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Short Paper

Isolation, cloning and expression of the *Brucella melitensis* Omp31 gene

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Summary

Brucellosis is a zoonotic disease transmitted to humans either from animals or from their products. Although brucellosis can be found worldwide, the Mediterranean Basin, South and Central America, Eastern Europe, Asia, Africa, the Caribbean, and the Middle East have currently been listed as high-risk regions. The genus *Brucella* is classified in at least nine species. *Brucella melitensis* is the global pathogenic species of *Brucella*. The outer membrane protein 31, (Omp31) from *B. melitensis* is considered as a protective immunogen and an important candidate vaccine. Contamination of purified Omp31 protein by biochemical methods has made some restrictions in practical experiments. In this study, the Omp31coding gene of *B. melitensis* Rev 1 strain was inserted in pET32b(+) plasmid with extra His-tag sequence. The integrity of the constructed plasmid was confirmed using restriction enzyme mapping and sequencing. Omp31 was expressed after induction with IPTG in *Escherichia coli* BL21. Recombinant Omp31 (rOmp31) was purified by chromatography through Ni-agarose. The electrophoresis showed successful purification and immunoblotting confirmed immunereactivity of rOmp31. Obtained rOmp31 could be used as a research experimental tool in protection assays to find its potential as a vaccine candidate.

Key words: Omp31, Brucellosis, *Brucella melitensis*, Cloning

Introduction

Brucellosis, an infectious zoonotic disease affecting mammalians, is caused by different species of the genus *Brucella* (Delpino *et al*., 2007), and remains endemic in many developing countries, such as the Mediterranean, Western Asia, North Africa and Latin America (Cassataro *et al*., 2007a). Brucellosis causes important economic losses in livestock by abortions and is known as a potentially life-threatening multi-system disease in human (Sauret and Vilissova, 2002).

Brucella are gram-negative facultative intracellular bacteria that are classified into different species within the genus *Brucella* (Vizcaino *et al*., 1996; Mantur *et al*., 2007).

Brucella melitensis and *B. abortus*, which are mainly involved in ovine and bovine brucellosis, respectively, are the pathogenic species of *Brucella* (Cloeckaert *et al*., 1996).

Vaccination may be the most economical means of controlling the brucellosis (Cassataro *et al*., 2007b). The attenuated strains such as *B. melitensis* Rev 1, *B. abortus* S19 and RB51 are used to control brucellosis in domestic animals. These currently available live vaccines present serious side effects, which restrict their use as ideal vaccines. The most crucial of these drawbacks is that the live vaccines, whilst attenuated, remain fully virulent for

human. In addition, the vaccination is not fully efficacious and interferes with the diagnosis of field infection (Guilloteau *et al*., 1999; Yang *et al*., 2005). A whole killed vaccine comprising smooth *B. melitensis* H38 strain induced protection similar to that given by the Rev 1 strain, but its use was prevented due to the development of local lesions at the site of inoculation (Guilloteau *et al*., 1999).

The identification of *Brucella* sp. protective antigens is essential for the development of future subcellular vaccines, which avoid the drawbacks of live-attenuated vaccines.

The outer membrane proteins (OMPs) of *Brucella* sp. were initially identified in the early 1980s (Dubray and Bezard, 1980) and have been extensively characterized as potential immunogenic and protective antigens (Cloeckaert *et al*., 1995; Cassataro *et al*., 2007c).

The major OMPs were classified according to their apparent molecular mass (Cloeckaert *et al*., 1992). Three major OMPs, Omp25 (25-27 kDa), Omp2b (36-38 kDa) and Omp31 (31-34 kDa) have been identified (Dubray and Bezard, 1980; Verstreate *et al*., 1982; Cloeckaert *et al*., 2002).

Southern blot hybridization has shown the Omp31 gene is present in all *Brucella* sp. except *B. abortus* (Cloeckaert *et al*., 2002). Omp31 has been found as an immunodominant antigen in the serological immune response in *B. ovis* infected rams (Kittelberger *et al*., 1995) and the most exposed OMPs on smooth strains of *B. melitensis* (Bowden *et al*., 1995). These characteristics support Omp31 as a promising subunit vaccine candidate against brucellosis.

In this study, the *B. melitensis* Omp31 gene has been cloned, expressed in *Escherichia coli* (*E. coli*) BL21 and purified.

