MOLECULAR DETECTION OF HEXOKINASE 1 GENE IN THE BLOODSTREAM FORM OF *TRYPANOSOMA BRUCEI BRUCEI* (FEDERE ISOLATE)

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ABSTRACT

Hexokinase 1 (EC:2.7.1.1) which belongs to the Hexokinase 2 superfamily is the first key regulatory enzyme in the glycolytic pathway of *Trypanosoma brucei brucei*. Therefore the enzyme has great significance in metabolism of the parasite particularly for the purpose of generating ATP and nucleic acid precursors. There is increasing interest in the study of enzymes in parasites for various purposes. This research therefore attempted to detect the presence of the hexokinase 1 gene in *Trypanosoma brucei brucei* (Federe isolate) which is prevalent in Nigeria. The parasites were grown in rats, harvested and purified using diethyl amino ethyl (DEAE) cellulose chromatography. From the genomic DNA isolated the hexokinase 1 gene was amplified through Polymerase Chain Reaction. The amplicon was sequenced and studied using some Bioinformatics tools. The 1401bp gene sequence (Accession number MH 198230) obtained translated into 464 amino acid sequence. The primary nucleotide sequence of the gene compares well with *Trypanosoma brucei* TREU 927 *and T. brucei gambiense*DAL972. The predicted molecular mass of the polypeptide as calculated using *ExPASy* is 51,918.50Da (~52kDa) giving the hexameric protein a molecular weight of around 311.5kDa which differs from the mammalian hexokinase The Isoelectric point (pI) was

predicted as 9.64 indicating that at neutral pH, the enzyme has a net positive charge. These revealed that the *T.b.brucei* hexokinase 1 is active in the bloodstream form of the parasite.

Keywords: Hexokinase 1, *Trypanosoma brucei brucei,* gene sequence

INTRODUCTION

African trypanosomiasis is mainly caused by *Trypanosoma brucei* species and is estimated to affect about 70 million people in Africa. This disease results in great loses. It has been estimated that about $ 4.5 billion is loss annually from animal fatality (Franco *et al.*, 2014; Tesfaye *et al.*, 2012; Simarro *et al.*, 2012). It has also been reported that the disease brings about death if not treated (Franco *et al.*, 2014). Therefore there is a need to further understand the biochemistry of the parasite. One of the biochemical elements in *T. b.brucei,* hexokinase 1, is the first key regulatory enzyme of the glycolytic pathway which serves as the only source of ATP and nucleic acid precursors for the parasite in the infective stage and differs from the host enzyme. The parasites in the mammalian bloodstream depend solely on glycolysis because of its poorly developed mitochondrion which lacks active components of the citric acid cycle and the electron transport chain (Joice *et al.,* 2013). This study was designed to detect the hexokinase 1 gene of *T.b.brucei* (Federe isolate) as a potential target for drug or vaccine development.

MATERIALS AND METHODS

Reagents

The reagents used were of analytical grade and purchased from Sigma, BDH POOLE, England, Whatman, USA; Pharmacia Fine Chemicals and Amresco Life Science, Fountain Parkway,Solon. DNA extraction kit was purchased from ZYMO RESEARCH. *Taq* polymerase and High Fidelity Polymerase Enzyme Mix were bought from Promega, USA and Fermentas, respectively. Molecular size marker was purchased from Roche, Mannheim Germany. Oligonucleotide primers were synthesized by Inqaba biotec Industry, Pretoria South Africa. The GeneAmp PCR System9700 for gene amplification was obtained from Applied Biosystems, Indonesia and the Gel Documentation System was obtained from Synegene® Inc. Indonesia. Sequencing analyses were performed by Macrogen in Netherlands.

Growth of Trypanosomes in Rats

The *Trypanosoma b. brucei (Federe isolate)* was obtained from the Veterinary and Livestock Studies Department of the Nigerian Institute for Trypanosomiasis Research, Vom, Plataeu State, Nigeria. Healthy rats were lightly anaesthetized and infected intraperitoneally with the parasite (about $1x10^4$ parasites/ml) sourced from tail vein blood of the *Trypanosoma brucei brucei* infected donor rat reservoir at a massive parasitemia ($1x10^6$ parasite/ml) in 0.3ml of PSG (pH 7.8). Parasitemia was monitored daily from the second day after the infection by bleeding the tail of each animal and the blood observed under X400

magnification of a phase contrast microscope with fields examined and the mobile parasites counted.

