

# Enolase, a plasminogen receptor isolated from salivary gland transcriptome of the ixodid tick *Haemaphysalis flava*

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**Abstract** Enolase, a multifunctional protein, is shown to act as a plasminogen receptor that contributes to fibrinolysis, which plays an important role in preventing the formation of blood clots during tick feeding. The study of enolase genes provides opportunities to develop a potential antigen target for tick control. So far, enolase has been identified in only a few species of ticks. Knowledge of the exact mechanisms of plasminogen activation and fibrinolysis by enolase as a plasminogen receptor is limited. Here, we cloned the enolase full-length complementary DNA (cDNA) from the salivary glands of *Haemaphysalis flava*, expressed it, and analyzed the function of the recombinant *H. flava* enolase. The enolase cDNA was 1988 bp in length and encoded 433 amino acid residues. It contained two domains and some highly conserved functional motifs including an assumed membrane re-association region “AAVPSGASTGI.” The enolase exhibited 83.3 % amino acid similarity to that of the putative enolase of *Ixodes ricinus*, and 85 % to that of *Ornithodoros moubata* enolase. After eukaryotic expression in insect cells, Western blot analysis showed that the mouse antiserum against the hexahistidine-tagged recombinant enolase protein recognized a band of approximately 48 kDa. The recombinant enolase bound human plasminogen in a dose-dependent manner and enhanced plasminogen activation in the presence of host tissue plasminogen activator (t-PA), most probably to promote fibrinolysis and maintain

blood flow at the host–tick interface. Real-time quantitative polymerase chain reaction (qPCR) analysis showed that the expression level of enolase in salivary glands was significantly higher than in other tested tissues. Although the enolase was expressed in all developmental stages, it had the highest expression in the rapid blood feeding period of ticks. These findings indicate that the enolase might play an important role in blood feeding of *H. flava*.

**Keywords** *Haemaphysalis flava* · Enolase · Plasminogen receptor · Gene expression

## Introduction

Ticks are obligate hematophagous ectoparasites and are known to suck a large amount of blood during a prolonged period of feeding. In order to maintain blood fluidity, ticks must modulate the host’s hemostatic responses (Chmelar et al. 2012; Maritz-Olivier et al. 2007; Francischetti 2010), including platelet aggregation, vascular constriction, coagulation, and fibrinolysis. Among these, fibrinolysis is the process of converting plasminogen into plasmin by plasminogen activators including tissue plasminogen activator (tPA) and urokinase (Cesarman-Maus and Hajjar 2005), which is very important to prevent the formation of blood clot during tick feeding. Thus far, only a few tick molecules which regulate plasminogen activation and fibrinolysis have been identified, such as metalloproteases (Francischetti et al. 2003) and longistatin (Anisuzzaman et al. 2011). Our knowledge of profibrinolytic molecules from ticks at the molecular level is relatively limited despite significant progress made in the past.

Enolase is a key glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (Pancholi 2001). In many microorganisms (Mundodi et al.

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2008; Nogueira et al. 2012), enolase is localized on the cell surface, where it assists in microbial dissemination within hosts (Nogueira et al. 2012; Itzek et al. 2010; Nogueira et al. 2012). Remarkably, enolase is also expressed on the surface of a variety of eukaryotic cells (such as epithelial, endothelial, and hematopoietic cells), where it is shown to act as a plasminogen receptor (Toledo et al. 2012; Floden et al. 2011; de la Torre-Escudero et al. 2010).

Enolase of ticks may be involved in the anti-hemostatic process as profibrinolytic plasminogen receptors. Cloning and characterization of the enolase would help to more fully understand the tick–host relationship and its physiology roles in ticks, which is essential for better tick control. Enolase from the saliva of the argasid tick *Ornithodoros moubata* has been identified and confirmed by plasminogen binding and activation assays (Díaz-Martín et al. 2013). However, information on enolase from ticks is very scanty, and the exact mechanisms of plasminogen activation and fibrinolysis by enolase as a plasminogen receptor need to be evaluated further.

The hard tick *Haemaphysalis flava* is widely prevalent in many countries and feeds on blood from various kinds of mammals and birds (Fujimoto 2006). This tick transmits various disease agents including *Francisella tularensis* (Ozawa et al. 1982), *Rickettsia* (Someya et al. 2015; Fournier et al. 2002), and *Ehrlichia* (Rar et al. 2010), which may cause significant vector-borne diseases (e.g., encephalitis, Ko et al. 2010; Lyme borreliosis, Moon et al. 2013; and spotted fever, Ishiguro et al. 2008), endangering agricultural production and

the health of humans and animals. Recently, we found a number of enolases in the saliva of the *H. flava* tick through the LC-MS/MS method, raising the question of what is the function of enolase in the tick–host interface.

Here, we report the cloning, expression, and functional analysis of the enolase gene from the salivary gland transcriptome of *H. flava*. A phylogenetic tree was constructed based on the enolase amino acid sequences available in GenBank. The recombinant enolase protein bound plasminogen and enhanced plasminogen activation and plasmin generation induced by t-PA. Additionally, the enolase was shown to be highly expressed in salivary glands.