Materials and Methods

Bacterial strains and growth conditions

The heat killed bacteria of *B. melitensis* vaccine strain Rev 1 was obtained from the Brucellosis department of Razi Vaccine and Serum Research Institute (Hesarak, Karaj, Iran). *Escherichia coli* strain Top10 (Stratagene, USA) was used for initial cloning, sequencing and maintenance of different DNA fragments.

A prokaryotic expression vector pET32b(+) (Novagene, USA) was used for recombinant protein production. The recombinant plasmid was transformed into *E. coli*, BL21 (DE3) pLysS (Stratagene, USA) as host strain. LB broth or LB agar was supplemented when required with 100 µg/mL ampicillin. All chemicals were purchased from Merck Company (Germany).

Sera were collected from five patients who were diagnosed with a history of brucellosis in Dr. Taheri Medical Diagnostic Laboratory, Mashhad, Iran. Sera from five healthy volunteers were considered to act as negative control. The pooled sera of the subjects as positive and negative controls were prepared individually.

DNA extraction, PCR amplification and cloning of *B. melitensis* Rev 1 Omp31 gene

The killed *B. melitensis* Rev 1 was digested enzymatically using proteinase K (100 µg/ml) and a 1% (w/v) sodium dodecyl sulfate (SDS) mixture. The treated bacteria were incubated 30 min at 37°C then subjected to phenol/chloroform extraction. The DNA was finally ethanol (95%) precipitated, washed with 70% ethanol, air-dried and resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 7.5). The purity of the obtained DNA was verified by NanoDrop ND-100 spectrophotometer.

Full length open reading frame (ORF) of the Omp31 gene was amplified by PCR with *Pfu* DNA polymerase from the extracted DNA by the forward primer that has *EcoR* I restriction site (5'-ATAGAATTCGATGAA GTCCGTAATTTTGGCGTCCAT-3') and the backward primer with restriction site for *Xho* I (5'-TATTGGAGCTCGAGGAACTT GTAGTTCAGACCGACGC-3'). These primers were selected according to the reported Omp31 nucleotide sequence deposited in the NCBI GenBank database and were synthesized by GenFanAvaran Co., Tehran, Iran.

PCR reaction was performed in a 25 µl volume containing 250 ng of template DNA, 0.5 µM of each primer, 2.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphates, 1 × PCR buffer and 1.5 unit of *pfu* DNA polymerase. The following condition was used for the amplification: hot start at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 3 min, annealing at 65°C for 45 s and extension at 72°C for 1 min. Then a final extension step of 10 min at 72°C was performed. The PCR products were analyzed by electrophoresis on 1% agarose gel in TBE buffer and visualized by ethidium bromide staining on UV transilluminator.

The predicted 723-bp PCR product was purified from the agarose gel by *AccuPrep*™ Plasmid Mini Extraction kit (Bioneer, Korea) according to the manufacturer's instruction. The purity of the eluted PCR product was checked by NanoDrop ND-100 spectrophotometer.

After electrophoresis of the PCR product on low melting agarose gel 1%, the amplified PCR product was purified with the conventional alkaline lysis method and subjected to two-step digestion with *EcoR* I/*Xho* I restriction enzymes (Fermentas, Lituania). After each digestion, the product was electrophoresed on low melting agarose gel 1% and then purified. The purified digested PCR product was cloned into the pET32b(+) plasmid, in which the recombinant protein includes a six-histidine tag (His-tag) at the C-terminal end for easier purification. Standard techniques for these steps such as plasmid DNA preparation, ligation, competent cell preparation and transformation were followed, as described previously (Sambrook and Russell, 2001).

The resulting construct, pET32b + Omp31, was transformed into competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA) using transformation kit (Fermentas, Lituania).

Ampicillin-resistant colonies were grown until OD 600 = 1.0 in LB medium containing 50 µg/ml of Ampicillin, at 37°C with agitation. The fidelity of TOP10 *E. coli* transformants was verified by PCR reaction using specific primers of Omp31 that had been previously used for amplification.

The plasmids were purified and analyzed by restriction enzyme digestion using *EcoR* I and *Xho* I. After evaluation on agarose gel 1%, the purified plasmids were subjected to sequencing (Seq Lab, Germany).

Expression and purification of rOmp31

For expression of Omp31, the purified pET32b + Omp31 construct was transformed in *E. coli* BL21 (DE3) cells (Novagen, USA) by transformation kit (Fermentas, Lithuania) and selected on LB ampicillin plates. To ensure the integrity of the plasmids in the selected colonies, the plasmids were purified and then subjected to restriction enzyme digestion and PCR.

