

# Cloning and molecular characterization of tick kynureneine aminotransferase (HIKAT) from *Haemaphysalis longicornis* (Acar: Ixodidae)

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**Abstract** A complementary DNA coding a novel kynureneine aminotransferase (KAT) molecule from *Haemaphysalis longicornis* tick embryo was cloned and characterized. The transcription of the *HIKAT* occurs at all stages during tick development as well as in the midgut, salivary glands, ovary, and synganglion of adult ticks, and protein expression levels increased during the blood-feeding course. The HIKAT gene without signal peptide was successfully expressed as a glutathione *S*-transferase fusion protein in soluble form, which is capable of catalyzing the transamination of kynureneine and 3-hydroxykynureneine to kynurenic acid and xanthurenic acid, respectively. The purified recombinant HIKAT showed dose-dependent inhibition effect on the growth of equine babesial parasite, *Babesia caballi*, in vitro culture. All results suggested that a specific HIKAT is present in tick and HIKAT may play an important physiological role in *H. longicornis*. This is the first report of a member enzyme of tryptophan pathway in Chelicerata.

## Introduction

Kynureneine aminotransferase (KAT; EC. 2.6.1.7) is an enzyme involved in tryptophan catabolism. In mammalian

cells, the essential amino acid tryptophan is degraded primarily by the kynureneine pathway, a cascade of enzymatic steps containing several biologically active compounds (Stone and Darlington 2002; Cooper 2004; Schwarcz 2004). Metabolites of this pathway have been shown to be involved in many diverse physiological and pathological processes (Cooper 2004; Schwarcz 2004). In particular, the levels of kynureneine and its product kynurenic acid (KYNA) are altered in several neurodegenerative diseases including the Huntington's disease (Beal et al. 1990; Guidetti et al. 2000), Alzheimer's disease (Widner et al. 2000), schizophrenia (Schwarcz et al. 2001; Erhardt et al. 2003, 2007) and acquired immunodeficiency syndrome (AIDS) dementia (Guillemin et al. 2005). In mammalian brain, KYNA is produced irreversibly from L-kynureneine by the action of kynureneine aminotransferases (KAT), which are capable of catalyzing transamination of kynureneine in vitro. In mammals, four proteins arbitrarily named as KAT I, II, III, and IV have been considered to be involved in KYNA synthesis in the central nervous system (CNS; Guidetti et al. 2000, 2007; Han et al. 2004). These proteins are all pyridoxal-5'-phosphate-(PLP)-dependent enzymes (Han et al. 2008). In the result of the study on tryptophan metabolism in mosquito, Fang et al. (2002) determined that the tryptophan to xanthurenic acid (XA) pathway via kynureneine and 3-hydroxykynureneine (3-HK) intermediates is the major catabolic pathway for tryptophan in *Aedes aegypti* mosquitoes. Their data suggest that *A. aegypti* has a transaminase that has a higher specific activity to 3-HK than to kynureneine (Li and Li 1997). The sequence of *A. aegypti* kynureneine aminotransferase (AeKAT)1 is 47% identical with that of human KAT I and 51.9% identical with that of human KAT III (Yu et al. 2006). AeKAT and a glutamine/phenylpyruvate aminotransferase from thermophilic bacteria *Thermus thermo-*

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*philus*, homologs of mammalian KAT I, have also been systematically characterized (Han and Li 2004; Hosono et al. 2003).

In this study, we cloned novel tick kynurenine aminotransferase (HIKAT) from *Haemaphysalis longicornis* tick embryo complementary DNA (cDNA) library and describe molecular characterization of this gene on the kynurenine pathway.

## Materials and methods

### Ticks

The parthenogenetic Okayama strain of the tick *H. longicornis* has been maintained by feeding on rabbits and mice for several generations in our laboratory (Fujisaki 1978).

### Identification of HIKAT cDNA

The *H. longicornis* kynurenine aminotransferase (HIKAT) was identified from expressed sequence tags (EST) constructed from egg cDNA library. Plasmids, containing a KAT gene encoding insert, were extracted using the QIAGEN DNA Purification kit (Qiagen, USA) and the inserts were sequenced by the big dye terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). To assign putative identifications to the KAT sequences, BLAST alignment (Altschul et al. 1997) was performed to compare with sequences available in the GenBank (Benson et al. 2002) and CDART architecture (Geer et al. 2002) to identify conserved domains. The full-length open reading frame (ORF) sequence of KAT cDNA was confirmed by sequencing three separate clones amplified from the egg cDNA library with T7 and T3 plasmid-specific promoters, and HIKAT-F1 and HIKAT-F2 primers, which were designed based on the cDNA-derived product sequence with MacVector software (Oxford Molecular, Madison, WI, USA). Sequence analysis and amino acid translations of the HIKAT sequence were determined using the MacVector software. A dendrogram showing the relationship between members of KATs was generated by pairwise alignment of several KATs with MacVector computer program (Oxford Molecular, CA, USA). Putative signal peptide cleavage sites with GENETYX software version 7 (version 7.3; Genetyx Corp., Tokyo, Japan) and potential N-glycosylation sites were determined by the prediction servers NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc>), respectively. Theoretical molecular mass and isoelectric

points were determined by PeptideMass (<http://us.expasy.org/tools/peptide-mass.html>; Wilkins et al. 1997).

### Expression of the recombinant HIKAT cDNA in *Escherichia coli*

A 1248-bp polymerase chain reaction (PCR) fragment from *H. longicornis* KAT containing an open reading frame was inserted into the EcoRI site of pGEX-4T-3 plasmid to generate recombinant plasmid pGEX-4T-KAT. Restriction enzyme analysis was performed to identify the construct containing the insert in the correct orientation. The recombinant HIKAT (rHIKAT) was expressed in *E. coli* (DH5 $\alpha$ ) and expressed as glutathione S-transferase (GST) fusion protein and designated GST-HIKAT protein. The resulting *E. coli* cells were washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100–PBS, sonicated, and then centrifuged at 12,000 $\times g$  for 10 min at 4°C. Supernatants containing the soluble GST fusion protein were purified with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions and later treated with thrombin protease (Amersham Pharmacia Biotech) at the elution step to remove the GST tail from the fusion protein. Thrombin protease cleavage was used to combine with glutathione–Sepharose 4B according to the manufacturer's instructions. The extracted recombinant HIKAT without GST was dialyzed in PBS overnight at 4°C, re-suspended in PBS at 500 µg/ml concentration, and then stored –80°C until use.

### Production of anti-recombinant GST-fused KAT serum

Female mice (BALB/c, 8 weeks old) were immunized intraperitoneally three times at 2-week intervals with 100 µg of the recombinant fusion protein in Freund's complete or incomplete adjuvant (Difco). Sera were collected from immunized mice 10 days after the last immunization and were stored at –80°C until use.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing and non-reducing conditions was performed as described by Laemmli (1970). After SDS-PAGE, the proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane. The blots were incubated in the primary anti-HIKAT sera (1:200 dilution) and positive signals detected with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:2,000 dilution; Amersham Pharmacia Biotech, UK) in 3,3'-diaminobenzidine tetrahydrochloride.

