metabolic re-programming in cancer. Akt/serine/threonine kinase is often upregulated in tumor cells.

Over the years, several researches have been conducted focusing on viral proteins, which are responsible for viral replication. Viruses are non-living entities and do not have their own metabolism, thus it relies on the host cellular mechanism to fulfil their energy requirement and production of virions. Viruses are also dependent on the generation of ATP and macromolecular precursors for critical processes, including genome replication, viral protein synthesis, membrane production, and virion assembly (Sanchez, & Lagunoff, 2015). Researchers have focused on investigating how virus alters the host cellular metabolism to replicate themselves using the host cellular metabolomics, where the metabolomics profile indicates the viral modulation in host cell (Delgado, Sanchez, Camarda, & Lagunoff, 2012; Fontaine et al., 2015). It has been reported that dengue viral infection triggers dramatic changes in cellular metabolism, particularly in centre carbon utilization pathway. This alteration of carbon source by infected cell might increase available energy for viral replication, as well as provide cellular substrate for the survival of virus inside the human's body (Sanchez, & Lagunoff, 2015). However, very few studies have focused on the impact of glycolytic pathway on dengue infected cell. Recently, it has been shown that glycolysis has a great impact on DENV replication. Fontaine and colleagues conducted an experiment on human fibroblasts (HFFs) mock cells and DENV-infected cells at different time points during the primary infection (Fontaine et al., 2015). It was observed that glucose metabolism was significantly altered during DENV infection, particularly glucose utilization. They concluded that HKII is highly expressed in DENV-infected cells, and glucose metabolism promotes DENV replication. In this study, we aimed to produce a substantial amount of pure human hexokinase protein through efficient HKII expression using three-step protein purification methods; Immobilized Metal Ion Affinity Chromatography, Size Exclusion

Chromatography. This protein will be utilized in downstream analyses and subsequent HKII inhibition analysis.

EXPERIMENTAL SECTION

Materials

The complementary DNA (cDNA) of human Hexokinase II (Accession Number: NM_000189 and Clone ID: OHu25460C) was purchased (from GenScript (NJ 08854, USA), QIAEX II gel extraction kit for DNA extraction was purchased from Qiagen (Germany), restriction enzymes were purchased from New England Biolabs (USA), *E.coli* host DH5 α (Cat No 18265017), BL21 (DE3) (Cat No 69450) and BL21 (DE3) pLysS (Cat No 69451) as well as pET28b (Cat no 69865-3) vector was purchased from Novagen (USA). All buffer components Tris-HCl (Trizma® hydrochloride Cat. No: T594) MgCl₂, (Magnesium Chloride Hexahydrate Cat. No. 1058331000), NaCl (Sodium chloride Cat. No 1064041000), Imidazole (Cat. No I202-100G), ATP (Adenosine 5'-triphosphate disodium salt hydrate Cat. No: A26209-1G), NADP (β -Nicotinamide adenine dinucleotide phosphate disodium salt Cat. No 10128031001), G-6PDH (Glucose-6-Phosphate Dehydrogenase, Grade-I Cat. No.10127655001), Ampicillin (Cat.No A9393-5G), Kanamycin (Cat No 60615-6g), IPTG (Isopropyl β -D-1-thiogalactopyranoside, Isopropyl β -D-thiogalactosid Cat. No I6758), Terrific broth (Cat No SD7035), Luria-Bertani broth (Cat no L3522), Glucose (Cas number 50-99-7) and PCR kit were purchased from Sigma-Aldrich (Merck, Germany). Primers for PCR were obtained from Apical Scientific (Malaysia). DNA ladder (Cat No N3232L) and prestained protein marker (Cat No PM2510) were from New England Biolabs (USA).

Cloning of HKII in pET28b

The pET28b-HKII vector was constructed by using the full length gene encoding human HKII (2745bp) that was amplified with forward primer F1(5'-GGTCGGGATCCGATTGCCTCGCATCTG-3'; BamHI site is underlined and bold) and reverse primer (3'-GAGGCTGGACAGCGAAAGCTTGCGG-5'; HindIII site is underlined and bold), using polymerase chain reaction (PCR) with the synthesized cDNA as a template. PCR reaction was carried out in 50 μ L of reaction volume consisting of 2 \times PCR buffer (20 mM Tris-HCl, 22 mM KCl, 22 mM MgCl₂, 22 mM NH₄Cl, 5% Glycerol, 0.05% Tween), 0.2 mM dNTPs, 10 μ M of each primer, and 1.25 U of Taq DNA polymerase. The reaction was set at pH 8.9, 25 °C temperature, while denaturation, annealing and extension were carried out at 94 °C for 30s, 55 °C for 50s and 72 °C for 1min 30s respectively for 30 cycles. The PCR product was purified using 1% agarose electrophoresis. Concomitantly, pET28b was digested with BamHI/HindIII restriction enzymes and purified using QIAEX II gel extraction kit. The PCR product and the

