Parasitology Research (2019) 118:1511–1518
https://doi.org/10.1007/s00436-019-06279-w

IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Heterologous overexpression of active hexokinases from microsporidia *Nosema bombycis* and *Nosema ceranae* confirms their ability to phosphorylate host glucose

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Received: 2 November 2018 / Accepted: 27 February 2019 / Published online: 13 March 2019

Abstract
The secretion of hexokinases (HKs) by microsporidia followed by their accumulation in insect host nuclei suggests that these enzymes play regulatory and catalytic roles in infected cells. To confirm whether HKs exert catalytic functions in insect cells, we expressed in *E. coli* the functionally active HKs of two entomopathogenic microsporidia, *Nosema bombycis* and *Nosema ceranae*, that cause silkworm and honey bee nosematoses. *N. bombycis* HK with C-terminal polyHis tag and *N. ceranae* enzyme with N-terminal polyHis tag were cloned into pOPE101 and pRSET vectors, respectively, and overexpressed. Specific activities of *N. bombycis* and *N. ceranae* enzymes isolated by metal chelate affinity chromatography were 29.2 ± 0.5 and 60.2 ± 1.2 U/mg protein at an optimal pH range of 8.5–9.5. The kinetic characteristics of the recombinant enzymes were similar to those of HKs from other parasitic and free-living organisms. *N. bombycis* HK demonstrated Km 0.07 ± 0.01 mM and k_{cat} 1726 min^{-1} for glucose, and Km 0.39 ± 0.05 mM and k_{cat} 1976 min^{-1} for ATP, at pH 8.8. *N. ceranae* HK showed Km 0.3 ± 0.04 mM and k_{cat} 3293 min^{-1} for glucose, and Km 1.15 ± 0.11 mM and k_{cat} 3732 min^{-1} for ATP, at the same pH value. These data demonstrate the capability of microsporidia-secreted HKs to phosphorylate glucose in infected cells, suggesting that they actively mediate the effects of the parasite on host metabolism. The present findings justify further study of the enzymes as targets to suppress the intracellular development of silkworm and honey bee pathogens.

Keywords Microsporidia · *Nosema bombycis* · *Nosema ceranae* · Hexokinase · Heterologous expression · Enzyme assay

Introduction

Microsporidia are intracellular parasites related to fungi; they infect a broad range of hosts from protozoa to humans (Didier and Weiss 2008). The prevalence of microsporidia among animal phyla indicates their long adaptation to the intracellular environment. Sequencing of microsporidia genomes demonstrated a unique minimization of parasite metabolic machinery (Katinka et al. 2001; Wiredu et al. 2017) and acquisition of unique transporters to effectively exploit an infected host cell (Tsaousis et al. 2008; Heinz et al. 2014; Dean et al. 2018). These data, and the fact that most microsporidia develop in direct contact with cytoplasm of infected cells, suggest the ability of these parasites to control host molecular programs and biochemical pathways.

Hexokinase (HK) is one of the microsporidial enzymes potentially involved in this process. Previously, it has been reported that, unlike other components of glycolysis, HK is highly expressed at early stages of intracellular development of the microsporidium *Nematocida parisii* (Cuomo et al. 2012). In the same study, the authors further showed that secretion signals of six microsporidian HKs directed the secretion of a reporter enzyme in the *Saccharomyces cerevisiae* secretion trap system. Further, it was reported that secretion of *Paranosema* (*Antonospora*) *locustae* HK into infected cells of locust *Locusta migratoria* fat bodies is followed by its accumulation in host nuclei (Senderskiy et al. 2014). The presence of secretory HKs in infected host cells has been demonstrated for two microsporidian species

Section Editor: Kevin S.W. Tan

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of *Nematocida* genus (Reinke et al. 2017). The authors expressed ascorbate peroxidase in the cytoplasmic or nuclear compartments of the nematode *Caenorhabditis elegans*, followed by mass spectrometric analysis of biomolecules biotinylated by the enzyme, and identified 82 parasitic proteins that interacted with the infected host cell. Secretion of the microsporidium *Trachipleistophora hominis* HKs into rabbit kidney RK13 cells has been demonstrated using immunofluorescence and immunoelectron microscopy (Ferguson and Lucocq 2018). Labeling was observed on the membrane structures of the secretory apparatus of the parasites as well as on the plaque matrix, an amorphous structure around the parasite (parasite vacuole) extending into the host cytoplasm; however, no labeling was observed on the host nuclei. The metabolic activity of *T. hominis* HKs was indirectly confirmed using a fluorescent analog of glucose (2-NBDG) whose signal increased in the enzyme-containing plaque matrix zone. Recently, HK secretion followed by its accumulation in the host nuclei was observed in the microsporidium *Nosema bombycis*—a pathogen of silkworms (Huang et al. 2018).

