

Enzymatic and biological characteristics of enolase in *Brucella abortus* A19

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Abstract *Brucella abortus* is the etiological agent of brucellosis, a disease causing human public health problems as well as major economic losses in domestic animal industries. In this study, the *enolase* gene of *B. abortus* A19 was cloned, sequenced and expressed in *Escherichia coli* BL21. Bacterial-expressed enolase protein (His-eno) was purified and its ability to catalyze the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP) (hereon referred to as enolase activity) was analyzed. Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of the reaction was determined to be 2.0×10^{-3} M and $178 \mu\text{M l}^{-1} \text{min}^{-1}$, respectively. Factors influencing the enolase activity of His-eno, such as pH, the presence of metal ions and temperature were investigated in vitro. The results showed that His-eno exhibited maximal enolase activity in pH 8.5 reaction buffer containing 10 mM MgSO_4 at 37°C. In addition to studying the enzyme activity, binding assays were performed to provide insights into the function of His-eno on pathogenesis and immunity. His-eno exhibits fibronectin-binding ability in immunoblotting assay, suggesting that enolase may play a role in *B. abortus* colonization, persistence, and invasion of host tissue. Furthermore, Western blot demonstrated His-eno's binding ability to 34 bovine *B. abortus* positive sera, suggesting that future studies may find enolase a useful as a diagnostic marker or a vaccine candidate for brucellosis.

Keywords *Brucella abortus* A19 · Enolase · Enzyme activity · Fibronectin-binding ability

Introduction

Brucella abortus, a facultative intracellular Gram-negative bacterium, is the most important *Brucella* species involved in bovine brucellosis, which is characterized by abortion and infertility of infected animals [1–3]. Brucellosis is also a zoonotic disease and can infect humans through contact with contaminated animal products [4]. *Brucella* does not have classical virulence factors such as exotoxins, cytolytins, capsule, fimbria, flagellum, plasmids, lysogenic phages, antigenic variation, endotoxic lipopolysaccharide (LPS), and inducers of host cell apoptosis [5]. A key step in the pathogenesis of *B. abortus* is its ability to invade phagocytic and non-phagocytic host cells. Virulent *Brucella* organisms can infect both nonphagocytic and phagocytic cells. The mechanism of invasion of nonphagocytic cells is not clearly established. However, a 41 kDa surface protein (SP41) and the O antigen of the LPS molecule have been reported to be involved in *Brucella*'s adherence and invasion of host cells [6, 7]. A considerable number of fibronectin-binding proteins from various bacterial species have been reported, most of these proteins were shown to be involved in colonization, invasion and adhesion to epithelial cells, which is a critical early step in infection [8]. Enolase (2-phospho-D-glycerate hydrolase) is an enzyme of the glycolytic pathway, which catalyzes a reversible conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP). Recent studies suggested that enolases from eukaryotic and prokaryotic cells possess fibronectin-binding activity and play such roles in pathogenesis [9, 10], however, there has been no report regarding *B. abortus* enolase yet.

We were particularly interested in studying the enolase protein in *B. abortus* is because: (1) Enolase is reported as an important enzyme of the glycolytic pathway, therefore,

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we intent to understand this enzyme to find ways to control the infections by blocking the sugar metabolism pathway in *B. abortus* by targeting enolase. (2) Enolase has been found on the cell surface in a variety of microorganisms where it plays a critical role in bacterial colonization, persistence and invasion of host tissue [11–13] or acts as an immunogenic protein [14–17], so it is extremely important to study enolase from pathogenesis and immunity points of view. Therefore in this study, we cloned, expressed and characterized *B. abortus* enolase in hope to understand its roles in *B. abortus* metabolism, pathogenesis and immunity.

