

# Expression, purification, and biological characterization of *Anaplasma phagocytophilum* enolase

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## Summary

The obligate intracellular bacteria *Anaplasma phagocytophilum* is the etiological agent of human granulocytic anaplasmosis (HGA), an acute febrile tick-borne disease. *A. phagocytophilum* has a complex lifecycle within both vertebrate reservoirs and tick vectors, and employs a range of different molecules to infect and multiply within the host cells. Enolase is an essential glycolytic enzyme in intracellular glucose metabolism, but is also a multifunctional protein expressed on the pathogen surface, that binds to and promotes plasminogen conversion to plasmin. In this study, we generated recombinant ApEno protein (rApEno), and confirmed that rApEno retains its enzymatic activity. Furthermore, we demonstrated that rApEno binds to human plasminogen, and that this binding could be significantly reduced in the presence of lysine analogs ( $\epsilon$ -aminocaproic acid). Additionally, rApEno promotes plasminogen to plasmin conversion in the presence of plasminogen activator. In conclusion, *A. phagocytophilum* enolase is a multifunctional protein which can catalyze the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate, and facilitate binding to host plasminogen.

**Keywords:** *Anaplasma phagocytophilum*, enolase, catalytic ability, plasminogen

## 1. Introduction

Human granulocytic anaplasmosis (HGA), caused by the obligate intracellular bacteria *Anaplasma phagocytophilum*, is an emerging tick-borne zoonosis in the United States, Europe, and Asia (1). *A. phagocytophilum* is primarily transmitted to humans via bites from infected ixodid ticks; however, transmission has also been reported to occur through blood transfusion and contact with infected mammal blood (2). After infection, the patient generally presents unexplained nonspecific symptoms such as fever, chills, headache, and myalgia, which may occur in the presence of abnormal laboratory features, which may include leukopenia, thrombocytopenia, and/or mildly elevated

liver enzymes (3).

*A. phagocytophilum* is a significant tick-borne pathogen in terms of public health, and improving our understanding of its transmission methods is vital to enabling the development of appropriate control measures. *A. phagocytophilum*'s natural infectious cycle is dependent on the presence of infected vertebrate reservoir hosts and ixodid tick vectors (4). Within the vertebrate host, the bacteria colonizes neutrophils, but also infects other cells of myeloid and nonmyeloid origin. Of interest, it has also been reported to infect and multiply in several different ixodid tick tissues, including the midgut and salivary gland cells (2). Over recent decades, several molecules used by *A. phagocytophilum* to infect and multiply within vertebrate host cells have been well characterized (4). Moreover, a growing numbers of reports suggest that *A. phagocytophilum* can modulate tick gene and protein expression to facilitate bacterial acquisition and/or transmission in the tick vector (5,6). However, little information is available about the specific bacterial molecules playing key roles in tick gut and salivary gland infection.

Enolase is a key cytoplasmic glycolytic enzyme

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in intracellular glucose metabolism, and catalyzes the reversible dehydration of 2-phospho-D-glycerate (2-PGA) to phosphoenolpyruvate (PEP) in the penultimate step of glycolysis (7). In recent years, enolase has also been identified on the surface of a variety of eukaryotic cells, and there acts as a plasminogen receptor promoting conversion to plasmin when in the presence of plasminogen activator (8). Furthermore, it has been confirmed that surface-expressed enolase on vector-borne pathogens plays an essential role during pathogen invasion of vector gut by binding mammalian plasminogen (9,10).

In the present study, we characterized the protein structure of *A. phagocytophilum* enolase (ApEno), and then generated recombinant ApEno (rApEno) via an *E. coli* expression system. Subsequently, we confirmed that rApEno catalyzes 2-PGA dehydration to PEP. Additionally, we demonstrated that rApEno binds to and promotes plasminogen conversion to the plasmin active form. Our results provide further evidence that these molecules likely contribute to *A. phagocytophilum*'s ability to infect tick vectors. However, further research is needed in order to fully elucidate ApEno's role during the bacterial infection process.