digested pET28b were ligated using T4 DNA ligase with 1:3 ratios (vector: insert) at room temperature for 30min, resulted in successful recombinant pET28b-HKII construct. The plasmid was transformed into *E.coli* strain DH α using standard condition (heat-shock 42 °C for 40s-60s; incubate 37 °C for 1h). The transformed cells were plated on LB agar plate containing 100 μ g. μ L⁻¹ kanamycin for pET28b-HKII construct and incubated overnight at 37 °C. Each plate contains approximately 50 colonies and the culture plates were stored at 4 °C. The constructs were verified by colony PCR, restriction enzyme digestion and DNA sequencing.

Expression of Recombinant HKII protein:

The construct (pET28b-HKII) was re-transformed into *E.coli* expression host strain BL21 (DE3) on LB agar plate following the same condition of transformation in DH α with antibiotics kanamycin (100 μ g. μ L⁻¹). A single colony was picked from an overnight- grown LB plate and was inoculated into 5 mL of TB media containing 100 μ g. μ L⁻¹ kanamycin. The cultures were left overnight (18 -19h) at 37 °C in the incubator shaker at 250 rpm. The following day, the cultures were sub-cultured into 100mL fresh TB media containing 100 μ g. μ L⁻¹ kanamycin and was incubated at 37 °C for 4-6h in an incubator shaker at 150 rpm. Once the OD₆₀₀ reached 0.6-0.8, 0.5 mM IPTG was added to induce protein expression, before being incubated for 18h-19h at 18 °C, 150 rpm in an incubator shaker. The cultures were later centrifuged at 8000 rpm for 10min at 4 °C, before the cell pellets were re-suspended with lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, 10% glycerol), pH 8.0 and homogenated approximately for 10 min until the pellet was dissolved. Then, sonication was conducted for 5 min with 30 sec bursts and 45% amplitude. The lysed cells were centrifuged at 10,000 rpm for 15-20 min, in order to analyse the level of protein expression, which was conducted by using 10% SDS-PAGE.

Dot-Blot analysis:

A nitrocellulose membrane was used to place HKII and block with TBS buffer (20 mM Tris-HCl and 150 mM NaCl pH 8.0) containing 5% skim milk, incubated at room temperature for overnight. The following day the membrane was washed with TBS for 6 times for 5 min and immersed with TBS buffer containing skim milk and primary antibody penta-his-antibody (1:25000), prior to incubation for 1 h on a shaking platform. Later, the membrane was washed again 4 times for 5 min and immersed with TBS buffer containing secondary antibody horseradish peroxidase (HRP)- conjugated monoclonal antibody (1:2000), before being incubated for 1h.

Purification of HKII protein

The pET28b-HKII construct has shown good expression in *E.coli* strain BL21(DE3) which was

Chosen for large-scale expression and purification process. Human hexokinase II (HKII) protein purification has been conducted using two step of purification process; Immobilised Metal Ion Affinity Chromatography (IMAC) and Size Exclusion Chromatography (SEC).

Immobilised Metal Ion Affinity Chromatography (IMAC)

The construct pET28b-HKII expressed in BL21(DE3) was cultured in 2 L TB media with $100 \mu\text{g}\cdot\mu\text{L}^{-1}$ kanamycin, with expression condition was set at 18°C for 18 h-19 h in the presence of 0.5mM IPTG to induce protein expression. Cell pellets obtained from 2 L culture was re-suspended in lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, 10% glycerol), pH 8.0 and sonication was conducted for 10 min with 5s bursts. The lysed cell was centrifuged at 10,000 rpm for 30 min at 4°C to collect soluble and insoluble fractions. The soluble fractions were passed through $0.22 \mu\text{m}$ filter (Millipore) before being loaded onto the 5 mL HisTrap™ HP (GE Healthcare) that was charged with 0.1M Ni^{2+} on an ÄKTA Avant (GE Healthcare Life Science). The column was washed with 3 column volume (CV) of distilled water and equilibrated with 5 CV of binding buffer (50 mM Tris-HCl, 150 mM NaCl and 25 mM imidazole), pH 7.4. Sequentially, the sample was injected by using a syringe fitted to the luer connector into the column, where the non-binding molecules were washed out with 10 CV binding buffer (50 mM Tris-HCl, 150 mM NaCl and 25 mM imidazole,) pH 7.4. HKII protein was eluted as fractions of 5 mL by 20 CV of elution buffer (50 mM Tris-HCl, 150 mM NaCl, 300 mM imidazole). All steps were performed by maintaining the flow rate at 5 mL/min. The eluted protein was concentrated using Vivaspin protein concentrator spin column 10,000 MW cut off (GE Healthcare) before proceeding to Size Exclusion Chromatography step.