Secretion of HKs by many microsporidia species into infected cells suggests their essential role in host-parasite relationships. In yeast (Petit et al. 2000; Moreno and Herrero 2002), cancer cells (Neary and Pastorino 2010), and plants (Moore et al. 2003), this enzyme was discovered to be a bifunctional protein that has a hexose-phosphorylating activity and plays a regulatory role in cell metabolism. Elucidation of the regulatory functions of microsporidia HKs is challenging; however, it is not as difficult to confirm their fermentative activity. The requirement for such enzymological study is supported by the evidence that finding of conserved, functionally significant residues in the active center of HK via computational methods is not sufficient to prove its activity. Completely inactive HK2 of the protozoan parasite *Trypanosoma brucei* is 98% identical to highly active HK1, and seven substitutions lie within the C-terminal peptide consisting of 18 amino acid (a/a) residues (Morris et al. 2006). Furthermore, we have been unable to detect HK activity in cells and spores of the microsporidium *Paranosema grylli* in previous studies (Dolgikh et al. 1997; Dolgikh 2000).

Here, we overexpressed HKs in *Escherichia coli* and purified functionally active enzymes of microsporidia *N. bombycis* and *Nosema ceranae* that cause silkworm and honey bee nosematoses. Kinetic characteristics of recombinant proteins were found to be comparable with parameters of HKs from other organisms, strongly suggesting an active role for microsporidia enzymes in the phosphorylation of host glucose and in mediating the effects of the parasite on the metabolism of infected cells.

Material and methods

DNA constructs for heterologous expression of HKs in *E. coli*

N. bombycis and *N. ceranae* spores were obtained from the microsporidia collection of Prof. Irma V. Issi affiliated with the All-Russian Institute of Plant Protection (St. Petersburg, Pushkin). Genomic DNA extraction has been described previously (Tokarev et al. 2018). The genes were amplified using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, MA). The following primers were designed for PCR amplification of HK-encoding genes without 5′-regions encoding predicted signal peptides (SP) responsible for protein secretion (the restriction sites are underlined, 5′-3′ sequences): NcHKF CAACCATGGGTTATGATGTACCA GGTTACACTAA; NcHKR1 TCAGCGGCCGCATTCAT TGTTTTTTTCGGTTTCACA; NcHKR2 TCAGCGG CCGCTTAATGATGATGATGATGATTCATTGTTT TTTTCGGTTTCACA; NcHKR3 TCACTGCAGTTAA TTCATTGTTTTTTTCGGTTTCACA; NbHKF CAACCAT GGGTGAATTAATTAAGACATTGGGAAA; NbHKR1 TCAGCGGCCGCATAAATAAT TCGATGTAAAGTAT; NbHKR2 CCAGCGGCCGCTTAATGATGATGAT GATGATGATAAATAATTCGATGTAAAGTAT.

To insert HK-encoding fragments into the pOPE101 vector (Progen, Germany), we used NcHKF/NcHKR1, NcHKF/NcHKR2, NbHKF/NbHKR1, and NbHKF/NbHKR2 combinations of primers. The PCR products were gel purified, digested with NcoI/NotI restriction enzymes, and inserted into the vector linearized by the same enzymes. To express *N. ceranae* HK in the pRSET expression vector (Thermo Fisher Scientific, MA), protein-encoding sequence was amplified with NcHKF/NcHKR3 primers, inserted into a pIEx-4 plasmid (Merck Millipore, Germany) at NcoI/PstI restriction sites and re-cloned into pRSETb vector at NcoI/HindIII sites. All constructed plasmids were sequenced to verify the correct amplification and insertion of HK-encoding fragments.

Heterologous expression and isolation of recombinant proteins

To express HKs in pOPE101 vector, the constructed plasmids were transformed into *E. coli* XL-1Blue MRF' cells via electroporation at 1700 V using Electroporator 2510 (Eppendorf, Germany). Positive colonies from plates with selective 2 × YT-GA medium containing 0.1 M glucose and 0.15 mg/mL ampicillin were inoculated in 25 mL of the same liquid medium. After growing in an orbital shaker at 220 rpm and 37 °C until OD_{600} 0.4, cells were harvested by centrifugation at 3000×*g* for 15 min and transferred into 25 mL of 2 × YT containing ampicillin and 0.04 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by cultivation for 4 h at

25 °C. To express *N. ceranae* HK in pRSETb vector, plasmids with the inserted gene were transformed into *E. coli* BL21(DE3)-derived C41 cells (Miroux and Walker 1996) by electroporation, and colonies selected on LB plates containing 0.15 mg/mL ampicillin were inoculated in 25 mL of the same liquid broth. The culture was grown to OD_{600} of 0.4, and expression was induced by addition of 0.1 mM IPTG (final concentration) followed by cultivation for 15 h at 25 °C.

