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Cloning, Expression and Immunoactivity of Periplasmic Binding Protein, FepB

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Abstract: The gene coding for ferric enterobactin biding 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 2 distinct bands, with molecular masses of 31 and 34 kDa. 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. Polyclonal antibody raised against the recombinant protein reacted with bacterial FepB. The successful production of antibody by periplasmic product of FepB gene can find a room for further research aimed at broad spectrum vaccines production. Identification and purification of FepB helped reveal its appropriate molecular mass. Reaction of the recombinant FepB antiserum with bacterial FepB finds its immunoactive contribution to protection against Gram negative bacteria harbouring the FepB protein. The serum of immunized mice was capable of protecting mice from live bacterial challenge. The recombinant protein FepB has a protective effect against *E. coli* O157:H7 on mice and might be useful as an effective vaccine.

Key words: *E. coli*, FepB, ferric enterobactin, periplasmic binding protein

INTRODUCTION

Iron is required for the growth of nearly all prokaryotic and eukaryotic cells, with notable exception including the bacteria *Borrelia burgdorferi* and *Lactobacillus plantarum* (Fischbach *et al*., 2006; Posey and Gherardini, 2000). Although, iron is the fourth most abundant element in the earth's crust, the availability of iron is greatly reduced by its insolubility at physiological pH. In the presence of oxygen, Fe^{2+} is rapidly oxidized to Fe^{3+}, which precipitates as a polymeric oxyhdroxide (Andrews *et al*., 2003; Faraldo-Gomez and Sansom, 2003; Schalk *et al*., 2004). Under reducing or acidic condition, the iron equilibrium shifts from the ferric Iron to the ferrous Iron that is more easily available for microorganisms (Cartron *et al*., 2006; Grass, 2006). 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 (Cartron *et al*., 2006; Sprencel *et al*., 2000). *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. The esterase hydrolyses the ester bonds of internalized ferric enterobactin producing dihydroxybenzoylserin which, functions as a weak siderophore. Importantly, the estrase also seems to function as a ferric-enterobactin-specific reductase reducing the ferric iron carried by enterobactin leading to release of iron from the siderophore (Andrews *et al*., 2003). Enterobactin is exported through the inner membrane by EntS, a major facilitator-subfamily exporter and through the outer membrane by a TolC-dependent mechanism (Bleuel *et al*., 2005; Furrer *et al*., 2002). FepA from *E. coli* is a 724-residue integral outer membrane protein that transports ferric enterobactin into the periplasm (Buchanan *et al*., 1999; Postle, 1999). Transport of ferric enterobactin across periplasmic membrane is mediated by periplasmic binding protein and associated cell membrane transporter (Postle and Larsen, 2008). Transcription of the transport gene, FepB and possibly the transport operon, FepDGC is directly anticlockwise and the biosynthesis operon enterobactin

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is transcribed clockwise (Brickman *et al*., 1990). Almost all binding proteins possess bilobate structure resembling a venus flytrap in which the 2 domains are linked by 2 or 3 β-strand. Each lobe is independently folded and is composed of a mixed α/β structure. In most periplasmic binding proteins these connecting β-strand form a region that allows the 2 lobes to twist with respect to each other and entrap the substrate in the cleft between the 2 domains (Clarke *et al*., 2000; Koster, 2001; Krewulak *et al*., 2005). Native FepB functions in the periplasm to facilitate the transfer of ferric enterobactin through the 2-protein inner membrane channel FepDG into cytoplasm by FepC catalyzed ATP hydrolysis (Chenault and Earhart, 1991; Shea and McIntosh, 1991). The 318-amino-acid pro FepB contain a cleavable leader sequence, has a calculated molecular mass of 34.3 kDa and is associated with the cytoplasmic membrane. The 292-amino-acid mature FepB protein has a calculated molecular mass of 31.6 kDa and exists in the periplasm (Sprencel *et al*., 2000). An alignment of the FhuD, FecB, FepB and Btuf amino acid sequence demonstrated a striking similarity along the entire length. Especially noteworthy is the existence of several motifs, often including proline residues, which may be structurally important. Structural relatedness suggests a common origin for the periplasmic protein of Iron transport family. These data support the idea that production of antibody against FepB may contribute to protection against gram negative bacteria (Koster, 1991). On the basis of the above criteria the present study was designed focusing on the cloning and expression of the FepB gene and its immunoactivity.

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 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 μg mL^{-1} ampicillin.

Construction of plasmid: 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-′ATTGAATTCGTGAGACTCGCCCC-3′and5′-TACCAAGCTTAAACAGCGCCTTAAG-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^{-1}) DNA, 1 μL of each primer (20 PM), 250 μM of each dNTP, 40 mM KCL, 40 mM $MgCl_2$ and 1 μL of 2.5u Taq DNA polymerase in a final volume of 50 μL on 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) agrose 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 μg mL^{-1} ampicillin (Golub, 1988). 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 μg mL^{-1} ampicillin. The culture was shaken at 220 rpm 37°C overnight and used to inoculate 100 mL of LB broth with 50 μg mL^{-1} ampicillin in a 500 mL flask. At an optical density at 600 nm approximately 0.6, 1 mM isopropyl β-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^{-1} and incubated on ice for 30 min. The lysate was then sonicated using a sonicator equipped with a microtip. After the sonication step (6, 10 sec at

200 W with a 10 sec cooling period between each burst), the lysate was centrifuged at 10000×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 10 mM 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 using bovine serum albumin as a standard.