Size exclusion chromatography (SEC)

The column, Superdex™ 200 10/300 (GE Healthcare) was equilibrated with 2 CV of buffer (50 mM Tris-HCl, 150 mM NaCl), pH7.4 at 0.65 mL/min flow rate. Subsequently, $500 \mu\text{L}$ of concentrated sample was loaded into the column with a flow rate maintained at 0.5 mL/min. Finally, the HKII protein was eluted as fractions of 1.5 mL with 1 CV of the same buffer, with the whole process conducted on ÄKTA explorer (GE Healthcare, Life science). The eluted fractions were analysed using SDS-PAGE and assayed for activity. Eluted protein fractions were quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) to measure the concentration of the pure HKII. The sample were aliquot and stored at -20°C with 50% glycerol for further downstream analyses.

Hexokinase II activity measurement

The conventional HKII assays were conducted according to modified protocol from Scheer et al,

1978. The HKII reaction was coupled to the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) reaction and the NADP^+ reduction was monitored at 340nm using Ultrospec™ 3100 Pro UV/visible spectrophotometer (GE healthcare Life Science). The 1mL reaction mixture contained 57 mM Tris-base (pH 7.4), 10 mM MgCl_2 , 0.8 mM NADP, 5.0 mM ATP, 0.5 mM glucose (substrate), 2.5 U/mL G6P-DH with the final addition of 0.01mg/mL HKII. The blank tube was prepared with Tris-base buffer, while the negative control was prepared in the presence of all reagents except for HKII enzyme. The enzyme activity was measured by the following the rate of NADPH production at 340 nm in 1cm cuvette at RT for 15 min. The absorbance was increased with 15s interval till 12 min and plotted data has shown liner graph which indicates the activity of human HKII protein.

RESULTS AND DISCUSSION

Construction of Recombinant pET28b-HKII

A full-length cDNA encoding human HKII was obtained from Genscript (Accession Number: NM_000189 and Clone ID: OHu25460C). The length of the open reading frame (ORF) sequence is 2751 bp containing 971 amino acids, while the molecular mass of HKII protein is 102 kDa. Based on the cDNA sequence of HKII, a set of primers were designed to amplify the region corresponding to HKII, where restriction sites *HindIII* and *BamHI* were added directly to the 5' and 3' ends of the primers. The same restriction enzymes were used to digest the vector pET28b. Subsequently, the amplified HKII gene (2.7 kb) was purified and later ligated to pET28b (5.3 kb) by utilising the *HindIII* and *BamHI* restriction sites. The successful cloning procedure was later confirmed by colony PCR (**Figure 1A**), and further evaluated by double digestion with *HindIII* and *BamHI*, which clearly has shown the separation of the vector (5.3 kb) as well as HKII gene, which is 2.7 kb in size (**Figure 1B**). The plasmid map is shown in **Figure 1 (C)** and the correct sequence was later confirmed by DNA sequencing.

Expression of recombinant HKII

To determine the expression level of HKII, pET28b-HKII was transformed into *E.coli* expression hosts, BL21 (DE3). In the present study, several expression parameters were included during expression trials; temperature, strains, growth media and IPTG concentrations. The HKII protein was expressed at 18°C with induction by 0.5 mM IPTG for 19h. The expression was determined by SDS-PAGE analysis, where protein bands that are 102 kDa in size, corresponding to HKII were observed. The presence of HKII protein was further confirmed through Dot blot analysis, which was exhibited by soluble expression of HKII when expressed in BL21 (DE3) system in TB media at 18°C for 19 h (**Figure 2A**). It should be noted that at higher temperature, which is at RT or 37°C , the level of HKII expression was lower, compared to a lower temperature (18°C). Temperature is one of the

crucial parameter that plays a vital role to express protein prior to IPTG induction, which increased protein folding ability and cell viability without the release of toxic substance in the cell cytoplasm (Vasina & Baneyx, 1997). The low temperature might have the ability to develop protein folding along with cell viability for HKII protein. In this work, TB, which is a rich nutrient growth media also aid in enhancing HKII expression in BL21 (DE3) system (Kwee, Hernandez, Chan, & Wrong 2012).