After cultivation, bacterial cells were pelleted at 3000×*g* for 15 min and sonicated in 1 mL of equilibration buffer solution (50 mM Tris-Cl (pH 7.5), 0 .3M NaCl, 10 mM imidazole) for immobilized metal ion affinity chromatography (IMAC). Equilibration buffer contained 1 mM phenylmethylsulfonyl fluoride (PMSF) as a serine protease inhibitor. Soluble fractions of homogenates centrifuged at 18,000×*g* for 20 min were diluted 1:10 with equilibration buffer solution and passed through columns with 0.25 mL of resin Roti®garose-His/Ni Beads (Carl Roth, Germany) equilibrated with the same buffer. After thorough washing, the recombinant proteins were eluted by equilibration buffer solution with 0.25 M imidazole. The first 0.6 mL of eluates were mixed with an equal volume of glycerol and stored at − 20 °C.

SDS-PAGE and immunoblotting

IMAC fractions (0.1 mL) were heated at 95 °C for 10 min with equal volume of 2 × sample buffer (125 mM Tris-Cl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol). Then, 8 μL of protein samples were separated by SDS-PAGE in 12% gel, stained by Coomassie Brilliant Blue, or transferred onto nitrocellulose membranes following the manufacturer's instructions for the electroblotting apparatus Mini-PROTEAN (Bio-Rad, CA). The membranes were blocked in TTBS (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.05% Tween-20) with 1% bovine serum albumin for 1 h at 25 °C, incubated for 2 h with monoclonal antibodies (Abs) against polyhistidine sequence conjugated with horseradish peroxidase (Sigma-Aldrich, MO) diluted 1:2000 with TTBS, and then washed with TTBS and TBS (TTBS without Tween-20). The peroxidase reaction was performed with 4-chloro-1-naphthol as a substrate.

Enzyme assays

HK activity was determined spectrophotometrically with an optical path of 10 mm at a wavelength of 340 nm and temperature 25 °C. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the phosphorylation of 1 μmol of D-glucose per minute at 25 °C and pH 8.8 in 1 mL of reaction mixture. The differential molar extinction of NADPH and $NADP^+$ at 340 nm equalled 6220 M^{-1} cm^{-1}.The assay mixture contained 0.1 M Tris-Cl (pH 8.8), 5 mM $MgCl_2$, 10 mM glucose, 5 mM ATP, 0.5 mM $NADP^+$, 0.2 U/mL glucose 6-phosphate dehydrogenase, and aliquot of recombinant protein. Michaelis constants (Km) were analyzed by activity assay at 7–9 varying ATP or glucose concentrations from 0.0375 to 20 mM. Km and Vmax were determined using GraphPad Prism 8.0.1 software. k_{cat} values (min^{-1}) were calculated as Vmax (μmol/min/L) divided by micromolar enzyme concentration (μmol/L). Optimal pH values for microsporidia HKs were defined in 0.1 M Tris-Cl (pH 7–8.8) or 0.1 M glycine-NaOH (pH 9–10) buffer solutions. Protein concentrations in HK preparations were determined by the Bradford method (Bradford 1976).

Results

Sequences of *N. bombycis* and *N. ceranae* HKs

Sequencing of fragments amplified from microsporidia genomic DNA and cloned in expression vectors confirmed that the *N. bombycis* HK gene encodes 406 a/a protein identical to the sequence EOB11276.1 deposited in GenBank, but without N-terminal 22 a/a SP. PCR-amplified gene of *N. ceranae* HK encoded 413 a/a protein devoid of 17 a/a SP, demonstrating a single a/a residue difference from the GenBank sequence XP_024330607.1 (Fig. 1a).

Heterologous expression of HKs

First, we attempted to express microsporidia HKs in *E. coli* XL1-Blue MRF' cells using the pOPE101 vector originally designed for the production of functional single-chain mini-antibodies under the control of the strong synthetic $P_{A1/04/03}$ promoter (Schmiedl et al. 2000). The encoding sequences were inserted in frame between a pelB-leader sequence for the secretion of heterologous proteins into the bacterial periplasmic space and a region encoding c-myc and polyHis C-terminal tags (Fig. 1b). The enzymes encoded by these plasmids were dubbed NbHKa and NcHKa. In addition, we constructed plasmids encoding recombinant proteins seamlessly fused with C-terminal polyHis peptide and devoid of c-myc tag (Fig. 1b). HKs encoded by these plasmids were designated as NbHKb and NcHKb.