Production of Periplasmic protein using Osmotic shock method: A 100 mL volume bacterial culture was centrifuged at 5000×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 MgSO4, autoclaved). After 20 min in an ice bath, the cells were pelleted by centrifugation at 5000×g for 20 min and the supernatant containing periplasmic fluid, was collected into new tube and then concentrated by freeze-drying (Sprencel *et al*., 2000).

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 5, 8-week-old mice. The animals were boosted with the same amount, emulsified in incomplete Freund's adjuvant after 2 weeks. The mice were then bled 7 days after the last injection and serum was collected.

Analysis of antibody response: Sera were assayed for antibody response against the recombinant protein by Enzyme-Linked Immunosorbent Assay (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 (Crowther, 1995).

Western Blot Analysis: 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 1 h at room temperature. The membrane was then washed 3 times with PBS-T (PBS+ 0.05% Tween-20, pH7.4) before incubation with diluted mouse anti-FepB (1:100) serum for 1 h. After 3 times washing with PBS-T, the membrane was incubated with mouse IgG conjugated with horseradish peroxidase for 1 h at room temperature. The membrane was then washed 3 times in PBS-T. The membrane 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.

Cross-reactivity between FepB and the recombinant protein anti serum: *E. coli* O157:H7 was inoculated in 2 100 mL of M9 media. One of these media contained 20 μM Iron, as iron rich medium. The cultures were shaken at 280 rpm at 37°C for 36 h and were then pelleted (5000 rpm/10 min). The supernatant was analyzed for the presence of siderophore with chrome azurole S (CAS) assay (Alexander and Zuberer, 1991). Periplasmic fraction was prepared from pellet by osmotic shock procedure and resolved on a 13% SDS- PAGE gel. The Western blotting was performed in order to confirm the cross-reactivity.

Immunization of mice: BALB/c mice were imminused with 15 μg of recombinant protein per mouse on days 0, 15 and 30, intraperitoneally injecting 0.2 mL of antigen/adjuvant mixture. Antibody response was determined by ELISA.

Lethal dose of *E. coli* O157:H7 to mice: *E. coli* O157:H7 at doses ranging from 2×10^6-1.7×10^9 cfu mL^{-1} were administered interaperitoneally to 5 BALB/c mice. The number of cfu in each of the dilutions was determined by plating on tryptic soy agar. Mortality in mice was recorded up to 1-week post challenge.

Mice challenge experiment: Immunized and control group mice were challenged with 200 μL injection containing 3.4×10^8 cfu. Five mice were used during each challenge experiment.

RESULTS

PCR amplification of fepB gene: The fepB gene of *E. coli* O157:H7 was successfully amplified by PCR and

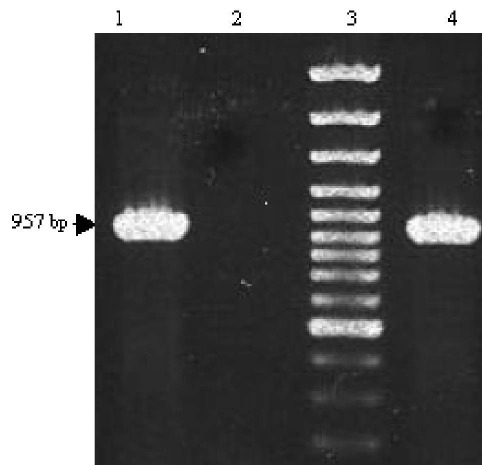

Fig. 1: PCR amplification of FepB gene of *E. coli* O157: H7. PCR product was visualized on 1% agarose. Lanes 1, 4, FepB gene; Lane 3, molecular marker; Lane 2, negative control

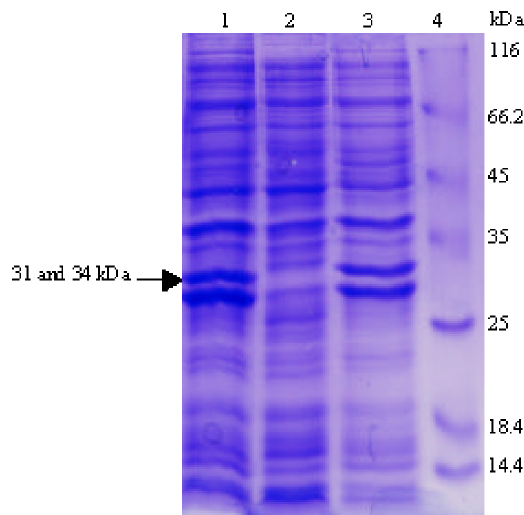

Fig. 2: SDS-PAGE profiles of the total proteins of BL21 (DE3) transformed with pET23a (+) -FepB. Lanes 1 and 3, positive control; Lane 2, negative control; Lane 4, protein marker

cloned into pET23a (+) vector (Fig. 1). 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