## Materials and methods

### Ticks and tissue collection

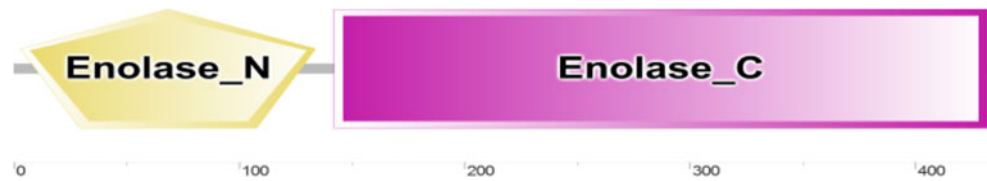
Nymph and adult female *H. flava* ticks used in this study came from Xinyang City of Henan Province, China. The ticks were reared by feeding on hedgehogs in our laboratory. All experimental procedures involving animals were approved and performed in accordance with guidelines of the Institutional Animal Care and Use Committee of Hunan Agricultural University. Whole ticks, salivary glands (SG), midguts (MG), ovaries (O), and crusts (C) of *H. flava* at different blood feeding phases were collected with forceps and washed in sterile ice-cold phosphate-buffered saline (PBS) with the aid

**Table 1** Sequences of primer used in this study

Primers	Nucleotide sequences (5'–3')	Size (bp)	Application
GSP3 outer	F: CAACATTCAGGACAACAAGGAG	941	3-RACE
3'race outer	R: TACCGTCGTTCCACTAGTGATTT		
GSP3 inner	F: TGAACGCAGCAACCAAGATT	456	5-RACE
3'race inner	R:CGCGGATCCTCCACTAGTGATTTCACTATAGG		
UPM	F: CTAATACGACTCACTATAGGGC	1302	expression
GSP5 outer	R: TGTAGAACTCGGAGGCGGCGACG		
NUP	F: AAGCAGTGGTATCAACGCAGAGT	104	qRT-PCR
GSP5 inner	R: CTCATCAATGGCAGTCTGGTCTG		
M13-47	F: CGCCAGGGTTTTCCAGTCAC	–	clone sequencing
RV-M	R: GAGCGGATAACAATTTACACAGG	117	reference
enolase-ORF	F: CGCGGATCCATGCATCACCATCACCACCC GATCAAGAGGATATACGC		
enolase-plasmid	R: CCCAAGCTTCTACTCAGGGTGGCGGAAGTTC F: TATTCCGGATTATTCATACC	–	
enolase-bacmid	R: ACAAATGGTATGGCTGA F: CCCAGTCACGACGTTGTA AAAA	–	
enolase-qPCR	R: AGCGGATAACAATTTACACAGG F:CAGGAGGCCATCAACCAG	104	qRT-PCR
actin	R:GATCGTACTTGCCGTCCTTG F: CAAGGAGAAGCTTTGCTACGTC R: CCGATGGTATGACCTGAC	117	reference



**Fig. 2** Analyses of the conserved structure domains of *H. flava* enolase. There are two predicted domains of *H. flava* enolase (Enolase-N and Enolase-C)



Total RNA was isolated from SGs using TRIzol Reagent (Invitrogen, USA). For the 5'-RACE, reverse transcription was carried out using the SMARTScribe reverse transcriptase and 5'-CDS Primer A. Polymerase chain reaction (PCR) amplification was performed according to the instruction of the SMARTer RACE cDNA Amplification Kit (Clontech, Canada, USA). For the 3'-RACE, reverse transcription and PCR amplification were performed using 3'-Full RACE Core Set with PrimeScript RTase (Takara, Japan) according to manufacturer's instructions.

The reverse transcription (RT)-PCR products were separated by 1.5 % agarose gel electrophoresis. Target bands were recovered and purified by a gel extraction kit (TransGen, Beijing, China) and then cloned into the PMD18-T vector (Takara, Japan). Three or more clones were sequenced using vector universal primers (M13-47 and RV-M, shown in Table 1) by the Shanghai Sangon Biological Engineering Technology Corporation in China. The sequences were edited and assembled into a full-length cDNA clone through the DNAMAN version 6.0 software.

### Bioinformatics analyses

The amino acid sequence of enolase was deduced using the Expert Protein Analysis System (<http://www.expasy.org/>). Homologous search of the predicted amino acids was performed using the BLAST program. The enolase protein domain features and several motifs were predicted, respectively, using the following servers: <http://smart.embl-heidelberg.de/> and <http://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi>. Multiple amino acid sequences of enolase from hematophagous arthropod species and *Homo sapiens* (as an out-group) were aligned using the program Clustal W version 2.0. The phylogenetic tree of the enolase proteins from different species was constructed using MEGA version 5.0 software with the neighbor-joining (NJ) method.