Transformation of *E. coli* by these constructs and subsequent heterologous expression, followed by sonication of bacteria and purification of soluble recombinant products by IMAC, revealed the efficient production of both *N. bombycis* HKs (Fig. 2, lanes 3, 4). The induction of *N. bombycis* HK expression in 25 mL of bacterial culture at OD_{600} 0.4 followed by cultivation for 4 h at 25 °C enabled the isolation of approximately 0.12 mg of microsporidia enzyme. Surprisingly, despite the successful purification of these proteins on Ni-resin, anti-polyHis Abs showed poor recognition of NbHKa (Fig. 2, lane 4) and did not stain NbHKb (Fig. 2,

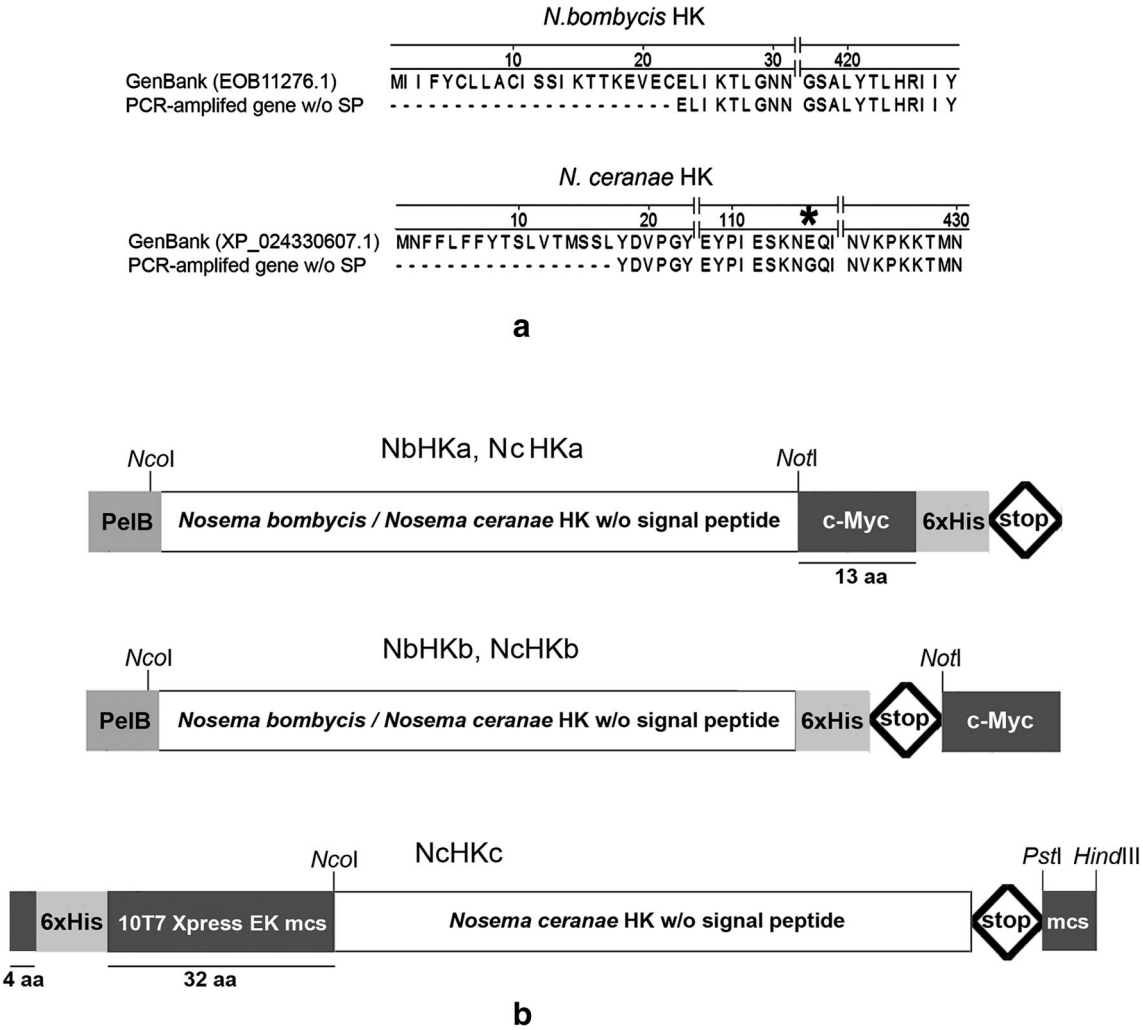

Fig. 1 Sequences of microsporidian HKs expressed in *E. coli*. **a** PCR-amplified genes of *N. bombycis* and *N. ceranae* enzymes encode 406 a/a and 413 a/a proteins without N-terminal signal peptides. The difference in single a/a residue between the cloned *N. ceranae* gene and GenBank sequence XP_024330607.1 is marked with an asterisk. **b** The recombinant proteins encoding by *HK* genes inserted into the pOPE101 vector in frame between a pelB-leader sequence and a region encoding c-myc and polyHis C-terminal tags were dubbed NbHKa and NcHKa. Recombinant proteins in the same vector fused with C-terminal polyHis peptide and devoid of c-myc tag were dubbed NbHKb and NcHKb. *N. ceranae* HK encoded by gene inserted in pRSETb vector in frame with N-terminal 4 kDa peptide containing polyHis sequence was denoted as NcHKc