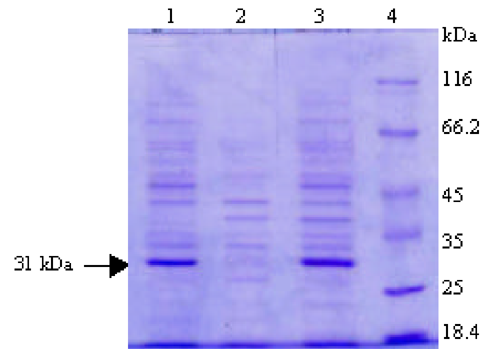

Fig. 3: SDS-PAGE analysis of periplasmic extract shows a single FepB band. Protein expression was induced with 1 mM IPTG for 6 h. Lanes 1, 3, positive control; Lane 2, negative control and Lane 4, molecular marker

FepB were over-produced. After IPTG induction, the molecular masses of the total proteins of BL21 (DE3) transformed with pET23a (+) -FepB revealed here were about 31 kDa and 34 kDa (Fig. 2). After His-tag affinity chromatography, the recombinant protein purified with SDS-PAGE analysis revealed 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. 3).

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 that was showed antiserum determined the recombinant protein.

Western blot analysis of the recombinant protein with mouse polyclonal antibody: The immunogenicity of the recombinant protein was further analyzed by western blot with antiserum of survival mouse after inoculation. Mouse polyclonal antibody was specifically reacted with the recombinant protein, whereas, no reactivity was observed with control (Fig. 4). Polyclonal antibodies raised against the recombinant FepB was reacted with bacterial FepB of periplasmic extract on Western Blot Assay (Fig. 5).

Mice immunization: A 18×10^{7} cfu mL^{-1} was determined as LD_{100} of *E. coli* O157:H7 for BALB/c mice. By a week, all control mice were dead but immunized ones stayed alive. Post mortem examinations were performed on the dead animals and *E. coli* were isolated from liver and spleen. The immunized mice remained alive even at doses higher than the lethal dose.

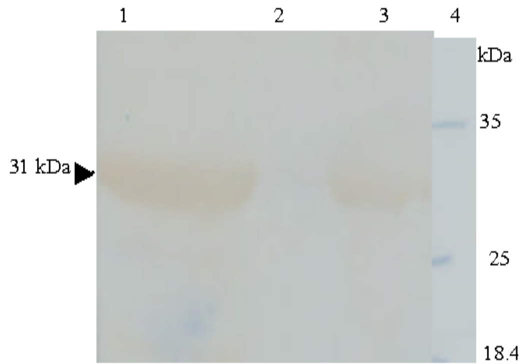

Fig. 4: Western blot analysis of the recombinant protein with mouse antiserum. Lane 1, cleared lysate; Lane 2, negative control; Lane 3, purified FepB; Lane 4, protein marker

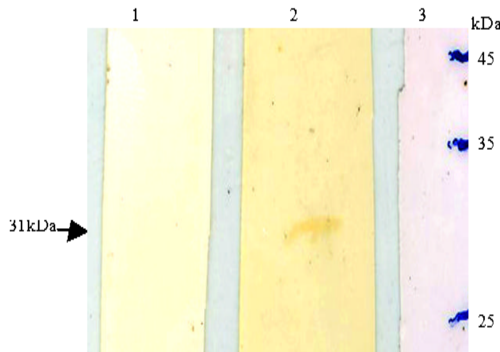

Fig. 5: Western blot analysis of bacterial FepB protein with antiserum against the recombinant FepB. Lane 1, periplasmic extraction of iron rich medium; Lane 2, periplasmic extraction of iron restricted medium; Lane 3, protein marker

DISCUSSION

The FepB gene, which is transcribed from right to left, encodes a periplasmic binding protein that is essential for the uptake of ferric enterobactin by *E. coli* (Hook-Barnard *et al*., 2007). DNA sequence analysis confirmed the presence of a 26-amino-acid hydrophobic signal peptide at the amino terminus of FepB (Ozenberger *et al*., 1987). 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 3 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 2 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 posttranslational phosphorylation of fepB (Elkins and Earhart, 1989; Pierce and Earhart, 1986; Sprencel *et al*., 2000). 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 2 bands at approximately 31 kDa and 34 kDa. The findings suggest that 2 forms of FepB of 31 kDa and 34 kDa exist, ruling out diverse forms of FepB. The ferric binding protein (FbpA) of *Neisseria meningitidis* and its homologous proteins in other bacteria act by transporting iron across periplasm (Ferreiros *et al*., 1999). In this study, the serum of immunized mice was capable of protecting mice from live bacterial challenge.

CONCLUSION

It can be concluded that the recombinant protein FepB has a protective effect against *E. coli* O157:H7 on mice and might be useful as an effective vaccine against *E coli* O157:H7 or probably other Gram negative bacteria harbouring FepB gene. These findings suggest production of extracellular outer membrane vesicles (MVs) by *E. coli*. These membrane vesicles contain periplasmic proteins (Mashburn-Warren and Whiteley, 2006).

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