Protein synthesis was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) in a culture of bacteria with an OD 600 of 0.6. Bacteria were incubated for 3 h at 37°C then harvested by centrifugation (3000 g, 20 min, 4°C) and stored at -80°C. The pellet from a 100 ml bacterial culture was resuspended in lysis buffer (Tris 50 mM, EDTA 5.0 mM, urea 8.0 M, pH = 8.0) and subjected to three freeze-thaw cycles using liquid nitrogen. Cell lysate was subjected to centrifugation at 9000 g for 15 min at 4°C to separate the supernatant containing soluble materials from the pellet. Both the supernatant and the pellet were evaluated by SDS-PAGE to analyse the expression of rOmp31.

Expressed protein was purified by chromatography through Ni-agarose (Invitrogen), from the insoluble phase of lysate using Guanidine hydrochloride 6 M to dissolve the pellet, according to the manufacturer's protocol.

Briefly, two ml of Ni-NTA resin was packed into a syringe, washed and equilibrated in 10 column volumes of deionized water, followed by 10 column volumes of binding buffer (pH = 7.8, $K3(PO_4)$ 50 mM, NaCl 400 mM, KCl 100 mM, 10 mM Imidazole, 10% Glycerol, 0.5% Triton X-100). The filtered supernatant of lysate insoluble phase through a 0.45 µm membrane was loaded onto a Ni-NTA column and then washed with 10 column volumes washing buffer containing 20 mM Imidazole. Target protein was eluted using an Imidazole gradient (100-500 mM) in the binding buffer. Subsequently, the eluted

solution containing protein was collected and dialyzed against 50 mM phosphate buffer.

The purified rOmp31 was analyzed by SDS-PAGE gel and Coomassie brilliant blue staining. The concentration of purified protein was determined by Bradford method.

Electroblotting of purified rOmp31 and bacteria total lysate onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) was performed following electrophoresis on 12% SDS-PAGE with the reducing agent 2-mercaptoethanol (2-ME).

The blots were incubated with 2% BSA for 16 h at 4°C and after blocking were exposed for 3 h at room temperature to the positive and negative pooled sera (diluted 1:5 in PBS), individually. The membranes were washed three times and then anti human immunoglobulin/horseradish peroxidase conjugate (Sigma) (1:2000 diluted in BSA 1%) was added. After 1 h incubation at room temperature and washing, diaminobenzidine (DAB) as chromogen was employed for visualization.

Results

Amplification, cloning and sequencing of DNA coding for Omp31

Amplification of Omp31 gene produced a main fragment of approximately 723 bp. This PCR product was cloned successfully in the pET32b(+) expression vector. The integrity of the constructed vector pET32b + Omp31b was confirmed by restriction digestion analysis. The sequencing of constructed plasmid with specific primers Omp31 and T7 primer were performed, and the sequence of the amplified Omp31 deposited in GenBank under accession number: GQ403950.

Expression and purification of rOmp31

The rOmp31 protein was expressed immediately after induction with IPTG. The expected recombinant fusion protein of ~46 kDa was detected after induction of the culture with IPTG. Most of it was found to be localized inside the inclusion bodies (pellet of cell lysate) in the cells. The maximum amount of rOmp31 was obtained 8 h after induction by IPTG at 37°C with agitation (Fig. 1).

After purification of rOmp31 by Ni-NTA Affinity Chromatography, the yield of the purified solution was estimated by Bradford method about 0.4 μg/ml of culture.

Immunoblotting with sera of positive pooled serum revealed a specific reactivity with purified rOmp31 produced in *E. coli* cells and showed the functional expression in the prokaryotic system (Fig. 2).

Discussion

In this study the expression vector pET32b(+) was used to construct expressing vector, pET32b + Omp31. The pET System is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli* (Novagen's pET System Manual and Novagen, Technical Bulletin 009). Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cells. T7 RNA polymerase is so active that, when fully induced, almost all of the cell's resources are converted to target gene expression (Novagen's pET System Manual and Novagen, Technical Bulletin 009).

Our target gene, Omp31, is initially cloned using TOP10 *E. coli* as a host that does not contain the T7 RNA polymerase.

By transferring the plasmid pET32b + Omp31 into *E. coli* BL21 DE3, expression was induced by the addition of IPTG. This vector encodes the 109 amino acids *trxA* (Trx•Tag™) domain immediately upstream from His•Tag sequences that allow easy purification, quantification and detection of target proteins (Meng *et al*., 2009). A second His•Tag domain follows the cloning sites, allowing C-terminal fusions by cloning in the appropriate continuous reading frame. The His•Tag sequence is very useful as a fusion partner for protein purification. His•Tag fusion proteins can be affinity purified under fully denaturing conditions (Novagen's pET System Manual and Novagen, Technical Bulletin 009).