### Recombinant protein expression in Sf9 cells and purification

Enolase-ORF primers (Table 1) with added restriction enzyme sites (shown in italics and bold, forward and reverse primer with the BamHI and HindIII site, respectively) and 6× His tag (the underlined part shown in forward primer) were designed to amplify the open reading fragment (ORF) of the enolase gene. The purified PCR products and expression vector pFastBac1 (Invitrogen, USA) were digested with restriction enzymes BamHI and HindIII (Thermo Scientific, USA) and ligated with T4 DNA ligase (Thermo Scientific, USA) to form the recombinant plasmid pFastBac1-enolase that was transformed into DH5α competent cells. The cells were cultured on LB agar plates with 100 mg/ml ampicillin at 37 °C. Plasmid DNA was extracted and sequenced using enolase-plasmid primers (Table 1). The proper recombinant plasmid DNA was then transformed into DH10Bac competent cells to generate the recombinant bacmid DNA which was extracted and analyzed by PCR using enolase-bacmid primers (Table 1) to confirm the correctness of transposition. The resulting bacmid was transfected into *Spodoptera frugiperda* (Sf9) cells to generate recombinant baculovirus using the Cellfectin reagent according to the Bac-to-Bac baculovirus expression system (Invitrogen, CA, USA) protocol. The recombinant virus was harvested and used to infect insect cells for expression of recombinant enolase proteins (HfEno). After 3 days of suspension culture at 27 °C, the expression products were obtained, separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane for Western blot analysis.

In order to analyze the function of enolase, purification of recombinant proteins was performed. The infected cells were collected by centrifugation and treated in binding buffer (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM Tris-HCl, pH 7.9). The

**Table 2** The predicted domains and motifs of *H. flava* enolase

	Name	Amino acids	Position (AA)
Domains	Enolase_N		3–134
	Enolase_C		142–432
Motifs	Mg <sup>2+</sup> binding	S, D, E, D	40, 245, 294, 319
	substrate (2-phosphoglycerate) binding	H, E, K, HRS, K	158, 210, 344, 372–374, 395
	the assumed membrane re-association	AAVPSGASTGI	33–43

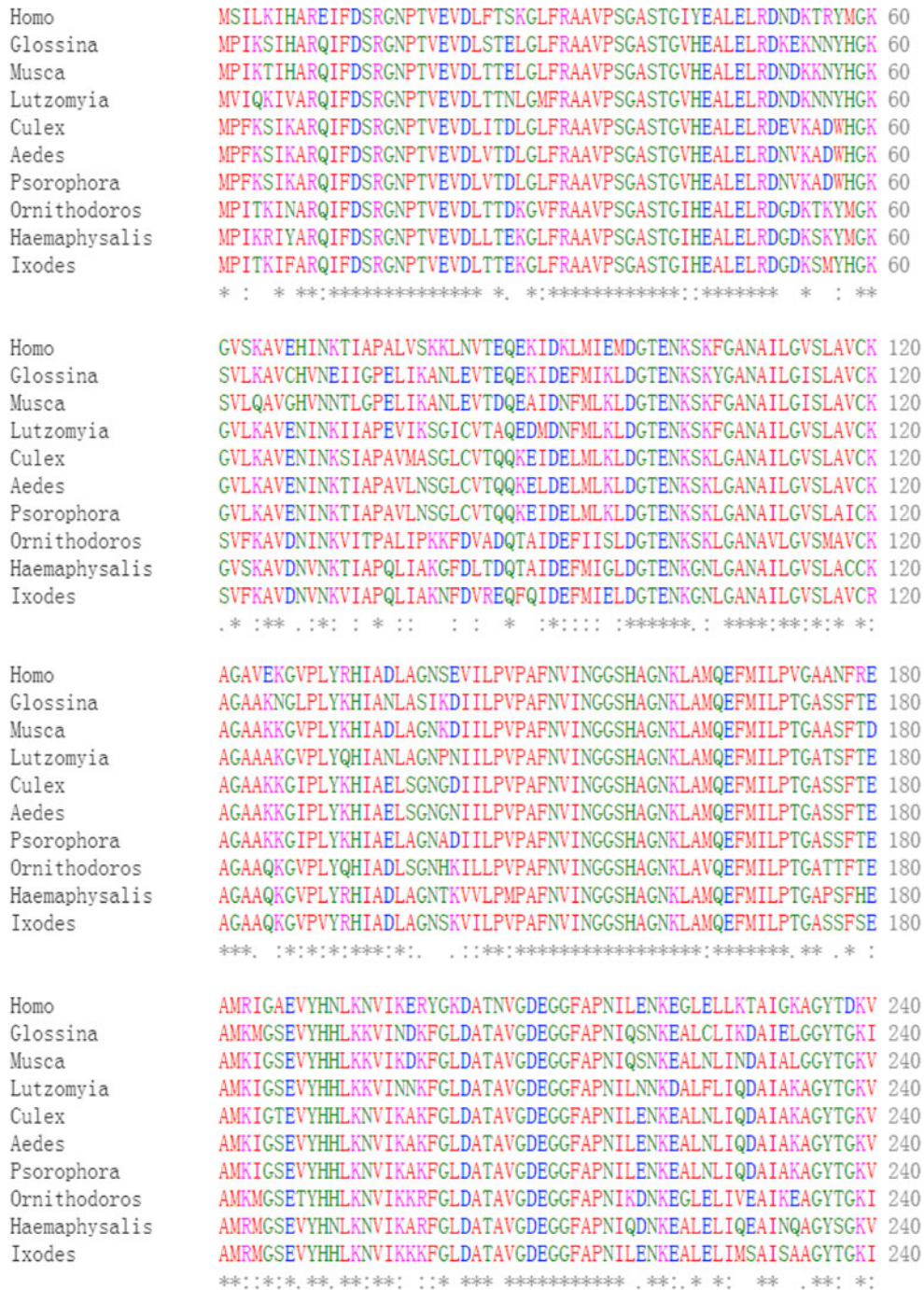


supernatant was obtained by centrifugation at 4 °C, 18,000g. Histidine-tagged HfEno was purified through a nickel affinity column. Urea was removed by washing the column with washing buffer (60 mM imidazole, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl. and 10 mM Tris-HCl, PH 7.9). After that, the recombinant protein was eluted with elution buffer (250 mM imidazole, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, and 300 mM NaCl, pH 7.9) and

then used for plasminogen binding and plasminogen activation assays.

