

Vibrio parahaemolyticus enolase is an adhesion-related factor that binds plasminogen and functions as a protective antigen

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Received: 24 July 2013 / Revised: 12 December 2013 / Accepted: 12 December 2013 / Published online: 16 January 2014
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Abstract *Vibrio parahaemolyticus*, an emerging food and waterborne pathogen, is a leading cause of seafood poisoning worldwide. Surface proteins can directly participate in microbial virulence by facilitating pathogen dissemination via interactions with host factors. Screening and identification of protective antigens is important for developing therapies against *V. parahaemolyticus* infections. Here, we systematically characterized a novel immunogenic enolase of *V. parahaemolyticus*. The enolase gene of *V. parahaemolyticus* ATCC33847 was cloned, sequenced, and expressed in *Escherichia coli* BL21. Enzymatic assays revealed that the purified recombinant *V. parahaemolyticus* enolase protein catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate. Western blot analysis showed that *V. parahaemolyticus* enolase was detectable in the extracellular, outer membrane (OM) and cytoplasmic protein fractions using antibodies against the recombinant enolase. Surface expression of enolase was further confirmed by immunogold staining and mass spectrometry (liquid chromatography–tandem mass spectrometry) analysis of OM protein profiles. Notably, *V. parahaemolyticus* enolase was identified as a human plasminogen-binding protein with the enzyme-linked immunosorbent assay. The values obtained for adherence and inhibition suggest a role of surface-exposed enolase in epithelial adherence of *V. parahaemolyticus*. We further showed

that enolase confers efficient immunity against challenge with a lethal dose of *V. parahaemolyticus* in a mouse model. To our knowledge, this is the first study to demonstrate the plasminogen-binding activity of enolase that is an adhesion-related factor of *V. parahaemolyticus*. Our findings collectively imply that enolase plays important roles in pathogenicity, supporting its utility as a novel vaccine candidate against *V. parahaemolyticus* infection.

Keywords *Vibrio parahaemolyticus* · Enolase · Enzyme activity · Adhesion · Vaccine candidate

Introduction

Vibrio parahaemolyticus, a Gram-negative motile bacterium inhabiting marine and estuarine environments, is commonly found free swimming, attached to underwater surfaces or commensally associated with different shellfish species (McCarter 1999; Shinoda 2011). The microbe is a leading cause of acute gastroenteritis after consumption of contaminated raw or undercooked seafood and occasionally causes wound infections in humans worldwide (Shimohata and Takahashi 2010; Su and Liu 2007). In approximately 5 % of cases, *V. parahaemolyticus* gastrointestinal infection progresses to septicemia and may be fatal for immunocompromised patients, including those with leukemia, liver disease, and individuals infected with human immunodeficiency virus (Baker-Austin et al. 2010; Qadri et al. 2003; Yeung and Boor 2004).

V. parahaemolyticus produces an array of factors associated with bacterial virulence, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), and type III secretion system (T3SS) (Broberg et al. 2011; Park et al. 2004; Yeung and Boor 2004; Nishibuchi et al. 1986). The global dissemination of this pathogen underscores the importance of understanding its numerous virulence factors and

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5471-z) contains supplementary material, which is available to authorized users.

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their effects on the human host. Microbial adhesion to host tissues is the initial event of most infectious process (Navarre and Schneewind 1999). Recent studies demonstrated that enolases from several pathogenic species, such as *Aeromonas hydrophila* (Sha et al. 2009), *Streptococcus pneumoniae* (Whiting et al. 2002), *Mycoplasma suis* (Schreiner et al. 2012) and *Borrelia burgdorferi* (Toledo et al. 2012), possess fibronectin-binding activity and affect bacterial virulence through different mechanism(s), playing critical roles in bacterial colonization, invasion, and adhesion to epithelial cells, which constitute significant early steps of infection.

Previous reports have shown that several pathogens capture plasminogen (Plg), which allows bacteria to acquire surface-associated proteolytic activity that facilitates invasion and dissemination in the infected host (Marcos et al. 2012; Kolberg et al. 2006). In particular, enolase plays a major role in microbial recruitment of Plg, a single-chain glycoprotein with a molecular mass of 92 kDa that is converted to plasmin in vivo (Song et al. 2012). Enolase is a ubiquitous enzyme that catalyzes the dehydration of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP) in the final steps of the catabolic glycolytic pathway (Han et al. 2012). Enolase is considered a multifunctional protein, in view of its contribution to several biological and pathophysiological processes in addition to its innate glycolytic and metabolic activities (Díaz-Ramos et al. 2012; Ghosh and Jacobs-Lorena 2011; Pancholi 2001). Considerable evidence has shown that enolase present on the surface of Gram-positive (Zhang et al. 2009; Ehinger et al. 2004) and Gram-negative (Han et al. 2012; Sha et al. 2009) bacteria, fungi (Marcos et al. 2012), and protozoa (Holmes et al. 2010; Mundodi et al. 2008) mainly acts as a receptor for plasminogen and functions as a mediator of microbial virulence (Avilán et al. 2011; Pancholi 2001; Pancholi and Chhatwal 2003).

Elucidation of the factors associated with virulence is a fundamental prerequisite for the search for new strategies targeting *V. parahaemolyticus* challenge. Enolase is found in both the cytosol and cell membrane in *Paracoccidioides brasiliensis*, and the presence of surface enolase is closely related to bacterial adherence to the host cell, where it interacts with Plg and facilitates microbial dissemination within hosts (Nogueira et al. 2010). Moreover, enolases of some bacteria (e.g., *Streptococci* and *Aeromonas*) have been characterized as protective immunogenic proteins (Feng et al. 2009; Sha et al. 2009; Zhang et al. 2009). However, Plg-binding activity has not been reported for *V. parahaemolyticus* enolase, and its role in cell adhesion is yet to be established. Therefore, it is important to examine enolase from the pathogenic and immunological points of view.

In the present study, we identified and characterized a functional *V. parahaemolyticus* enolase, with the aim of understanding its role in bacterial metabolism, pathogenesis,

immunity, and infection. The potential of enolase for development as a novel vaccine antigen against *V. parahaemolyticus* was also investigated.

Materials and methods

Bacterial strains and growth conditions

The *V. parahaemolyticus* strain ATCC33847, obtained from the Chinese Veterinary Culture Collection Center (CVCC), was grown on thiosulfate–citrate–bile salt–sucrose (TCBS) medium (Oxoid Ltd., Hampshire, UK) and incubated for 24 h at 37 °C. For polymerase chain reaction (PCR), bacterial adhesiveness, and virulence assays in mice, a single bacterial colony from TCBS agar was inoculated into brain heart infusion broth (BHI, Oxoid, UK) supplemented with 2 % NaCl and grown overnight at 37 °C with constant shaking. Luria-Bertani (LB) agar supplemented with 3 % NaCl was used for bacterial counting. The expression vector pET-28a (+) was purchased from Novagen (Madison, WI, USA). Prestained protein marker was obtained from MBI (Fermentas Inc., USA). All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA). Laboratory *Escherichia coli* strains, DH5 α and BL21, were used as nonadherent and noninvasive recipients of pET-28a (+) plasmids expressing recombinant protein.