The findings from the Dot blot analysis has confirmed that construct pET28b-HKII expressed in BL21(DE3) in TB media provides relatively good signal that confirmed the presence of HKII protein in the induced samples (Figure 2B). Taken together, both the SDS-PAGE and Dot blot analysis have suggested that pET28b-HKII construct transformed in BL21(DE3) has shown the best HKII expression in TB media, thus this condition was chosen for large-scale expression of HKII.

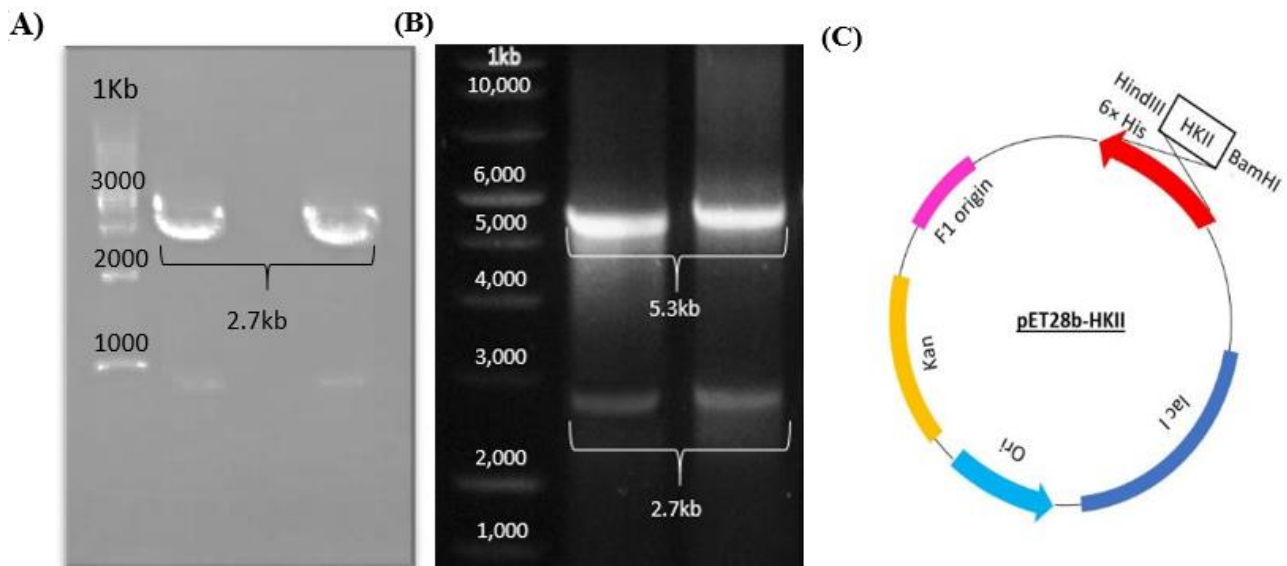


Figure 1: (A) Colony PCR indicating the presence of HKII gene, which is 2.7kb in size. (B) Double digestion with *HindIII* and *BamHI* of pET28b-HKII construct, which shows the bands corresponding to pET28b (5.3 kb) and HKII (2.7 kb). (C) The map of pET28b-HKII recombinant plasmid.

