Cloning, expression and immunogenicity of ferric enterobactin binding protein Fep B from *Escherichia coli* O157:H7

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Abstract The gene coding for ferric enterobactin binding protein from *E. coli* O157:H7 was amplified. This gene was cloned and expressed as C-terminal His (6)-tagged protein. The SDS-PAGE analysis of the total protein revealed only two distinct bands, with molecular masses of 31kDa and 34kDa. The Ni-NTA chromatography purified FepB and the osmotically shocked periplasmic fraction of IPTG induced cells showed only a single band of 31 kDa. Polyclonal mouse antibody was raised against the recombinant protein during 4 weeks after immunization. Western blot analysis of the recombinant FepB with mouse antiserum revealed a single band of 31 kDa. Identification and purification of FepB helped reveal its appropriate molecular mass. Polyclonal antibody raised against the recombinant protein reacted with bacterial FepB. The recombinant protein FepB could have a protective effect against *E. coli* O157:H7 and might be useful as an effective vaccine.

Keywords FepB · *E. coli* · Periplasmic binding protein · Ferric enterobactin

Introduction

Iron is required for the growth of nearly all prokaryotic and eukaryotic cells, with notable exception of the bacteria *Borrelia burgdorferi* and *Lactobacillus plantarum* [1]. Vertebrates use iron deprivation as an important natural defense against microbial pathogens [2]. Iron, an essential nutrient for the growth of bacteria, serves as a cofactor for enzymes, as a redox center in electron carriers such as cytochromes and as a global biosynthetic and the metabolic system [3]. The mechanisms by which microbes acquire iron during infection are of considerable interest and are best characterized in bacterial pathogens [4]. For example, many pathogenic bacteria produce siderophores that bind to ferric iron with high affinity, and many are able to utilize ferritin, transferrin, lactoferrin, heme and heme-containing proteins. In many species, the mechanisms of iron acquisition have been elucidated in detail and preferences for specific iron sources during infection are being identified [5]. *Escherichia coli* excrete the catecholate siderophore enterobactin in response to iron deprivation, to solubilize iron prior to transport. In *E. coli*, the utilization of ferric enterobactin requires the esterase protein which is encoded by the *fes* gene. Enterobactin is exported through the inner membrane by EntS, a major facilitator-subfamily exporter, and through the outer membrane by a TolC-dependent mechanism [6]. FepA from *E. coli* is a 724-residue integral outer membrane protein that transports ferric enterobactin into the periplasm [7]. Native FepB functions in the periplasm to facilitate the transfer of ferric enterobactin through the two-protein inner membrane channel FepDG into cytoplasm by FepC catalyzed ATP hydrolysis [8]. The 318 amino acid pro FepB contain a cleavable leader sequence,

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has a calculated molecular mass of 34.3 kDa, and is associated with the cytoplasmic membrane. These data support the idea that production of antibody against FepB may contribute to protection against gram-negative bacteria [9]. On the basis of the above criteria the present study was designed focusing on the cloning and expression of the FepB gene and its immunogenicity.

Materials and methods

Chemicals and enzymes

T_4 DNA ligase was purchased from Fermentase. Restriction endonucleases were obtained from Cinagene (Iran). All other chemical reagents were obtained from Sigma (USA) or Merck. Gel purification kit and plasmid extraction kit were obtained from Bioneer (Korea). Ni-NTA agarose was from Qiagen. Primers were synthesized by Bioneer.

Bacterial strains, plasmids and culture media

E. coli (ATCC 43889) O157:H7 and *E. coli* O157:H7 were procured from Shahed University and Bu-Ali hospital and reference centre (Tehran) respectively. The plasmid pET23a (+) was the Novagen product (USA). *E. coli* BL21 (DE3) was purchased from Cinagen (Iran). *E. coli* strains were cultivated in Luria-Bertani (LB) broth or LB agar at 37°C. *E. coli* transformants were grown on LB medium containing 50 μgml^{-1} ampicillin.