Cloning, expression, and purification of *V. parahaemolyticus* enolase

The enolase gene was amplified with PCR using the primers Eno-F (5'CCAGGATCCATGTCTAAGATCGTTAAAGT T3') and Eno-R (5'GCAGAGCTCTTAAGCTTGGCCTTTA ACT3'). Primers were designed according to the enolase gene sequence of *V. parahaemolyticus* RIMD 2210633 (NC_004603) and contained *Bam*HI and *Sac*I sites (underlined) at the 5' and 3' ends, respectively. PCR was performed in a thermal cycler using the genomic DNA of *V. parahaemolyticus* ATCC33847 obtained using a Genomic DNA isolation kit (Tiangen, Beijing, China) as the template under the following conditions: denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension for 1.5 min at 72 °C for a total of 30 cycles. The 1,302-bp PCR product corresponding to enolase obtained was digested with *Bam*HI/*Sac*I and cloned into the corresponding sites of pET28a (+). The resulting plasmid, pET28a-eno, was used to transform *E. coli* DH5 α (Invitrogen, Carlsbad, CA, USA). Sequencing was performed on a 3070xl DNA analyzer (Applied Biosystems, Foster City, CA, USA), and DNA analysis was performed with DNASTAR software (DNASTAR Inc., Madison, WI, USA). Protein was induced in *E. coli* BL21 (DE3, Stratagene, La Jolla, CA, USA) transformed with pET28-eno at 37 °C for

4 h with 0.1 mmol/L isopropyl b-D-1-thiogalactopyranoside (IPTG). After sonication on ice (99 times for 5 s at 400 W with 10-s intervals between repeats, Scientz, JY92-IIN), bacterial lysates were subjected to centrifugation at 10,000g for 30 min for the removal of insoluble pellets. The acquired supernatant was purified using a His-Bind Purification Kit (Novagen), according to the manufacturer's instructions. Fractions of recombinant protein were filtered through a 0.22- μ m membrane (Millipore) and stored at -80°C . Protein quantitation was performed with a BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA).

Enolase activity and kinetics assay of *V. parahaemolyticus* enolase

Enzymatic assays for *V. parahaemolyticus* enolase were performed using standard procedures (Han et al. 2012; Feng et al. 2009; Esgleas et al. 2008) with modifications. Enolase activity was determined by measuring the conversion of 2-PGE to PEP. To address the capability of *V. parahaemolyticus* enolase to convert 2-PGE to PEP, 1 mM 2-PGE was added to a reaction buffer (100 mM HEPES buffer, pH 8.5, 7.7 mM KCl, 10 mM MgSO_4 , prewarmed to 25°C); 10 μ g of purified enolase protein was then added to initiate the reaction. PEP release was measured at 240 nm on a spectrophotometer at 1-min intervals for 10 min. Rabbit muscle enolase (Sigma) was used as the positive control. The kinetics of enolase activity was expressed as PEP production. To study *V. parahaemolyticus* enolase enzyme kinetics, different amounts of 2-PGE (0.25, 0.5, 1.0, 1.5, and 2.0 mM) were used for assay. Michaelis constant (K_m) and maximum reaction velocity (V_{\max}) for enolase were determined from double-reciprocal Lineweaver–Burk plots.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot

Recombinant enolase (10 μ g) was resolved on a 12 % (v/v) polyacrylamide vertical slab gel with a 5 % (v/v) stacking gel. After electrophoresis, one gel was stained with CBB R-250, and the other was applied for electrotransfer to a PVDF membrane (Amersham Pharmacia Biotech) using a semidry transfer cell (Bio-Rad) with a blotting buffer (39 mM glycine, 48 mM Tris, 0.037 % (w/v) SDS, 20 % methanol) at 15 V for 15 min. The membrane was blocked with a blocking buffer [5 % skimmed milk, 0.05 % Tween-20 in phosphate-buffered saline (PBS)] for 2 h at room temperature, washed with PBS containing 0.05 % Tween-20 (PBST), and incubated with convalescent-phase serum from a mouse clinically infected with the *V. parahaemolyticus* strain ATCC33847 (1:300) for 1 h, followed by incubation with anti-mouse IgG (whole-molecule) peroxidase conjugate (Sigma). Cross-reacting protein bands were visualized using a DAB Substrate Kit (Thermo). All experiments were performed in triplicate.

Antibody production

One milliliter (1 mL) of purified recombinant enolase (1 mg/mL) emulsified in Montanide ISA 50 V (SEPPIC, France) at a ratio of 1:1 was employed for subcutaneous immunization of two New Zealand rabbits (Slack Shanghai Laboratory Animal Co., Ltd., China), two times at 2-week intervals. Blood samples were collected when the second booster injection was administered 10 days later. The titers of rabbit antiserum against *V. parahaemolyticus* enolase were evaluated using enzyme-linked immunosorbent assay (ELISA). ELISA plate wells were coated with purified enolase (1 μ g/well). Well contents were reacted with serial dilutions (1/2,000 to 1/32,000) of rabbit anti-enolase antibody, followed by HRP-conjugated anti-rabbit IgG antisera (1:4,000, Sigma). Reactions were developed with tetramethylbenzidine substrate solution (Sigma) and terminated with 2 M H_2SO_4 , and OD_{450} of each well was read on an ELISA reader (BioTek, USA). The highest dilution of sera with $\text{OD}_{450} > 2.1$ times that of negative control wells was valued as ELISA titers. All samples were examined in triplicate.

Preparation of *V. parahaemolyticus* protein fractions and localization of enolase

To determine the distribution of *V. parahaemolyticus* enolase, extracellular proteins, cytoplasm proteins, and outer membrane proteins (OMPs) from *V. parahaemolyticus* strains ATCC 33847 were obtained as previously described (Ni et al. 2010; Sha et al. 2003), with modifications. Briefly, after centrifugation at 10,000g for 20 min, the supernatant of *V. parahaemolyticus* overnight cultures was collected and filtered through a 0.22- μ m-pore-size filter to remove residual bacteria. Then, 10 μ g/mL of a cocktail of protease inhibitors was added (Merck, USA) per milliliter of supernatant. The extracellular proteins were kept in the supernatant. The cell pellets were washed three times, resuspended in Tris–HCl (0.02 mol/L, pH 7.5), and sonicated in the ultrasonic disintegrator (600 W). After sonication of the cells, debris was removed by centrifugation at 10,000g for 20 min. The supernatant was ultracentrifuged at 150,000g for 1 h, and then, the supernatant obtained contained cytoplasm proteins. The pellet was resuspended into 0.5 % sodium lauroyl sarcosine and incubated overnight at 4°C . After ultracentrifugation at 150,000g for 45 min, the pellet (OMPs) was resuspended in distilled water with a proteinase inhibitor. The extracellular and cytoplasm protein fractions in the supernatant were precipitated using trichloroacetic acid (TCA) and acetone, respectively. Protein samples or bovine serum albumin (BSA, negative control) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting, rabbit antiserum against *V. parahaemolyticus* enolase (1:600) was used as the primary antibody and goat anti-rabbit

IgG (whole-molecule) peroxidase conjugate (Sigma) as the secondary antibody. Blots were developed with a DAB Substrate Kit. Experiments were repeated three times.