## 2. Materials and Methods

### 2.1. Animals and ethics statements

All mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and maintained at the Xuzhou Medical University Animal Center. This study was carried out in strict agreement with the guidelines for the Care and Use of Laboratory Animals as defined by the Xuzhou Medical University Laboratory Animal Ethics Committee. All protocols involving mice were approved by the Xuzhou Medical University Laboratory Animal Ethics Committee (Permit Number: 201547).

### 2.2. Reagents

All reagents were purchased from Sigma-Aldrich, CO (St. Louis, USA) unless otherwise specified.

### 2.3. ApEno characterization

*A. phagocytophilum* str. HZ enolase amino acid sequences were obtained from the NCBI protein database (11,12). The enolase signature sequence and other domains were defined using ScanProsite online software (<http://prosite.expasy.org/scanprosite>), and MotifScan online software ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)). Multiple protein sequences were aligned and analyzed using the MUSCLE multiple sequence alignment tool from the European Bioinformatics Institute (EMBL-EBI) (<http://www.ebi.ac.uk/Tools/msa/clustalo>).

### 2.4. ApEno cloning, recombinant expression, and purification

The gene fragment encoding the entire ApEno coding sequence was synthesized by GENEWIZ® (GENEWIZ Suzhou, China). *Bam*HI and *Xho*I restriction sites were added to ApEno 5' and 3' ends, respectively, to facilitate cloning into the expression vector. ApEno was then cloned into the pGEX-4T-2 expression vector (TransGen Biotech, Beijing, China), and transformed into *Escherichia coli* BL21 Star (DE3) competent cells. Recombinant protein expression and purification were performed as previously described (13). In brief, *E. coli* cells were induced overnight with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and then lysed with a sonicator. Following centrifugation, the supernatant was purified on a GSTrap 4B column. Finally, the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining.

### 2.5. Mouse polyclonal antibody against rApEno

Polyclonal antibodies against rApEno were generated as previously described (13). Briefly, 50  $\mu$ g purified rApEno protein was mixed with MONTANIDE™ ISA 70 VG adjuvant (Seppic, Puteaux, France), and then subcutaneously injected into four- to six-week-old female Balb/c mice at two-weekly intervals. One week after the third immunization, mouse blood samples were isolated and centrifuged to obtain antibodies. Finally, antibody titer was measured using an indirect enzyme-linked immunosorbent assay (ELISA), and antibody specificity evaluated by western blot.

### 2.6. rApEno enzymatic activity

Purified rApEno enzymatic activity was determined by direct monitoring of absorbance enhancement at 240 nm using a Synergy HT spectrophotometer (Bio-Tek Instruments, Winooski, USA) as previously described (14). In brief, 1 mM 2-PGA was incubated with different concentrations of rApEno (2.5, 5, 10, and 20 ng/ $\mu$ L) in a 30°C preheated reaction buffer (100 mM HEPES buffer, 7.7 mM KCl, 10 mM MgSO<sub>4</sub>, pH 7.0). The continuous assay was measured every 3 min for a period of 60 min.

In order to calculate the Michaelis-Menten constant ( $K_m$ ) of rApEno, purified rApEno protein was incubated with differing 2-PGA concentrations (1, 1.5, 2, 4, 6, 8, and 10 mM) in reaction buffer. To analyze the effects of pH and temperature on rApEno activation, the assay was performed using a reaction buffer system at different pH values (3, 4, 5, 6, 7, 8, 9, 10, and 11) and temperature (4, 22, 30, 37, 50, 60, 70, and 80°C). Reactions were initiated by the addition of rApEno (1  $\mu$ g/reaction) diluted in the corresponding buffer. GST protein was used as a negative control.

