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Expression and purification of the major outer membrane protein (OmpH) of *Pasteurella multocida* P52 from *Escherichia coli*

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ABSTRACT

Porin H (OmpH) is the major outer membrane protein in the envelope of *Pasteurella multocida*. The gene *ompH*, encoding major outer membrane protein was amplified by PCR excluding the region coding for signal peptide and cloned in the pQE32 prokaryotic expression vector. The recombinant OmpH was expressed as a fusion protein with 6-His tag at N-terminal in *E. coli* M15 cells transformed with recombinant plasmid pQE32-*ompH*. The expressed protein was purified from *E. coli* and characterized by SDS-PAGE and western blot analysis. The fusion recombinant protein eluted had a molecular mass of about 37 kDa. The expressed recombinant protein was confirmed with western blot analysis using RGS-His antibody and anti-*P. multocida* serum raised against whole cell lysate.

Key words: haemorrhagic septicaemia, outer membrane protein, *Pasteurella multocida*, recombinant protein

Introduction

Protein H, or porin H (OmpH), is the major outer membrane protein in the envelope of *Pasteurella multocida* (LUGTENBERG et al., 1986). This protein has been purified and characterized as a porin because it is structurally and functionally related to the superfamily of porins of Gram negative bacteria (CHEVALIER et al., 1993). OmpH is a homologue of the P2 porin of *Haemophilus influenzae*. The role of OmpH in protective immune response has been studied in recent past. A monoclonal antibody (mAb) against this protein was found to passively protect mice against *P. multocida* infection (VASFI

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MARANDI and MITTAL, 1997). The purified native OmpH was shown to induce protective immunity in chickens against homologous strain challenge (LUO et al., 1997). The gene (*ompH*) encoding this protein was cloned and sequenced and was found to be distributed among all the serotypes of *P. multocida* causing fowl cholera (LUO et al., 1997). Sequence alignment and secondary structure predictions helped in the development of synthetic peptide vaccine mimicking the conformational epitopes of native protein OmpH that provided protection in chickens against bacterial infection (LUO et al., 1999). LEE et al. (2007) cloned and expressed OmpH from a pathogenic strain of *P. multocida* causing atrophic rhinitis. Recombinant OmpH proteins were found to be antigenic and provided strong protection against *P. multocida* infection, indicating recombinant OmpH as a useful vaccine candidate antigen for *P. multocida*.

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease of cattle and buffaloes caused by the *Pasteurella multocida* serotype B:2 in Southern and Southeastern Asia and E:2 in the countries of tropical Africa (DE ALWIS, 1992). It is one of the economically important diseases of livestock in India. Although very little work has been done on the outer membrane proteins (OMPs) of *P. multocida* B:2 and their role in protective immune response, various workers have reported different predominant and specific polypeptides of *P. multocida* capsular type B (PATI et al., 1996; SRIVASTAVA, 1998; TOMER et al., 2002; BASAGOUDANAVAR et al., 2006; ARORA et al., 2007). No comparable report is, however, available regarding OmpH of the serotype B:2 of *P. multocida* causing HS in India.

In the present study, we expressed and purified the major outer membrane protein of *P. multocida* P52.

Materials and methods

Bacterial strain. Virulent *P. multocida* (serotype B:2) vaccine strain P52 was obtained from the Division of Standardization, Indian Veterinary Research Institute, Izatnagar, India. The strain was maintained on blood agar medium.

Genomic DNA extraction. P. multocida genomic DNA was isolated by chemical lysis method using lysozyme, ethylene diamine tetra acetic acid (EDTA) and sodium dodecyl sulphate (SDS) by the method of SAMBROOK and RUSSELL (2001). The DNA was purified using phenol, chloroform mixture. DNA in the aqueous phase was precipitated and spooled out with 3M sodium acetate (pH, 7.0) to a final concentration of 0.3 M and 2 volumes of chilled absolute ethanol at -20 °C for 1 h. The precipitated DNA was pelleted, washed, air dried and dissolved in Tris (10 mM) EDTA (1 mM) buffer, pH 8.0. Purity and concentration of DNA was determined by UV/VIS spectrophotometer. The optical density (OD) of diluted DNA sample was read at A_{260} and A_{280}. The resolution and purity

of genomic DNA was further analysed by agarose gel electrophoresis in a 0.8% agarose gel.