One-dimensional SDS-PAGE of OMPs and protein identification

In order to determine whether enolase is localized in the outer membrane of *V. parahaemolyticus*, OMPs of strain ATCC33847 were separated on a 1D SDS-PAGE using an acryl amide concentration of 5 % for the stacking gel and 12 % for the running gel. Separated protein bands in the SDS-PAGE gel were visualized with Coomassie Brilliant Blue. The protein band of about 48 kDa was excised and sent to Shanghai Applied Protein Technology Co. Ltd. (Shanghai, China) for tryptic in-gel digestion and liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC-MS/MS profiles of the peptides from OMPs of the *V. parahaemolyticus* strain ATCC33847 were used for searching the sequence similarities to data from *V. parahaemolyticus* or closely related Gram-negative bacteria available in the National Center for Biotechnology Information (NCBI) database. MS/MS spectra were automatically searched against the NCBI database using the BioWorks Browser rev.3.1 (Thermo Electron, San Jose, CA, USA). Protein identification results were extracted from SEQUEST out files with BuildSummary. The peptides were constrained to be tryptic, and up to two missed cleavages were allowed. Carbamidomethylation of cysteine was treated as a fixed modification, whereas oxidation of methionine was considered as a variable modification. The mass tolerance allowed for the precursor ions and fragment ions was 0.8 and 2.0 Da, respectively. The protein identification criteria were based on Delta CN (≥ 0.1) and cross-correlation scores (X_{corr} , one charge ≥ 1.9 , two charges ≥ 2.2 , three charges ≥ 3.75). Protein identifications were accepted if they could be established at >95 % probability and contained at least two identified peptides.

Immunogold staining

The surface exposure of enolase on *V. parahaemolyticus* was examined using immunoelectron microscopy as previously described (Sha et al. 2003), with modifications. Briefly, after several washes with PBS, log-phase-grown cultures of the *V. parahaemolyticus* strain ATCC33847 were fixed with a mixture of 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) and embedded in LR White resin (London Resin Company Limited, England), according to the manufacturer's instructions. The ultrathin-sectioned LR White-embedded cells were reacted with mouse antiserum against *V. parahaemolyticus* enolase (prepared in our laboratory using the purified recombinant enolase from this study) diluted to 1/800, followed by colloidal gold-labeled

(9–11-nm particles) anti-mouse IgG (Sigma) at a dilution of 1:20. After several washes with PBS, bacteria were finally stained with aqueous 3 % uranyl acetate and examined with an electron microscope (TEM, Philips CM120) at an accelerating voltage of 80 kV. Bacteria coated with normal mouse antibodies served as a negative control.

Plasminogen-binding assays

An ELISA was performed to determine the plasminogen-binding ability of *V. parahaemolyticus* enolase. Wells of multiwell plates (Nunc) were coated with 1 μg purified enolase diluted in carbonate buffer, pH 9.6. In a parallel experiment, ELISA wells were coated with OMPs from *V. parahaemolyticus*. Plates were incubated at 4 °C overnight. After subsequent blocking with 1 % (w/v) BSA in PBS, different concentrations (0.0156–2 $\mu\text{g}/\text{well}$) of human Plg (R&D Systems) or BSA were added to enolase-coated wells. Reactions proceeded at 37 °C for 1 h, and wells were subsequently washed. Bound protein was detected using anti-Plg monoclonal (R&D Systems) and horseradish peroxidase (HRP)-conjugated secondary antibodies. The competition experiment was performed by adding rabbit anti-enolase antibody (serial dilutions from 1/500 to 1/64,000), prior to the addition of Plg. The reaction was visualized with 3,3',5,5'-tetramethylbenzidine (TMB) solution for 10 min and terminated with 2 M H_2SO_4 . OD_{450} was measured using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA) to determine binding activity. The assay was performed in triplicate, and data were reproducible, since the values did not vary by more than 5 % of the mean.

Indirect immunofluorescence assays

To visualize whether *V. parahaemolyticus* enolase specifically adheres to the surface of Hep-2 cells, immunofluorescence assays were conducted (Chen et al. 2011; Zhang et al. 2009) with specific modifications. Hep-2 cells cultured in 96-well cell plates were washed twice with PBS, fixed with cold acetone/methanol (1:1) for 20 min at -20 °C, and allowed to air-dry. Purified enolase (10 μg) or bovine serum albumin (blank control) was incubated with fixed Hep-2 cells at 37 °C for 1 h and washed three times with PBS (100 $\mu\text{L}/\text{well}$). Rabbit antibodies against enolase preadsorbed with Hep-2 cells were added and incubated for 30 min at 37 °C. Hep-2 cells were incubated with recombinant enolase, washed with PBS, and incubated with preimmunization rabbit serum, which was tested as a negative control. After washing, cells were incubated with 100 μL FITC-conjugated goat anti-rabbit IgG (1:200, Sigma) at 37 °C for 1 h. Finally, samples were washed three times and visualized under a confocal laser scanning microscope (Nikon). To determine only enolase, but not the fused peptide (His₆ tag) of the recombinant enolase

that contributes to protein binding to the cells, the His₆-tag was removed from the expressed target protein and followed by removal of thrombin using a Thrombin Cleavage Capture Kit (Novagen), according to the manufacturer's instructions. Protein was applied to the His-Bind Purification Kit (Novagen) as above for a second time, and flow-through fractions were collected and concentrated by ultrafiltration (Millipore Amicon Ultra 10 K device). The resulting cleaved protein was verified by SDS-PAGE and Western blot with an anti-His-tag monoclonal antibody (Beyotime Institute of Biotechnology, China), while its adherence ability to host cells was confirmed as above by indirect immunofluorescence assays.

Inhibition assays

To evaluate the potential role of enolase in *V. parahaemolyticus* adhesion to host cells, the inhibition assays were performed. Hep-2 cells were grown to confluence in 24-well tissue culture plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum at 37 °C in a 5 % CO₂-humidified atmosphere without antibiotics. After washing with PBS three times, increasing amounts of purified recombinant enolase (10–20 µg/well) were added to cells and incubated at 37 °C for 2 h. Cells treated with 20 µg/well BSA served as the control. All cells were washed with PBS three times, infected with the ATCC33847 strain at a multiplicity of infection (MOI) of 1:50 in DMEM without fetal calf serum, and incubated for 2 h at 37 °C in 5 % CO₂. Cells were washed with PBS to remove nonadherent bacteria and treated with a lysis buffer (PBS containing 0.1 % (v/v) trypsin and 1.0 % (v/v) Triton 100) at 37 °C for 5 min. Serial dilutions of this cell lysate were plated onto LB agar supplemented with 3 % NaCl. After incubation at 37 °C overnight, the numbers of colonies were determined. The percentage of inhibition of enolase to adherence was calculated as (number of CFU recovered in the enolase-treated cells/number of CFU recovered in the BSA-treated cells) × 100.

To investigate the effect of antienolase antibody to the adherence of the *V. parahaemolyticus* strain ATCC33847, bacteria were pretreated with either specific antienolase antibody or preimmune serum for 1 h at 37 °C. Pretreated bacteria were used to infect Hep-2 cells as described above. All experiments were performed in triplicate and repeated three times.

Animal challenge experiment

ICR mice (6 weeks old) were purchased from Slack Shanghai Laboratory Animal Co., Ltd., China. Before challenge, the median lethal dose (LD₅₀) for the *V. parahaemolyticus* strain ATCC33847 was measured to determine virulence in a mouse model, as described previously (Baffone et al. 2001; Vongxay et al. 2008). Briefly, bacteria were grown to the exponential

phase, collected, washed twice in PBS, and adjusted to the appropriate doses. ICR mice were inoculated intraperitoneally (i.p.) with 0.2 mL of each bacterial suspension containing different numbers of CFU. The numbers of bacterial CFU in injected inoculum were confirmed by plating on LB agar supplemented with 3 % NaCl. Negative controls were injected with PBS. Ten animals were used per dose, and mortality was monitored until 7 days postinfection. The experiment were repeated three times, and the results were averaged and calculated using the method of Reed and Muench (1938).

