Parasitology International 57 (2008) 354–361

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Molecular cloning and characterization of *Brugia malayi* hexokinase

Alok Ranjan Singh [a], Shweta Joshi [a], Rahul Arya [b], Arvind Mohan Kayastha [c], Kishore Kumar Srivastava [d], Lalit Mohan Tripathi [e], Jitendra Kumar Saxena [a,*]

[a] *Division of Biochemistry, Central Drug Research Institute, Lucknow-226001, India*
[b] *Virology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India*
[c] *School of Biotechnology, Banaras Hindu University, Varanasi-221005, India*
[d] *Division of Microbiology, Central Drug Research Institute, Lucknow-226001, India*
[e] *Division of Parasitology, Central Drug Research Institute, Lucknow-226001, India*

ARTICLE INFO

Article history:
Received 17 October 2007
Received in revised form 12 March 2008
Accepted 15 March 2008
Available online 9 April 2008

Keywords:
Brugia malayi
Hexokinase
Expression
Characterization
Filariasis

ABSTRACT

5′ EST from filarial gene database has been subjected to 3′ rapid amplification of cDNA ends (RACE), semi-nested PCR and PCR to obtain full-length cDNA of *Brugia malayi*. Full-length hexokinase gene was obtained from cDNA using gene specific primers. The elicited PCR product was cloned, sequenced and expressed as an active enzyme in *Escherichia coli*. Sequence analysis of *B. malayi* hexokinase (BmHk) revealed 59% identity with nematode *Caenorhabditis elegans* but low similarity with all other available hexokinases including human. BmHk, an apparent tetramer with subunit molecular mass of 72 kDa, was able to phosphorylate glucose, fructose, mannose, maltose and galactose. The K_m values for glucose, fructose and ATP were found to be 0.035±0.005, 75±0.3 and 1.09±0.5 mM respectively. BmHk was strongly inhibited by ADP, glucosamine, *N*-acetyl glucosamine and mannoheptulose. The recombinant enzyme was found to be activated by glucose-6-phosphate. ADP exhibited noncompetitive inhibition with the substrate glucose (K_i=0.55 mM) while, mixed type of inhibition was observed with inorganic pyrophosphate (PPi) when ATP was used as substrate (K_i=9.92 μM). The enzyme activity is highly dependent on maintenance of free sulfhydryl groups. CD analysis indicated that BmHk is composed of 37% α-helices and 26% β-sheets. The observed differences in kinetic properties of BmHk as compared to host enzyme may facilitate designing of specific inhibitors against BmHk.

1. Introduction

Filariasis (elephantiasis) caused by *Wuchereria bancrofti* and *Brugia malayi* is endemic in tropical and sub-tropical countries and annually affects about 120 million people worldwide [1]. The antifilarial Diethylcarbamazine (DEC) is effective against the microfilarial stage of the parasite, having little effect on the adult parasites [2,3]. In order to develop new effective antifilarial compounds for chemotherapeutic intervention, identification of new parasite specific chemotherapeutic target is a mandatory requirement. Hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1.) is the first key regulatory enzyme of glycolytic pathway [4]. The product of catalysis, glucose-6-phosphate (G6P), also serves as a precursor for pentose phosphate pathway which yields NADPH and pentose sugars [5,6]. While the former maintains the redox potential, the latter is utilized in biosynthesis of nucleic acids [7]. Since parasitic nematodes depend mainly on glycolysis for their energy metabolism, hexokinase represents an important putative target for antihelminthic development [8].

Hexokinases can be distinguished on the basis of their molecular weight and sensitivity to inhibition by the product G6P. Generally, hexokinases from non-mammalian organisms have a molecular weight of approximately 50 kDa. Some of these hexokinases viz., yeast hexokinase are not inhibited by physiological relevant levels of G6P [9], whereas hexokinases from various marine organisms, silkworm and the parasite *Schistosoma mansoni*, show reasonably potent inhibition [10–12]. The hexokinases of plants show variations in their molecular mass from 38–68 kDa [13–16]. In mammals three isozymes, hexokinase I, II, and III having molecular weight of approximately 100 kDa and exhibiting marked sensitivity to inhibition by G6P have been demonstrated [17–19]. The mammalian isozymes have been shown to be evolved by duplication and fusion of a gene encoding an ancestral 50 kDa G6P sensitive hexokinase [17,20–22].