**Plasminogen binding assays**

To determine whether HfEno binds plasminogen, an enzyme-linked immunosorbent assay (ELISA) was performed as



**Fig. 3** The alignment of amino acid sequences of enolases from *Haemaphysalis flava*, *Ixodes ricinus*, *Ornithodoros moubata*, *Glossina morsitans*, *Musca domestica*, *Culex quinquefasciatus*, *Psorophora albipes*, *Lutzomyia longipalpis*, *Aedes aegypti*, and *Homo sapiens*. The

amino acids conserved in all the sequences are labeled with *asterisks*. The conserved and semi-conserved substitutions are labeled with two and one points, respectively

Homo	VIGMDVAASEFFRSGKYDLDFKNSPD-DPSRYISPDLADLYKSFIDYPPVVSIEDPFDQD	299
Glossina	EIGMDVAASEFFKDGQYDLDFKNPSSDKSWLSPEKLLGLYQEFINEFPIVSIEDAFDQD	300
Musca	EIGMDVAASEFFKDCVYDLDFKNPNSDKAQWLPPAKLTDLYQEFIKEFPIVSIEDPFDQD	300
Lutzomyia	EIGMDVAASEFHKDGLYDLDFKNPNSDKSLWSPDKLTELMEFVKEFPIVSIEDPFDQD	300
Culex	EIGMDVAASEFHKDCKYDLDFKNPKSFKSAWLSLSPDSLEMYQGFIKDFPIVSIEDPFDQD	300
Aedes	EIGMDVAASEFHKDCKYDLDFKNPNSDKSAWLTPDALEGMVQGFIKDFPIVSIEDPFDQD	300
Psorophora	EIGMDVAASEFHKDCKYDLDFKNPNSDKSAWLSLSPDALEDMYQGFIKDFPIVSIEDPFDQD	300
Ornithodoros	EIGMDVAASEFYKNGKYDLDFKNPKSEKDHVDQAALQAIYEGFIKDYPIVSIEDPFEQD	300
Haemaphysalis	EIGMDVAASEFYKDKYDLDFKNPNSPKDYVVDKDALKAMYEEFISSYPIVSIEDPFDQD	300
Ixodes	DIGMDVAASEFYKCKYDLDFKNPNSNPGDFLERSLQRLYEGFIQDYPPVVSIEDPFDQD	300
	*****. :. * *****. * : : * : * * :. : * :. * * * * *	
Homo	DWGAWQKTASAGIQVVGDDLTVNPKRIAKAVNEKSCNCLLLKVNQIGSVTESLQACKL	359
Glossina	HWDASTITANTKIQIVGDDLTVNPKRIQTAVEKKACNCLLLKVNQIGVTESIKAHLL	360
Musca	HWDASNITANTTIQIVGDDLTVNPKRIQTAVEKKACNCLLLKVNQIGVTESIKAHLL	360
Lutzomyia	HWDASKMTAGTNIQIVGDDLTVNPKRIATAVEKKACNCLLLKVNQIGVTESIKAHNL	360
Culex	HWDAWAKITANTTIQIVGDDLTVNPKRIATAVEKKACNCLLLKVNQIGSVTESINAHLL	360
Aedes	HWDAWAKMTANTSIIQIVGDDLTVNPKRIATAVEKKACNCLLLKVNQIGVTESINAHLL	360
Psorophora	HWDAWAKITANTTIQIVGDDLTVNPKRIATAVEKKACNCLLLKVNQIGSVTESINAHLL	360
Ornithodoros	HWDAWTKFNATAKIQIVGDDLTVNPKRIQTAIDKKACNCLLLKVNQIGSVTEAIRAHQL	360
Haemaphysalis	DWDAWTAMNAATKIQIVGDDLTVNPKRIQMAVDKNACNCLLLKVNQIGSLTETIRAHQL	360
Ixodes	DWDAWSSMTSSNPIQIVGDDLTVNPKRIQMAVERGACNCLLLKVNQIGVTESIRAHLL	360
	. * * * :. : * : ***** * :. : ***** : * :. * * *	
Homo	AQANGWGMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLLRIEEELGS	419
Glossina	ARENGWGTMVSHRSGETEDSFIADLVVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGS	420
Musca	AKQNGWGTMVSHRSGETEDSFIADLVVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGA	420
Lutzomyia	AKSNGWGTMVSHRSGETEDTFIADLVVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGA	420
Culex	AKKNGWGTMVSHRSGETEDTFIADLVVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGS	420
Aedes	AKKNGWGTMVSHRSGETEDTFIADLVVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGS	420
Psorophora	AKKNGWGTMVSHRSGETEDTFIADLVVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGS	420
Ornithodoros	AKKNGWGTMVSHRSGETEDCTIADIVVGLGTGQIKTGAPCRSERLAKHNQLLRIEEELGS	420
Haemaphysalis	AKKSGWGTMVSHRSGETEDSTIADIVVGLSTGQIKTGAPCRSERLCKYNQLLRIEEQLGA	420
Ixodes	AKKNGWGTMVSHRSGETEDHTIADIVVGLSTGQIKTGAPCRSERLCKYNQLLRIEEQLGA	420
	* :. * * * . ***** * * : * * * * ***** * : * : * * * * * :	
Homo	KAKFAGRNFRNPLAK-	434
Glossina	TAKYAGKNFRSPLSPK	436
Musca	AAKFAGKFRKPK---	433
Lutzomyia	GAKFAGKFRKPK---	433
Culex	GAKFAGKFRHPQ---	433
Aedes	DAKFAGKNFRHPQ---	433
Psorophora	AAKFAGKFRHPQ---	433
Ornithodoros	KAVFAGKNFRHPA---	433
Haemaphysalis	NAVFAGKNFRHPE---	433
Ixodes	NAIYAGKNFRNPQ---	433
	* : * * :. * * *	