Forty-five female ICR mice were randomly assigned to three groups of 15 mice each. Mice in group 1 were immunized subcutaneously at multiple sites with 100 µL of purified recombinant enolase (500 µg/mL) emulsified in Montanide ISA 50 V (SEPPIC, France) at a ratio of 1:1. Mice received one booster injection after 14 days with the same concentration of antigen and were reimmunized after 7 days. Mice in group 2 immunized with the inactivated *V. parahaemolyticus* strain ATCC33847 served as the positive control. Specifically, bacterial strains were grown for 24 h in LB medium supplemented with 3 % NaCl with shaking (250 rpm) at 28 °C and inactivated with formaldehyde at a final solubility of 0.4 % for 24 h. The injection dose was 100 µL of inactivated whole-cell vaccine (1 × 10¹⁰ CFU/mL) diluted with ISA 50 V adjuvant (SEPPIC, France) at a ratio of 1:1. Mice in group 3 inoculated with PBS emulsified in the same adjuvant served as the negative control. After 1 week, each group was challenged with a lethal dose of 5 LD₅₀ of the log-phase *V. parahaemolyticus* strain ATCC33847 in 0.5 mL PBS. Mice were monitored and scored for survival for 7 days. All animal infection experiments were approved by the Animal Ethics Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (no. SYXK<HU>2011-0116).

Statistical analysis

Antibody levels are expressed as geometric mean ELISA units of *n* independent observations ± standard deviations. Data were analyzed using Student's *t* test, and statistical significance was accepted at *P* < 0.05. Statistical analyses for in vitro and in vivo experiments were determined using the chi-square test with SPSS 16.0, and significance was established at *P* values of < 0.05.

Results

Expression and immunogenicity of *V. parahaemolyticus* enolase

The enolase gene (*eno*) was amplified from the *V. parahaemolyticus* strain ATCC33847. The open reading frame of *eno* gene is 1,302 bp in length encoding an enzyme

of 433 amino acids with a calculated molecular mass of 48 kDa. DNA sequence analysis showed that the amplified enolase gene displays 100 % identity with *V. parahaemolyticus* RIMD 2210633 (GenBank: BA000031). BLAST analyses indicated that the sequence is highly homologous to the protein sequences of enolase from a variety of other *Vibrio* species (85 to 98 %). Recombinant fusion protein *V. parahaemolyticus* enolase tagged with hexa histidine (His₆) at its N-terminus was successfully expressed in *E. coli* BL21 and purified with the His-Bind Purification Kit (Novagen, Madison, WI, USA), which was observed as a 52-kDa band in SDS-PAGE analysis (Fig. 1). Western blot analysis showed that *V. parahaemolyticus* enolase has a good immunoreactive to convalescent phase serum (1:300) from a mice clinically infected with *V. parahaemolyticus* (Fig. 1), suggesting that enolase may be an infection-associated antigen.

Antiserum against *V. parahaemolyticus* enolase was obtained from rabbits on day 10 after the booster injection and tested for ELISA titers as 1:32,000, which indicated that *V. parahaemolyticus* enolase has strong immunogenicity.

Enzymatic activity and kinetics assay of *V. parahaemolyticus* enolase

V. parahaemolyticus enolase activity in catalyzing the conversion of 2-PGE to PEP was similar to that of rabbit muscle enolase, as determined with the spectrophotometric assay. The production of PEP increased with time for both *V. parahaemolyticus* enolase and rabbit muscle enolase, and *V. parahaemolyticus* enolase reached maximal activity at 5 min, which was earlier than that of rabbit muscle enolase (positive control) at 9 min (Fig. 2a). The Michaelis–Menten plot produced using *V. parahaemolyticus* enolase kinetics data with varying substrate concentrations showed that *V. parahaemolyticus* enolase is able to fully convert 2-PGE to PEP for all five substrate concentrations, clearly implying that recombinant enolase retains enzymatic activity. The K_m and V_{max} values were determined as 1.9 mM and 154 $\mu\text{M}/\text{min}$, respectively (Fig. 2b).

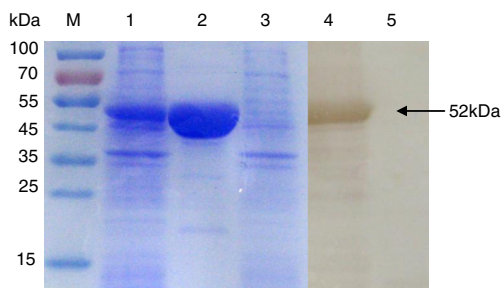


Fig. 1 SDS-PAGE and Western blot analysis of the recombinant *V. parahaemolyticus* enolase expressed in *E. coli* BL21. Lane M, MW marker (SM0671, Fermentas); lane 1, pET28a-enolase in *E. coli* BL21, induced; lane 2, purified recombinant enolase; lane 3, pET28a-enolase in *E. coli* BL21, uninduced; lane 4, Western blot of recombinant enolase with convalescent sera from mouse against *V. parahaemolyticus* ATCC33847; lane 5, Western blot of BSA as the negative control

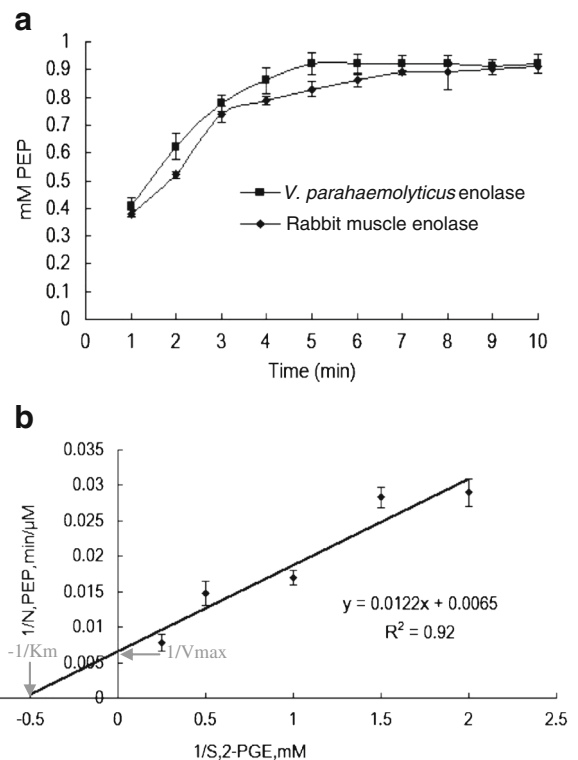


Fig. 2 Enzymatic characterization of enolase. **a** The production of PEP increased with time for both recombinant *V. parahaemolyticus* enolase and rabbit muscle enolase (positive control). **b** Determination of V_{max} and K_m for *V. parahaemolyticus* enolase by means of a Lineweaver–Burk plot (double-reciprocal plot). Data from three independent assays were fitted to the Equation $V = V_{max}S/(S + K_m)$. The values found were the $V_{max} = 154 \mu\text{M}/\text{min}$ and $K_m = 1.9 \text{ mM}$. Data shown here were the mean values \pm standard deviations

Localization of *V. parahaemolyticus* enolase

To determine in which portion of the cell, the majority of the enolase was located, Western blot, LC-MS/MS, and immunoelectron microscopy were performed. The extracellular, outer membrane, and cytoplasmic protein fractions from *V. parahaemolyticus* culture were prepared and reacted with rabbit antiserum against recombinant *V. parahaemolyticus* enolase. Western blot analysis showed that a band corresponding to the size of enolase protein (~48 kDa) was detected in all the above fractions (Fig. 3). Further, OMP preparations from *V. parahaemolyticus* were separated by 1D SDS-PAGE (Fig. 4a), and the protein band of about 48 kDa was excised and digested with trypsin. The resultant peptides were then analyzed with the help of LC-MS/MS. A total of 68 proteins were predicted to be localized to the outer membrane of *V. parahaemolyticus*, including maltoporin, OmpTolC, long-chain fatty acid transport protein, OmpU, OmpA, OmpK, polar flagellar assembly protein FliH, and so on (date not shown). As expected, enolase was also identified in the OMP preparations.