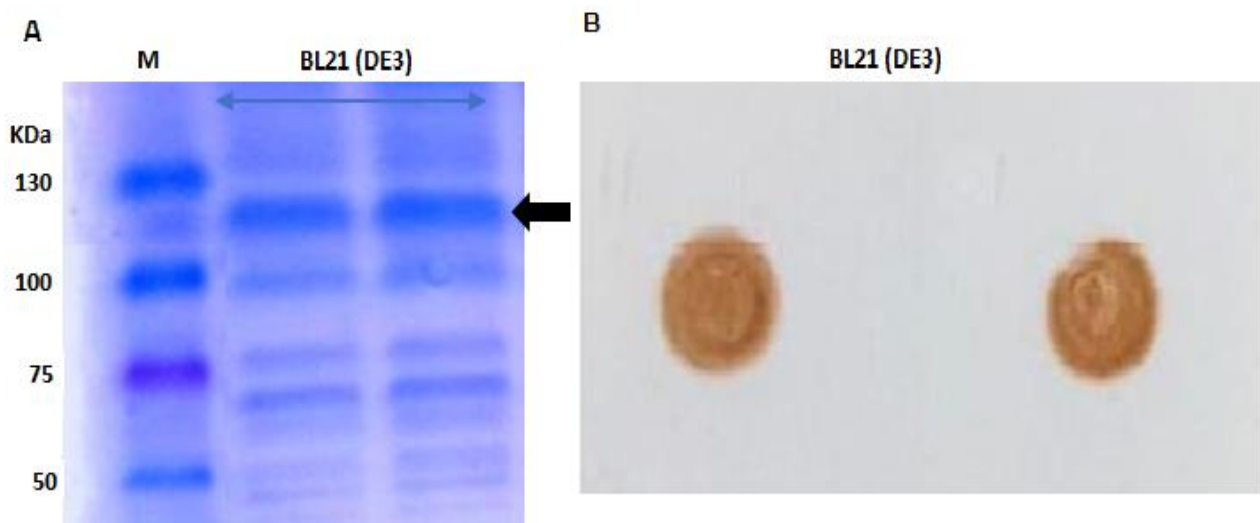


Figure 2: (A) HKII expression at 18 °C for 19 hours in TB growth media. A 10% SDS-PAGE is shown with 2-3µg IPTG induced samples containing HKII. M indicates pre-stained protein marker. The sequential lanes are expressed HKII. (B) Dot blot analysis of expressed HKII protein sfzwasfd`3ff3fghgwgqsgf12f`d2gwfsg3on a nitrocellulose membrane. The sequential dots are pET28b-HKII in BL21(DE3). Black arrow indicates the expected HKII protein band (102 kDa).

Purification of Recombinant HKII

Following the expression of bacterially-expressed HKII, 24.36 g pellet of dry cell was obtained and later disrupted for protein purification. Taking advantage of the His-Tag engineered at the N-terminal of HKII protein, the first stage of purification step was conducted via Immobilized Metal Ion Affinity Chromatography (IMAC). Affinity chromatography is one of the most powerful method, whereby the protein of interest is purified by specific binding properties to an immobilized ligand. The recombinant HKII protein was purified using IMAC performed by using HisTrap™ HP column charged with Ni²⁺ ions. **Figure 3A and B** shows the elution profile of HK2 through the HisTrap™ HP column, while SDS-PAGE analysis

(**Figure 3C**) revealed the presence of HKII in the fractions corresponding to the eluted peak at A280 nm. Fractions containing HKII proteins were pooled from initial IMAC purification and concentrated with Vivaspın protein concentrator spin column (GE Healthcare) prior to Size Exclusion Chromatography (SEC) step. SEC separates molecules by differences in size as they passed through a SEC resin-packed column. In this experiment, a 5mL fractions corresponding to pure recombinant HKII from the initial IMAC purification was applied onto a Superdex 200 10/300 GL column, and HKII was eluted in distinct peaks corresponding to HKII protein and later confirmed by SDS-PAGE analysis (**Figure 4A and 4B**). The purification table for HKII is shown in **Table 1**.

Table 1. Recombinant human HKII protein purification table.

Purification stages	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg)	Yield (%)	Fold Purification
Crude extract	184.8	323.4	1.25	100	1.0
Immobilized metal affinity chromatography	4.8	100.3	20.89	31.01	11.9
Size exclusion chromatography	0.42	33.98	80.90	10.50	46.2