Fig. 3 (continued)

described previously (de la Torre-Escudero et al. 2010; Floden et al. 2011). Ninety-six-well plates were coated overnight (4 °C) with 0.5 µg/well of HfEno, casein, or bovine serum albumin (BSA) diluted in carbonate buffer, pH 9.6. BSA was included as a negative control, and casein as a positive control, which was reported to bind plasminogen (Heegaard et al. 1997). After coating, the wells were washed three times with PBS–0.05 % Tween 20 (PBST) and blocked for 2 h at room temperature with PBS–3 % BSA. The wells were washed three times again and then incubated for 1 h at 37 °C with

varied amounts (0.5 to 3 µg/well) of the human plasminogen diluted with PBS. After three new washes with PBST, the wells were incubated (for 1 h at 37 °C) successively with goat anti-human plasminogen (Acris Antibodies) diluted 1/2000 in PBST and horseradish-peroxidase-conjugated anti-goat IgG (Sigma) diluted 1/5000 in PBST. Tetramethylbenzidine (TMB) was used as a chromogenic substrate for peroxidase. Reactions were stopped by addition of 100 µl/well of 2 N H<sub>2</sub>SO<sub>4</sub>, and absorbance was read at 450 nm in the Microplate Reader (Thermo Scientific).



## Plasminogen activation assays

Plasminogen activation assays were performed in 96-well plates according to a protocol described by de la Torre-Escudero et al. (2010). Reaction mixtures in each well contained 1 µg HfEno or 1 µg BSA (negative control), 2 µg human plasminogen, 3 µg plasmin-specific chromogenic substrate (D-Valyl-L-leucyl-L-lysine 4-nitroanilide dihydrochloride, Sigma), 15 ng human tissue-type plasminogen activator (tPA), and PBS to a final volume of 100 µl. At the same time, several control experiments were also run in which tPA or HfEno was removed. Plates were incubated at 37 °C for 2 h and the hydrolysis of the chromogenic substrate was measured by reading absorbance at 405 nm. Each reaction was run in quadruplicate and the means and standard errors were calculated per condition.

## Real-time qPCR analysis

To evaluate the expression levels of enolase in different tissues and at different developmental stages of *H. flava*, the total RNA samples were prepared from different tissues (SG, salivary glands; MG, midguts; O, ovaries; C, crusts) and various developmental stages (EN, engorged nymph ticks; 1/4 EA, quarter-engorged adult ticks; 1/2 EA, semi-engorged adult ticks; EA, engorged adult ticks) of *H. flava*. cDNA was generated with PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Osaka, Japan) using the protocol provided by the manufacturer. Primers for quantitative PCR (qPCR; shown in Table 1) were designed utilizing the Roche Universal Probe Library Assay Design Center (<https://www.roche-applied-science.com>). SYBR Green real-time PCR was carried out on an Applied Biosystems 7300 Real time PCR system (Applied Biosystems, USA). The actin gene was used as a reference gene. Amplification reactions were performed in a final volume of 20 µl including 2 µl cDNA template, 0.4 µl ROX reference dye, 0.4 µM forward/reverse primer, and 10 µl 2 × SYBR qPCR Mix (Takara, Osaka, Japan). All reactions were accompanied by proper controls (such

as no template control). Reaction conditions were 95 °C for 30 s, followed by 40 cycles consisting of 95 °C for 5 s, and 60 °C of the annealing temperature for 31 s. The  $2^{-\Delta\Delta C_t}$  method (Schmittgen and Livak 2008) was used to analyze the relative expression levels of the enolase gene.

## Results and discussion

### Sequence analysis

The present study identified the full-length *H. flava* enolase cDNA which is 1988 bp in length. The complete cDNA included a start codon at nucleotides 158–160, a stop codon at nucleotides 1457–1459, and an ORF of 1302 nucleotides encoding a protein of 433 amino acids (shown in Fig. 1), without signal peptide. The 5' noncoding sequence was 157 bp in length and the 3' untranslated region contained 529 bp and ended with a 15-bp poly (A+) tail that began 31 bp downstream from AATAAA. The sequence of the enolase gene of *H. flava* has been submitted to the GenBank database under the accession number KM191327.