Purification of Bloodstream Form *T. brucei brucei* from Rat

At peak parasitemia, the circulating blood was withdrawn by cardiac puncture under terminal anaesthesia using a syringe loaded with a small volume of PSG containing heparin. The whole blood was centrifuge at 1,250 × *g* for 15 minutes at 4°C resulting in separation of the sample into three different layers. The white Buffy coat containing the parasite formed between the red blood cells and the plasma layer. The Buffy layer was carefully removed using a Pasteur pipette and re-suspended in a small volume of phosphate buffered saline with glucose (PSG, pH = 7.8) and thereafter purified using diethyl aminoethyl cellulose (DEAE) anion exchanger column (DE-52, Whatman) as described by Lanham and Godfrey (1970). The eluate containing the trypanosomes in centrifuge tubes were centrifuged for 10mins at 1600 *x g* in a bench top centrifuge, and the supernatant was discarded. The trypanosome pellets were then washed by gentle resuspension in 1ml of PBS (pH 7.4) and transferred to microcentrifuge tubes and centrifuged for 5mins at 1600 *x g*. After a final 5mins centrifugation at 1600 *x g*, the supernatant was removed, the trypanosomes were pooled and the pellet was resuspended in PBS (pH 7.4) for subsequent analysis.

DNA Isolation and Polymerase Chain Reaction (PCR)

The genomic DNA was extracted from the resulting trypanosome cell

pellets using the ZR Fungal/Bacterial DNA MiniPrep (ZYMO RESEARCH) following manufacturer's instruction. The following primers (Forward 5'-ATGTCTAGACGCCTAAACA ATATCC-3' and Reverse 5'-TTACTTGTCGTTCACCACCA TTGCG-3') which were designed based on the sequence obtained from GeneDB were used to amplify the gene sequence of *T. brucei brucei* hexokinase 1 gene. The PCR reaction was performed in a 50µl reaction mixture which contained 27µl of nuclease free water, 10 µl of 5 X phusion buffers, 1 µl of dNTPS, 1.5µl of DMSO, 2.5 µl of forward primer, 2.5 µl of reverse primer, 0.5 µl of *Taq* DNA polymerase (Promega) and 5.0 µl of the genomic DNA of T.brucei brucei. The tubes containing the mixture will be subjected to 30 cycles of amplification in a thermocycler using the following Amplification conditions: Polymerase activation 94°C for 5 min; Denaturation 94°C for 30 s; Annealing 48°C for 30 s; Extension 72°C for 1 min. Step 2-4 were repeated for 30 cycles and Final extension was carried out at 72°C for 5 minutes. The PCR product was then subjected to 1.5% agarose gel electrophoresis (1.5 % w/v) and the results documented using Gel Documentation System (Synegene® Inc. Indonesia).

DNA Sequencing and Bioinformatics Analysis

The *T. brucei brucei* Hexokinase 1 DNA amplified was then sent to macrogen in Netherlands for sequencing. Sequence of isolated *T. brucei brucei* Hexokinase I *was* analyzed using some BLAST tools provided by NCBI

(http://www.ncbi.nlm.nih.gov/). The physico-chemical parameters of *T. brucei brucei* Hexokinase I was analyzed using ExPASy (http://www.expasy.org/).

RESULTS AND DISCUSSION

The result from the gel electrophoresis showed that the *T. brucei brucei* (Federe isolate) hexokinase 1 gene band (lanes S1 & S2) has open reading frame (OFR) that is less than 1650bp but far above 1000bp (Figure 1), this was thereafter confirmed by sequencing the gene which showed that it contained 1401bp (Figure 2). This size was more than 1350bp obtained by Chen *et al.*,(2014) for hexokinase from Clonorchis sinensis but closer to that of *T. cruzi* (1416 bp) as reported by Cáceres *et al.*,(2003) .