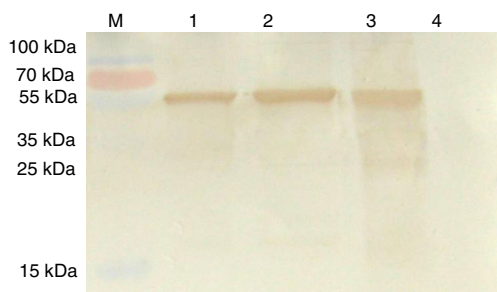
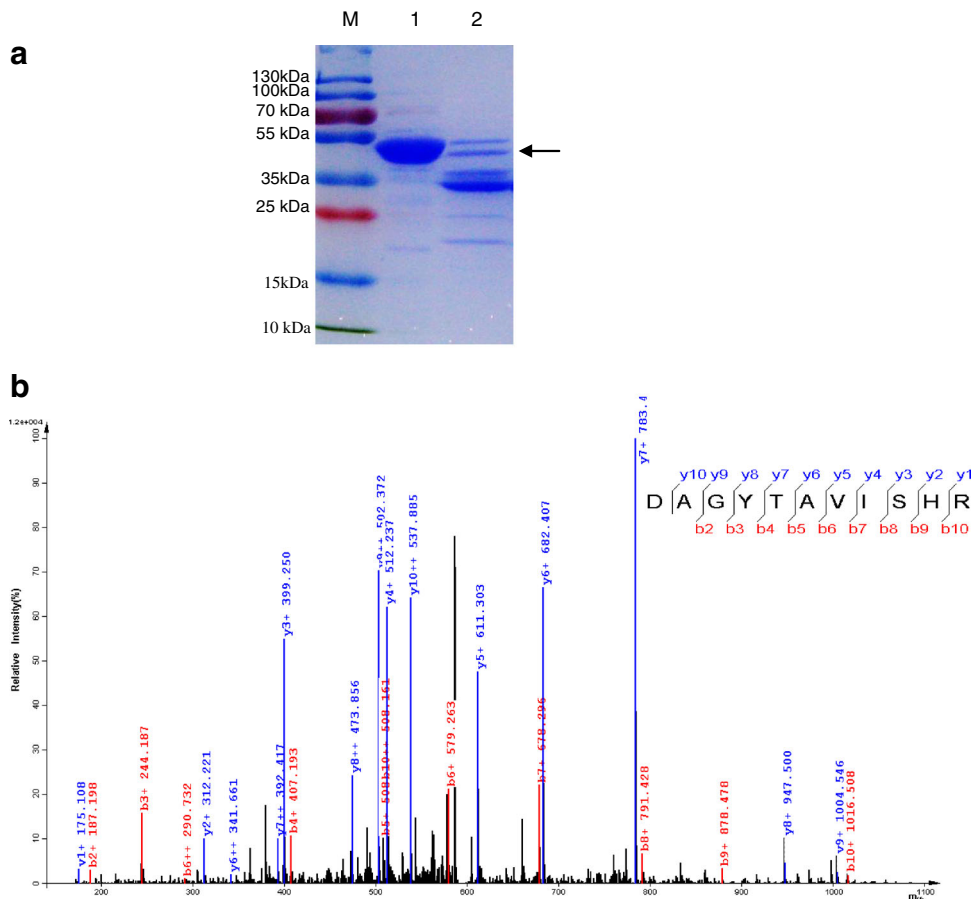


Fig. 3 Western blot analysis of enolase in different cell fractions from *V. parahaemolyticus*. Lane 1, cytoplasmic protein fractions; lane 2, outer membrane protein fractions; lane 3, extracellular protein fractions; lane 4, BSA, negative control; lane M prestained protein marker (SM1811, Fermentas)

Fig. 4 The localization of enolase in outer membrane. **a** SDS-PAGE analysis of purified recombinant enolase (lane 1) and OMPs of *V. parahaemolyticus* strain ATCC33847 (lane 2). The band pointed by the arrow was cut from the gel and further analyzed with the help of LC-MS/MS. Numbers on the left refer to commercial molecular weight markers (SM1811, Fermentas). **b** Simplified LC-MS/MS spectrum of the DAGYTAVISHR peptide. **c** Amino acid sequence of *V. parahaemolyticus* enolase. Identified peptide sequences are highlighted in yellow, which cover 17.3 % of the full-length sequence of enolase



c *V. parahaemolyticus* strain ATCC33847 enolase (Genbank accession number: KF835607)

MSKIVKVLGR	EIIDSRGNPTV	EAEVHLEGGF	VGMAAAPSGA	STGSREALEL
RDGDKARFLG	KGVLKAIKAV	NGPIAEALVG	KDAKDQAID	AVMIELDGTE
NKSKFGANAI	LAVSLANAKA	AAAAGMPLY	EHIAELNGTA	GQFSMPLPMM
NIINGGEHAD	NNVDIQEFMI	QPVGAATLKE	AVRMGAEVFH	NLAKV LKSKG
YNTAVGDEGG	FAPNLKSNAE	ALEVIAEAVA	AAGYELGKDV	TLAMDCAASE
FFDKEAGIYN	MKGEGKTFTS	EEFNHYLAGL	VEQFPVSIIE	DGLDESDWDG
FAHQTQLLGD	KIQLVGDDL	VTNTKILAEG	IEKGIANSIL	IKFNQIGSLT
ETLAAIKMAK	DAGYTAVISH	RSGETEDATI	ADLAVGTAAG	QIKTGSMRSR
DRVAKYNQLI	RIEALGERA	PFNGLKEVKG	QA.	

Simplified LC-MS/MS spectrum of a peptide of amino acid sequence of *V. parahaemolyticus* enolase, and the sequence coverage was shown in Fig. 4b, c. The *eno* gene sequence has been deposited in GenBank under the accession number KF835607.

In order to further examine the distribution of enolase, the *V. parahaemolyticus* strain ATCC33847 was fixed and stained with primary antibodies against *V. parahaemolyticus* enolase followed by gold-labeled secondary antibodies. Immunoelectron microscopy confirmed the surface and intracellular locations of enolase on bacterial cells (Fig. 5a). In contrast, no labeling was observed for the negative control (Fig. 5b).