Materials and methods

Culturing of bacteria

B. abortus A19 was obtained from Chinese Veterinary Culture Collection Center (CVCC) and cultured in tryptic soy agar (TSA, Difco, NJ) or tryptic soy broth (TSB, Difco, NJ) at 37°C with 5% CO₂. *Escherichia coli* strains DH5 α (Invitrogen, Carlsbad, CA) and BL21 (DE3, Stratagene, La Jolla, CA) were grown on Luria–Bertani (LB) agar plates or broth at 37°C and transformants were selected on media supplemented with 50 μ g/ml kanamycin. The expression vector pET-28a was purchased from Novagen (Madison, WI). Restriction enzymes were from MBI Fermentas (Hanover, MD). All chemicals used in this study were of analytical grade and purchased from Sigma (St. Louis, MO).

Preparation of bacterial genomic DNA of *B. abortus* A19

Brucella abortus A19 was cultured on TSA plates, harvested in saline (0.85% NaCl solution) and heat-killed at 80°C for 30 min in a water bath. Chromosomal DNA was isolated using Wizard Genomic DNA Purification Kit according to the manufacturer's protocol (Promega, Madison, WI).

Expression and purification of *B. abortus* A19 enolase

The *enolase* gene was amplified by polymerase chain reaction (PCR) using primers Eno-F (5'-CGCGGATCC ATGACTGCAATCATCGACAT-3') and Eno-R (5'-CGCGTTCGACTTAGAGCAACTTCAGTGCAC-3'). The primers were designed according to the *enolase* gene sequence of *B. abortus* 2308 (NC_007618) with *Bam*HI and *Sal*I site (underlined) inserted. PCR product was *Bam*HI/*Sal*I-digested and cloned into *Bam*HI/*Sal*I sites of pET 28a (+)

(Novagen). The resulting plasmids, pET28a-Eno, was used to transform *E. coli* BL21. Expression of His-tagged enolase (His-eno) was induced by 1 mM IPTG, analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The recombinant protein His-eno was purified using HisTrap chelating high-performance columns (Amersham Pharmacia Biotech, Piscataway, NJ) and was quantitative determined using a BCA protein assay kit (Pierce, Rockford, IL).

Enolase activity and kinetics assay of His-eno

His-eno activity was determined by measuring the conversion of 2-PGE (Sigma) to PEP (Sigma) at 25°C as described [9] with modifications. A standard assay consists of adding 1 mM 2-PGE to 1.0 ml reaction buffer (100 mM HEPES buffer, pH 8.5, 7.7 mM KCl, 10 mM MgSO₄, pre-warmed to 25°C), 15 μ g of His-eno was then added to initiate the reaction. The reaction was monitored spectrophotometrically by measuring absorbance at 240 nm for the production of PEP at 1 min intervals for 10 min. The kinetics of the enolase activity was expressed as the production of PEP. Rabbit muscle enolase (Sigma) was used in place of His-eno as a positive control for the assay. The enzyme activity was also measured with 2.5, 5.0, 10.0 and 20.0 μ g of His-eno to test whether enzyme quantity is related to the activity.

To study enolase enzyme kinetics, varying concentrations of 2-PGE substrate (0.5, 1.0, 1.5, or 2.0 mM) were used for the assay. Michaelis–Menten kinetics of His-eno showed that His-eno was able to fully convert 2-PGE to PEP for all four substrate concentration levels. V_{max} and K_m for His-eno were determined from double-reciprocal Lineweaver–Burk plots.

Effects of pH, metal ions and temperature on enolase activity of His-eno

Modifications to the standard spectrophotometry enzyme kinetics assay were made to study the effect of pH, metal ions, and temperature on His-eno activity. As for the pH experiment, reaction buffers with pH values ranging from 5.0 to 10.0 in 0.5 increments were used for the assay. To examine the effect of metal ions, 10 mM of MgSO₄ in the original reaction buffer was replaced with 10 mM of ZnSO₄, MnCl₂, AlCl₃, NiSO₄, CuSO₄, HgCl₂, CrCl₃ or nothing. To test the thermostability of His-eno, the enzyme was pre-incubated for 1 h at 25, 37, 50, 62 or 75°C prior to the assay. Enolase activity in each of the above tests were compared to that of a standard reaction at 25°C involving 1.0 mM 2-PGE substrate, 15 μ g of His-eno, and the

standard reaction buffer at pH 8.5 containing 10 mM MgSO₄. All the reactions were performed in triplicate.