2.7. Plasminogen binding analysis

The analysis was performed in 96-well plates as previously described (10). In brief, each well was coated overnight at 4°C with 100 µL PBS containing 0.04 µg/µL human plasminogen. Non-specific binding sites were blocked with 2% BSA in PBS, then each well was incubated with 100 µL PBS containing 0.04 µg/µL rApEno for 2 h at 37°C. Following three washes with PBST (0.05% (V/V) Tween-20 in PBS), bound protein was detected using mouse anti-rApEno antibody and goat alkaline phosphatase (AP)-conjugated secondary antibodies. Finally, the wells were washed three times with PBST before the AP substrate was added and optical densities (OD) were read at 450 nm using a Bio-Tek plate reader. GST was used as a negative control. In addition, to analyze the role of lysine residues in plasminogen- enolase interactions, differing concentrations (0-100 mM) of lysine analog ε-aminocaproic acid (ε-ACA) were added together with rApEno to the plasminogen-coated plates.

2.8. Plasminogen activation assay

As previously described (15), 96-well plates were coated overnight with 4 µg rApEno or GST in 100 µL PBS at 4°C. Subsequently, plates were blocked and washed three times as above, and then 4 µg/well of human plasminogen was added and incubated for 2 h at 37°C. Wells were washed three times with PBST, and then 4 ng/well of human urokinase plasminogen activator (uPA) was added. Subsequently, 0.3 mM plasmin-specific substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride in PBS was added. Following overnight incubation at 37°C, absorbance was read at 405 nm using a Bio-Tek plate reader.

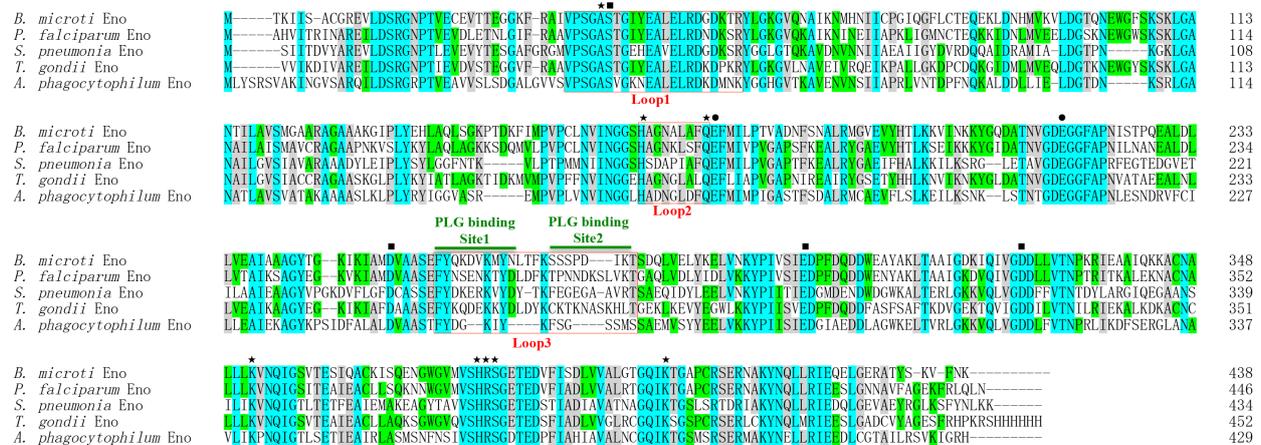
2.9. Statistical analysis

All data were collected from three independent experiments and presented as mean ± standard deviation (SD). Statistical comparisons of enzyme activity, plasminogen binding, and activation of rApEno were performed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. *p* values < 0.05 were considered to be significant and data analysis was performed with Prism 5.0 (GraphPad Software, Inc. USA).