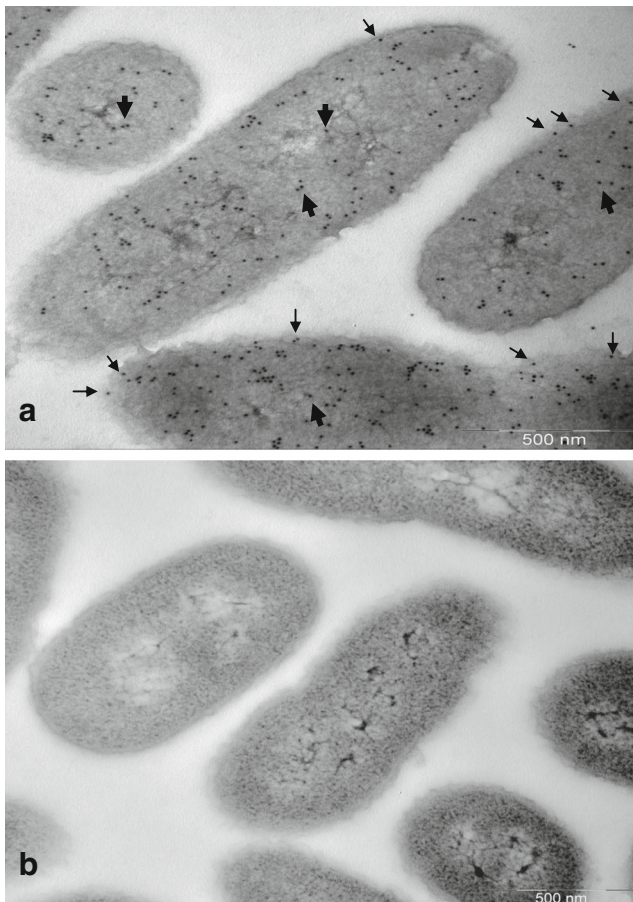


Fig. 5 Surface localization of enolase on *V. parahaemolyticus*. **a** Immunogold electron microscopic detection of the location of enolase (original magnification $\times 58,000$) on *V. parahaemolyticus* pretreated with mouse anti-enolase antibody followed by colloidal-gold-labeled anti-mouse IgG. Colloidal gold particles were localized at the surface of bacterial cells (indicated by thin arrows) and in the cytoplasm of bacterial cells (indicated by thick arrows). **b** Normal mouse serum used as a negative control. Bars, 500 nm

V. parahaemolyticus enolase binds plasminogen

ELISA analysis revealed specific concentration-dependent interactions between Plg and immobilized *V. parahaemolyticus* enolase. A similar pattern was observed in wells coated with *V. parahaemolyticus* outer membrane proteins. Binding was detected using anti-plasminogen and secondary antibodies. The differences between Plg (0.0156 to 2 $\mu\text{g}/\text{well}$) and control (BSA, negative control) were significant ($P < 0.05$) (Fig. 6a, b). Competition experiments were performed by adding *V. parahaemolyticus* enolase rabbit polyclonal antibodies to the system. The addition of increasing concentrations of anti-enolase antibody led to a dose-dependent decrease in Plg binding to *V. parahaemolyticus* (Fig. 6c).

Inhibition of bacterial adherence

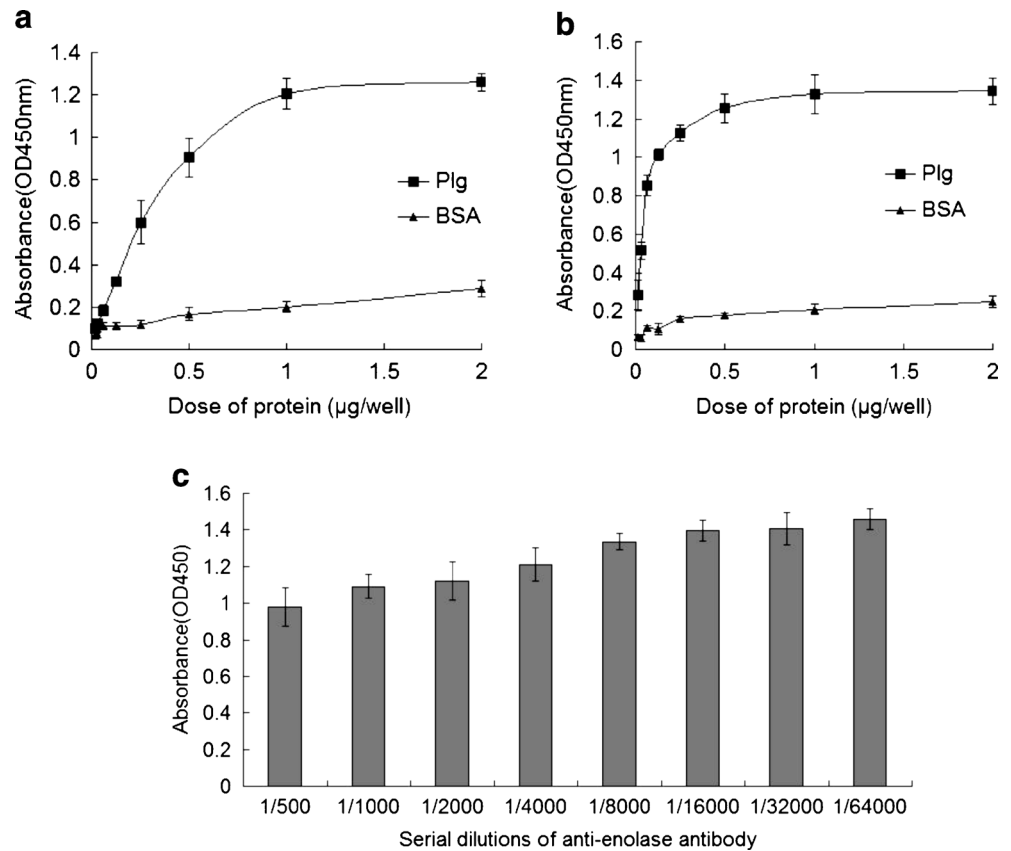
Immunofluorescence analyses were conducted to determine whether *V. parahaemolyticus* enolase contributes to bacterial adherence. Significant green fluorescence was detected from the surface of Hep-2 cells incubated with *V. parahaemolyticus* enolase (Fig. 7a), while no specific green fluorescence was observed from negative controls (Fig. 7b, c). To exclude the possible contribution of His₆ tag in the adherence, we removed the tag from the recombinant enolase using the Thrombin Cleavage Capture Kit. SDS-PAGE and Western blot analyses showed that the cleaved protein was successfully obtained, and its purity was $>95\%$ (Fig. S1). The immunofluorescence assay indicated that the resulting cleaved protein could still contribute to protein binding to Hep-2 cells (Fig. S2).

Further, Hep-2 cells were treated with increasing amounts of purified *V. parahaemolyticus* enolase before adherence of *V. parahaemolyticus* to cells. *V. parahaemolyticus* adherence to Hep-2 cells was inhibited by enolase in a dose-dependent manner. At 15 $\mu\text{g}/\text{well}$ enolase, we observed $\sim 50\%$ inhibition of *V. parahaemolyticus* adherence (Fig. 8a). To exclude the possibility that *V. parahaemolyticus* enolase prevents a bacterial adhesin from interacting with its receptor on the epithelial cell surface, bacterial enolase was blocked with a specific anti-enolase antibody. As shown in Fig. 8b, anti-enolase antibody treatment decreased the adhesion of *V. parahaemolyticus* to Hep-2 cells to $\sim 63\%$ compared with the control. Our results collectively indicate that enolase is a *V. parahaemolyticus* adherence-related factor.