### Collection of tick tissues

Adult *H. longicornis* were fed on the ears of rabbits (Fujisaki 1978). Ticks were recovered from the rabbit 3 days post-infestation. The salivary glands, midgut, synganglion, and ovary from unfed and partially fed ticks were immediately dissected out under a microscope (Fujisaki 1978). Dissected organs were separately homogenized mechanically in PBS using a pellet pestle® (Kontes, Japan). The homogenized tissues were ultrasonicated on ice and then centrifuged at 8,000×*g* for 10 min. The supernatant was recovered and stored at –80°C as lysate antigen until use.

### Indirect fluorescent antibody test

Dissected midguts of ticks (4 days post-engorgement) were fixed in a fixation buffer (4% paraformaldehyde and 0.1% glutaraldehyde in PBS, pH7.4) overnight at 4°C. After fixation, the midgut was embedded in OCT compound (Tissue-Tek®, USA) for making frozen sections. Frozen sections (approximately 10 µm thick) were cut on a Leica CM 3050 cryostat and put on poly-L-lysine-coated glass slides. For immunostaining, the slides were blocked with PBS buffer containing 5% skim milk (Wako Pure Chemical Industries, Osaka, Japan) overnight at 4°C. Sections were then incubated for 1 h at 37°C with a mouse anti-HIKAT antiserum or mouse anti-GST antiserum (as a negative control) at a dilution of 1:250. The slides were thoroughly rinsed with PBS buffer and reacted with Alexa Fluor® 594 goat anti-mouse IgG secondary antibody (Invitrogen, CA, USA; 1:2,000). After reaction with the secondary antibody, the slides were washed, mounted in mounting medium (Vectashield®, Vector, CA, USA), and then covered with coverslips. The images were recorded using Leica TCS-NT confocal laser scanning microscope (Leica, Heidelberg, Germany), and image processing was conducted by using Adobe Photoshop® Ver. 4.0J software (Adobe Systems, CA, USA).

### Expression analysis by reverse transcriptase polymerase chain reaction

In order to determine the KAT gene expression in different development stages, total RNA extracted from egg (embryo), larval, nymphal, and adult ticks was used to purify the total RNA and then analyzed by reverse transcriptase polymerase chain reaction (RT-PCR), using one-step RNA PCR kit (Takara, Japan). To confirm the KAT gene expression in different tissues, salivary glands, midgut, ovary, and synganglion were dissected from 4-day-fed adult tick and subjected to RT-PCR analysis. About 1 µl of the RT products were used in a PCR reaction with gene-specific primers (sense primer, GAGGTGATCATCATTGAGCCCTTCTTGAT; anti-sense

primer, CCCACTGTGCCTGAGGGGGGCTAC). Positive control RT-PCR reactions were carried out using tick actin primers to confirm the cDNA integrity (accession no. AY254898, sense primer, GGTTGCCGCCCTGGTGG TTGA; anti-sense primer, GCCGCACGATTCCATACC CAGG). The series of RT-PCRs were performed in 50 µl of a mixture containing 1 µg of RNA, 100 pmol of oligonucleotides, one-step RNA PCR buffer, 5 mM of MgCl<sub>2</sub>, 1 mM of dNTPs, 1 U of RNase inhibitor, 5 U of AMV RTase XL, and 5 U of AMV-optimized Taq DNA polymerase. The reverse transcription reaction was carried out at 50°C for 30 min, and then PCR was repeated for 30 cycles under the following conditions: 30 s of denaturation at 94°C, 30 s of primer annealing at 65°C, and 2 min of elongation at 72°C. The PCR products were subjected to electrophoresis in a 1.5% agarose gel in TBE buffer.

### KAT activity assay

All chemicals were purchased from Sigma Chemical Company unless otherwise specified. KAT activity assay was based on the methods described in previous reports (Okuno et al. 1990; Li and Li 1997). Briefly, a typical reaction mixture of 100 µl containing 5 mM L-kynurenine or 5 mM 3-HK, 10 mM pyruvate, 70 µM PLP, and varying amounts of the protein sample was prepared using 200 mM phosphate buffer, pH7.5. The mixtures were incubated for 10 min at 30°C, and the reactions were stopped by adding an equal volume of 10% trichloroacetic acid. Supernatants were obtained by centrifugation of the reaction mixture at 15,000×*g* at 4°C for 10 min and analyzed by HPLC-UV at 330 or 340 nm for KYNA and XA, respectively.

### pH and temperature effect on KAT activity

The effect of pH on HIKAT activity was determined by preparation of the typical reaction mixture in 200-mM acetate buffer (pH4.0–5.0), phosphate buffer (pH6.0–7.0), and tris buffer (pH8.0–9.0), respectively. The amount of KYNA produced in the reaction mixture was quantified by spectrophotometer analysis. To determine the effect of temperature, the typical reaction mixture was incubated at 16°C, 25°C, 30°C, 37°C, 45°C, 55°C, 60°C, 70°C, and 80°C for 5 min prior to the addition of 2 µM of the enzyme preparation and continuously incubated for 10 min after the addition of purified rHIKAT. Enzyme activity was measured by a spectrophotometer at 330 nm.

### Substrate specificity

The possible transamination activity of HIKAT to other amino acids at varying concentrations (1.5–25 mM) was

determined to replace kynurenine and pyruvate as an amino group acceptor in the typical reaction mixture.

#### Growth inhibitory assays of rHIKAT against *Babesia caballi* cultured in vitro

The in vitro growth-inhibitory assays were conducted as described previously (Bork et al. 2003). One hundred microliters of infected equine erythrocytes (RBCs) was diluted with non-infected RBCs to obtain 1% parasitemia in a 0.1-ml volume, and the mixture was subsequently suspended in 0.9 ml of a suitable growth medium supplemented with the indicated concentrations of recombinant KAT. The suspension was added to 24-well culture plates (Nunc, Roskilde, Denmark), and the plates were incubated in a humidified atmosphere of 5% CO<sub>2</sub> gas water-jacketed incubator at 37°C for 4 days. During the incubation period, the overlaid culture medium was replaced daily with 0.9 ml of a fresh medium containing the rHIKAT at different concentrations. In parallel, GST proteins were used as controls. All experiments were carried out in triplicate wells (for each concentration) and in three separate trials to obtain conclusive results. Parasite growth in Giemsa-stained thin blood smears with approximately 1,000 RBCs was monitored every 12 h for

4 days and determined on the basis of the morphological appearance.

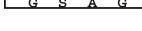
#### Nucleotide sequence accession number

The sequence of KAT gene of *H. longicornis* has been submitted to the GenBank database under accession number AB428569.

## Results

#### Molecular cloning and sequence analyses of HIKAT cDNA

A cDNA clone encoding *H. longicornis* kynurenine aminotransferase from EST constructed from embryo cDNA library of *H. longicornis* was sequenced. The full-length cDNA, as shown in Fig. 1, is 1717 bp, including an intact ORF encoding an expected protein with 415 amino acids. The full sequence has two in-frame stop codons at nucleotide numbers 260 and 263 of the 5'-untranslated region and a complete ORF of 1245-bp starting and ending at nucleotide numbers 306 and 1554, respectively. The 3'-untranslated region has a typical eukaryotic consensus polyadenylation signal (AATAAA) located 12-bp upstream of the poly A tail (Fig. 1).