Amplification of ompH gene by polymerase chain reaction (PCR). The gene for OmpH was amplified in PCR using gene specific oligonucleotide primers (forward: 5'-TCAGGATCCCAGCAACAGTTTACAATCAAGA-3', reverse: 5'-CTACCCGGGTCCATTCCTTGCAACATATTGA-3') based on the sequence information reported earlier (LUO et al., 1997). Primers were designed to add restriction sites for *Bam*HI and *Sma*I at the 5'ends to facilitate cloning. PCR was performed using 20 ng of genomic DNA along with forward and reverse primers (60 pmol each), 0.1 mM of dNTPs, 1.5 mM $MgCl_2$ and 3 units of Pfu DNA polymerase in 1 x reaction buffer. The amplification cycle was 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 1 min and amplification at 72 °C for 1 min. The PCR amplified product was analysed on 1% agarose gel along with DNA molecular weight marker.

Cloning of ompH gene into prokaryotic expression vector pQE32. The amplified PCR product was digested with restriction endonucleases *Bam*HI and *Sma*I as per the manufacturer's instructions. 1 µL (i.e. 0.8 µg) pQE32 vector was also digested with *Bam*HI and *Sma*I for ligation. Both vector and insert DNA prepared were gel eluted using a minelute gel extraction kit following the manufacturer's instructions. The gel purified *ompH* gene fragment was ligated to pQE32 plasmid between *Bam*HI (cohesive) and *Sma*I (blunt) restriction sites. The ligated plasmid was transformed into *E. coli* DH5α competent cells. The recombinant pQE32 plasmids (pQE32-ompH) were isolated by miniprep method of plasmid isolation (Sambrook and Russell, 2001). The presence of *ompH* gene and its orientation in the pQE32-ompH plasmid was checked by restriction digestion with *Eco*RI, *Hind*III and *Pst*I restriction endonucleases followed by analysis of the digested products on 1% agarose gel. The positive clone was designated as pEOMPH.

Expression and purification of recombinant OmpH in E. coli M15 host. The pEOMPH plasmid with ompH gene was transformed into *E. coli* M15 expression host following instructions from the manufacturer. The pEOMPH plasmid carrying *E. coli* M15 colony was grown at 37 °C in Luria Bertani (LB) broth containing 100 µg/mL ampicillin and 25 µg/mL kanamycin. When OD_{600} of the broth reached 0.6, it was induced with 1mM of isopropyl-β-D-galactopyranoside (IPTG) and cells were allowed to grow further for 4-6 h. The induced *E. coli* cells were then harvested by centrifuging the broth at 1,100 x g for 10 min. The recombinant OmpH with 6 histidine residues at the N-terminal end of the protein was purified under denaturing conditions using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. For this, a Ni-NTA Fast Start Kit was used as per the manufacturer's instructions. Briefly, the bacterial pellet was resuspended in lysis buffer (8M urea, 0.1 M NaH_2PO_4, pH 8.0). Incubation was carried out at room temperature for 60 min by gently swirling the cell suspension. Lysis was complete when the suspension

was translucent. Lysate was centrifuged at 1,100×g for 10 min at room temperature to pellet the cellular debris. Cell lysate supernatant containing the recombinant protein was applied to the column. The recombinant protein bound to Ni-NTA agarose was washed twice with wash buffer (8M urea, 0.1 M NaH_2PO_4, pH 6.3) and eluted with elution buffer (8M urea, 0.1 M NaH_2PO_4, pH 4.5).

Characterization of recombinant OmpH. The recombinant protein was analysed in SDS-PAGE and western blot analysis. The eluted protein was separated on 12% SDS-PAGE along with induced and non induced bacterial cells and stained with Coomassie brilliant blue. For western blot analysis, the recombinant protein was separated in SDS-PAGE, electroblotted to nitrocellulose and probed with anti-Pasteurella serum raised against whole cell lysate of *P. multocida* and anti-His antibodies (Anti-His Antibody Selector Kit). There were three antibodies, viz, Penta-His, Tetra-His and RGS-His.

Results

Genomic DNA of P. multocida. The A_{260}:A_{280} ratio of the extracted DNA from *P. multocida* P52 was 1.811 indicating high purity of DNA without RNA and protein contamination. The concentration of DNA was 8. 827 μg/mL.

Amplification and cloning of the ompH gene. PCR amplification of the ompH gene of *P. multocida* P52 yielded the expected product of 1.2 kb (Fig. 1, Lane 1). For prokaryotic expression of the ompH gene, the amplified fragment was cloned into pQE32 expression vector between *Bam*HI and *Sma*I sites. The positive clone pEOMPH yielded a linear plasmid on digestion with *Bam*HI (Fig. 2, Lane 5). The orientation of the insert fragment was checked by digestion of pEOMPH plasmid with *Eco*RI, *Hind*III and *Pst*I as it generated fragments of approximately 483 bp, 285 bp and 689 bp, respectively by releasing the part of insert ompH gene fragment (Fig. 2, Lane 4, Lane 2 and Lane 3).