Cloning of fep B gene

The gene coding for ferric enterobactin binding protein (FepB) from *E. coli* (ATCC 43889) O157:H7 was amplified from its genomic DNA via polymerase chain reaction. The forward and reverse primers 5'-ATTGAATTCGTGAG-CTCGCCCC-3' and 5'-TACCAAGCTTAAACAGC-GCCTTAAG-3' respectively were used to amplify the fepB gene with the stop codon truncated in order to be fused with a downstream His-tag sequence on the vector (EcoRI and Hind III restriction enzyme sites underlined). PCR conditions consisted of 5 μl (50 ng/ μl) DNA, 1 μl of each primer (20 picomole), 250 μM of each dNTP, 40 mM KCL, 40mM MgCl_2 and 1 μl of 2.5u *Taq* DNA polymerase in a final volume of 50 μl in a thermal cycler (Techne gradient). The PCR procedure was as follows: initial denaturation at 94°C for 5 min and 30 cycles of 1 min at 94°C, 1 min at 57°C, 1 min at 72°C, followed by additional 5 min at 72°C. The amplified DNA products were electrophoresed on a 1.0% (w/v) agarose gel. The PCR products (957 bp for fepB) were purified using a PCR purification kit according to the

manufacturer's instruction. PCR product was digested with EcoRI and Hind III and cloned into pET23a (+) vector digested with the same endonucleases. The new construct was named pET23a – fepB. The recombinant DNA was transformed into *E. coli* strain BL21 (DE3), an expression host. The transformants were selected on LB agar containing 50 $\mu\text{g ml}^{-1}$ ampicillin. After mini-scale isolation of the plasmid DNA using plasmid extraction kit, the presence of open reading frame (ORF) was confirmed by restriction analysis and by sequencing (Gene Service).

Expression and purification of FepB protein

A single colony from cells was used to inoculate 5 ml of LB broth containing 50 $\mu\text{g ml}^{-1}$ ampicillin. The culture was shaken at 220 rpm 37°C overnight and used to inoculate 100 ml of LB broth with 50 $\mu\text{g ml}^{-1}$ ampicillin in a 500 ml flask. At an optical density of 600 nm approximately 0.6, 1mM isopropyl -D-thiogalactoside (IPTG) was used for induction. The culture was further shaken at 37°C for 4 h and harvested by centrifugation at 5000 rpm at 4°C for 10 min. The pellets were suspended in phosphate-buffered saline (PBS, pH 7.4) and boiled at 100°C for 5 min with the sample buffer solution. Total proteins were analyzed by SDS-PAGE on 13 % (w/v) gel and stained with coomassie brilliant blue. Recombinant FepB was purified by nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography. The cell pellet was thawed at 4°C and resuspended in lysis buffer, followed by addition lysozyme to 1 mg/ml and incubated on ice for 30 min. The lysate was then sonicated using a sonicator equipped with a microtip. After the sonication step (six 10 s at 200 W with a 10 s cooling period between each burst), the lysate was centrifuged at 10000 $\times$ g for 25 min at 4°C to pellet the cellular debris. The supernatant was loaded onto a Ni-NTA agarose column, pre-equilibrated by 5 ml lysis buffer containing 10mM imidazole. The FepB protein was eluted by a stepwise procedure, using buffers containing 20, 40, 90 and 150 mM imidazole. The fractions were collected and analyzed by SDS-PAGE gel. Pure protein fractions were pooled, and then concentrated by freeze-drying. Protein concentration was initially determined by Bradford method.

Production of periplasmic protein using osmotic shock method

A 100 ml volume bacterial culture was centrifuged at 5000 $\times$ g for 15 min, and the supernatant was decanted. The pellet was resuspended with 40 ml hypertonic media (20% sucrose, 30 mM Tris-HCL, pH 8, autoclaved) and was incubated at room temperature for 20 min. The cells were pelleted by centrifugation and resuspended in 2 ml of

hypotonic solution (5 mM MgSO_4 , autoclaved). After 20 min in an ice bath, the cells were pelleted by centrifugation at $5000 \times g$ for 20 min, and the supernatant containing periplasmic fluid, was collected into new tube and then concentrated by freeze-drying.