The prediction of domain features and several motifs by some web servers indicated that the *H. flava* enolase included Enolase-N and Enolase-C domains (Fig. 2) and possessed the following motifs (Table 2): Mg<sup>2+</sup> binding (S40, D245, E294, and D319), substrate (2-phosphoglycerate) binding (H158, E210, K344, HRS372-374, and K395), and an assumed membrane re-association region (a conservative hydrophobic region, AAVPSGASTGI, 33–43). The molecular characteristic of *H. flava* enolase showed similar structural features that are present on the enolase from the soft tick *O. moubata* (Díaz-Martín et al. 2013).

Enolase belongs to a new class of protein, called moonlighting protein (Paludo et al. 2015; Spurbeck et al. 2015), which has multiple functions. Enolase could be secreted in the cytosol, extracellular, and at the cell surface. Similar to other enolases,

**Table 3** Comparison of amino acid sequence of *H. flava* enolase with other known orthologous genes (% of identity)

Accession number	Species	<i>A. a.</i>	<i>C. q.</i>	<i>G. m.</i>	<i>H. f.</i>	<i>H. s.</i>	<i>I. r.</i>	<i>L. l.</i>	<i>M. d.</i>	<i>O. m.</i>	<i>P. a.</i>
XM_001653700	<i>Aedes aegypti</i>	<b>100</b>	96.1	82.2	77.1	75.5	77.4	86.4	83.8	76.9	97.2
XM_001842566	<i>Culex quinquefasciatus</i>	96.1	<b>100</b>	82.2	76.4	75.5	76.9	85.7	83.6	76.9	97.0
EZ423104	<i>Glossina morsitans</i>	82.2	82.2	<b>100</b>	74.4	71.9	76.7	82.9	88.9	73.9	82.4
KM191327	<b><i>H. flava</i></b>	77.1	76.4	74.4	<b>100</b>	73.1	83.6	75.1	76.2	82.2	77.4
AK223192	<i>Homo sapiens</i>	75.5	75.5	71.9	73.1	<b>100</b>	74.3	74.3	73.4	73.1	76.2
GADI01005028	<i>Ixodes ricinus</i>	77.4	76.9	76.7	83.6	74.3	<b>100</b>	76.2	77.1	79.9	76.9
EU124610	<i>Lutzomyia longipalpis</i>	86.4	85.7	82.9	75.1	74.3	76.2	<b>100</b>	86.1	74.8	85.9
KA647336	<i>Musca domestica</i>	83.8	83.6	88.9	76.2	73.4	77.1	86.1	<b>100</b>	75.1	85.0
GU594041	<i>Ornithodoros moubata</i>	76.9	76.9	73.9	82.2	73.1	79.9	74.8	75.1	<b>100</b>	76.7
GALA01001134	<i>Psorophora albipes</i>	97.2	97.0	82.4	77.4	76.2	76.9	85.9	85.0	76.7	<b>100</b>

Boldface entries refer to 100 % identity

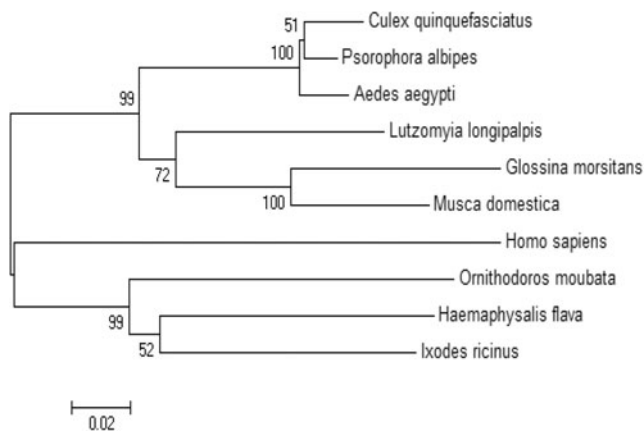
*H. flava* enolase lacks the conventional N-terminal signal peptide and membrane anchor segment, raising the question as to how the secreted enolase re-associates with the cell surface (Avilán et al. 2011). This situation is very common and can also be found in enolases from eukaryotic and bacterial organisms (Floden et al. 2011). In a study by Pancholi (2001), the conserved hydrophobic region “AAVPSGASTGI” of the enolase might play a very important role in membrane association.