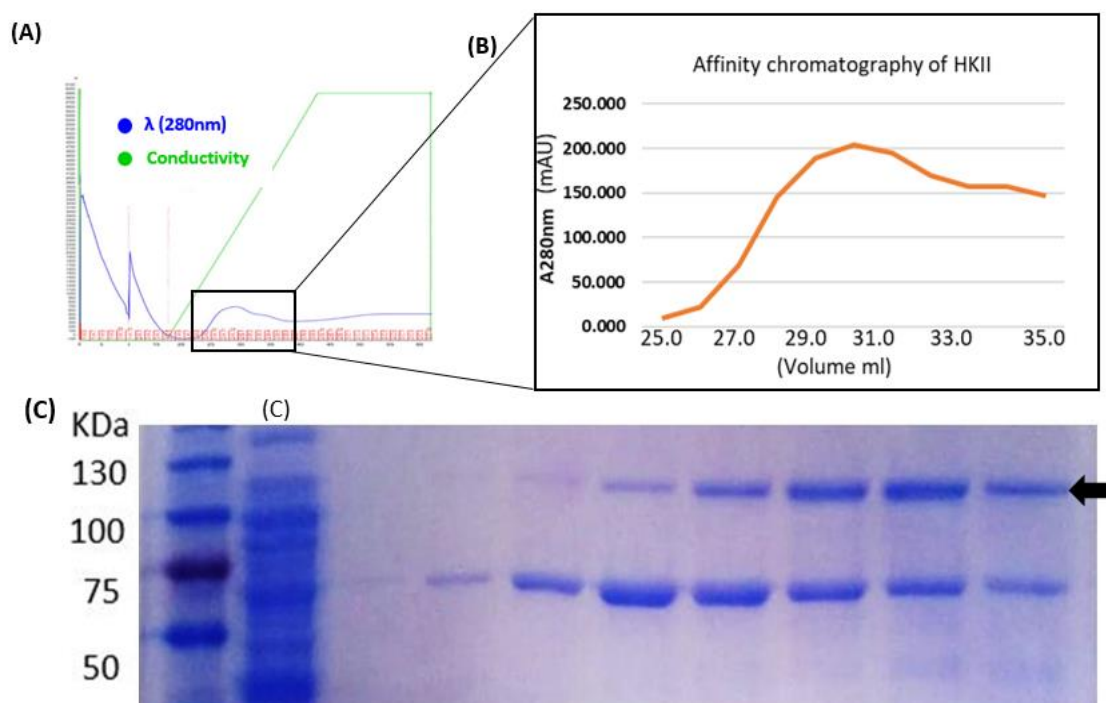


Figure 3: Purification of recombinant HKII protein by using Immobilized Metal Ion Affinity Chromatography (IMAC) (A) chromatographic profile of the recombinant HKII protein by IMAC. The circle indicates the fractions containing HKII (B) The eluted fractions containing HKII protein which produces peak from fractions 25mL to 35mL. (C) Purified HKII protein was analysed on 10% SDS-PAGE. C indicates crude sample as a control, with the black arrow indicates the purified HKII band (102 kDa).