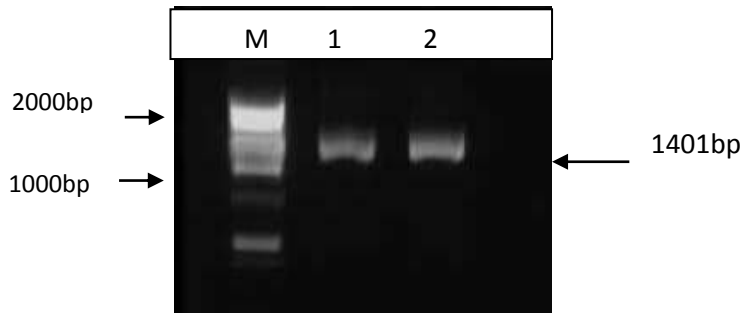

Figure 1: Analysis of PCR amplicon on 1% agarose gel elctrophoresis of *T. b.brucei Hexokinase 1* gene. S1 and S2= *T. b. brucei* Hexokinase 1 gene run in duplicate; M= Molecular marker

The NCBI BAST of the sequence yielded a good similarity (88%) with hexokinase sequences of genes of *Trypanosoma brucei brucei* TREU927(AJ437577.1); *Trypanosoma brucei Gambiense(FN554973.1) ;* Trypanosoma cruzi (XM841212.1). The Taxonomy report shows that the gene sequence is of an organism that is a Trypanozoon summarised in Table 1.

ATGTCTAGAC GCCTAAACAA TATCCTCGAA CACATCTCGA TCCAGGGAAA TGATGGTGAG
ACTGTGCGTT CTTTTTATGT TACGTTGCAC GCCCTCACCA ACCAACCTTT TCTGAGTGTC
GAGTCTATGC GACAGATCAT GACATACCTC CTGTACGAGA TGGTGGAGGG TCTTGAGGGT
CTTCACACCA CCCTCTCCAT TTTACCATGT ACCCGACGCG GACCCTACGC CCTGGCCTTC
TTCTCCCCCT TTGACCTCTG TGGTTCCATT TTCTGTGTGT TGCGTGGCCA TGCAAGGAGG
GTCCCGTGGT GTTTTCCTCT CCTTCTGCAT TCAAAATCCC CAAATATGCC CTTGAGGGTA
ACGCCACCGA TCTGTTTGGC TTCATTGCAT CCAATGTGAA AAAACCCCTG GAACTCTCGT
ACCATGGAAA CTCGTGCACC TGAGGACCTC AATCGCACAG TTCCTCTTGG GTTTACCTTC
AGTTTCCCCG TGGAGCAGAC GAAGGTTAAC CGTGGTGTGC TTATCCGGTG GACGAAGGGC
TTCAGCGGAT GCTATGTGAT TGCCCTTCTT CGCGCTGCTT TTGGGAAATT TAGCCTAAGT
GTCAATGTTG TGGCTTTGTG CAACGACCCC TTTTGAACTT TAATTTCGCA TTACTTTAAG
GACCCTGAGG TACAGGTTGG TGTGATTATC GGCACTGGTC CAGATGCGTG CTACTTTGAC
AGGGCCTCTG CTGTGACAAA AGACCGTGCC TTTGCTGCTC GTGAGTCAGC ACTTACTCCC
ATCAATATAC AAAGCCTCCA TTTTGACTCC GAGTACCGGT ATGTCCTCTA CAACAAATTT
CAACTTGTAT ATCGACGATC CGTCGTTAAA CAAATGTCAA CATGCTCTAG AGAAGATATA
TCCCGCATGT ATCGCGGTAA AATCTCCCGC CGCGTTATTG TGCACCTTTC GTCTATAAGC
CGCCTTCCTG CGGCACTCCA GACGGCTTTT GGACAACCGG GTGTCGTTTT AATCCCCATT
TCCCCGGAAT CACCAGTGCT TCACCTTAAG CCCCGACTTC AGTTCACTCG CAGCACGATC
CAGAAGGTGT GTGGTGTTGA CGTGCAGTCA ATTGAAGACC TTCGCATCAT TCGCGATGTG
TGCCGCCTTG TCCGTGGGAG GGCTGCGCAA CTCTCTGCTT CCTTCTGCTG CGCTCCACTG
GTTAAGACTC AAACACAGGG CCGTGCAACT ATTGCAATTG ACGGCTCCGT GTTTGAGAAG
ATTCCGTCAT TCCGCCGCGT CTTGCAGGAC AACATCAACC GTATCCTTGG CCCTGAGTGC
GATGTCAGGG CCGTTCTCGC AAAGGATGGC AGTGGAATTG GTGCTGCATT TATTTCCGCA
ATGGTGGTGA ACGACAAGTA A

Figure 2: Primary Nucleotide Sequence of *Trypanosoma brucei brucei (Federe isolate)* hexokinase 1 gene (Accession number MH 198230).