### Comparison of amino acid sequence homology among animal enolases

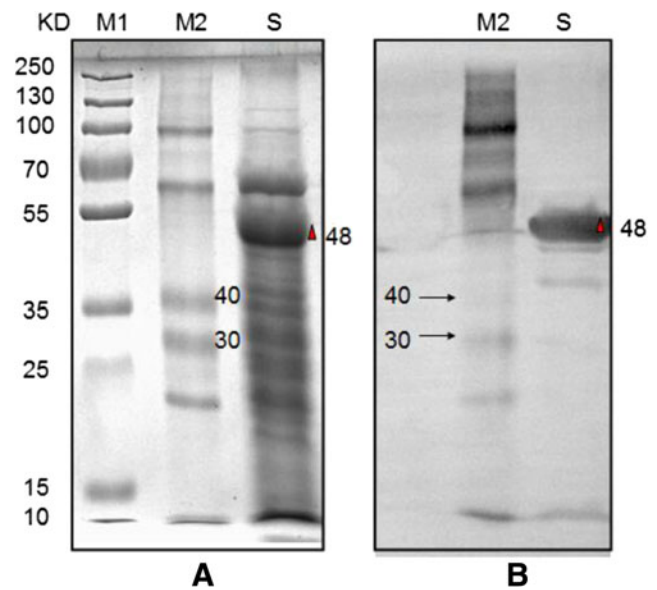
The multiple alignments (shown in Fig. 3) of the deduced amino acid sequences of *H. flava* enolase with those of other hematophagous arthropods (*Ixodes ricinus*, *O. moubata*, *Glossina morsitans*, *Musca domestica*, *Psorophora albipes*, *Culex quinquefasciatus*, *Lutzomyia longipalpis*, *Aedes aegypti*) and *H. sapiens* were performed using the Clustal W software. The comparison indicated that enolase was a highly conserved protein in the above mentioned organisms, encoding 433 amino acids except *H. sapiens* (434 amino acids) and *G. morsitans* (436 amino acids). *H. flava* enolase had high similarity with enolases of *I. ricinus* (GADI01005028, 83.3 % identity) and *O. moubata* (GU594041, 82.2 % identity), ranging from 74.4 to 77.4 % identity with those of insects, and 73.1 % identity with *H. sapiens* enolase (Table 3).

### Phylogenetic analysis

The phylogenetic tree (shown in Fig. 4) of the enolase proteins from different species based on the neighbor joining method



**Fig. 4** Phylogenetic relationship among the enolase gene of *H. flava* and other species. Neighbor joining trees were generated from the homologies of the enolase sequences using MEGA version 5.0. Accession numbers used are as follows: *H. flava* (KM191327), *Ixodes ricinus* (GADI01005028), *Ornithodoros moubata* (GU594041), *Glossina morsitans* (EZ423104), *Musca domestica* (KA647336), *Culex quinquefasciatus* (XM\_001842566), *Psorophora albipes* (GALA01001134), *Lutzomyia longipalpis* (EU124610), and *Aedes aegypti* (XM\_001653700). *Homo sapiens* (AK223192) was used as an out-group

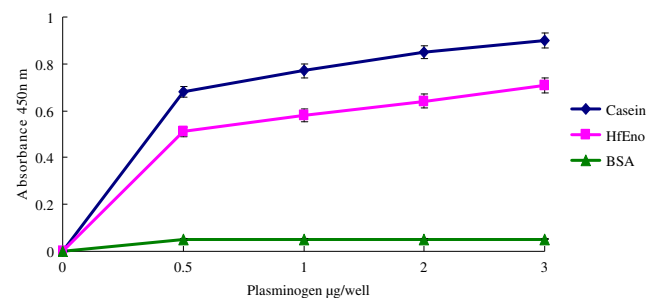


**Fig. 5** SDS-PAGE (a) and Western blot analysis (b) of expressed recombinant *H. flava* enolase. M1 and M2 represent two different molecular weight markers. S represents recombinant protein enolase/His from infected Sf9 cells. The red arrowheads indicate the position of the expressed targeted proteins (color figure online)

revealed that it was composed of four main clusters. The *H. flava* enolase formed a cluster with the *I. ricinus* and *O. moubata*, the *A. aegypti*, *C. quinquefasciatus*, and the *P. albipes* enolase formed another cluster, the *G. morsitans*, *M. domestica*, and *L. longipalpis* formed the third cluster, and the human (*H. sapiens*) formed a single clade. These results revealed that enolases from ticks were distant from those of insects and confirmed the close relationship among the ticks *H. flava*, *I. ricinus*, and *O. moubata*.

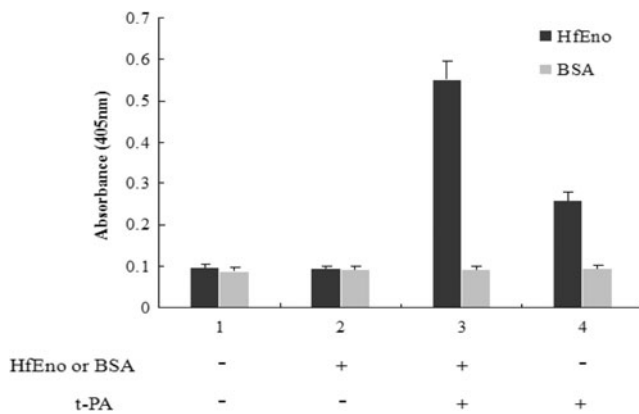
### Expression of the enolase in Sf9 cells

The enolase protein was expressed by the Bac-to-Bac baculovirus expression system. The *H. flava* enolase cDNA was cloned into the expression vector pFastBac1. After transposition and transfection, the enolase protein was expressed in



**Fig. 6** *H. flava* enolase binds plasminogen. Binding of increasing amounts (0.5–3 µg/well) of plasminogen to 0.5 µg/well of HfEno, 0.5 µg/well of casein (positive control), and 0.5 µg/well of BSA (negative control). Each point is the mean of four replicates and standard error





**Fig. 7** Activation of human plasminogen by *H. flava* enolase. Plasminogen activation assays with HfEno (black bars) or BSA (white bars, negative control) alone, or together with tPA. Proteolytic activity was measured by absorbance at 405 nm. The data represent the means and standard errors from four different experiments with four replicates per condition

Sf9 cells as a His-fusion (enolase/His) protein. The hexahistidine-tagged recombinant enolase had an estimated molecular weight of around 48 kDa, which was in accordance with the size observed by SDS-PAGE after Coomassie Blue staining (Fig. 5a). The corresponding band was also revealed by Western blot analysis (Fig. 5b). An enolase protein with a similar molecular weight has been reported in the argasid tick *O. moubata* (Díaz-Martín et al. 2013).