1	GAGGGGGCAGCCCGCGTAGTACGCGAAGGTTACTCTTGCACTCTCGCCTTGCTGCACCGCAATTGTCAGACAGCTGTGCCGTACTCATCAGGTGCACCCACAACCGGCTTCCTCTG	120
241	GGTACTGCATATCGCACTGTTAGTACTTCCGGACGTCGTGTCGCTAGCAAGCTCAGATGCTTAAACATCTCAAGTGTCTTGTCCAACATCCTCGAGCAGATACGCTCCCTA	240
	CTGGGGAAACAAACCGCGTTGATAAAACCCCGCTGCTGCTGCTCTGTCAGTCACCCAGACTAAATGGCAAGATGGAATTGGCAAAGCGGGTCCAGGGATGCAAGACAGTGTCTGG	360
	M G K M E L A K R V Q G M Q D S V W	18
361	GTGGAGTTCATACAGCTTGCTCAGGAAACCAAGCCTGCAACCTGGGGCAGGGGTTCTGACTTCCCTCACCAGAATTGTTGTCAAAGGCCTCCAGGAGGCTGCCTCGGGCAATGCT	480
19	V E F I Q L A Q E T K P  AV N L G Q G F P D F P P E F V V K A L Q E A A S G N A	58
481	CTTCACCACTACACGCCGGGATACGCCACTTGCCTGGTGAATGCGCTGCCAAGATGTAATCGGCCCTCACGGTGCAGATCGACCCCCAGAAGGAGGTGCTGGTACGGTGGGG	600
59	L H O Y T R G Y G H L R L V N A L A K M Y S R L T G R Q I D P Q K E V L V T V G	98
601	GCTTACGAGGGCCTACTGCAACATGCTGGGCCCTGGTAACACCCGGGGAGGAGGTGATCATCATTGAGCCCTCTTGATTGCTATGAGCCATGACCCCTGATGGGGAGGAGTCCA	720
99	A Y E A L Y C T M L G L V N P G E E V I I I E P F F D C Y E P M T L M A G G V P	138
721	GCTTCATCCCCCTCCGGCTAGAAAGCAGGGGAAATTCCAGTGCTGACTGGGTGCTCGACCCCTGAGGAGCTGGCTTCAAGTTCACATCCAAAACAAAATTGATCATTGTCACACT	840
139	V F I P L R P R K Q G Q I S S A D W V L D P E E L A S K F T S K T K L I I V N T	178
841	CCTCACACCCCACCTGGGAAGGTTCTCCCGACAAGAACATGGAATGATAGCAGAGCTTGCAAGAACGACGATGTGATCTGATCATGGATGAGGTTATGAGTGGATCATTTATGAG	960
179	P H N P L G K V F S R Q E L E M I A E L C K K H D V I C I M D E V Y E W I I Y E	218
961	GGTCCAAGCACATCCGAATGAACACATTGCTGTGTTGAGGAAAGAACATAACCATGGAAAGTGCCTGGAAAGACCTTCAGCATAACCGGCTGGAAAGGTTGGTGGCGTACGGTCCA	1080
219	G S K H I R M N T L P G M W E R T I T  G S A G K T F S I T G W K V G W A Y G P	258
1081	TCAACACCTGTTGAAGGGCTGCAGATGATTCAACAGAACACTGTGTCTACACCCCTCGGCCACCTCTGCAGGAAGCGATTGCGCTGGGGCTGGAAAAAGAACATGGACGGACTGGTACCCCC	1200
	S H L L K G L Q M I H Q N C V Y T L G T L L Q E A I A V G L E K E M D R T G D P	298
1201	ACGGGCTACTGGAACCGACCTGTCGAGCTCCCTGAGAGAACGCGCACAGATCTGCCCTCCTCAACTCTGCTGCGCTAGCAGCCACTGTGCTCTGAGGGGGCTACTTCTCATCTGC	1320
299	T G Y W N D L S S S L Q K K R D T I C G F L N S V G M T P T V P E G G Y F L I C	338
1321	GACTTTCCAAGCTGCTTCAAAGATAAAGCTGAAAGGCAATGAGGCCAAGGATTACCTGTTGCGAAGTGGTTGCTAGGACCAAGAACGCTGAGGGCATCCCCCTAGTCCTTCTAT	1440
339	D F S K L A S K I K L E G N E P K D Y L F A K W L S R T K K L Q G I P P S A F Y	378
1441	GGTCCAGAGCACAAAGCTCTTGCAGGATTGATACGGTTCTGTTTATTAGGATGACGCAACTCTAAAGGCTGAGACATCTGCTGAGCTAAAGAACATGCTTAAGTTG	1560
379	G A E H K P L A Q D L I R F C F I K D D A T L K K A A D I L A E L K ♦ N M S *	415
1561	GGGTACTGGAAAATGCCCTCAACTGAAACAGCTTGCCTTCAATTGTTATCAGTGGTACTACAGGGGAAT  AACGGTTGACAACCAAAAAAAAAAAAAAA 1680	
1681	AAAAAAAAAAAAAAAAAAAAAA 1717	

**Fig. 1** Nucleotide sequence and deduced amino acid sequence of HIKAT. The *underlined* regions used for sequencing primers. The arrow indicates the potential cleavage site of leader sequence. *Boxed amino acid sequence* represents the putative PLP binding site. The

asterisk represents the stop codon (TAA). *Boxed nucleotide sequence* represents the polyadenylation signal (AATAAA) at the 3'-untranslated region. *Diamond* indicates the potential N-linked glycosylation site

### Deduced amino acid sequences

The ORF of the isolated clone codes 415 amino acid residues with predicted molecular mass of 46,479 (Fig. 1). A putative signal peptide cleavage site was identified between residues 30 (P) and 31 (V) of the preprotein that resulted in a mature polypeptide of 385 amino acids with a theoretical isoelectric point (*pI*) of 7.35 and molecular weight ( $M_w$ ) of 43.1 kDa. A highly conserved pyridoxal-phosphate (PLP) binding site (Gly-Ser-Ala-Gly-Lys-Thr-Phe-Ser) is located between Gly-219 and Ser-226 (Figs. 1 and 2).

## Amino acid sequence homologies

Using ClustalW program of multiple sequence alignment, amino acid sequence comparison shows that the tick clone shares high identity with KAT from *Drosophila melanogaster* (53%), *A. aegypti* (49%), *Mus musculus* (49%), *Rattus norvegicus* (48%), and *Homo sapiens* (48%; Fig. 2). BLASTP analysis of the predicted polypeptide sequence against all non-redundant databases accessed through NCBI revealed a significant score with members of the KAT family of humans and insects. The modular composition of the protein has aminotransferase domain similar to other KAT of human insects by simple Modular Architecture.