lane 3) during immunoblotting. Another surprising result of this experiment was that a very low rate of synthesis of both variants of *N. ceranae* recombinant HKs was observed. Despite the identical conditions of heterologous expression and purification of the recombinant proteins of two microsporidia species, only trace amounts of *N. ceranae* enzyme were present in the analyzed IMAC fractions (Fig. 2, lanes 1, 2). This could not be attributed to the low protein solubility in *E. coli* because the expression of *N. ceranae* HKs in XL1-Blue MRF' cells was not accompanied by the accumulation of insoluble inclusion bodies (IBs).

Subsequently, the *N. ceranae* HK gene was cloned into the pRSET vector designed for expression of foreign proteins under the control of a strong promotor from the T7 bacteriophage. The gene was cloned in frame with a N-terminal 4 kDa peptide (42 a/a) containing a polyHis tag (Fig. 1b). The HK encoded by this plasmid was dubbed NcHKc. Transformation of C41 *E. coli* cells by this construct resulted in the accumulation of a recombinant enzyme in the form of insoluble IBs. Further, IMAC of soluble fraction of sonicated bacteria followed by SDS-PAGE and western blot analysis indicated that a significant part of the enzyme remained soluble in bacteria and could be isolated using a Ni-resin (Fig. 2, lane 5). *N. ceranae* HK expression in 25 mL of bacterial culture for 15 h at 25 °C yielded approximately 0.5 mg of purified enzyme. In this case, anti-polyHis Abs clearly demonstrated the N-terminal tag of the recombinant protein (Fig. 2, lane 5).

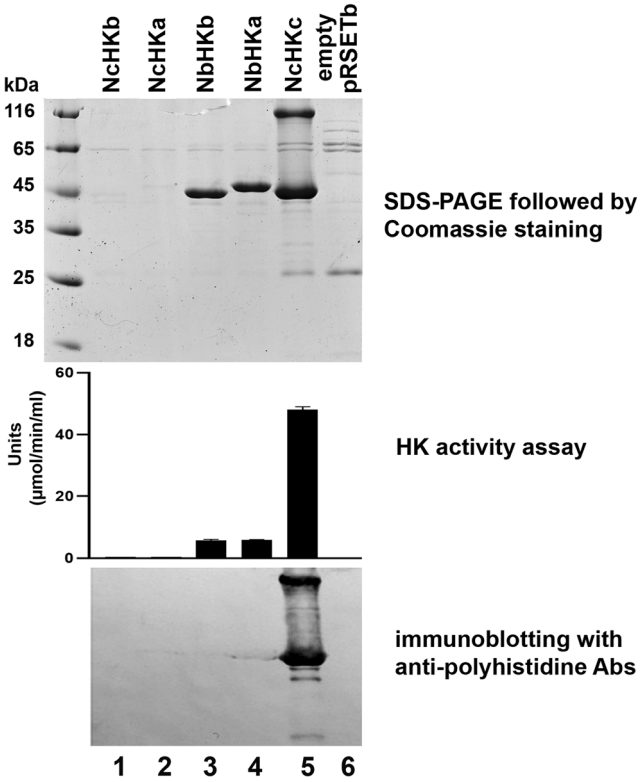

Fig. 2 Analysis of *N. bombycis* and *N. ceranae* purified HKs by SDS-PAGE, immunoblotting, and enzyme activity assay. Recombinant proteins from 25 mL of bacterial cultures were isolated by IMAC as 0.6-mL fractions with protein concentration 0.2 mg/mL (NbHKa, NbHKb) and 0.8 mg/mL (NcHKc). Eluates (4 μL) were analyzed by SDS-PAGE and immunoblotting. In enzyme activity assay, mixture concentrations of NbHKa, NbHKb, and NcHKc were 0.1 μg/mL

Activities and kinetic properties of HKs

The spectrophotometric method based on the reduction of $NADP^+$ through a coupled reaction with glucose-6-phosphate dehydrogenase demonstrated the glucose-phosphorylating activity of microsporidia HKs for the first time. Specific activities of IMAC fractions were 29.2 ± 0.5 μmol/min/mg protein (U/mg protein) for NbHKa, 28.2 ± 1.6 U/mg protein for NbHKb, and 60.2 ± 1.2 U/mg protein for NcHKc. Activities of NcHKa and NcHKb (Fig. 2, lanes 1, 2) did not exceed 3% of NbHKa and NbHKb values and were two orders lower than the activity of NcHKc. The control fraction purified from C41 cells transformed with empty pRSETb vector did not show any activity (Fig. 2, lane 6). For further analysis of recombinant proteins, we used IMAC-purified fractions of NbHKa and NcHKc.