3. Results

3.1. Anaplasma phagocytophilum enolase

An online search for *A. phagocytophilum* str. HZ proteomic sequences identified a protein (accession number: ABD44357) comprising 429 amino acids encoded by 1, 290 nucleotides. This protein was predicted to be a soluble 46.2 kDa polypeptide with a pI of 5.60, and presented an enolase signature motif spanning residues 338-351 (<sup>338</sup>VLIKPNQIGTLSET<sup>351</sup>). Following protein sequence analysis, eight conserved 2-PGA binding residues (Ala<sup>47</sup>, His<sup>161</sup>, Gln<sup>169</sup>, Lys<sup>341</sup>, His<sup>369</sup>, Arg<sup>370</sup>, Ser<sup>371</sup>, and Lys<sup>392</sup>), two conserved dehydration residues (Glu<sup>170</sup> and Glu<sup>211</sup>), and four conserved Mg<sup>2+</sup> binding amino acids (Ser<sup>48</sup>, Asp<sup>248</sup>, Glu<sup>289</sup>, and Asp<sup>316</sup>), were identified. Moreover, <sup>43</sup>VPSGASVKGNEALELRDKDMNK<sup>64</sup> (loop 1), <sup>161</sup>HADNGLDFQ<sup>169</sup> (loop 2), and <sup>254</sup>FYDGGKIYKFSGSSMS<sup>268</sup> (loop 3) were predicted to form the catalytic loop. In addition, two predicted plasminogen-binding motifs were characterized at <sup>254</sup>FYDGGKIY<sup>260</sup> and <sup>262</sup>FSGSSM<sup>267</sup> (Figure 1). The protein sequence also carried phosphorylation and

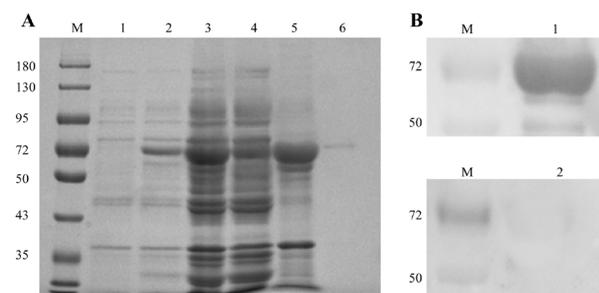


**Figure 1. ApEno sequence characterization.** Comparison of enolase amino acid sequences from *Babesia microti* (XP\_012649799), *Plasmodium falciparum* (AAA18634), *Streptococcus pneumoniae* (AOG56016), *Toxoplasma gondii* (3OTR\_A), and *Anaplasma phagocytophilum* (ABD44357), was performed with the MUSCLE multiple sequence alignment tool. Stars, circles, and rectangles were used to mark the residues interacting with 2-PGA, dehydration residues, and residues interacting with Mg<sup>2+</sup> ions, respectively. Catalytic loops are marked with boxes, and plasminogen (PLG) binding sites are indicated with horizontal green lines.

N-glycosylation sites, but no trans-membrane domains, signal peptides, nor plant-like insertions.

### 3.2. rApEno expression, purification, and characterization

The synthesized full-length enolase coding sequence was cloned into the pGEX-4T-2 plasmid with an in-frame GST-tag as described above. rApEno expression was induced in *E. coli* and bacterial extracts were assessed by SDS-PAGE with Coomassie blue staining.



**Figure 2. rApEno expression, purification, and characterization.** (A) rApEno expression and purification were assessed by SDS-PAGE with Coomassie blue staining. M: Pre-stained protein ladder, Lane 1: Total cellular proteins of pGEX-4T-2 BL21 (DE3) cells, Lane 2: Total cellular proteins of non-induced pGEX-4T-2-ApEno BL21 (DE3) cells, Lane 3: Total cellular proteins of induced pGEX-4T-2-ApEno BL21 (DE3) cells, Lane 4: Supernatant of pGEX-4T-2-ApEno BL21 (DE3) cell lysate, Lane 5: Sediment of pGEX-4T-2-ApEno BL21 (DE3) cell lysate, Lane 6: GST-tagged purified recombinant ApEno protein. (B) Anti-rApEno serum specifically recognized purified recombinant ApEno. M: Pre-stained protein ladder, Lane 1: Purified GST-tagged rApEno proteins were immunoblotted with mouse polyclonal anti-rApEno serum. Lane 2: Purified GST-tagged rApEno proteins were immunoblotted with normal mouse serum.