Research Tool database searches (<http://smart.embl-heidelberg.de>). A dendrogram showing the relationship between members of KATs was generated by pairwise alignment of several KATs with MacVector computer program (Oxford Molecular; Fig. 3). The dendrogram demonstrates the relationships of proteins based on their similarity in amino acid sequences and also implies the evolutionary relationships among these proteins. In the phylogenetic tree, *HIKAT* clusters with insect KATs and distantly related to mammalian and bacterial KATs.

## Cloning and expression of recombinant KAT in *E. coli*

The HIKAT gene with no signal peptide sequences was ligated into the bacterial expression vector pGEX-4T-1 into *Eco*RI site and the KAT was then successfully expressed as a GST fusion protein with an expected size of 45 kDa (Fig. 4). The rHIKAT was expressed as a soluble form and then purified by affinity chromatography using Sepharose 4B columns.

## Identification of tick native KAT protein

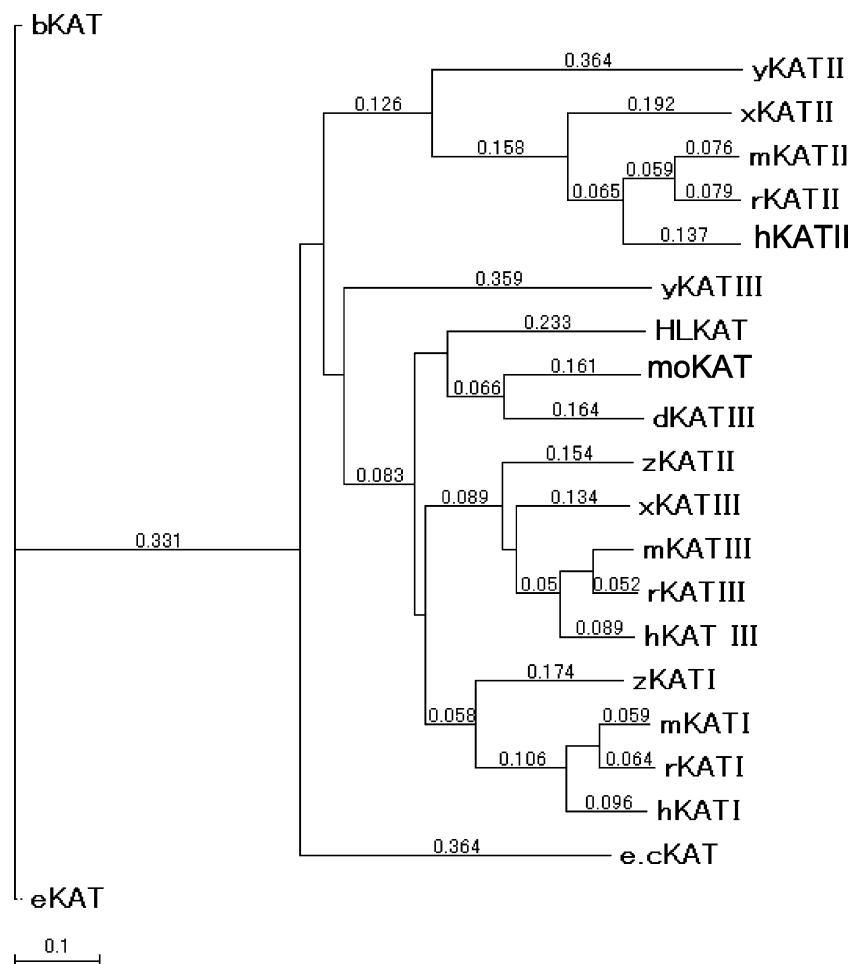
Polyclonal anti-rKAT serum was prepared from the mouse and used to test for the presence of native KAT in different developmental tick stages and in the midgut,