Expression and purification of recombinant OmpH protein. After characterization, the recombinant plasmid pEOMPH was transformed into the *E. coli* M15 expression host and induced with IPTG for recombinant protein expression. The expressed protein was purified under denaturing conditions using Ni-affinity chromatography and the eluted proteins were analysed on 12% SDS-PAGE (Fig. 3). Both induced and non induced *E. coli* harbouring pEOMPH did not show the presence of recombinant protein (Fig. 3, Lanes 1-5). The second and third aliquots of the eluted recombinant purified protein showed the presence of the target protein band (Fig. 3, Lanes 9 and 10). The poly histidine tag associated eluted recombinant protein had an approximate molecular weight of 37 kDa.

The immunoblots treated with anti-*P. multocida* serum, showed cross reactivity with *E. coli* lysate as the induced *E. coli* harbouring pEOMPH showed a number of coloured bands (Fig. 4, Lanes 3). The purified recombinant protein also reacted with the anti-*P. multocida* serum in the blot (Fig. 4, Lane 4). Blot results with anti-His antibodies showed

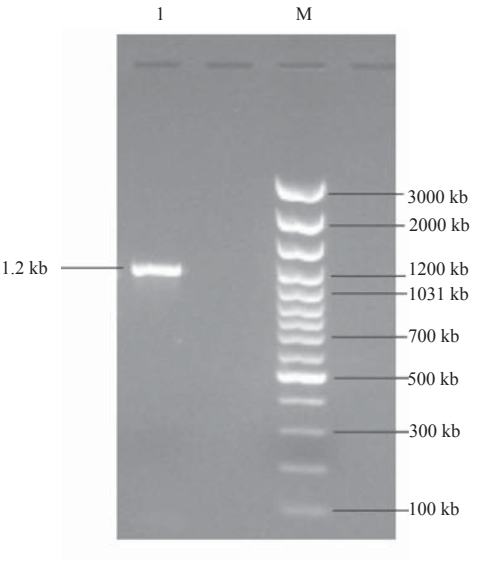

Fig. 1. Agarose gel electrophoresis of the *ompH* gene of *Pasteurella multocida* P52; Lane1: PCR amplified *ompH* gene. M:100 bp DNA ladder

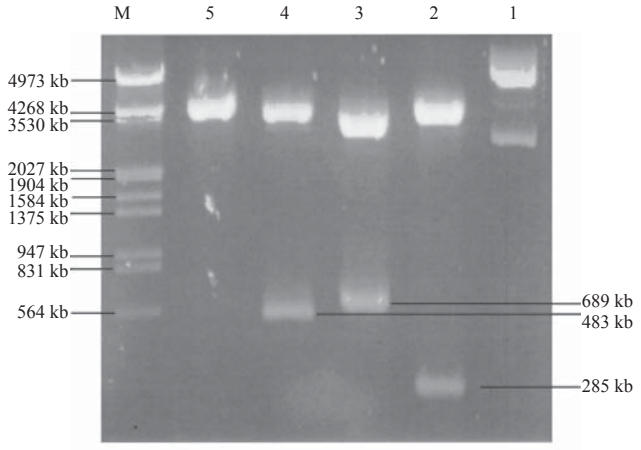

Fig. 2. Restriction enzyme digestion of the cloned *ompH* gene of *Pasteurella multocida* in pQE 32 Vector. Lane 1: Uncut plasmid DNA; Lane 2: *Hind*III digested plasmid. DNA; Lane 3: *Pst*I digested plasmid DNA; Lane 4: *Eco*RI . digested plasmid DNA; Lane 5: *Bam*HI digested plasmid DNA; M: Marker.