Preparation of rabbit anti-serum against His-eno

Rabbit anti-serum against His-eno was prepared by immunizing two New Zealand female rabbits (female, 2–3 kg) subcutaneously with 500 µg of His-eno emulsified in Montanide ISA 50 V (SEPPIC, France), three times at 4-week intervals. Blood samples were collected 2 weeks after the third injection. The titers of immunized sera were evaluated by enzyme-linked immunosorbent assay (ELISA) according to the standard methods, with a coating concentration of 100 ng purified His-eno per well on a 96 well ELISA plate.

Preparation and Western blot analysis of *B. abortus* A19 membrane proteins

To determine whether *B. abortus* A19 enolase was exported to the cell membrane, bacterial membrane proteins were isolated and analyzed by Western blot as previously described [18] with modifications. Briefly, mid-logarithmic phase *B. abortus* A19 in 200 ml TSB culture were collected by centrifuging at 8,000×g for 15 min at 4°C. The pellets were washed with phosphate buffered saline (PBS, pH 7.4), re-suspended in buffer A (15 mM Tris-HCl, pH 8.0, 0.45 M sucrose, 8 mM EDTA, and 0.4 mg/ml lysozyme), incubated at room temperature for 30 min, and centrifuged at 8,000×g for 15 min again. The pellet was re-suspended in buffer B (50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, and 2 mM PMSF), chilled on ice and sonicated (10 s at 400 W, for a total of 30 times with 5 s breaks in between). Then the samples were centrifuged twice at 3,000×g for 15 min to pellet intact cells. The supernatant was ultra-centrifuged at 45,000 rpm for 90 min and the pellet was collected and re-suspended in 12 ml buffer C (50 mM Tris-HCl, pH 7.6 with 2 mM PMSF). The protein was quantitative determined using a BCA protein assay kit (Pierce, Rockford, IL).

For Western blot, protein sample (10 µg/lane) was subjected to SDS-PAGE, then transferred onto PVDF membranes (Amersham Pharmacia Biotech) using a semi-dry transfer cell (Bio-Rad) at 15 V for 15 min. The membrane was blocked with 5% skim milk in PBS-0.05% Tween 20 (PBST) at 4°C for overnight and washed three times in PBST and then incubated with rabbit anti-serum against His-eno (1: 3,000) at room temperature for 2 h. After washing, the membrane was incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Sigma, 1:5,000) at room temperature for 1 h. The membrane was

washed and then developed with 3, 3'-diaminobenzidine (DAB, Sigma) until optimum color development was observed.

Binding ability of His-eno to human fibronectin or bovine *B. abortus* positive sera by Western blot

For Western blot, 10 µg purified His-eno was loaded into each lane of a 12% acrylamide gel and subjected to SDS-PAGE. The proteins were then electrophoretically transferred onto a PVDF membrane and the membrane was blocked with 5% skim milk in PBST at 4°C for overnight and washed with PBST.

To determine the ability for His-eno to bind to human fibronectin (Sigma), the membrane was incubated with 10 µg/ml of human fibronectin in PBST at room temperature for 2 h. After washing, the membrane was incubated with monoclonal anti-human fibronectin antibody (Sigma, 1:5,000) in PBST at room temperature for 2 h, followed by incubation with HRP-conjugated anti-mouse IgG (Sigma, 1:10,000). The blotting band was detected using an ECL kit (Amersham Pharmacia Biotech) as described in the manufacturer's protocol.