<i>H. longicornis</i>	1	MGMKMELAKEVQCMQDSVVVEFIFIQLAQETKPVNLGQGPFD	40	
<i>A.aegypti</i>	1	MMNLRNHNSVGAIRTAVVQLQDLQNIVSNKSSALTGVAVSVHQRQQIRT--MSSTSNETMHNKNDLPLKRYQGSTKSVWVYIQLAAQYPLNLGQGPFDY	97	
<i>Homo sapiens</i>	1	MFLAQRSRLCSLSGRAKFLKTISSS-KILGFSTSAKMSLKFNTNAKRIEGLDSNVNWIETFKLAADPSVNLGQGPFDY	75	
<i>D.melanogaster</i>	1	MLRSCQITLSAAKRSLRQFQFLQA--LRHQQTAKMKEFDLPLKRLQGSTPVSWNBYIALAMQVKPLNLGQGPFDY	73	
<i>Rattus norvegicus</i>	1	MLLAQRRRLFSLGCRAKPKITIYSS-KVLGLSTSAAKMSLKFNTNAKRIEGLDQNWNVFETFKLAADPSVNLGQGPFDY	75	
<i>Mus musculus</i>	1	MLLAQRRRLISLGCRSKPKITIYSSSKVLGCLTSAAKMSLKFNTNAKIEGLDSQNWNVFETFKLAADPSVNLGQGPFDY	76	
		*****	*****	
<i>H. longicornis</i>	41	PPPEFFVVKALQEAASGN--ALHQYTRGYGHLLRVLNALAKMYSRLTRQIDPQEVLTVGAYEALYCTMLGLVNPEEEVIIPEFFDCYEPMLTMAAGGP	138	
<i>A.aegypti</i>	98	HAPKYASNALAAAANSPDPSANQYTRGFPHPLVQALKSYQSVDRTINPMTEVSVTGAYEASVATIQGHDEDEGVIIIEPNFDCYEPMVKAAGGIP	197	
<i>Homo sapiens</i>	76	SPPTYVKEELSKIAAIDS--LNQYTRGFPHPSLVKALSYLYEKLYQKQIDSNEKEIIVTVGAYGSLFNFNIQALIDEDEGVILIVPFYDCYEPMVRMAGATP	173	
<i>D.melanogaster</i>	74	AAPEYVTHSLADIKEQNPLHQQYTRGYGHVRLNALSKLYSGLVKEKLNPPLSDILITSGAYEALYSTIMGHQDVDEGVIIIEFFDCYEPMVKMAAGGP	173	
<i>Rattus norvegicus</i>	76	TLPSPYVQEELSKAAFIDN--LNQYTRGFPHPSLVKALSYLYEKLYQKQIDSNEKEIIVTVGAYGSLFNFNIQALIDEDEGVILIVPFYDCYEPMVKMAAGGP	173	
<i>Mus musculus</i>	77	SPPTYVKEELSKAAFIDN--MNQYTRGFPHPALVKALSYLYEKLYQKQIDSNEKEIIVTVGAYGSLFNFNIQALIDEDEGVILIVPFYDCYEPMVRMAGATP	174	
	*	*****	*****	
<i>H. longicornis</i>	139	VF1BLRPRKQGQ--ISSADWVLDPPEELASKETSTKLIIVNTPHNPGLKVFSRQELEMIAELCKKHVICIMDEVYEWIIYEGSKHIRMNLTPGMWERTI	236	
<i>A.aegypti</i>	198	RN1PSKPKNKTG--TISSADWVSNNESSEALNNKEPKMIIINTPHNPNGKVMDRAESEVVANSCKKWNVLCVSDEVYEHMVFPFEEHIRCTLPGMWERTI	296	
<i>Homo sapiens</i>	174	VF1PLRSKPVYGRKNSSDWTLDQPELESKENSSTKAIILNTPHNPNGKVNREELQVIADLCIHKTYTLICISDEVYEWLVSQGNKHLIAITPGMWERTI	273	
<i>D.melanogaster</i>	174	RFVFLKLRKTEG-PISSADWVLDDAEFLESNSTKMTIILNTPHNPNGKVNRELELIAELCRWNVLCVSDEVYEWLFVDFGEHIREHCTLPGMWDRTL	272	
<i>Rattus norvegicus</i>	174	VF1PLRSKRTDGKWTSSDWTFNPOQELESKESSTKAIILNTPHNPNGKVNREELQVIADLCIHKTYTLICISDEVYEWLVSQGNKHLIAITPGMWDRTL	273	
<i>Mus musculus</i>	175	VF1PLRSKPTDGKWTSSDWTFPRELESKESSTKAIILNTPHNPNGKVNREELQVIADLCIHKTYTLICISDEVYEWLVSQGNKHLIAITPGMWDRTL	274	
	*	*****	*****	
<i>H. longicornis</i>	237	TIGSAGKTFSSITGVKVGWAWGPSPHLLKGQLMIHQNCVYTILGTLLOEAIAVGLKEKMDRTGPTGVWNLSSSIQKKRDTTCGFLNSVGMTPTVPEGGYFL	336	
<i>A.aegypti</i>	297	TIGSAGKTFSSLTGWKIGWAWGPSPALSKNLMQVMHQNCVYTATCP1QEAIAVGFTESKRKSPCYFNFSISGESMAKRDYMASFLAEVGNNPTVPGGGYFM	396	
<i>Homo sapiens</i>	274	TIGSAGKTFSSITGVKLGWSIPGPNHLIKHLQTVQQNTIYTCTPLQEAQAFWIDIKRMDDEPECFCNFSLSPKELEVVKDRMVRLLSVELGLKHPIVPDGGYFI	373	
<i>D.melanogaster</i>	273	TIGSAGKTFSSITGVKLGWSIPGPNHLIKHLQTVQQNTIYTCTPLQEAQAFWIDIKRMDDEPECFCNFSLSPKELEVVKDRMVRLLSVELGLKHPIVPDGGYFI	372	
<i>Rattus norvegicus</i>	274	TIGSAGKTFSSITGVKLGWSIPGPNHLIKHLQTVQQNTIYTCTPLQEAQAFWIDIKRMDDEPECFCNFSLSPKELEVVKDRMVRLLSVELGLKHPIVPDGGYFI	373	
<i>Mus musculus</i>	275	TIGSAGKTFSSITGVKLGWSIPGPNHLIKHLQTVQQNTIYTCTPLQEAQAFWIDIKRMDDEPECFCNFSLSPKELEVVKDRMVRLLSVELGLKHPIVPDGGYFI	374	
	*****	*****	*****	
<i>H. longicornis</i>	337	IICDFSKLASKIKLEG--NEPKDYLFAKWLSTSRTKLLQGIPPSAFYGAEHKPLAQDLIIFCFIKDDATLKKAAIDLAEIKNMSN	416	identity
<i>A.aegypti</i>	397	VADWSSSDSKVDLTQETDARKDYRFTKWMTKTSVGLQGIPPSAFYSEPNKHLGEDFVRYCFFKKDENLQKAAEILRKWKGS	477	similarity
<i>Homo sapiens</i>	374	IADWSSLDPLDSLDMK--NNEPYDVKFVKWMTKHKLSAIIPVSAFCNSETKSQFEEFKVRCFCIHKDSTDAAEELIKAWSVQKS	454	49%
<i>D.melanogaster</i>	373	IADWSPLAGKIDSLPSSDPLKDKHRYDVKFVKWMTKHKLSAIIPVSAFCNSETKSQFEEFKVRCFCIHKDSTDAAEELIKAWSVQKS	450	11%
<i>Rattus norvegicus</i>	374	IADVSSLGVFDLSDVK--SDEPYDVKFVKWMTKHKLSAIIPVSAFCNSETKSQFEEFKVRCFCIHKDSTDAAEELIKAWSVQKS	454	48%
<i>Mus musculus</i>	375	IADVSSLGADLSDMN--SDEPYDVKFVKWMTKHKLTAIPVSAFCNSETKSQFEEFKVRCFCIHKDSTDAAEELIKAWSVQKS	455	14%

**Fig. 2** Multiple sequence alignment of HIKAT with proteins from six species. Amino acids identical to all KATs are indicated by asterisks. Dark gray boxes with black letters show identical amino acids and

*light gray boxes with black letters* show similar amino acids. Sequence in *blue box* shows a highly conserved PLP binding site

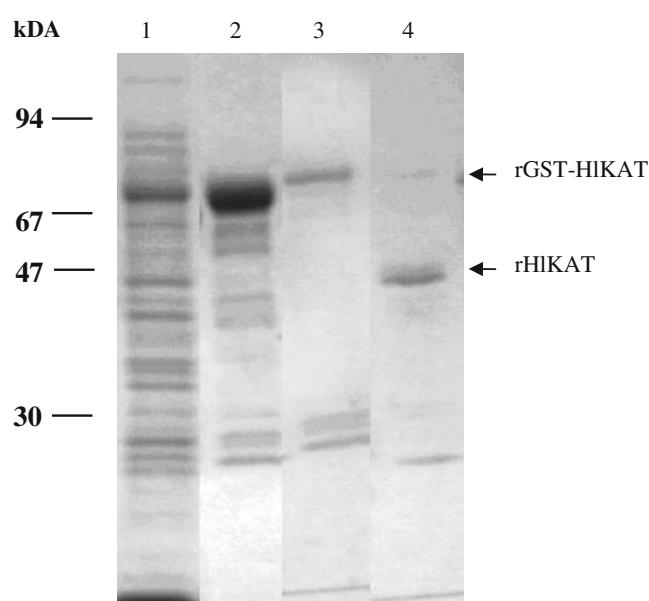
**Fig. 3** Phylogenetic analysis of aligned KATs by ClustalW. KATs found in *Haemaphysalis longicornis* (HLKAT) (AB428569), human KAT I, hKAT I (CAI15414); human KAT II, hKAT II (NP\_872603); human KAT III, hKAT III (NP\_001008661); mouse KAT I, mKAT I (NP\_765992); mouse KAT II, mKAT II (NP\_035964); mouse KAT III, mKAT III (AAQ15190); rat KAT I, rKAT I (AABK26163); rat KAT II, rKAT II (NP\_058889); rat KAT III, rKAT III (NP\_001015037); zebrafish KAT I, zKAT I (NM\_213359); zebrafish KAT II, zKAT II (AL922034); *Drosophila melanogaster* KAT III, dKAT III (NP\_788640); yeast KAT II, yKAT II (NP\_588566); yeast KAT III, yKAT III (CAB11066); *Escherichia coli* KAT, e.KAT (YP\_853509); *Aedes aegypti* mosquito KAT, moKAT (AAK97625); *Xenopus* KAT II, xKAT II (CAJ83707); *Xenopus* KAT III, xKAT III (AAH51239); bacteria KAT, bKAT (BAD70763) and *Thermus thermophilus*, eKAT (IV2D\_A)