Production of polyclonal mouse antibody against FepB

The recombinant protein was emulsified with complete Freund's adjuvant and 15 μg protein was injected intraperitoneally to five 8-week-old BALB/c mice. The animals were boosted with the same amount, emulsified in incomplete Freund's adjuvant after two weeks. The mice were then bled seven days after the last injection and serum was collected. Antibody response was determined by enzyme-linked immunosorbent assay (ELISA).

Analysis of antibody response

Sera were assayed for antibody response against the recombinant protein by ELISA. In short, 96-well microtiter plates were coated with 4 μg purified recombinant FepB protein. Serial dilutions of the sera with the first dilution 1:50 were added to wells of the plates. Antibodies binding to the antigen were detected using anti-mouse IgG conjugated with horseradish peroxidase (HRP) at 1:2000 dilution. The color was developed with ortho-phenylenediamine (OPD) for 30 min and the reaction stopped with 2.0 M H_2SO_4 . The plates were read with a microplate reader at 492 nm. Results were considered positive if the absorbance was at least double of the control sera and antibody titer were scored as the highest positive dilution.

Western blot analysis

E. coli O157:H7 was inoculated in two 100 ml of M9 media. One of these media contained 20 μM iron, as iron rich medium. The supernatant from the pelleted cultures (5000 rpm/10 min) was analyzed for the presence of siderophore with chrome azurole S (CAS) assay [10]. Periplasmic fraction was prepared from pellet by osmotic shock procedure and resolved on a 13% SDS-PAGE gel. Protein samples were electrophoresed on 13% SDS-PAGE gel, and then electroblotted onto nitrocellulose membrane at a constant current of 300 mA at 4°C for 1.5 h. The membrane was incubated in the blocking buffer of 3% bovine serum albumin, with gentle shaking for one hour at room temperature. The membrane was then washed three times with PBS-T (PBS+ 0.05% Tween-20, pH7.4) before incubation with diluted mouse anti-FepB (1:100) serum for 1h. After three times washing with PBS-T, the membrane was incubated with mouse IgG conjugated with

horseradish peroxidase for 1h at room temperature. The membrane was then washed three times in PBS-T. The membrane was visualized with diaminobenzidine (DAB) substrate until brownish bands were observed. Color development was then terminated by washing in PBS. As a control, the negative control sera were treated with the same protocol.

Results

PCR amplification of fepB gene

The fepB gene of *E. coli* O157:H7 was successfully amplified by PCR and cloned into pET23a (+) vector (Fig. 1a). This construct was confirmed by restriction enzyme analysis and by DNA sequencing. The pET23a (+) - fepB was transformed into BL21 (DE3) *E. coli* cells for the overexpression of the recombinant FepB with a C-terminal His-tag. Stop codon of fepB gene was truncated in order to be fused with a downstream His-tag sequence on the vector.

Expression and purification

A brownish pellet was obtained after IPTG induction. The recombinant proteins FepB were over produced. After IPTG induction, the molecular masses of the total proteins of BL21 (DE3) transformed with pET23a (+)-FepB were about 31 kDa and 34 kDa (Fig. 1b). After His-tag affinity chromatography, the recombinant protein purified with SDS-PAGE analysis showed a single band at approximately 34 kDa. After IPTG induction, the SDS-PAGE analysis of periplasmic extract revealed a single band at approximately 31 kDa (Fig. 1c).

Analysis of antibody response

Compared with the negative control group injected with PBS, significantly high antibody was detected in the positive control group by ELISA. Highest OD of test serum and the control were 0.787 and 0.1 respectively.

Western blot analysis of the recombinant protein with mouse polyclonal antibody

The immunogenicity of the recombinant protein was further analyzed by western blot. Mouse polyclonal antibody was specifically reacted with the recombinant protein, whereas, no reactivity was observed with control (Fig. 1d). Polyclonal antibodies raised against the recombinant FepB was reacted with bacterial FepB of periplasmic extract on western blot assay (Fig. 1e).