In this study, the *Xho* I and *EcoR* I cloning sites were chosen for insertion. Therefore, His•Tags sequences were incorporated in the expressed protein. These additional tags increased the size of the expressed protein about 20 kDa. The sequencing and PCR results confirmed the integrity of the cloning. Comparing the sequencing result, which has been deposited in the GenBank database (accession number: GQ403950) with the databases and using basic local alignment search tool (BLAST) software (data not shown) confirmed the fidelity of our work.

The rOmp31 was purified successfully and could be used as a vaccine candidate and for further studies.

It had already been reported that recombinant *B. melitensis* Omp31 extract was highly immunogenic in the *B. ovis* mouse model of infection (Estein *et al*., 2003) and rams as the natural host against *B. ovis* (Estein *et al*., 2004).

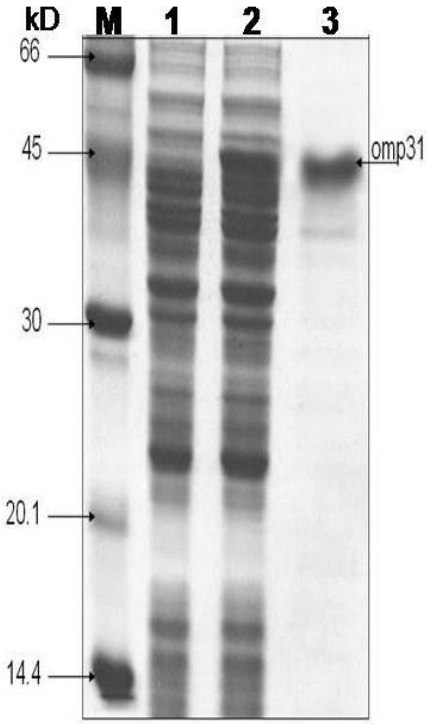

Fig. 1: SDS-PAGE (12%) analysis of purified rOmp31 from insoluble fraction of lysate bacteria stainded with Coomassie blue. M: Molecular weight marker, Sigma. 1: Insoluble bacterial cell lysates before IPTG inducing. 2: Bacterial cell lysate after IPTG inducing, rOmp31 as an extra 46-kD band is seen. 3: Purified rOmp31, a major 46-kD single band is visualized

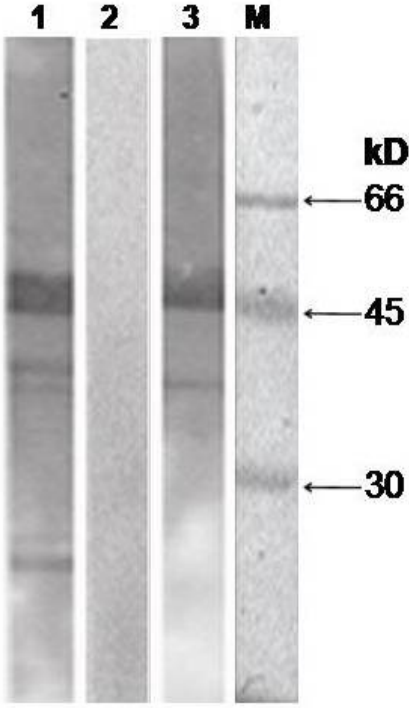

Fig. 2: Western blots analyses of insoluble bacterial cell lysate and purified rOmp31 to evaluate the immunoreactivity of rOmp31. 1: Insoluble bacterial cell lysate induced with IPTG was exposed to positive pooled human serum. The reactivity with newly expressed rOmp31 is seen. 2: Purified rOmp31 on blot was exposed to negative pooled human serum, no reactivity was visualized. 3: Purified rOmp31 on blot was exposed to positive pooled serum, the specific reactivity with rOmp31 is seen. M: Molecular weight marker, Sigma

Culling of animals positive to serological and/or bacteriological tests is the first controlling measure. Vaccination is recommended in high incidence of brucellosis (Renukaradhya *et al*., 2002).

Initial practical steps toward making new vaccines are production, extraction, and purifying the vaccine candidates. In this study, one of the most important vaccine candidates against *B. melitensis* was made available by production of a recombinant *E. coli* expressing the Omp31 gene as a fusion protein to easy purification. Exploring the immunogenicity and potential use of this protein is our main focus.

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