The activity of either microsporidia enzymes was the highest at pH 8.5–9.5 (Fig. 3) and significantly decreased outside this range. Analysis of kinetic characteristics showed lower affinity of *N. ceranae* HK for glucose and ATP compared with that of the *N. bombycis* enzyme (Fig. 4). NcHKc and NbHKa Km values for ATP were 1.15 ± 0.11 mM and 0.39 ± 0.05 mM, respectively. *N. bombycis* HK showed four times higher affinity for glucose compared with the *N. ceranae* enzyme. Km values for this substrate were 0.07 ± 0.01 mM and 0.3 ± 0.04 mM in the case of NbHKa and NcHKc, respectively. In contrast, specific activity, Vmax, and k_{cat} (turnover number) of *N. ceranae* HK were approximately twofold higher than the corresponding values for the *N. bombycis* enzyme. NcHKc displayed Vmax values of 72.1 ± 1.8 U/mg protein and 63.6 ± 1.6 U/mg protein for ATP and glucose, whereas the corresponding values for NbHKa were 38.2 ± 1.5 U/mg protein and 33.3 ± 0.7 U/mg protein, respectively. The k_{cat} values of *N. ceranae* and *N. bombycis* HKs for ATP were 3732 min^{-1} and 1976 min^{-1}, respectively. For glucose, the k_{cat} values of NcHKc and NbHKa were 3293 min^{-1} and 1726 min^{-1}, respectively.

Discussion

In this study, functionally active HKs of two entomopathogenic microsporidia were overexpressed in bacteria *E. coli*. As evidence suggests, *N. ceranae* and *N. bombycis* enzymes are secretory proteins (Cuomo et al. 2012; Huang et al. 2018), predicted signal peptides were removed in their recombinant forms. Heterologous expression and IMAC purification, followed by enzyme assays, showed that specific activities of microsporidial recombinant proteins were comparable to the activity of natural HK purified from bakers' yeast *S. cerevisiae* (Simon et al. 1985).

The slightly alkaline pH optima showed for *N. bombycis* and *N. ceranae* enzymes were also demonstrated in the case of coccidian *Eimeria tenella* (Sun et al. 2016), plant *Zea mays* (Doehlert 1989), and trematode *Clonorchis sinensis* (Chen et al. 2014) HKs. At the same time, HKs of yeast *S. cerevisiae* (Sols et al. 1958), and protozoan parasites *T. brucei* (Nwagwu and Opperdoes 1982), *Trypanosoma cruzi* (Cáceres et al. 2003), *Plasmodium falciparum* (Harris et al. 2013), and *Toxoplasma gondii* (Saito et al. 2002) exhibited maximal activity at neutral pH values between 7.0 and 8.8. As the pH optimum of immobilized yeast HK was shifted by 1.5 U to a more alkaline value than that of the soluble enzyme (Simon et al. 1985), additional tags could modify the properties of microsporidial recombinant proteins.

Both microsporidia HKs exhibited Km values for ATP that were similar to those of four enzymes of apicomplexan parasites (Table 1). Additionally, the affinity of *N. bombycis* recombinant HK for glucose was the highest among all enzymes presented in the table, barring the *T. cruzi* natural form and *T. gondii* enzyme with the GST-tag removed. Notably, the natural (untagged) forms of enzymes may have higher affinity for substrates than their recombinant homologs. *T. cruzi* recombinant HK displayed lower affinity for glucose and ATP than the natural enzyme, and the removal of the N-terminal tag