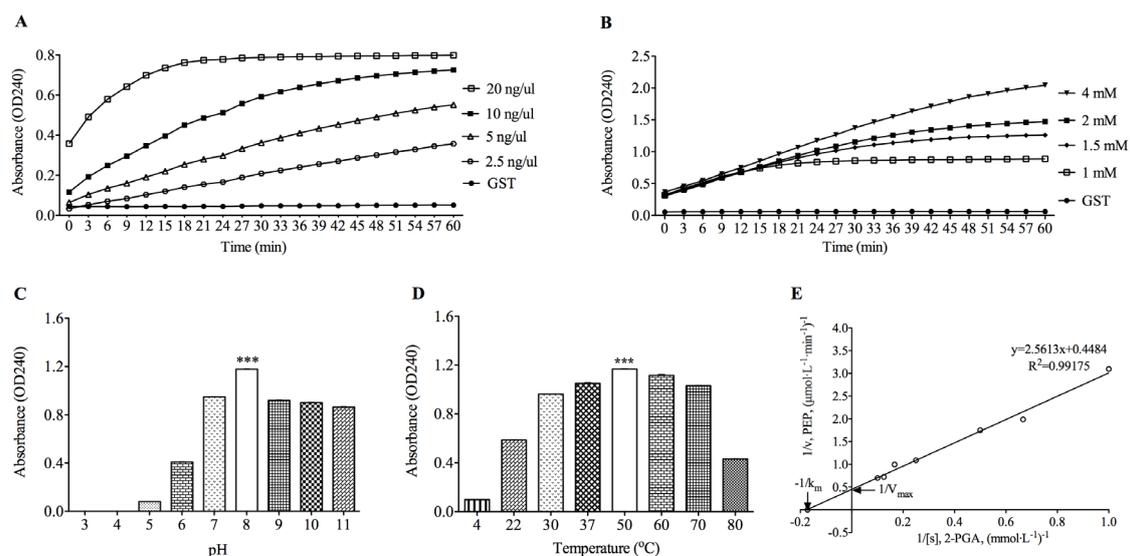
A prominent band of approximately 72 kDa, GST-tagged rApEno, was observed in the induced cell lysate. Soluble GST-tagged rApEno protein was purified to homogeneity from bacterial lysates, producing a single band (Figure 2A). Anti-rApEno mouse serum, but not normal mouse serum, specifically recognized purified rApEno via western blot (Figure 2B).

### 3.3. rApEno enzymatic activity

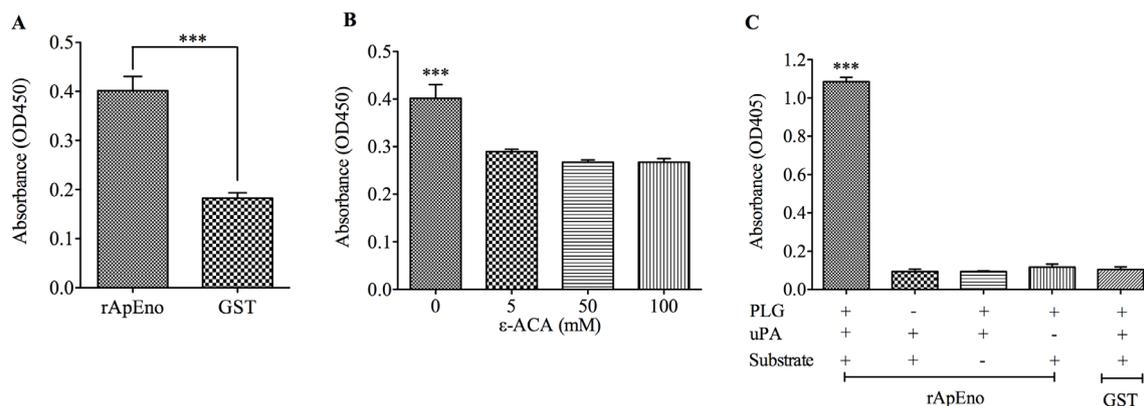
The classical enzymatic activity of purified rApEno was evaluated by monitoring its ability to catalyze 2-PGA to PEP. Results showed that the catalytic activity of rApEno increased with increasing concentrations from 2.5 to 20 ng/ $\mu$ L (Figure 3A). The catalytic activity of rApEno also increased when 2-PGA concentration rose from 1.0 to 4.0 mM (Figure 3B). Moreover, the Michaelis constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) of rApEno was determined to be 6.053 mM and 21.87  $\mu$ mol/L/min, respectively, by applying double-reciprocal Lineweaver-Burk plots (Figure 3C). This analysis also showed that the enzyme's optimal pH and temperature were 8.0 and 50°C, respectively (Figure 3D and 3E).