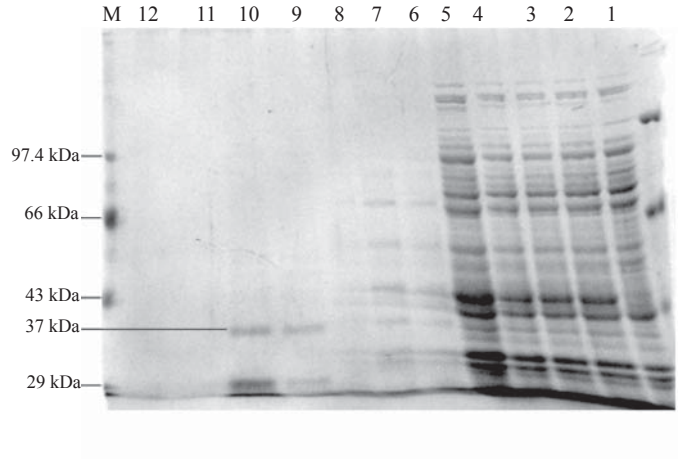

Fig. 3. Expression and purification of recombinant OmpH protein from pQE32-*ompH* plasmid transformed *E. coli* M15 cells. Lane1: non-induced cell lysate; Lane 2: induced (2 h) cell lysate; Lane 3: induced (3 h) cell lysate; Lane 4: induced (4 h) cell lysate; Lane 5: induced (over-night) cell lysate; Lane 6: cell lysate supernatant; Lane 7: flow through; Lane 8: wash fraction; Lane 9-12: elute fractions; M: protein molecular weight marker.

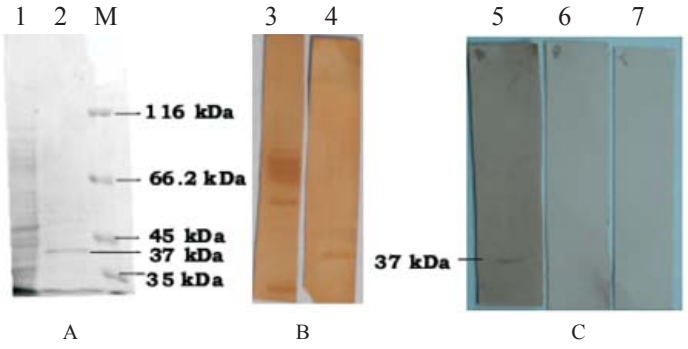

Fig. 4. SDS-Page (A) and Western blot analysis of purified recombinant OmpH protein with anti - *Pasteurella* serum (B) and anti-His antibodies (C). Lane 1: induced *E. coli* lysate; Lane 2: purified recombinant OmpH Protein; Lane 3: induced *E. coli* lysate probed with anti - *Pasteurella* serum; Lane 4: recombinant OmpH probed with anti - *Pasteurella* serum; Lane 5: recombinant OmpH probed with RGS-His antibodies; Lane 6: recombinant OmpH probed with Penta-His antibodies; Lane 7: recombinant OmpH probed with Tetra-His antibodies; M: protein marker.

the detection of 6xHis-tagged recombinant protein with only RGS-His antibodies (Fig. 4, Lane 5), whereas no band on the blot was observed with Penta-His and Tetra-His antibodies (Fig. 4, Lanes 6 and 7).

Discussion

HS is an important disease of cattle and buffaloes. For effective control of the disease, an efficacious and longer duration immunity vaccine is required. Identification and characterization of important immunogens of the bacteria would not only help in designing an improved vaccine but also would help in developing a test for protection status of vaccinated animals. OMPs have been identified as playing an important role in protection. This study aimed to perform expression and purification of the major outer membrane protein (OmpH) of *P. multocida* P52 (serotype B:2), the strain used in vaccines against haemorrhagic septicaemia in India to make available the protein in pure form and the desired amount.

The result of PCR amplifying a product of the expected size of 1.2 kb reflects the conserved nature of the *ompH* gene among the *P. multocida* serotypes. LUO et al. (1997) successfully amplified ompH genes from all the serotypes of *P. multocida* associated with fowl cholera. A single PCR product with a similar molecular size was obtained from each strain.

Cloning for expression was performed using pQE vector for N-terminal 6x His tag constructs. RE digestion of PCR product and pQE32 vector was done using *Bam*HI and *Sma*I, the restriction sites for which are present in the multiple cloning site of vector and at the terminal ends of the PCR product. The amplified fragment was cloned into pQE32 expression vectors between these *Bam*HI and *Sma*I restriction sites. The *E. coli* M15 host strain carrying the pREP4 repressor plasmid was used for the stable propagation of the expression construct as it has a higher repressor level.