This is the first report of cloning, expression and characterization of hexokinase from any human filarial parasite. The results obtained in the present study indicate significant differences between the hexokinase of filarial parasite and the homologous enzyme from

Abbreviations: BmHk, *Brugia malayi* hexokinase; 3′-RACE, rapid amplification of cDNA ends; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; PPi, inorganic pyrophosphate.

* Corresponding author. Tel.: +91 522 2625932; fax: +91 522 2623405.
E-mail address: jkscdri@yahoo.com (J.K. Saxena).

1383-5769/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2008.03.004

host, allowing us to propose hexokinase as a potential target for the design of specific inhibitors against *B. malayi*.

2. Materials and methods

2.1. Materials

All the reagents were procured from Sigma (St. Louis, MO, USA). Anti-His IgG and Ni–NTA agarose were purchased from Qiagen (Germany). IPTG, prestained markers and restriction enzymes BamHI and HindIII were purchased from MBI Fermentas (Hanover, Maryland USA). λ DNA EcoRI/Hind III double digested marker was procured from Bangalore Genei, India. PCR®4-TOPO cloning vector was obtained from Invitrogen (USA). pTriEx-4, expression vector was purchased from Novagen.

2.2. Maintenance of B. malayi infection

Mastomys coucha (mastomys) was used as an experimental host and *Aedes aegypti* as a vector for maintaining sub-periodic strain of *B. malayi* in laboratory as reported earlier [23]. Four–five day old mosquitoes (*A. aegypti*) were fed on donor mastomys with high microfilaraemia. On the 10th day, these fed mosquitoes were crushed gently and put inside the funnel of Baermann Apparatus over two layers of muslin cloth. Infective larvae (L_3) were collected from the other end of the tube in Ringer's Solution (0.24 g $CaCl_2$, 9.0 g NaCl, 0.42 g KC1, 0.42 g $NaHCO_3$, 1.0 g D-glucose; pH 7.4 for 1.0 l). Larvae were washed thoroughly with 10 ml medium containing antibiotic–antimycotic solution [200 U of penicillin, 200 µg of streptomycin and 0.5 µg amphotericin B/ml (GIBCO, Invitrogen)], and active, mature infective larvae (L_3) were used for infecting the animals.

2.3. Infection and collection of microfilariae and adult B. malayi

6–8 week old male mastomys were inoculated with 200 L_3 of *B. malayi*. The infective larvae developed into adult parasites and microfilariae appeared in the blood of infected animals after 90 days. The adult parasites were collected from peritoneal cavity of infected animals in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 5.3 mM Na_2HPO_4 and 1.8 mM KH_2PO_4). The microfilariae were separated as reported earlier [23].

2.4. Preparation of homogenate of microfilariae and adult B. malayi

Adult *B. malayi* and microfilariae obtained from infected male mastomys were washed several times with PBS and soluble extract was prepared according to the method of Hamilton et al. [24]. The adult filarial parasites (100 mg) were homogenized in 250 µl of PBS containing protease inhibitor cocktail (Sigma) in a glass tissue grinder and volume was made up to 500 µl. The homogenized material was sonicated (20 cycles; 20 s pulse at 25 W with 1 min interval after each pulse) (Ultrasonic processor, Heat system Inc., Model-XL-2020) at 4 °C. Homogenate was centrifuged at 12,000 ×*g* for 30 min at 4 °C. Microfilariae separated from infected animals were centrifuged at 8000 ×*g* to obtain a pellet. The pellet was suspended in PBS to obtain a 10% (w/v) suspension and processed after sonication as reported above. Protein in supernatants was determined by Bradford's method [25].

2.5. cDNA synthesis and polymerase chain reaction (PCR)

Total RNA of *B. malayi* was isolated according to the protocol provided with the NucleoSpin RNA extraction kit (Clontech BD Biosciences, USA). The quality of isolated RNA was checked on 1.0% (w/v) agarose gel. Undegraded RNA preparation showing two distinct bands of 28S and 18S RNA was used for reverse transcription reaction. About 20 ng RNA was subjected to cDNA synthesis using cDNA Cycle Kit (Invitrogen, USA). The synthesized cDNA was stored at −80 °C.