### Enolase binds human plasminogen

Previous studies have shown that enolase is a receptor of plasminogen (Jolodar et al. 2003; Mundodi et al. 2008) and is one of the well-known plasminogen binding proteins found in many organisms (Nogueira et al. 2012; de la Torre-Escudero et al. 2010). These reports prompted us to investigate whether the enolase of *H. flava* could bind plasminogen. To accomplish this, multiwell plates were coated with HfEno, casein (positive control protein), or BSA (negative control protein), and then plasminogen binding assays were performed by ELISA. The results indicated that the HfEno bound human plasminogen in a dose-

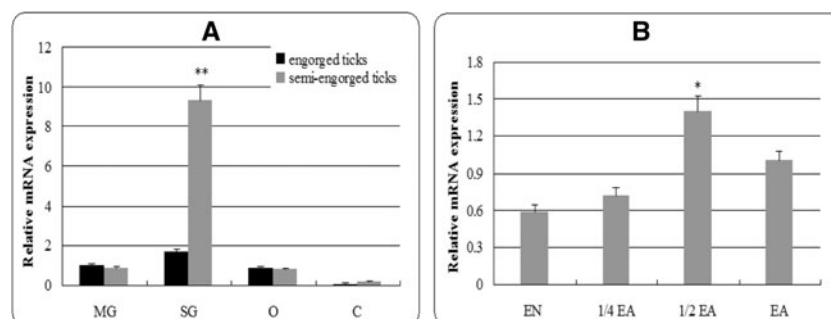
dependent manner, and this binding increased with the increasing concentration of plasminogen in the reaction mixture (Fig. 6), which is in agreement with a previous report (Díaz-Martín et al. 2013). Previous studies (de la Torre-Escudero et al. 2010; Nogueira et al. 2012) have pointed out that lysine residues may perform important functions in plasminogen binding. The casein also bound plasminogen significantly as a positive control, and BSA did not bind plasminogen at all as a negative control, as expected. When comparing the activity of the recombinant enolase and casein, casein had a higher absorbance than HfEno, indicating that HfEno may have less plasminogen binding ability than casein.

### Plasminogen is activated by HfEno in the presence of tPA

Plasminogen activation with subsequent generation of plasmin is responsible for the degradation of intravascular clots and is mediated by the physiological plasminogen activator t-PA (Lijnen 2001). To determine if enolase-bound plasminogen can be converted to the active plasmin, we carried out activation assays. Figure 7 shows that HfEno-bound plasminogen was converted to active plasmin in the presence of a physiological activator tPA (Reaction 3) and the generation of active plasmin was increased by HfEno (compared with reaction 4), whereas there was no generation of plasmin without tPA (reaction 2), indicating that HfEno could influence the plasminogen activation but lacked the ability to activate plasminogen by itself. These results suggested that plasminogen can be activated by HfEno in the presence of tPA, which are consistent with the findings reported by others (de la Torre-Escudero et al. 2010; Díaz-Martín et al. 2013). Enolase is different from other plasminogen activators, or at least as regards longistatin from *Haemaphysalis longicornis* (Anisuzzaman et al. 2011), which activates fibrin clot-bound plasminogen into plasmin in the same way as tPA.

### Expression pattern of the tick *H. flava* enolase gene

Expression pattern of the enolase gene in different tissues and at different tick developmental stages of *H. flava* was analyzed



**Fig. 8** Real-time quantitative PCR analysis of enolase gene expression in different tissues (a) and at different developmental stages (b) of *H. flava*. SG, salivary glands; MG, midguts; O, ovaries; C, crusts; EN, engorged

nymph ticks; 1/4 EA, quarter-engorged adult ticks; 1/2 EA, semi-engorged adult ticks; EA, engorged adult ticks. The y-axis shows the relative mRNA expression levels. \* $P < 0.05$ , \*\* $P < 0.01$

by real-time PCR. The results revealed that enolase expression was much higher in salivary glands (Fig. 8a) than in midguts, ovaries, and crusts of *H. flava*. The enolase was expressed in all developmental stages (Fig. 8b). The expression level of enolase reached the peak in semi-engorged adult ticks (1/2 EA), indicating that enolase had the highest expression in the rapid blood feeding period, suggesting that enolase is very important for tick blood feeding.

## Conclusion

In this study, we have characterized the enolase from the hard tick *H. flava*. *H. flava* enolase, as a plasminogen receptor, is able to bind plasminogen and enhance its activation in the presence of t-PA. The gene expression study indicated that the enolase is important for tick blood feeding, which may offer a novel target for controlling ticks.

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