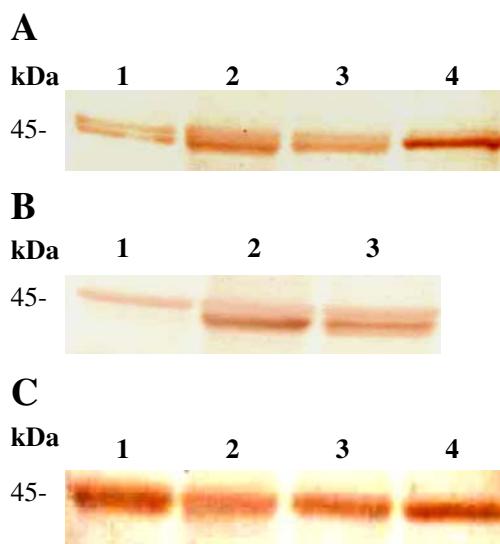
salivary gland, ovary, and synganglion of adult ticks. The lysates of tick samples were analyzed by Western blotting using mouse antiserum against rKAT. As shown in Fig. 5a, a specific strong band of approximately 45 kDa was detected in eggs, larval, nymphal, and adult tick (Fig. 5a), tick lysate of unfed, 2nd day of blood feeding, and 3rd day of blood feeding (Fig. 5b), and midgut, salivary gland, ovary, and synganglion of adult tick (Fig. 5c). Control anti-rGST serum did not show any band in the samples (data not shown). These results show that ticks of all developmental stages and the midgut, salivary gland, ovary and synganglion of partially fed adult tick express KAT protein. Tick lysate on the 3rd day of blood feeding showed stronger band than those unfed and on the 2nd day of blood feeding.

#### Localization of HIKAT in midgut epithelial cells

To examine the localization of HIKat12 in the cytoplasm of midgut epithelial cells, we performed indirect fluorescent antibody test using the anti-GST-HIKAT antibody. As shown in Fig. 6, the antisera strongly reacted with midgut



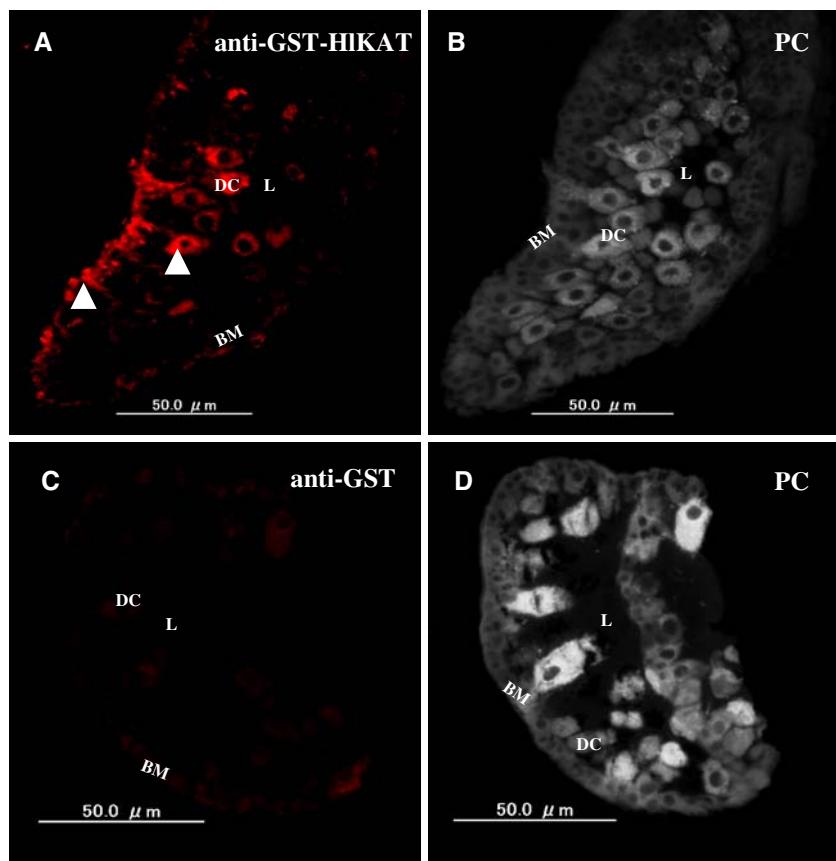
**Fig. 4** Expression of HIKAT in *E. coli* with GST fusion protein. Lane 1 expression of GST-HIKAT in *E. coli*; 2 GST-HIKAT protein in soluble fraction; 3 purified GST-HIKAT; 4 purified rHIKAT after cutting with thrombin



**Fig. 5** Western blot analysis of native HLKAT. **a** Lane 1 egg extract, 2 larval extract, 3 nymph extract, 4 adult female extract. **b** Lane 1 unfed tick extract, 2 2-day-fed tick extract, 3 3-day-fed tick extract. **c** Lane 1 midgut, 2 salivary gland, 3 ovary, 4 synganglion

epithelial cells and basement membranes while the control serum shows no specific reaction with the midgut section. This result confirms that the HLKAT protein has a strong expression in the midgut of partially fed adult tick.

**Fig. 6** IFAT showing endogenous HIKAT in the midgut of 3-day-fed adults. **a** HIKAT was detected by the mouse anti-GST-HIKAT antibody and visualized by red fluorescence conjugated anti-mouse IgG. Note that some aggregated red colors indicate positive reaction in the digestive cells and basement membrane (arrowheads). **b** Phase contrast image of **a**. **c** The control panel shows that anti-GST mouse antibody did not bind to any cells. **d** Phase contrast image of **c**. *BM* basement membrane, *DC* digestive cell, *L* lumen of midgut, *PC* phase contrast images. Bars=50 μm

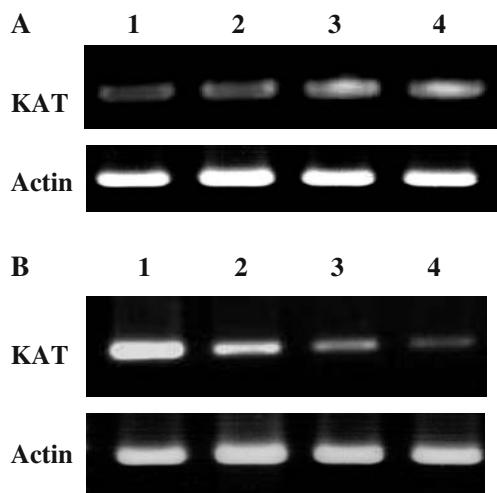


#### Transcript analysis of KAT mRNA by RT-PCR

To determine the expression profiles of the KAT gene, total RNA samples from different tick development stages and different tissues were subjected to RT-PCR. As shown in Fig. 7a, such as the positive control tick actin gene, KAT mRNA transcripts were detected in eggs, larvae, nymphs, and adults. Different tissues from partially fed adult ticks, including the salivary glands, midgut, ovary, and synganglion, were found to transcribe this gene. Compared with other tissues, this gene seems to express more richly in the tick salivary gland and midgut (Fig. 7b). The negative control gene, *Babesia equi* EMA-1 gene, was not amplified in any samples. These results indicated that the KAT gene was expressed throughout the developing stage and in the salivary glands, midgut, ovary, and synganglion in tick.