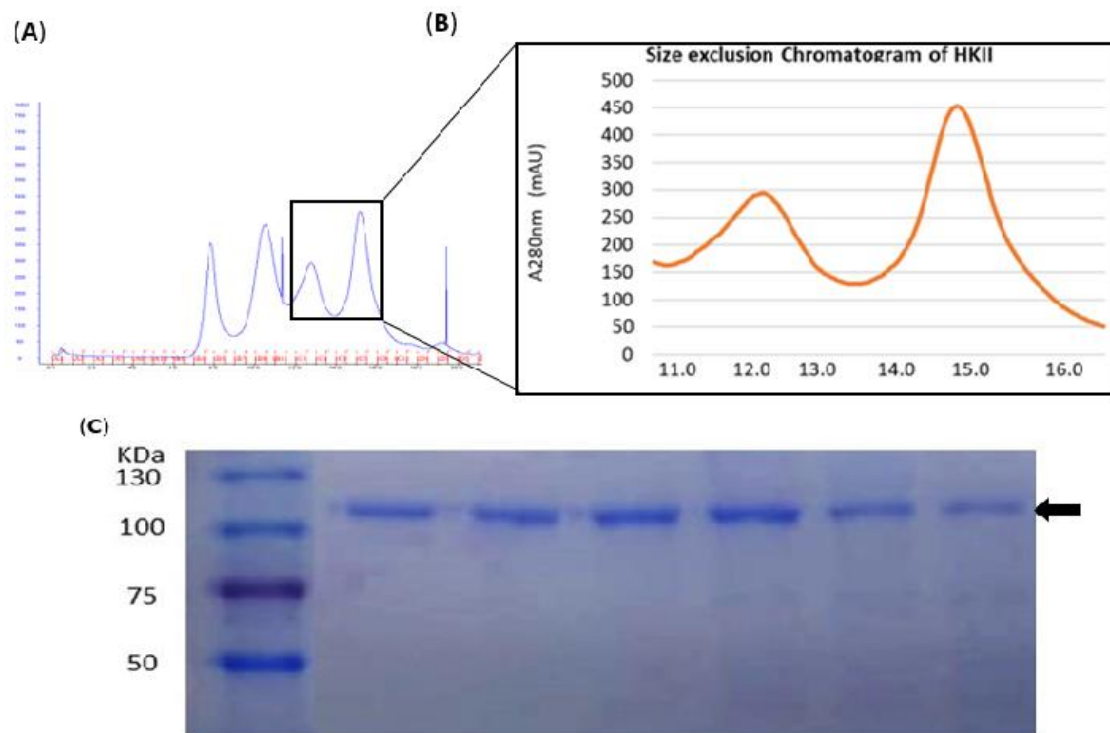


Figure 4: Purification of recombinant HKII protein by using Size Exclusion Chromatography (SEC) (A) chromatographic profile of the recombinant HKII protein by SEC. The circle indicates fractions containing HKII (B) The eluted fractions containing HKII protein from fractions 11mL to 16mL. (C) Purified HKII protein was analysed on 10% SDS-PAGE, with the black arrow indicates the purified HKII band (102 kDa).

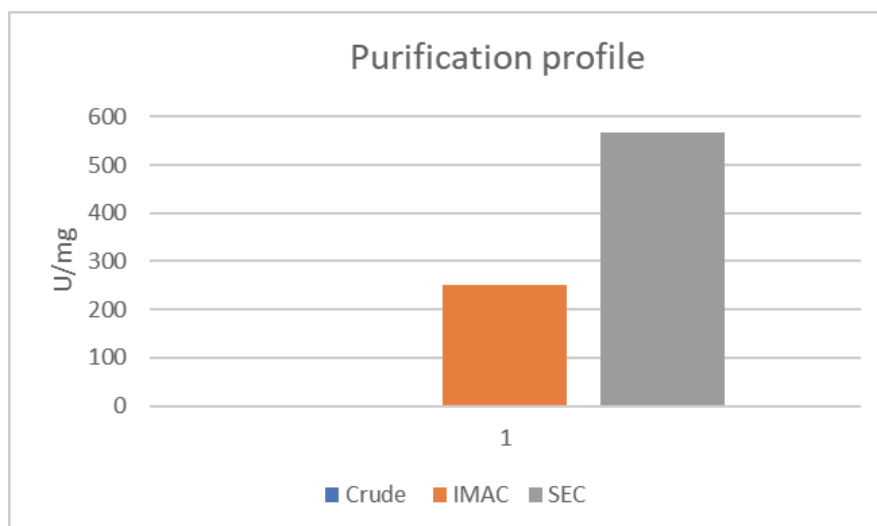


Figure 5. Measuring the specific activity of human HKII from each purification step.

The purpose of protein purification is to obtain pure target protein, where purification table summarises the progress of protein purification. In this work, a two-step purification protocol for HKII purification was established, comprising IMAC, followed by SEC as a polishing step. Purification rank and activities were scrutinized during the whole experiment by using the HKII coupled enzyme assay (Table 1). Each step of the purification process bears the significance to obtain active form of HKII, where contaminants such as

bacterial proteins were washed out through washing steps that were evaluated by SDS-PAGE analysis. Using the low temperature (18 °C) expression method, the final yield of functional and active HKII protein reached approximately 80mg from 2L of *E.coli* culture with specific activity 80.90 U.mg⁻¹ and the purity 10.50%.

Currently, there are very limited studies reported on recombinant HKII expression and purification in bacterial system, and the inability to quantify the

amount of active enzyme has led us to produce recombinant HKII using pET system in bacteria. The pET system is one of the efficient system to develop expression of recombinant protein in *E.coli*, where the target genes are under the control of bacteriophage T7 promoter. Several research has reported on cloning and characterization of hexokinase isoforms, mostly HKI and HKII from different species, such as from plant, yeast, fungi and those genes mostly expressed in bacteria and yeast systems.