To determine the binding ability of His-eno to bovine *B. abortus* positive serum, the membrane was incubated with the bovine serum at room temperature for 2 h. The membrane was washed and incubated with peroxidase-labeled affinity purified antibody to bovine IgG (H + L) (1:3,000, KPL, Gaithersburg, MD) in PBST at room temperature for 1 h. The blotting bands were detected using an ECL kit as described above. A total of 34 bovine *B. abortus* positive sera, which were tested as *B. abortus* positive infection by a government approved brucellosis testing method, from Shanghai Center for Animal Disease Control and Prevention, China were tested. A serum sample from a healthy bovine was used as the negative control.

Results

Expression and purification of *B. abortus* A19 enolase

DNA sequence analysis of the PCR amplified *B. abortus* A19 enolase gene showed that the 1,278 bp gene has 100% sequence identity with that of *B. abortus* 2308 (NC_007618). Recombinant fusion protein His-eno was successfully expressed in *E. coli* BL21 as shown by a 49 kDa band in the SDS-PAGE analysis (Fig. 1, lanes 2 and 3). After purification with HisTrap chelating high-performance columns (Amersham), a single band of His-eno was identified (Fig. 1, lane 1).

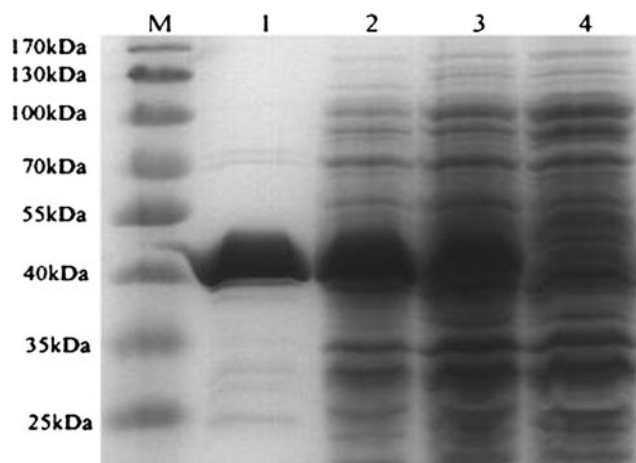


Fig. 1 Expression and purification of His-eno were identified with SDS-PAGE followed by Coomassie blue staining. *M* pre-stained protein marker (SM0671, Fermentas). *Lane 1* a 49 kDa band of purified His-eno with HisTrap chelating high-performance columns (Amersham Pharmacia Biotech). *Lane 2 and 3* total cellular proteins of *E. coli* BL21 transformed with expression plasmids pET28a-Eno. A 49 kDa expressed His-eno band in each lane was shown. *Lane 4* Total cellular proteins of *E. coli* BL21 transformed with pET28a. No band of 49 kDa His-eno showed

Enolase activity and enzyme kinetic assay of His-eno

His-eno's activity in catalyzing the conversion of 2-PGE to PEP was higher than that of rabbit muscle enolase as determined by the spectrophotometry enzyme kinetics assay (Fig. 2). His-eno activity is positively correlated with enzyme concentration of 2.5, 5, 10 or 20 μg . Michaelis–Menten plot was produced using the His-eno kinetics data with varying substrate concentrations. The plot was fitted to the equation $V = V_{max}S/(S + K_m)$ (Fig. 3), Michaelis Constant (K_m) and Maximum Reaction Velocity (V_{max}) were determined to be 2.0×10^{-3} M and $178 \mu\text{M l}^{-1} \text{min}^{-1}$, respectively, indicating high catalytic capability