### 3.4. rApEno binds to human plasminogen and promotes its activation

ELISA binding assays were used to analyze the binding activity of rApEno to human plasminogen. The analysis showed that rApEno significantly binds to human plasminogen compared to the negative control ( $p < 0.0001$ ) (Figure 4A). Interestingly, rApEno binding



**Figure 3. rApEno enzymatic activity and affecting factors.** (A) Influence of different rApEno concentrations on enzymatic activity. (B) Influence of different 2-PGA concentrations on enzymatic activity. (C) rApEno  $K_m$  and  $V_{max}$  values were calculated with a double-reciprocal plot as 6.053 mM and 21.87  $\mu$ mol/L/min, respectively. (D) rApEno activity in a buffer system spanning a pH range 3-11, enzymatic activity was maximal at pH 8.0. (E) rApEno activity in a buffer system covering a 4 - 80°C temperature range, enzymatic activity was maximal at 50°C. Relative activity of rApEno was measured by OD240 absorbance. All data shown represent the mean  $\pm$  SD from three independent experiments. Significant differences as compared to other conditions are denoted by \*\*\* for  $p < 0.0001$ .



**Figure 4. rApEno binds to human plasminogen and promotes its activation.** (A) Relative rApEno binding activity to human plasminogen (PLG) was measured by absorbance at OD450, and GST served as a negative control for nonspecific binding. (B) rApEno binding activity was reduced in the presence of lysine analog ( $\epsilon$ -ACA). (C) rApEno-coated 96-well plates were incubated with PLG, uPA, and/or a plasmin-specific chromogenic substrate, and GST served as a negative control. Proteolytic activity was monitored by absorbance at OD405. All data shown represent the mean  $\pm$  SD from three independent experiments. Significant differences compared to other conditions are denoted by \*\*\*for  $p < 0.0001$ .

was significantly reduced with the addition of the lysine analog  $\epsilon$ -ACA, which acts as an efficient binding competitor ( $p < 0.0001$ ) (Figure 4B). As reported, enolase promotes plasminogen activation to plasmin. Our results confirmed that rApEno dramatically promoted plasmin activation in the presence of uPA, when compared to the GST negative control ( $p < 0.0001$ ) (Figure 4C).

#### 4. Discussion

Enolase is a multi-functional protein present in both prokaryotes and eukaryotes. As a cytoplasmic protein, enolase plays an important role in intracellular glucose metabolism; but as a surface expressed protein, it also plays a crucial role in several biological and pathophysiological processes: *e.g.* as a plasminogen receptor (7). In our study, we characterized a 46.2 kDa protein containing enolase signature motif residues, obtained from the gram-negative bacteria *A. phagocytophilum* str. HZ. In order to evaluate the potential catalytic and plasminogen-binding motif, enolase amino acid sequences from different hosts were aligned using MUSCLE multiple sequence alignment tools. As shown in Figure 1, ApEno possesses both 2-PGA binding residues and catalytic loops when compared to *Babesia microti* enolase and *Toxoplasma gondii* enolase (13,16). Furthermore, ApEno also possesses a conserved internal plasminogen-binding motif "FYDGIYKFKFSGSSMS" located between amino acids 254 and 268, when compared to *Streptococcus pneumoniae* surface enolase (17). However, neither plant-like pentapeptide nor dipeptide insertions – which play crucial roles in  $k_{cat}/K_m$  and dissociation into monomers – were conserved in ApEno (18,19). Taken together, these results provide vital information for the further refinement of ApEno's function.