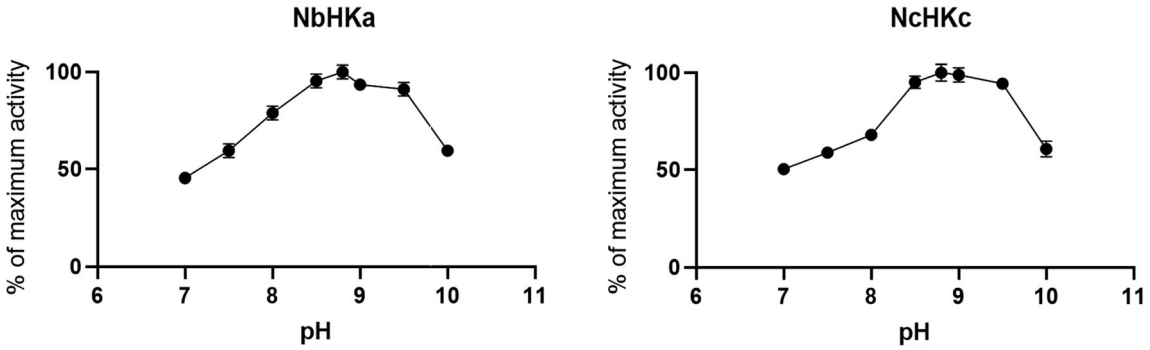

Fig. 3 Effect of pH on the activity of *N. bombycis* and *N. ceranae* HKs. A 0.1 M Tris-Cl (pH 7–8.8) or 0.1 M glycine-NaOH (pH 9–10) buffer solutions contained 5 mM $MgCl_2$, 10 mM glucose, 5 mM ATP, 0.5 mM NADP, 0.2 U/mL glucose 6-phosphate dehydrogenase, and 0.1 μg/mL recombinant protein. Bars represent standard errors of the means based on reactions in triplicate

reduced its Km values (Cáceres et al. 2003). The k_{cat} values of *N. bombycis* and *N. ceranae* HKs were about 10 times higher than those of *T. gondii* and wild potato *Solanum chacoense* HKs (Saito et al. 2002; Claeyssen et al. 2006). However, these values were about 10 times lower than the k_{cat} of *E. tenella* (Sun et al. 2016), *P. falciparum* (Harris et al. 2013), *T. brucei* (Morris et al. 2006), and *T. cruzi* (Cáceres et al. 2003) enzymes. The turnover numbers of microsporidial recombinant proteins were similar to the k_{cat} of apicomplexan parasite *Cryptosporidium parvum* HK (Yu et al. 2014) as well as human and mouse glucokinases (Antoine et al. 2009; Pino et al. 2007).

Kinetic properties of microsporidia recombinant HKs show that *N. bombycis* and *N. ceranae*, the most practically important entomopathogenic microsporidia, have retained active glucose-phosphorylating enzymes during their adaptation to intracellular lifestyle. According to the phylogenetic system based on molecular analysis of the small subunit ribosomal