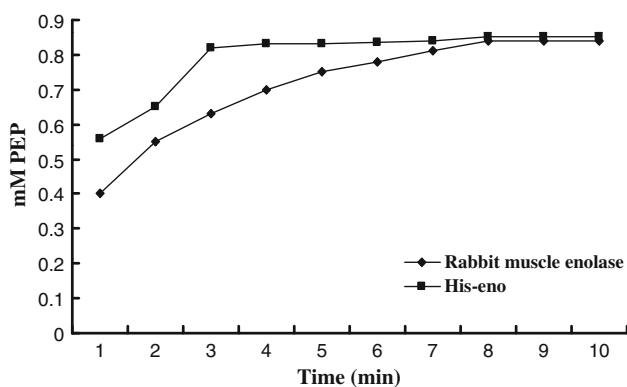


Fig. 2 Enolase activity was determined by measuring the conversion of 2-PGE to PEP. His-eno reached maximal enolase activity at 3 min, which was earlier than that of rabbit muscle enolase (positive control) at 10 min. The kinetics of the enolase activity was expressed as the production of PEP

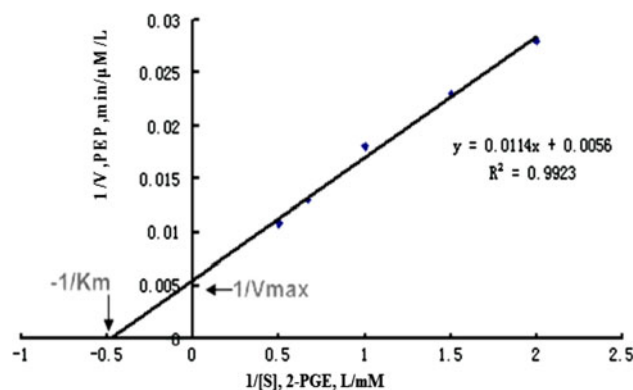


Fig. 3 Enzymatic characterization of purified His-eno. The conversion-rate (V) of 2-PGE (S) to PEP was measured at 240 nm using 15 μg of His-eno and various concentrations of 2-PGE (0.25–2 mM). Measurements were performed at 1 min intervals for 10 min. Data from three independent assays were plotted by the method of Michaelis–Menten and fitted to the equation $V = V_{max}S/(S + K_m)$. K_m and V_{max} for His-eno were determined to be 2×10^{-3} M and $178 \mu\text{M l}^{-1} \text{min}^{-1}$, respectively, by means of a Lineweaver–Burk plot (double-reciprocal plot). Data shown here were the mean values \pm standard deviations

of His-eno for the dehydration of 2-PGE to PEP under our experimental condition.

Effects of pH, metal ions and temperature on enolase activity of His-eno

His-eno exhibited maximal activity at pH 8.5 (Fig. 4). Comparing to having 10 mM Mg^{2+} in the reaction buffer, the relative activity of His-eno increased 10.6% when Mg^{2+} was replaced by Zn^{2+} of the same concentration (Table 1). Replacement with other ions such as Ni^{2+} , Al^{3+} and Mn^{2+} or no salt in the buffer reduced enzyme activity by 90.5, 31.2, 9.6 and 57.0%, respectively, whereas replacement of Mg^{2+} by Cu^{2+} , Hg^{2+} or Cr^{2+} completely inhibited the reaction (Table 1). The thermostability assay of His-eno revealed maximal enolase activity at 37°C. His-

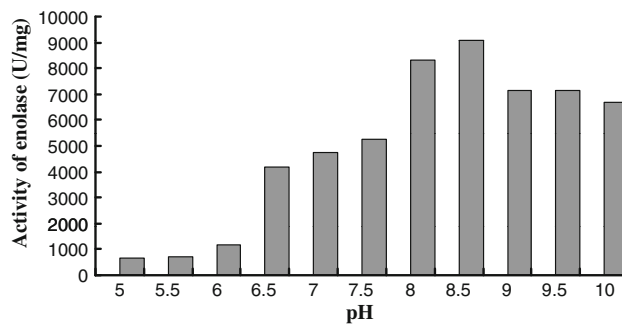


Fig. 4 Effect of pH on enolase activity of His-eno. The maximal enolase reactivity of His-eno occurred at pH 8.5, with reaction temperature of 37°C and 10 mM MgSO_4 in the reaction buffer. One unit is defined as the conversion of 1 μmol of substrate 2-PGE per minute at 37°C