To biologically characterize ApEno, a synthesized ApEno gene was cloned and expressed in *E. coli* cells. SDS-PAGE showed that rApEno could be expressed in the supernatant, which aided the purification and study of its biological characteristics. Expression products were purified, and Balb/c mice were immunized to generate anti-rApEno serum. Western blots showed that anti-rApEno mouse serum specifically recognized purified rApEno, where normal mouse serum did not (Figure 2). This suggested that the anti-serum presented high specificity and could be used in subsequent experiments.

Although it has long been recognized as a cytosolic protein important in sugar metabolism, enolase also catalyzes the dehydration of 2-PGA to PEP in the penultimate step in glycolysis (7). Here, our results also showed that purified rApEno catalyzed the conversion of 2-PGA to PEP in reaction buffer with 10 mM  $Mg^{2+}$  at a wide range of pH values and temperatures. Interestingly, rApEno demonstrated the highest enzymatic activity at 50°C and pH 8.0 (Figure 3). Moreover, rApEno presented high  $K_m$  and  $V_{max}$  values during 2-PGA to PEP conversion, when compared to other infectious intracellular pathogens (13,20). This suggests that ApEno is highly adaptable to a changing surrounding environment. This may be due to the fact that the bacteria cannot use glucose as a carbon or energy source, but instead uses a partial glycolysis pathway which starts with fructose 1,6-biphosphate (2).

Many reports have demonstrated that enolase can also be expressed on the surface of organisms, and act as the plasminogen receptor (10,13,21-23). In order to characterize rApEno plasminogen binding, ELISA experiments were performed. The results showed that rApEno was also able to bind to human plasminogen, and that binding could be significantly reduced in the presence of lysine analogs ( $\epsilon$ -ACA) (Figure 4A and

4B). These results confirmed that lysine analogs play an essential role in enolase-plasminogen interactions (10,13,15). The active form of plasminogen is plasmin, a serine protease, which can degrade cell extracellular matrix to assist pathogen infection of hosts (24). Furthermore, enolase can also promote plasminogen to plasmin conversion in the presence of tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (13,15). In the present study, we showed that rApEno dramatically enhanced plasmin activation in the presence of uPA (Figure 4C). It has been reported that *Plasmodium falciparum* enolase antibody was able to block merozoite invasion of red blood cells (25) suggesting that enolase could play an important role in the host cell invasion process. Herein, we hypothesized that *A. phagocytophilum* may utilize enolase to promote plasmin generation, which then degrades host cell membrane sections, enabling granulocyte invasion. However, further research is needed in order to confirm ApEno's role during the bacterial infection process of host cells. Enolase has also been identified as an important candidate antigen for vaccines against pathogen infection (25-29). Therefore, further research is also required to evaluate ApEno's potential as a candidate vaccine target to control anaplasmosis infection.

In conclusion, our results demonstrate that enolase from *A. phagocytophilum* possesses a highly conserved active site and plasminogen-binding domains. Recombinant ApEno catalyzes the reversible dehydration of 2-PGA to yield PEP. Furthermore, rApEno can bind to and promote the activation of plasminogen. In addition, we also confirmed that lysine residues play an important role in ApEno and plasminogen interaction. Our results provide new evidence that further supports the identification of molecules that likely contribute to successful *A. phagocytophilum* infection of tick vectors.

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