Bacterial porin genes are sometimes difficult to clone in *E. coli* because foreign porins are usually lethal for *E. coli*. Initial attempts to clone the entire *ompH* gene into the expression vector were unsuccessful by a number of workers (LUO et al., 1997; LEE et al., 2007). This failure could be explained due to the leaking expression of the primary protein without IPTG induction, and lethality of recombinant porin protein in *E. coli*. The signal peptide in the primary protein helped to target OmpH to the outer membrane of *E. coli* that may cause osmotic destabilization of the cells or a change in the structural integrity of the outer membrane. In the present study, transformation of *E. coli* with recombinant pQE32 (pEOMPH), which contained the ompH gene for the mature protein without a signal peptide, was successful though the expression level of the recombinant gene was relatively low on induction of expression as it could not be detected in SDS-PAGE of induced cell lysate. Similar was the observation by GUPTA et al. (2006) in prokaryotic

expression and purification of recombinant bovine IFN-γ from *E. coli*. There was also no leaking expression of the primary protein without IPTG induction as non induced lysate also did not show the presence of protein in SDS-PAGE.

The recombinant OmpH with 6 histidine residues at the N-terminal end of the protein was purified under denaturing conditions using Ni-affinity chromatography. There were a large number of contaminating *E. coli* proteins in the pellet of induced cells. However, these proteins were not bound to Ni-NTA agarose and passed as unbounded protein in the flow through. The non specific proteins were removed in the wash buffer and the protein eluted in the elution buffer was purified as only specific protein was seen in SDS-PAGE. The second and third aliquots of the eluted recombinant purified protein showed the presence of a specific protein band. The fusion recombinant protein had a molecular mass of about 37 kDa produced by *E. coli*. LUO et al. (1997) found 40 kDa recombinant protein for the 37 kDa purified OmpH and it was the most abundant protein produced by *E. coli*. In native conformation, porin H is a homotrimer, stable in SDS at room temperature, and is dissociated into monomers upon boiling. The molecular mass of denatured monomers range between 34 and 42 kDa depending on the serotype and the electrophoretic system used for analysis (LUGTENBERG et al., 1986; CHEVALIER et al., 1993; LUBKE et al., 1994).

The immunoblots treated with anti-*P. multocida* serum, showed cross reactivity with *E. coli* lysate as both the induced and non induced *E. coli* harbouring pEOMPH showed coloured bands. This was because of the use of anti-*P. multocida* serum raised against whole cell lysate that cross reacted with the other Gram negative bacterial cell lysate. The purified recombinant protein also reacted with the anti-*P. multocida* serum in the blot. Blot results with anti-His antibodies showed the detection of 6xHis-tagged recombinant protein with only RGS-His antibodies, a monoclonal antibody specific to arginine-glycine-serine-4-his (His4), whereas no band on the blot was observed with Penta-His and Tetra-His antibodies. Individual 6xHis-tagged proteins are often recognized better by one anti-His antibody than by the others, possibly because of subtle differences in the exact conformation of the 6xHis tag and other parts of the protein in the vicinity of the tag. Non-reactivity of protein with other antibodies is possible as RGS is raised against 6xHis while others are against tetra or penta, so reactivity of recombinant protein may vary with stringency condition or dilution of conjugate antibody or tag is not exposed in expressed protein.

The isolation of pure OmpH from *Pasteurella multocida* represents a difficult challenge. Since the major outer membrane proteins and other bacterial porins are usually associated with LPS, which results in uncertainty in the immunological and functional characterization of them. Consequently, the production of recombinant OmpH in *E. coli*

and its subsequent purification would greatly facilitate the characterization of OmpH in the absence of interference from the contaminant molecules.

With the expression and purification of the ompH gene of *P. multocida* P52 in the present study, further work is needed to investigate the role of recombinant protein in protection studies and to study the antigenic properties of the recombinant OmpH as a candidate for vaccine.

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SAŽETAK

Porin H (OmpH) je glavni protein stanične stijenke bakterije *Pasteurella multocida*. Gen *ompH*, koji kodira njegovu tvorbu, isključujući područje za tvorbu signalnog peptida, bio je umnožen lančanom reakcijom polimerazom i kloniran u prokariotskom vektoru pQE32. Rekombinantni OmpH bio je izražen kao fuzijski protein sa 6-His tag na N-kraju u stanicama *E. coli* M15 transformiranima rekombinantnim plazmidom pQE32-*ompH*. Proizveden protein bio je pročišćen iz *E. coli* i identificiran SDS-PAGE-om i western blotom. Izdvojeni fuzijski rekombinantni protein imao je molekularnu masu oko 37 kDa. Identitet proizvedenog rekombinantnog proteina bio je povrđen western blot analizom uporabom protutijela za RGS-His i antiseruma za lizat cjelovite stanice *P. multocida*.

Ključne riječi: hemoragijska septikemija, *Pasteurella multocida*, protein stanične stijenke, rekombinantni protein