A 5′ EST, TC2209 was retrieved from *B. malayi* genome sequence reposited at www.tigr.org. This EST showed sequence similarity at 5′-end with known hexokinases in the NCBI GenBank database. From this EST, three internal gene specific primers (GSPs), Bm2209 F6 (5′-CAGAAGAAGGTCGATTTATTGC-3′), Bm2209 F7 (5′-GGT GAAGAGCT-GAAGACGGAG-3′), Bm2209 F8 (5′-CTATTCGATTACATAACAAAA TGC-3′), were designed for 3′-RACE reactions to deduce the 3′-end sequence of cDNA. PCR amplification of total cDNA prepared from *B. malayi* was performed using Bm2209 F6 and 3′ adaptor primer (AP) (5′-GGCCACGCGTCGAAGTACTTTTTTTTTTTTTTT TT-3′). The product obtained was subjected to semi-nested PCR using Bm2209 F7 or Bm2209 F8 as forward primer and 3′ adaptor primer which gave us 3′-end sequence of *B. malayi* hexokinase encoding cDNA. The sequence obtained from 3′-RACE was joined with that of EST to generate the full cDNA sequence. The full-length cDNA thus obtained was PCR amplified using forward primer BmHkF (5′-CGCGGATCCGATGCT AGGTTTGTTGA-CAATTAC-3′; BamHI site at 5′-end is underlined) and reverse primer BmHkR (5′-GGCAAGCTTATGGTCAGTGGATAAATGGATCAC-3′; HindIII site at 3′-end is underlined) which contained start as well as stop codons at 5′ and 3′ terminal ends, respectively. PCR reaction was carried out in 50 µl reaction volume consisting of 1× PCR buffer, 200 µM dNTPs, 10 pmol of each primer, 1.5 mM $MgCl_2$, 20–50 ng DNA and 1.0 U of Taq DNA polymerase. Denaturation and extension were carried out at 95 °C for 15 s, and 72 °C for 2 min, respectively, for 30 cycles in a thermocycler (PTC-100, MJ Research, U.S.A.). Annealing temperature varied among primer pairs. The full-length cDNA has been submitted to GenBank (Accession no. AY341346).

2.6. Cloning and expression of BmHk

PCR amplified fragment was gel purified and cloned into PCR®4-TOPO-TA cloning vector (Invitrogen, USA) resulting in the plasmid PCR®4-TOPO-BmHk. The gene was digested out from the plasmid with restriction endonucleases BamHI and HindIII and sub-cloned into pTriEx-4 vector (Novagen) resulting in plasmid pTriEx-BmHk. This plasmid was transformed into *Escherichia coli* DH5α cells. Transformants were screened through colony PCR and restriction digestion. Cloning of complete ORF of BmHk was finally confirmed by sequencing of the clone.

To study the expression of recombinant BmHk, plasmid pTriEx-BmHk was transformed in T7 RNA polymerase encoding *E. coli* strain Rosetta (DE3). Single colony from transformed plate was inoculated in 5 ml of Luria-Bertani (LB) broth containing 40 µg/ml chloramphenicol and 100 µg/ml ampicillin. Cells were grown for 14–16 h at 37 °C with shaking at 180 rpm. 1.0% (v/v) of overnight grown culture was inoculated into 500 ml of fresh LB containing 40 µg/ml chloramphenicol and 100 µg/ml ampicillin. Cultures were then grown at 37 °C until an optical density of 600 nm (OD_{600}) reached 0.4–0.6, at this stage culture was induced with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG). Cultures were then grown at 20 °C for 14–16 h before harvest. Cells were harvested by centrifugation at 8000 ×*g* for 10 min at 4 °C. Cell pellet was stored at −20 °C until used.

2.7. Protein purification

BmHk having 6x-His tag attached to its N and C terminals was over-expressed in *E. coli* strain Rosetta (DE3) by inducing cells with IPTG (0.5 mM) and purified by Ni^{2+}–nitrilotriacetic acid resin (Ni^{2+}–NTA). The frozen cells were thawed in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 0.1% (v/v) Tween-20 and 10 mM β-mercaptoethanol) containing protease inhibitor cocktail (Sigma) and lysed by pulse sonication. The supernatant obtained after centrifugation at 10,000 ×*g* for 45 min was loaded on Ni^{2+}–NTA column pre-equilibrated with lysis buffer and contaminating proteins were