#### KAT activity assay

The molecular mass of the rHIKAT protein was estimated to be about 71 kDa. After partial cleavage of rHIKAT with thrombin, the fusion protein was separated into the GST protein (26 kDa) and the HIKAT protein (45 kDa). The 45-kDa HIKAT (without GST tag) were assayed for enzyme activity (Fig. 4).



**Fig. 7** **a** Expression of HIKAT in different tick stages by RT-PCR. *1* Female adult, *2* nymph, *3* larva, *4* egg. Total RNA was extracted from adult, nymphal, and larval ticks after 3 days of infestation and eggs after 3 days of oviposition. **b** Expression of HIKAT in tick organs analyzed by RT-PCR. The predicted product sizes of HIKAT and actin genes are about 0.7 and 0.85 kb, respectively. *Lane 1* Midgut, *2* salivary gland, *3* ovary, *4* Synganglion. Actin is shown as an internal control

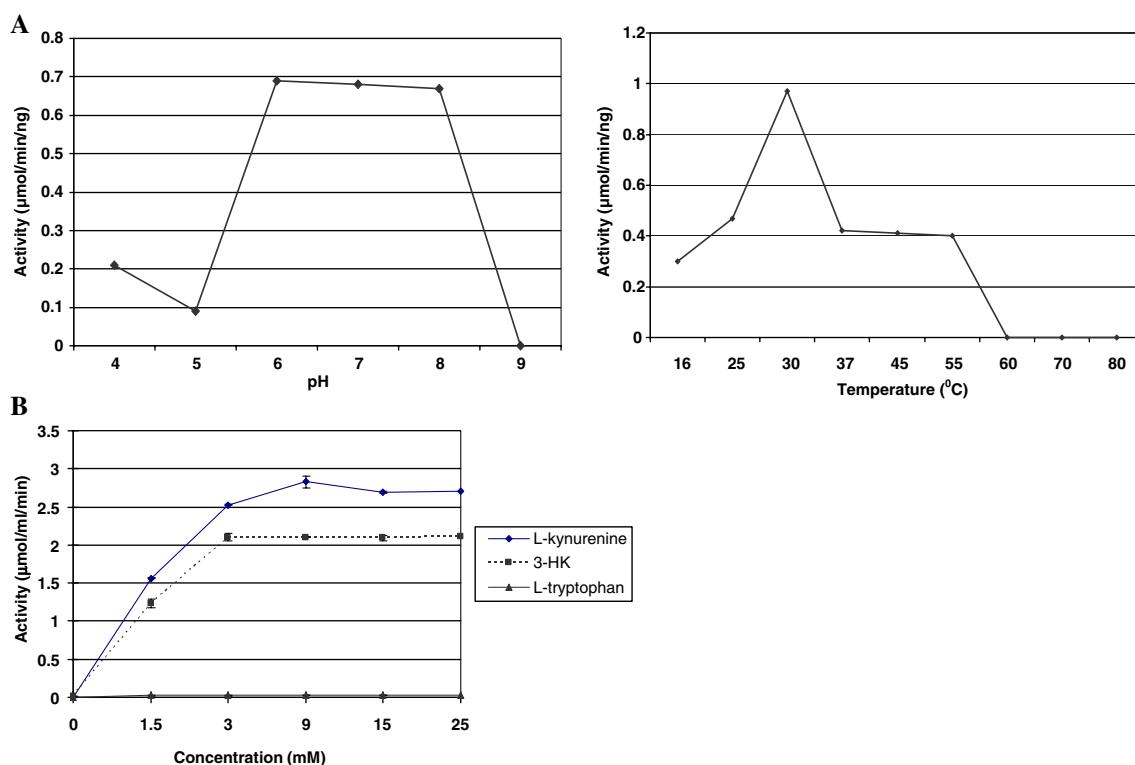
**Optimum pH** The influence of pH on the activity of the recombinant HIKAT was investigated over the pH range 4–9. The buffers that employed 200 mM phosphate buffer at pH 6–7 and 200 mM tris buffer at pH 8 showed optimal pH

(Fig. 8a). HIKAT showed maximum enzyme activity at pH 6–8, which is closed to tick physiological condition.

**Optimum temperature** The enzymatic activities were detected at temperatures from 16°C to 55°C (Fig. 8a). The maximum activity was exhibited at 30°C. The enzymatic activities started to decrease dramatically when the temperature was above 55°C.

**Kinetic properties** The  $K_m$ ,  $k_{cat}$ , and  $k_{cat}/K_m$  values for kynurenine, 3-HK, and tryptophan as substrates and pyruvate, 2-ketobutyrate,  $\alpha$ -keto- $\gamma$ -methylthiobutyrate, and pyruvic acid as co-substrates were measured. The substrate specificity of 2  $\mu$ g/ml of recombinant HIKAT at pH 7.5 for L-kynurenine showed  $K_m$  value of 111.60  $\mu$ M, a  $k_{cat}$  value of  $3.33 \times 10^3$  min $^{-1}$ , and  $k_{cat}/K_m$  value of 30 min $^{-1}$   $\mu$ M $^{-1}$ ; Table 1).

**Substrate specificity** The rHIKAT was capable of catalyzing the transamination both of kynurenine and 3-HK in the presence of pyruvate and PLP (Fig. 8b). Therefore, kynurenine and 3-HK were used as the amino group donor for subsequent biochemical characterization of the rHIKAT. It was found that the amino acceptors used in the reaction mixture had a major impact on the specific activity of rHIKAT.



**Fig. 8** **a** Effect of pH and temperature on HIKAT activity. HIKAT was incubated in the presence of 5 mM kynurenine and 10 mM pyruvate. The higher activity shown in the pH range from 6 to 8. The highest activity shown at 25–30°C. **b** Substrate specificity profile of HIKAT.

HIKAT was incubated in the presence of 1.5–25 mM kynurenine or 3-HK or L-tryptophan and 10 mM pyruvate. The substrate specificity of 2  $\mu$ g/ml recombinant HIKAT showed for L-kynurenine a  $K_m$  value of 111  $\mu$ M and  $k_{cat}$  value  $3.3 \times 10^3$  min $^{-1}$

**Table 1** Kinetic parameters of HIKAT towards different substrates and co-substrates

	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{min}^{-1} \mu\text{M}^{-1}$ )
Substrate specificity			
L-Kynurenine	111.60±12.3	3,330±146	30
3-Hydroxykynurenine	206.224±39.1	3,907±124	18.9
L-Tryptophan	0	0.00001	0
Co-substrate specificity			
Pyruvate	17.456±2.8	121.2±9.7	6.94
2-Ketobutyrate	0.217±0.02	0.1065±0.03	0.49
$\alpha$ -Keto- $\gamma$ -methylthiobutyrate	4.38±0.87	9.6798±1.32	2.21
Pyruvic acid	0	0.0009	0

*Co-substrate specificity* Among the tested co-substrates,  $\alpha$ -keto- $\gamma$ -methylthiobutyrate and pyruvate were more effective amino acceptors for the recombinant HIKAT (Table 1).