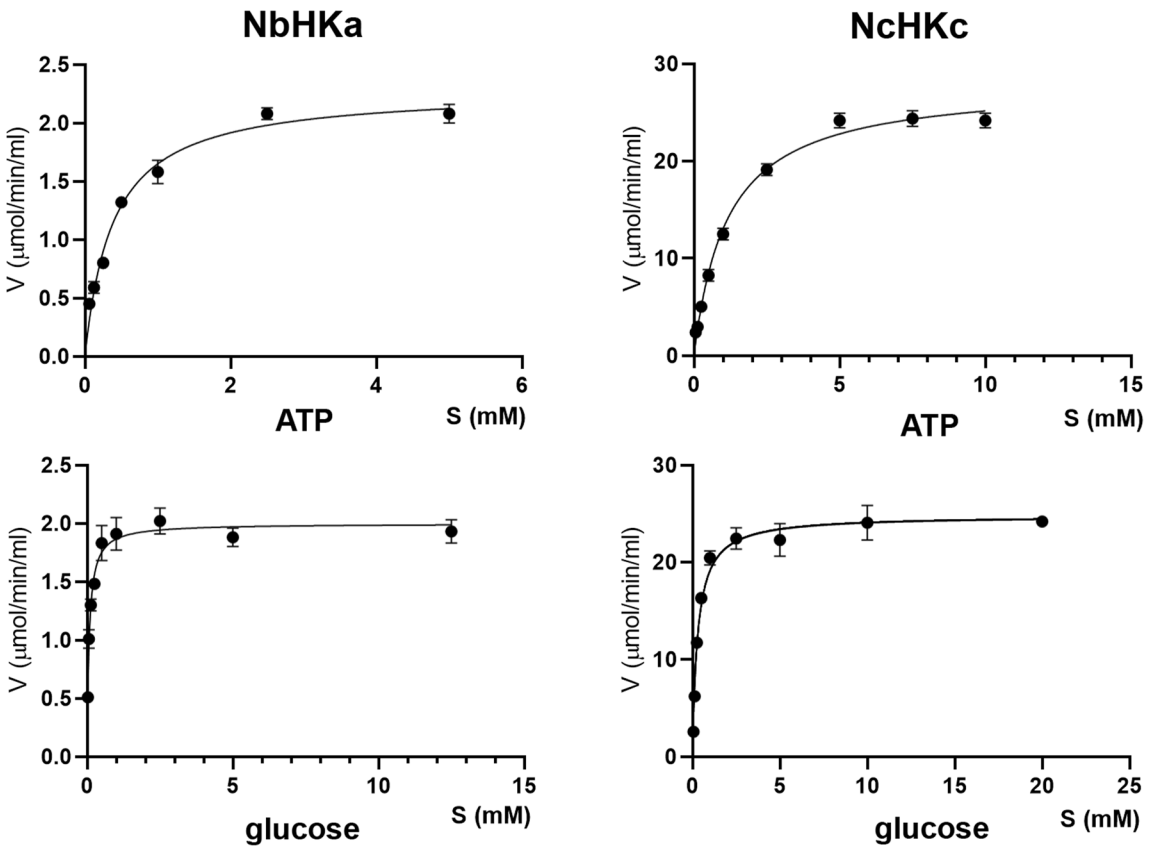

Fig. 4 Michaelis-Menten kinetics of microsporidia HKs towards ATP and glucose. Enzyme assay mixture contained 0.1 M Tris-Cl (pH 8.8), 5 mM $MgCl_2$, 10 mM glucose (Km for ATP) or 5 mM ATP (Km for glucose), 7–9 varied concentrations of substrates, 0.5 mM NADP, 0.2 U/mL glucose 6-phosphate dehydrogenase, and 0.06 μg/mL (NbHKa) or 0.2 μg/mL (NcHKc) recombinant protein. Bars represent standard errors of the means based on reactions in triplicate

Table 1 Michaelis constants for glucose and ATP of recombinant (R) and natural (N) hexokinases of some parasitic and free-living organisms

Species	Recombinant or natural enzyme	Km for glucose	Km for ATP	Reference
Cryptosporidium parvum	R	0.14/0.18	0.67	Yu et al. 2014
Toxoplasma gondii	R[a]	0.008	1.05	Saito et al. 2002
Plasmodium falciparum	R	0.62	0.66	Harris et al. 2013
Plasmodium falciparum	N	0.43	3.1	Roth 1987
Eimeria tenella	R	0.67	0.66	Sun et al. 2016
Trypanosoma cruzi	N/R	0.04/0.20	0.32/0.53	Cáceres et al. 2003
Trypanosoma cruzi	N	0.09	0.4	Racagni and Machado de Domenech 1983
Trypanosoma brucei	R	0.09	0.28	Morris et al. 2006
Trypanosoma brucei	N	0.017	0.116	Nwagwu and Opperdoes 1982
Saccharomyces cerevisiae	N	0.1	0.1	Sols et al. 1958
Saccharomyces cerevisiae				Kopetzki and Entian 1985
Isoform I	N	0.12	0.2	
Isoform II	N	0.23	0.15	
Clonorchis sinensis	R	0.116	0.459	Chen et al. 2014
Homo sapiens	R	0.34	1.02	Ardehali et al. 1996
Zea mays				Doehlert 1989
Isoform I	N	0.117	0.066	
Isoform II	N	0.750	0.182	
Nosema bombycis	R	0.07 ± 0.01	0.39 ± 0.05	Present study
Nosema ceranae	R	0.3 ± 0.04	1.15 ± 0.11	Present study

[a] GST fusion tag was removed using Factor Xa proteinase (Saito et al. 2002)

DNA (Vossbrinck and Debrunner-Vossbrinck 2005), these microsporidia belong to phylogenetic clade IV (Terresporidia) and lack alternative oxidase genes (Williams et al. 2010). It suggests that glycolysis accompanied by ATP synthesis may be problematic in terrestrial microsporidia. The main function of parasite HK is rather connected with its secretion into infected cells to phosphorylate host glucose. As previously suggested (Cuomo et al. 2012; Ferguson and Lucocq 2018), the generation of glucose-6-phosphate by this enzyme should increase host synthesis of ATP, other nucleotides, amino acids, and lipids necessary for parasite growth as well transport of new glucose molecules into infected cells. Microsporidia HKs in infected host cells may also facilitate the delivery of glucose-6-phosphate into cisternae of the host endoplasmic reticulum, which is often associated with parasite cells and may potentially be involved in relationships the two cells (Ferguson and Lucocq 2018). As mentioned in the introduction, microsporidia HKs may also be involved in the regulation of cell metabolism as bifunctional proteins. However, this feature of parasite enzymes requires further experimental evidence.

In addition to demonstrating the glucose-phosphorylating activity of *N. bombycis* and *N. ceranae* HKs, we identified the conditions for their efficient overexpression and isolation. Recently, an important role of HK in the proliferation of microsporidia *N. bombycis* was demonstrated by RNA interference; in this study, down-regulation of the expression of the enzyme suppressed parasite intracellular development (Huang et al. 2018). These data justify further study of microsporidia HKs as targets to suppress the intracellular development of silkworm and honey bee pathogens.

Funding information This work was supported by Russian Science Foundation (RSF 18-16-00054) and Russian Foundation of Basic Research (RFBR 18-34-00265mol_a).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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