Vaccine protective efficacy

The virulence of *V. parahaemolyticus* ATCC33847 was tested with mouse models. Mortality of animals was observed for 7 days after the challenge. The LD₅₀ value was 4.0×10^8 CFU/mouse with the strain ATCC33847. Three groups of 15 mice were injected with *V. parahaemolyticus* enolase, inactivated *V. parahaemolyticus* strain ATCC33847, and PBS, respectively. At 10 days after booster immunization, each group was challenged i.p. with 5 LD₅₀ of *V. parahaemolyticus* ATCC33847. At 1 day after inoculation, all mice in the negative control group died, and most exhibited marked clinical signs, such as ruffled hair, difficulties in breathing, and poor appetite. At 2 days after challenge, two mice died in both the group immunized with the inactivated *V. parahaemolyticus* strain ATCC33847 and the enolase-vaccinated group. Some of the surviving mice showed clinical signs of *V. parahaemolyticus* infection, but these were gradually convalescing. In both groups, 86.7% of mice (13/15) successfully survived the *V. parahaemolyticus* infection. From day 3, no deaths were observed until the end of the study, which was indicative of high protection from cause-specific mortality.

Fig. 6 Plasminogen binding assays. ELISA results indicated that plasminogen (Plg) (0.0156 to 2 $\mu\text{g}/\text{well}$) binds to purified recombinant enolase (a) or OMPs from *V. parahaemolyticus* (b) in a concentration-dependent manner. Binding of plasminogen is inhibited by increasing concentrations of rabbit antienolase antibody (in serial dilutions from 1/500 to 1/64,000) in a competition assay (c). Three independent experiments were performed in triplicate



Discussion

Enolase is a glycolytic enzyme that participates in a variety of normal cellular activities (Miles et al. 1991). The role of enolase in the pathogenesis of Gram-negative bacteria is far from clear. Moreover, no reports to date have focused on *V. parahaemolyticus* enolase and its encoding gene, although those from several other pathogenic organisms, including bacterial enolases, have been investigated (Agarwal et al.

2008; Bergmann et al. 2003; Ehinger et al. 2004). In the present study, enolase from *V. parahaemolyticus* ATCC 33847 was cloned, expressed, and characterized. Our enzymatic test results revealed that in vitro overexpressed *V. parahaemolyticus* enolase, a functional protein, successfully catalyzes the conversion of 2-PGE to PEP, indicating that recombinant enolase retains enzymatic activity. The kinetic coefficients K^m and V_{max} values for *V. parahaemolyticus* enolase were comparable with those published for the enolases of

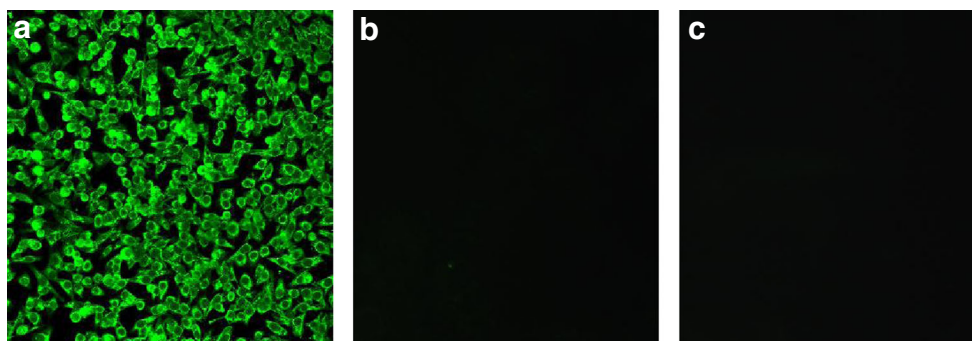


Fig. 7 Immunofluorescence assay (IFA). Adherence of enolase to Hep-2 cells was confirmed by an indirect immunofluorescence assay, which indicates enolase bound to the surface of the Hep-2 cell. Hep-2 cells were incubated with recombinant *V. parahaemolyticus* enolase and then with rabbit anti-enolase antibody (a) or with preimmunization rabbit serum (b),

or Hep-2 cells were incubated with BSA and then with rabbit anti-enolase antibody (c), stained with goat anti rabbit IgG-FITC before being examined with a confocal laser scanning microscope (original magnification $\times 400$)

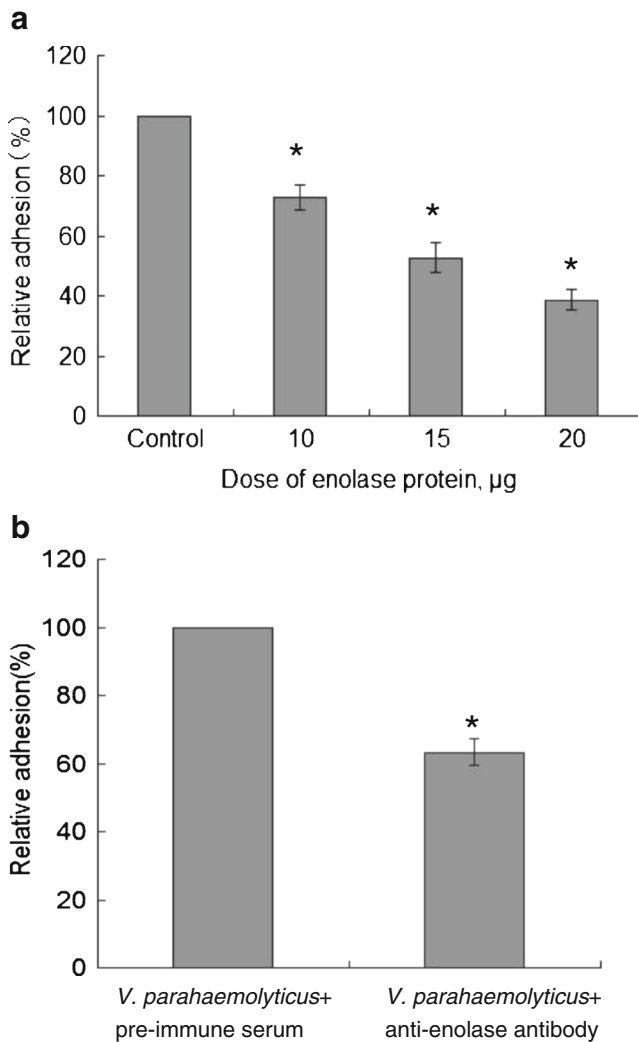


Fig. 8 Inhibition assays. **a** The recombinant enolase inhibits binding capacity of *V. parahaemolyticus* to Hep-2 cells. Dose-dependent inhibition of *V. parahaemolyticus* adherence to Hep-2 cells was determined in the presence of increasing amounts of purified enolase. **b** The anti-enolase antibody inhibits binding capacity of *V. parahaemolyticus* to Hep-2 cells. The *V. parahaemolyticus* strain ATCC33847 was incubated with preimmune serum (control) and with anti-enolase antibody, respectively. * $P < 0.05$ in comparison with the level of adhesion with control (considered to be 100 %)

several other pathogens such as *Brucella abortus* (Han et al. 2012) and *Streptococcus suis* (Feng et al. 2009).