#### Growth inhibitory assay of KAT against *B. caballi* cultured in vitro

The in vitro growth of *B. caballi* was significantly inhibited by rHIKAT-GST at 2, 4, and 20  $\mu\text{M}$  in the 3rd- and 4th-day cultures ( $P<0.01$ ; Student's *t* test) in comparison with the control 20  $\mu\text{M}$  GST (Fig. 9).

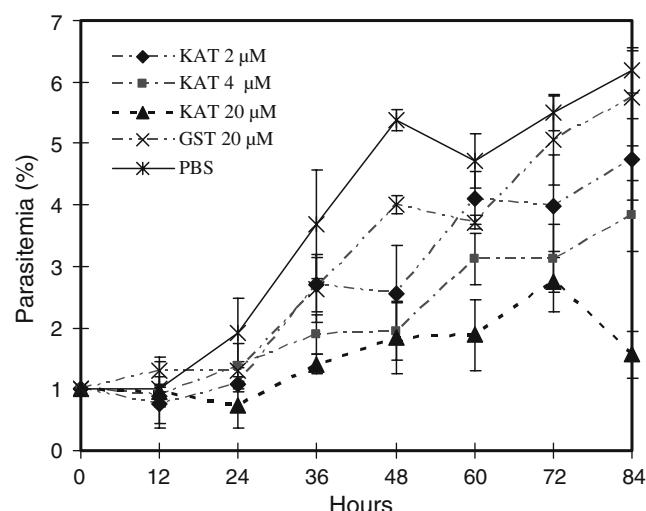
#### Discussion

In this paper, we described the cloning, DNA sequencing, expression, and characterization of novel *H. longicornis* kynurenine aminotransferase, HIKAT. In mammals, KYNA functions as a broad spectrum antagonist at ionotropic

excitatory amino acid receptors (NMDA receptors) in CNS, which protects these receptors from overstimulation by excitatory cytotoxins. Xanthurenic acid (4, 8-dihydroxyquinoline-2-carboxylic acid), a product of tryptophan catabolism, has been identified as mosquito-derived gametocyte activating factor (GAF) or mosquito exflagellation factor (MEF) (Billker et al. 1998; Garcia et al. 1998). XA has a two-ring structure and is a bio-product of the ommochrome pathway of the eye pigment of insects (Carter and Ranford-Cartwright 1998). Because of these reasons, KAT, the enzyme responsible for the production of KYNA and XA in mammals (Beal et al. 1990, 1992; Stone 2000) and in mosquito (Fang et al. 2002; Han et al. 2004, 2005), has attracted a considerable attention in the research area of vector-borne diseases.

The high-sequence similarity of the isolated *H. longicornis* clone with *A. aegypti* KAT and mammalian KATs and its functional biochemical characterization verified the presence of a specific KAT in *H. longicornis*, HIKAT, which is novel for Chelicerata, which includes ticks, spiders, and scorpions. HIKAT transcripts in all developmental stages similar to those obtained in *A. aegypti* (Fang et al. 2002; Han et al. 2004, 2005) and the HIKAT protein expression may depend on the blood-feeding course in adult tick, which may be explained by increased strength of the band in Western blot analysis. Different tissues from partially fed adult ticks, including the salivary glands, midgut, ovary, and synganglion, were found to transcribe HIKAT mRNA compared with other tissues, and HIKAT seems to express more richly in the tick salivary gland and midgut, which conforms again a role of gene expression in tick blood feeding. However, transcription of HIKAT which is quite weak in tick synganglion might show presence of a physiological low level of the HIKAT enzyme in tick brain.

Few studies reported about the role of tryptophan and its metabolism in ticks. Agyei et al. (1992) obtained positive reaction for some protein components including tryptophan in vacuoles of midgut digestive cells in tick only during blood feeding. This shows the tick as an organism ingesting tryptophan in the form of proteins, which are then



**Fig. 9** Inhibition effect of rHIKAT on *Babesia caballi* in vitro culture. Results show dose-dependent inhibition effect of HIKAT on *B. caballi*

hydrolyzed into the constituent amino acids in the digestive system (Agyei et al. 1992). It was considered that HIKAT is the first enzymatically active aminotransferase on the tryptophan pathway, particularly the kynurenine pathway-related genes in a tick. Our data suggest that HIKAT has a role to maintain kynurene to KYNA and 3-HK to XA, which is similar to that previously described for human, rats (Okuno et al. 1990; Perry et al. 1993, 1995); Alberati-Giani et al. 1995; Buchli et al. 1995), and mosquito (Fang et al. 2002; Han et al. 2004, 2005) KATs. HIKAT sharing high-sequence identity with mosquito (Fang et al. 2002; Han and Li 2004; Han et al. 2005) and mammalian KATs would logically be considered the probable enzyme responsible for the 3-HK to XA pathway in ticks. *A. aegypti*-specific 3-HK transaminase, which is more active to 3-HK than to kynurene, is present and the enzyme prevented the accumulation of 3-HK in mosquitoes during larval development. In contrary, our data showed that HIKAT is capable of catalyzing the transamination both of kynurene and 3-HK to kynurenic acid and xanthurenic acid, respectively, in the presence of pyruvate as an amino group acceptor. The enzyme activity of KAT I of rat brain was previously shown to have an alkaline optimum pH (Guidetti et al. 1997), and human KAT I exhibits high enzyme activity under neutral conditions (Han et al. 2004), suggesting that KAT I might be an important player in KYNA synthesis under physiological conditions. Similar to those reports, HIKAT showed maximum enzyme activity at optimal pH in range of 6–8 and at 30°C, close to tick physiological condition.

Our results demonstrate the presence of specific HIKAT in *H. longicornis* tick, which shares high-sequence identity with insect and mammalian KATs and shows similar biochemical activities. Both its substrate specificity and expression profile during tick development suggest that HIKAT may be responsible for maintaining a physiological level of KYNA and XA in tick during development. Further research is needed on the detection and role of tick kynurenes in tick biology and also tick–host interaction particularly neurodegenerative effect of tick kynurenes in tick lesion area.

The *in vitro* growth of *B. caballi* was significantly inhibited by rHIKAT-GST at 2, 4, and 20 μM in the 3rd and 4th days of culturing ( $P < 0.01$ ; Student's *t* test) in comparison with the control 20 μM GST. This inhibition might occur by changing tryptophan concentration in the culture media by adding KAT enzyme. Therefore, it was speculated the possibility that XA as a resultant product of HIKAT activity may have some GAF-like function on babesial developments in vector ticks.

In summary, all the results of this study demonstrate the presence of specific HIKAT in *H. longicornis* tick, which shares high-sequence identity with insect and mammalian

KATs and shows similar biochemical activities. Both its substrate specificity and expression profile during tick development suggest that the enzyme may be responsible for maintaining a physiological level of KYNA and XA in tick during development. This is the first report of a member enzyme of tryptophan pathway in Chelicerata.

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