Table 1: BLAST Results Showing Hexokinase 1 Sequence with significant alignments

Description of Organism	Max score	Total score	Query cover	E value	identity	Accession Number
Trypanosoma brucei gambiense DAL972 hexokinase, putative partial mRNA	1626	1626	100%	0.0	88%	XM_011779121.1
Trypanosoma brucei gambiense DAL972 chromosome 10, complete sequence	1626	3145	100%	0.0	88%	FN554973.1
Trypanosoma brucei brucei strain 927/4 GUTat10.1 hexokinase (Tb10.70.5820) partial mRNA	1620	1620	100%	0.0	88%	XM_817363.1
Trypanosoma brucei HK gene for hexokinase	1620	1620	100%	0.0	88%	AJ345044.1
Trypanosoma brucei gambiense DAL972 hexokinase, putative partial mRNA	1519	1519	98%	0.0	87%	XM_011779123.1
Trypanosoma brucei brucei strain 927/4 GUTat10.1 hexokinase (Tb10.70.5800) partial mRNA	1507	1507	98%	0.0	87%	XM_817364.1

The translated amino acids sequence (Figure 3) gives 464 amino acids, which compares well with 471 amino acids of *T. brucei brucei* hexokinase 1(XP_822456.1) in the NCBI data base and 474 amino acids sequence obtained by Willson *et al.*,(2002).

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MSRRLNNILE HISIQGNDGE TVRSFYVTLH ALTNQPFLSV ESMRQIMTYL   50
LYEMVEGLEG LHTTLSILPC TRRGPYALAF FSPFDLCGSI FCVLRGHARR   100
VPWCFPLLLH SKSPNMPLRV TPPICLASLH PMKNPWNSRT METRAPEDLN   150
RTVPLGFTFS FPVEQTKVNR GVLIRWTKGF SGCYVIALLR AAFGKFSLSV   200
NVVALCNDPF TLISHYFKDP EVQVGVIIGT GPDACYFDRA SAVTKDRAFA   250
ARESALTPIN IQSLHFDSEY RYVLYNKFQL VYRRSVVKQM STCSREDISR   300
MYRGKISRRV IVHLSSISRL PAALQTAFGQ PGVVLIPISP ESPVLHLKPR   350
LQFTRSTIQK VCGVDVQSIE DLRIIRDVCR LVRGRAAQLS ASFCCAPLVK   400
TQTQGRATIA IDGSVFEKIP SFRRVLQDNI NRILGPECDV RAVLAKDGSG   450
IGAAFISAMV VNDK
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Figure 3: Translated amino acids sequence of *T. brucei brucei* (Federe isolate) Hexokinase 1. KEY: **G** - Glycine **P** - Proline, **A** – Alanine, **V** – Valine, **L** – Leucine, **I** – Isoleucine, **M**

– Methionine, **C**–Cysteine, **F** – Phenylalanine,**Y** – Tyrosine, **W** – Tryptophan,**H** – Histidine, **K** - Lysine **R** – Arginine, **Q** – Glutamine, **N** – Asparagine, **E** - Glutamic Acid, **D** - Aspartic Acid **S** – Serine, **T** – Threonine

The molecular mass and pI of the polypeptide were calculated using *ExPASy software by* Swiss Institute of Bioinformatics **(**Gasteiger *et al.*, 2005). The predicted molecular mass of the polypeptide which exist in hexameric form in Trypanosomal hexokinase is 51,918.50Da (~52kDa) giving it a molecular weight of around 311.5kDa which appear to be different from mammalian hexokinase which exist as monomer (types A, B and C, 96-111 kDa; type D (glucokinase), 47 - ***55*** kDa). Hexokinase from *T. brucei* can therefore be classified as non-vertebrate hexokinases since vertebrate hexokinases all have a molecular mass of approximately 100 kDa (Ureta, 1982; Thompson *et al.*, 1994; Willson *et al* 2002). It was *T. brucei brucei* hexokinase 1 gene that was detected and studied.

ETHICAL APPROVAL

Animal experiments were carried out in accordance with the instructions for the care and use provided by the University of Jos, Nigeria .

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