Fig. 1a PCR amplification of FepB gene from *E. coli* O157: H7 showing FepB gene on lanes 1 and 4. Lane 2, negative control and Lane 3 is the molecular marker.

Fig. 1b SDS-PAGE profiles of the total proteins of BL21 (DE3) transformed with pET23a (+)-FepB. Lanes 1 and 3, positive control; Lane 2, negative control; Lane 4, protein marker.

Fig. 1c SDS-PAGE analysis of periplasmic extract showing a single FepB band. Protein expression was induced with 1 mM IPTG for 6 h. Lanes 1, 3, positive control; Lane 2, negative control and Lane 4, molecular marker.

Fig. 1d Western blot analysis of the recombinant protein with mouse antiserum. Lane 1, cleared lysate; Lane 2, negative control; Lane 3, purified FepB; Lane 4, protein marker.

Fig. 1e Western blot analysis of bacterial FepB protein with antiserum against the recombinant FepB. Lane 1, periplasmic extraction of iron rich medium; Lane 2, periplasmic extraction of iron restricted medium; Lane 3, protein marker.

Discussion

The emerging resistance of microorganisms to currently available antibiotics has raised great concerns regarding the battle against infections in man. The FepB gene encodes a periplasmic binding protein that is essential for the uptake of ferric enterobactin by *E. coli* [11]. DNA sequence analysis confirmed the presence of a 26-amino acid hydrophobic signal peptide at the amino terminus of FepB. The proFepB containing 318-amino acids which possesses a cleavable leader sequence, has a calculated molecular mass of 34.3 kDa, and is associated with the cytoplasmic membrane. The mature FepB protein having 292-amino acids has a calculated molecular mass of 31.6 kDa and exists in the periplasm. Previous studies reported that SDS-PAGE analysis of the periplasmic fraction revealed three distinct FepB bands, with molecular masses of 36.5, 33.5, and 31.5 kDa. Although the origin of these isoforms is unknown,

previous investigations eliminated two potential causes: FepB contains no cysteine, ruling out the presence of alternative, disulfide-stabilized forms, and double labeled experiments with ^{32}P and ^{35}S -labeled methionine did not demonstrate post-translational phosphorylation of fepB [12]. In the present study, FepB of *E. coli* O157:H7 was expressed and purified. SDS-PAGE analysis of purified FepB through a Ni-NTA column and periplasmic fraction of IPTG induced cells showed only a single band 31 kDa. Western blot analysis of purified recombinant FepB using Ni-NTA and bacterial FepB from periplasmic extract of iron limited environment revealed a single band of 31 kDa. The SDS-PAGE analysis of total proteins of IPTG induced cells showed two bands at approximately 31 kDa and 34 kDa. The findings suggest that two forms of FepB of 31 kDa and 34 kDa exist, ruling out diverse forms of FepB. The ferric binding protein (FepA) of *Neisseria meningitidis* and its homologous proteins in other bacteria act by transporting iron across periplasm [13]. In an experiment the large amounts of peptide injected into mice were well tolerated and that recently, one of the peptides has been successfully tested in patients for the detection of infections [14]. Although, the strongest antibacterial activity was observed for natural peptide, the high costs to synthesize this peptide under GMP conditions and, alternatively, the elaborate purification procedures to obtain natural antibacterial agent, limits its use for treatment and detection. In this respect, a selected periplasmic protein like FepB, offers a promising alternative. Its availability, ease of synthesis and production, as well as its strong protective activity towards microorganisms make this compound a promising candidate for antimicrobial therapy of infections. It can be concluded that the recombinant protein FepB could have a protective effect against *E. coli* O157:H7 and might be useful as an effective vaccine against *E. coli* O157:H7 or probably other gram-negative bacteria harboring FepB gene. These findings suggest production of extracellular outer membrane vesicles (MVs) by *E. coli*.

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