Table 1 Effect of metal ions on enolase activity of His-eno in vitro

Metal ions (10 mM)	Activity of enolase (U/mg) ^a	Relative activity (%) ^b
Mg ²⁺	9090.91	100.0
Control ^c	3909.09	43.0
Zn ²⁺	10054.55	110.6
Mn ²⁺	8218.18	90.4
Al ³⁺	6254.55	68.8
Ni ²⁺	863.64	9.5
Cu ²⁺	0	0
Hg ²⁺	0	0
Cr ³⁺	0	0

^a Enolase activity was determined by measuring the conversion of 2-PGE to PEP at 240 nm. The reaction mixtures consisted of 15 µg purified His-eno, 1 mM 2-PGE, reaction buffer (pH 8.5) with respective metal salts. The reaction was performed at 37°C for 1 h. One unit is defined as the conversion of 1 µmol of substrate 2-PGE per minute at 37°C

^b The activity of His-eno in the reaction buffer with 10 mM Mg²⁺ was valued as 100%

^c The activity of His-eno in the reaction buffer without metallic salts. The experiments were performed in triplicate

eno retained over 90% of the initial activity when pre-incubated at 50 or 62°C for 1 h. However, pre-incubation at 75°C caused His-eno to lose 100% activity (Fig. 5).

Preparation of rabbit anti-serum against His-eno and Western blot analysis of membrane proteins

Rabbit anti-serum against His-eno was isolated and tested for ELISA titers as 1:10,000. The anti-serum was used for Western blot analysis of *B. abortus* A19 membrane proteins,

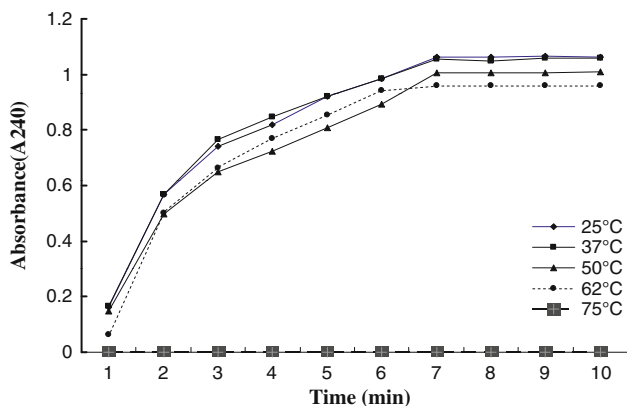


Fig. 5 Thermostability assay of His-eno. 15 µg of His-eno was pre-incubated at 25, 37, 50, 62 or 75°C, respectively for 1 h for the assay. The results showed that the enolase activity of His-eno was thermally stable at 25, 37, 50 and 62°C but was completely inhibited by a pre-incubation of the enzyme at 75°C. The experiment was performed in triplicate