In addition to its property as a glycolytic enzyme, the *V. parahaemolyticus* enolase sequence contains typical Plg-binding site motifs characteristic of other enolase proteins. The ability of *V. parahaemolyticus* enolase to bind Plg was examined using ELISA, and our results clearly suggest that *V. parahaemolyticus* enolase is a Plg-binding protein enolase and binds plasminogen in a dose-dependent manner. Several recent studies have demonstrated that enolase plays a major role in the microbial recruitment of Plg, and the Plg-binding property of bacteria, including pathogenic spirochetes, is suggested to be a contributory factor in tissue invasion and

survival in hosts (Bergmann et al. 2003; Crowe et al. 2003; Ehinger et al. 2004; Nogueira et al. 2010).

To function as a Plg-binding protein, enolase must localize to the microbial surface. Enolase had been shown to be present in both the soluble cytoplasmic and membrane fractions in several other bacterial species, such as *A. hydrophila* (Sha et al. 2009), *Bacillus anthracis* (Agarwal et al. 2008), *B. burgdorferi* (Toledo et al. 2012; Floden et al. 2011), *S. pneumoniae* (Kolberg et al. 2006), and *S. suis* (Esgleas et al. 2008). In the present study, we found that enolase was detectable in the extracellular, outer membrane, and soluble cytosolic fractions of *V. parahaemolyticus* by Western blotting. Further, we demonstrated its presence in the cytoplasm as well as localization on bacterial surface by LC-MS/MS analysis of OMP profiles and immunogold staining of *V. parahaemolyticus*. Diverse localization of enolase suggests its pivotal role as a glycolytic enzyme.

The exact mechanism by which enolase is secreted and translocated to the bacterial surface is still unknown. It is known that the amino acid sequence of enolase is absent for an N-terminal signal sequence. The findings from Yang et al. (2011) showed that protein structure, such as a hydrophobic α -helical domain of enolase, might be a contributing factor in enolase secretion of *B. subtilis*. A recent study from *Leptospira interrogans* (Nogueira et al. 2013) speculated that a secretion system might involve in enolase secretion, and once secreted by a yet-unknown mechanism, enolase probably localizes on the bacterial surface by reassociation. Nine protein secretion systems have been unravelled so far in Gram-negative bacteria (Chagnot et al. 2013). A secreted protein can be translocated across outer membrane through secretion systems. Besides, the contribution of the so-called nonclassical (NC) secretion and other protein trafficking mechanisms in bacterial colonization (e.g., allolysis, phage-mediated lysis, membrane budding) remains to be determined. Nevertheless, the molecular mechanism of how enolase is exported, secreted, or become bacterial cell-surface localization needs further investigation.

Although the mechanism underlying the translocation of protoplasmic enolase across cellular membranes remains unknown at present, in view of the collective data showing that *V. parahaemolyticus* enolase binds to human Plg, we propose that enolase on the cell surface of *V. parahaemolyticus* functions in the colonization and/or invasion of the pathogen. Accumulating data support the hypothesis that surface-related proteins are involved in bacterial pathogenesis via adhesion, invasion, and bacterial defense mechanisms (Boleij et al. 2011; Patti et al. 1994). The localization of enolase is consistent with the etiology of adherence to the host cells. In general, proteins localized on the cell surfaces of pathogenic bacteria may act as potential adhesins. Indeed, for many infectious agents, surface-displayed enolase is proposed to function as an adhesion (Marcos et al. 2012; Nogueira et al.

2012; Song et al. 2012). Undoubtedly, adherence to epithelial cells is important for *V. parahaemolyticus* to break through the first host barrier. To elucidate its possible role in the early events of *V. parahaemolyticus* infection, indirect immunofluorescence and inhibition assays were conducted in this study and *V. parahaemolyticus* enolase-mediated adherence to the Hep-2 cell surface was confirmed.

The virulence roles of microbial antigens can be effectively analyzed using molecular genetic approaches. However, our attempts to develop an enolase-deficient mutant of *V. parahaemolyticus* were unsuccessful (data not shown), possibly due to its indispensable involvement in glycolysis. It suggests that enolase is essential for bacterial life of *V. parahaemolyticus* and may thus be successfully employed as a diagnostic or protective antigen.

To investigate the immunological characteristics of *V. parahaemolyticus* enolase, an immunization assay and animal challenge experiment were performed. Our immunological data revealed strong antigenicity and immunogenicity of *V. parahaemolyticus* enolase. The recombinant protein enolase reacted strongly with convalescent phase serum from mouse clinically infected with *V. parahaemolyticus*, showing that enolase triggered an antibody response during the course of infection. In addition, immunoassay results showed that *V. parahaemolyticus* enolase is immunogenic in rabbits, and an ELISA titer of 1:32,000 could be achieved. Western blot analysis further revealed that the acquired anti-enolase antibody not only responds effectively to purified recombinant enolase but also specifically recognizes native enolase from *V. parahaemolyticus* cytosolic proteins or outer membrane proteins. Our data showing the generation of enolase-specific antibody responses in infected hosts as well as extracellular or microbial surface-associated localization of enolase suggest that the protein may facilitate the pathogen's infection in the host.

The issue of whether *V. parahaemolyticus* enolase exhibits strong immunogenicity and confers protective efficacy is of significant interest. Enolases from several pathogens have been identified as an important immunogenic protein and a protective antigen (Feng et al. 2009; Pal-Bhowmick et al. 2007; Sha et al. 2009; Zhang et al. 2009). For instance, mice immunized with purified recombinant enolase subjected to a lethal challenge dose of wild-type (WT) *A. hydrophila* were protected against mortality. López-Villar et al. (2006) reported that enolase of the pathogenic yeast *Candida albicans* constitutes an immunodominant antigen during invasive candidiasis. Moreover, Zhang et al. (2009) demonstrated that enolase of *S. suis* can be used as an immunogen to protect mice from infection. *V. parahaemolyticus* enolase could effectively serve as a vaccine candidate, as confirmed with the challenge study. Among mice immunized with purified recombinant enolase in our study, 86.7 % were protected against mortality from a lethal challenge dose of the TDH-positive *V. parahaemolyticus* strain ATCC33847. This relative protective efficacy was similar to

that with inactivated ATCC33847, confirming the suitability of *V. parahaemolyticus* enolase as a candidate subunit vaccine against bacterial infection. In addition, the enolase gene was detected in all 150 strains of *V. parahaemolyticus* isolated from seafood or diarrhea patients using PCR (data not shown), indicating that enolase is reasonably conserved among *V. parahaemolyticus*. BLAST analysis showed high similarity of *V. parahaemolyticus* enolase gene and amino acid sequences to homologs from other *Vibrio* species. The findings suggest that *V. parahaemolyticus* enolase may be a suitable candidate for developing vaccine against different *Vibrio* species.

In conclusion, our studies have clearly shown that *V. parahaemolyticus* enolase is a Plg-binding, surface-exposed protein facilitating bacterial adherence to Hep-2 cells, which possibly functions in colonization and/or invasion of bacteria into host epithelial cells and promotes pathogen–host interactions. Additionally, *V. parahaemolyticus* enolase plays an important role in pathogenicity and confers protective efficacy against *V. parahaemolyticus* infection, supporting its utility as an effective subunit vaccine for development.

Acknowledgments This work was funded by the National Key Technology Support Program (2012BAK08B07), Shanghai Municipal Science and Technology Commission technical standards (13DZ0502702), and Aquatic Three New Projects in Jiangsu Province (D2013-5-4).

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