Meanwhile, Plant SchKII cDNA was isolated from *Solanum chacoense*, a wild type potato and cloned into pProEx HT vector containing His-tag, and expression was conducted using *E.coli* bacterial system using culture media LB at 23 °C and 37 °C for 1h, induced by 0.6 mM IPTG (Jin et al., 2016). Jin and colleagues (Jin et al., 2016) observed that SchKII expression in cells were higher at 37 °C than at 23 °C, which declined the increment of SchKII levels in culture media at 37 °C, while at the same time HKII activity were extremely low in bacteria and in the medium when the expression was performed at 37 °C. Contrastingly, HKII activity was higher (5.6U. mL⁻¹) in bacteria at 23 °C. Another interesting hexokinase expression and activity has been investigated in plant *Solanum chacoense*, where HKI gene was isolated from Gala Apple, with pMD18-T and pET32a vectors were used for cloning and expression, respectively. In this study, RT-PCR was used to observe HKI gene expression in growing root, new leaves caulicle and flowers at the initial bloom stage and young fruits and the samples were collected 3,6,9,12,24 and 48h after treatment such as by salt stress, low temperature, and abscisic acid for expression analysis. The best expression was observed in roots team and young fruits after one day of treatment (Claeyssen et al., 2006). On the other hand, MdHXK1 gene was expressed in *E.coli* strain BL21 at 37 °C for 1, 3 and 6 h with 0.5 mM IPTG induction, where the expression level was low in prokaryotic than the eukaryotic system. Studies on hexokinase gene expression and characterization on *Arabidopsis*, *Oriza sativa* and *Physcomitrella patens* have led further research on HK of different species (Lehto et al., 1993). In the present study, cDNA HKII from human was cloned into pET28b system and was expressed in BL21(DE3) at 18 °C for 19 h with 0.5 mM IPTG induction. The best expression result was obtained at low temperature rather than 37 °C (data not shown), which is aligned with other plant HKII (Claeyssen et al., 2006; Jin et al., 2016). Two purification steps were performed to purify HKII protein from bacterial lysate, where the specific activity of HKII activity was determined 80.90 U.mg⁻¹, showing better yield and purity compared to purified HKII from plant (Claeyssen et al., 2006).

In the mammalian tissue, HKII plays crucial role as a biological catalyst to convert glucose to glucose-6-phosphate, as the first step in the glycolytic pathway. Most importantly, it was noted that HKII acts as the

main gate to initiate this metabolic pathway in mammalian cell (Wilson, 2003). HKII is primarily expressed in insulin-sensitive tissues such as skeletal and cardiac muscles, as well as adipose tissue, alongside its role in causing non-insulin-dependent diabetes mellitus, cancer and tumours, as well as to proliferate virus cells in host (Vogt et al., 2007). This disease hence urges research in producing recombinant human hexokinase II protein for therapeutic purposes. Due to the consideration of HKII dominancy on cancer, tumour cells as well as non-insulin dependent tissues in mammal, most of human HKII activities, expression pattern and gene structure research were conducted on the diseases affected cell itself rather than the HKII from rodent cell. Several research has been highlighted that purified rat skeletal muscle type II hexokinase shows the highest specific activity is 210 U/mg than any other expression and purification system (Vogt et al., 2007). Previous research work on human HKII protein expression and purification has been conducted using bacterial system at 17 °C for 24h, but the protein yield was lower than this reported study (Tanbin & Fuad 2019). As described earlier, HKII from other species has also been expressed at high temperature, however it was not fruitful in terms of yielding substantial amount of protein, with low activity at high temperature. Recently, Jeong and colleagues (Jeong et al., 2007) have cloned human hexokinase II into pET21b with a C-terminal His-tag, and the protein was expressed in *E.coli* BL21(DE3) at 18 °C and 37 °C with 1M sorbitol. They successfully obtained 78% HKII in soluble fractions when cultured in LB media, where 18 °C temperature alone has shown good expression rather than 37 °C, with a single IMAC step conducted for HKII purification (Jeong et al., 2007). We successfully recovered 11.9% HKII yield from the IMAC step, where the previous study recovered 19% yield of HKII from IMAC (Jeong et al., 2007). In this study, two-step of purification stage was performed to get pure and active human HKII protein, whereas Jeong and colleagues (2007) conducted one-step purification procedure to obtain pure human HKII. This method was used to enhance the production of bacterially-expressed HKII, where subsequently the purified HKII will be utilized for HKII inhibition analysis. Since it has been shown that HKII plays a crucial role for dengue virus replication, inhibition of HKII could be a great approach for the discovery of anti-dengue therapeutics.

CONCLUSIONS

In conclusion, we have successfully purified HKII and yielded active and pure protein that is functional for downstream purposes. The expression conditions for bacterially-expressed HKII has been optimized at 18 °C temperatures for 19 h, expressed in BL21 (DE3) strain, in the presence of 0.5mM IPTG. The bacterially-expressed HKII (~102 kDa) was then successfully

purified through a two-step purification procedure, with combination of IMAC and SEC where the specific activity of 566. 33U.mg⁻¹ and final yield of 10.5% of HKII were obtained through this method.

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