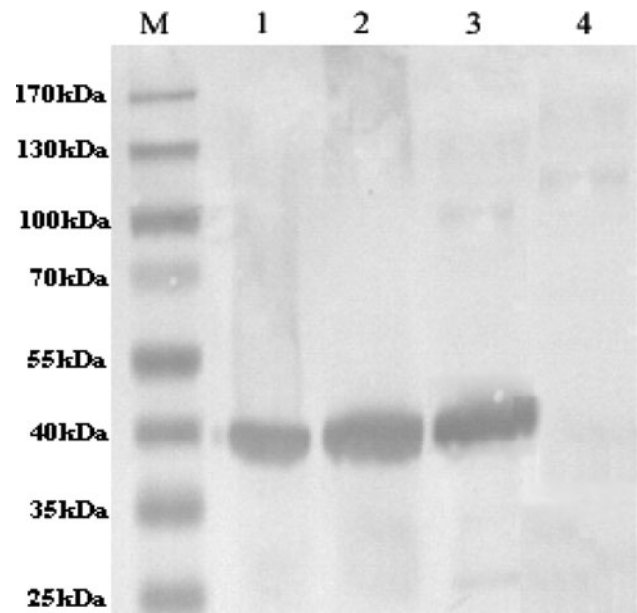


Fig. 6 Rabbit anti-serum against His-eno binds to membrane proteins of *B. abortus* A19 by Western blot. Lane M pre-stained protein marker (SM0671, Fermentas). Lane 1 immunoblot of total cellular proteins of *B. abortus* A19. Lane 2 immunoblot of isolated membrane protein of *B. abortus* A19. Lane 3 immunoblot of purified His-eno. Lane 4 no protein addition to the lane 4, showed no blot band to rabbit anti-serum against His-eno

using whole cell proteins and His-eno as the positive controls. Both total cellular proteins (lane 1) and membrane proteins (lane 2) of *B. abortus* A19 showed positive binding bands of 45 kDa (Fig. 6), suggesting that enolase is a membrane associated protein. Positive control of lane 3 with loading of His-eno showed a 49 kDa band. Lane 4 without any protein loading showed no binding band.

Binding ability of His-eno to human fibronectin or bovine *B. abortus* positive sera

The results of Western blot demonstrated that His-eno has both binding ability to human fibronectin (Fig. 7) and bovine *B. abortus* positive sera (Fig. 8, complete set of data not shown).

Discussion

In the present study, enolase of *B. abortus* A19 was cloned, expressed and characterized. The expressed recombinant enolase, His-eno, successfully catalyzes the conversion of 2-PGE to PEP with K_m and V_{max} values of 2.0×10^{-3} M and $178 \mu\text{M l}^{-1} \text{min}^{-1}$, respectively, at 25°C in pH 8.5 reaction buffer with 10 mM Mg²⁺.

His-eno maximal activity occurs at pH 8.5, a condition more suitable to alkaline condition, which might be due

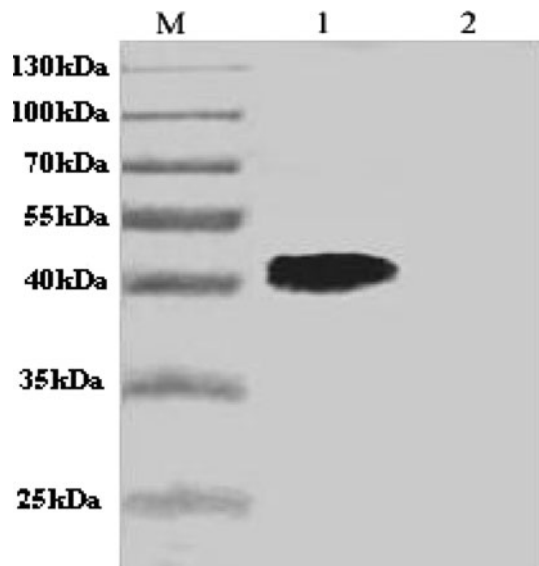


Fig. 7 Binding ability of His-eno to human fibronectin. Lane M pre-stained protein marker (SM0671, Fermentas). Lane 1 human fibronectin was immunoblotted with His-eno. Lane 2 no protein addition to the lane 2, showed no blot band to His-eno

to the structure and characteristics of enolase. Previous report indicated that the enolase of *Lactobacillus crispatus* were localized on the cell surface at pH 5 but released into the medium at alkaline pH, indicating that *lactobacilli* rapidly modify their surface properties in response to changes in pH [19]. It is possible that pH in the environment has similar effect on *B. abortus* enolase as well, which warrants future investigations for the biological significance. In addition, we demonstrated that Ni^{2+} , Cu^{2+} , Hg^{2+} and Cr^{3+} strongly inhibited the activity while Zn^{2+} and Mg^{2+} were the most potent activators. This result was consistent with a previous study showing that magnesium is a co-factor that stabilizes the enolase dimer formed by two subunits [20]. In this study, *B. abortus* enolase showed

also a relatively broad thermostability from 25 to 62°C that is beneficial in prompting the process of infection at different temperatures.

Membrane proteins of *B. abortus* A19 were isolated and subjected to SDS-PAGE, and rabbit anti-serum against His-eno was used for Western blot. The membrane proteins reacted with the rabbit anti-serum of His-eno, suggesting there is enolase protein in the isolated membrane proteins. Combined with the result of His-eno binding to human fibronectin, we believe *B. abortus* enolase play roles in the colonization and/or invasion of the pathogen. Further investigations of *B. abortus* enolase function on *brucella* colonization and invasion will be performed.

The major antigen that dominates the antibody response against *B. abortus* is the LPS, people used LPS as a marker to make diagnosis, however, there are cross-reactions of the *Brucella* O antigen with various bacteria, e.g. *Yersinia enterocolitica* O:9, *Salmonella urbana* group N, *Vibrio cholerae*, *Francisella tularensis*, *E. coli* O:157, and *Stenotrophomonas maltophilia* [21–23], therefore, serological diagnosis of brucellosis is still a challenge in human and animal disease. As *Brucella* is an intracellular parasite, the host immune response is not restricted to surface proteins, numerous outer and inner membranes, cytoplasmic, and periplasmic protein antigens of *B. abortus* have also been characterized, some are recognized by the immune system during infection and are useful in diagnostic tests [24–28]. To investigate whether there is enolase specific antibody in bovine *B. abortus* positive serum, we checked 34 such bovine sera by western blot analysis. All the sera tested showed binding bands to His-eno, suggesting that enolase is an immunogenic protein that may used as a diagnostic marker or potential vaccine candidate in the future.

In conclusion, bacterial expressed His-eno showed enolase activity of catalyzing the conversion of 2-PGE to PEP. His-eno has the ability to bind to human fibronectin

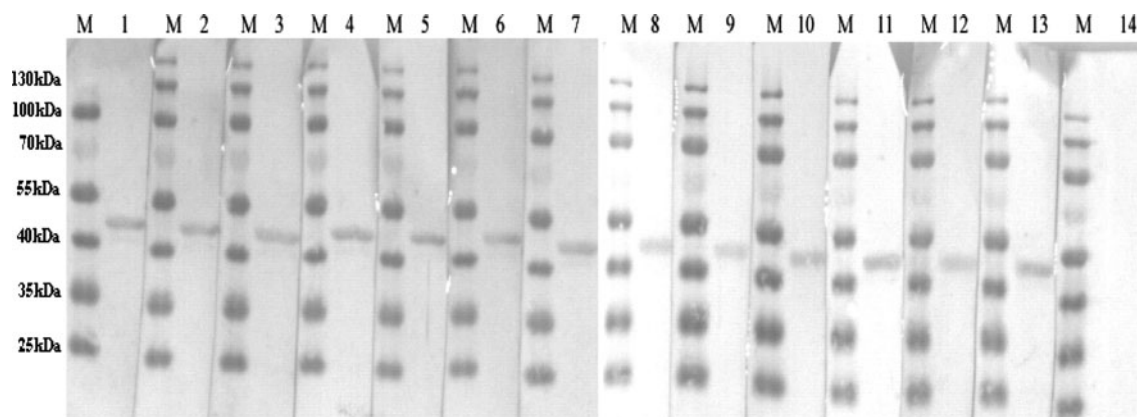


Fig. 8 Binding ability of His-eno to bovine *B. abortus* positive sera. Lane M pre-stained protein marker (SM0671, Fermentas). Lane 1–13 13 bovine *B. abortus* positive sera showed binding band to His-eno.

Data for other 21 bovine sera not shown but results were the same for all 34 sera. Lane 14 normal bovine serum showed no binding band to His-eno

and bovine *B. abortus* positive serum, which will benefit for future research concerning the role of enolase in *B. abortus*.

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