Original Article

In silico Design of Truncated Omp31 Protein of *Brucella melitensis:* Its Cloning and High Level Expression in *Escherichia coli*

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Abstract:

Introduction: Omp31 is animmunodominant and protective antigen conserved in *Brucella* species and a good candidate for vaccine design. **Methods:** The present study aimed at *in silico* design of the truncated Omp31 (TOmp31) using bioinformatic tools and to express the selected form in *Escherichia coli (E. coli)* **conclusion:** Various bioinformatically calculated scores for the model showed that the structure conformation of the truncated Omp31 is in the range of the native protein with the C-score, Z-score, TM-score and Ramachandran Z-score of the truncated form model being -0.53, -0.72 and -0.98 respectively. Amplification of TOmp31 produced a single fragment of approximately 345 bp which was cloned in the pET28a expression vector. The integrity of the constructed vector (pET28- TOmp31) was confirmed by PCR and restriction digestion analysis and the rTOmp31 was successfully expressed in *E. coli* BL21. SDS-PAGE analysis of the lysate from the induced *E. coli* carrying the TOmp31 construct and the purified protein showed the expected molecular mass of approximately 12 kDa. The yield of the purified protein was estimated at approximately 250 µg/ml. Anti-His antibody reacted with the purified protein in Western blot confirming its expression in the prokaryotic system. Future studies exploring the immunogenicity and cross-protection of the protein against *Brucella spp.* are underway. *Vac Res, 2014, 1 (1): 16 - 20*

Keywords: *Brucella*, Omp31, Modeling, Truncated, *In silico* analysis.

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INTRODUCTION

Brucellosis is the most common bacterial zoonotic disease worldwide, with over half a million infection in humans annually [1, 2]. Brucellosis causes high economic loss in livestock by abortion and is known as a potentially life-threatening multi-system disease in humans [3, 2]. It remains endemic in many parts of the world, including the Middle East, Africa, Latin America, Central Asia and several regions of the Mediterranean basin [4, 5]. The true incidence of human brucellosis is unknown and reported incidence in endemic areas varies widely from less than 0.01 to more than 200; however in Iran, it is 132.4 per 100,000 population [6]. Brucellosis results from infection by various species of *Brucella*, a Gram negative, facultative intracellular pathogen of the family *Brucellaceae* [4]. The six important species of *Brucella* are classified mainly based on their preferred hosts and pathogenicity: *B. melitensis* (sheep and goats), *B. abortus* (cattle and buffalo), *B. suis* (pigs), *B. ovis* (rams), *B. canis* (dogs) and *B. neotomae* (desert wood rat). *B. melitensis, B. abortus* and *B. suis* are the main pathogenic species worldwide [7]. In humans, brucellosis can be caused by *B. abortus, B. melitensis, B. suis* and rarely, *B. canis* or marine mammal *Brucella* [4]. Of these, *B .melitensis* causes the most severe and acute form of the disease in humans, with a milder form caused by *B. suis, B. abortus* and

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B. canis in descending order. Human brucellosis is usually prevented by controlling the infection in animals [4, 8].

The live attenuated smooth strains S19, Rev.1 *(B. melitensis)* and non-smooth RB51 *(B. abortus)* are used for animal vaccination of which S19 and Rev.1 have been effective for brucellosis in cattle, sheep and goats [4, 9]. These vaccines have the disadvantages of causing abortion in pregnant animals, being pathogenic for humans, resistant to streptomycin and inducing O-polysaccharide (O-PS) specific antibodies that interfere with the serological tests [8, 10]. However, no effective and safe vaccine against human brucellosis has been developed so far. Recent studies have concentrated on unraveling the mechanisms of protective immunity in this infection and development of an effective human brucellosis vaccine [7, 9, 11, 12]. Immunity against *Brucella* requires cell mediated responses, particularly the Th1 characterized by production of gamma interferon. These responses are best stimulated by live vaccines or protective proteins with adjuvants favoring cell-mediated immune responses [13]. To develop a human *Brucella* vaccine, proteins which are present in pathogenic strains, but absent in nonpathogenic strains are ideal targets. The web-based VIOLIN vaccine database containing many literature mining programs has identified 46 proteins that are conserved in all *Brucella* genomes and lack sequence similarity with human or mouse proteins [11].

Of these, the 31-kDa outer membrane protein (Omp31) of *B. melitensis* is considered as a priority antigen for developing a subunit vaccine [7, 9, 11, 12]. Omp31 is the only protective antigen and the most exposed OMP identified in smooth *B. melitensis* [7, 10, 14, 15]. Southern blot hybridization has shown that omp31 gene is present in all *Brucella spp*. and the coded protein is an immunodominant antigen in the serological immune tests [11, 15, 16].

In the present study, we aimed to design a truncated form of Omp31 (TOmp31) based on conserved areas and MHC I binding affinity and to perform protein structure modeling of the truncated form using bioinformatic tools, in order to express the protein in *E. coli* for future cross-protection studies against brucellosis.

MATERIALS AND METHODS

Strains, plasmids & media. The heat inactivated *B. melitensis* 16M was obtained from Pasteur Institute of Iran and prokaryotic expression vector pET28a (Novagene, USA) was used for recombinant protein production. This vector expresses the recombinant proteins fused with a six-histidinetag and a thrombin recognition site resulting in an increase of approximately 5 kDa in the size of the expressed proteins. *E. coli*, BL21 (DE3) (Stratagene, USA) was used as host for expression of the recombinant protein. Bacterial strains were routinely grown at 37°C in LB broth or agar (Merck, Germany), supplemented with 50 µg/ml of kanamycin as required.

***In silico* design.** Truncated Omp31 (TOmp31) design was based on multiple sequence alignment of full-length protein sequences from several *Brucella spp*. using CLUSTALW Multiple Sequence Alignment software (http://workbench.sdsc.edu/) [17] and the conserved areas of Omp31 protein sequence of *B. melitensis* and *B. abortus* was also determined using the same program. Immune Epitope Data Base (IEDB) analysis resource (http://www.iedb.org) was used for identification of immunogenic epitopes of *B. melitensis* Omp31 protein and peptides with high binding affinity for BALB/c mice MHC-I were predicted using EMBL MHC-I epitope predictor [18-21]. Finally, the location of the MHC good binders and the conserved area were matched to design the truncated form.

TOmp31 protein modeling was performed using I-TASSER server. I-TASSER is a hierarchical protein structure modeling approach based on the secondary-structure enhanced Profile-Profile threading Alignment (PPA) and the iterative implementation of the Threading ASSEmbly Refinement (TASSER) program (http://zhanglab.ccmb.med.umich.edu/I-TASSER) [22]. Validation and analysis of modeling results were made using protein structure analysis Prosa server (https://prosa.services.came.sbg.ac.at/prosa.php) and SPDBV software [23, 24]. The C-score (a confidence score for estimating the quality of predicted model) and TM-score (scale for measuring the structural similarity between two structures) were given in the I-TASSER result page. Z-score (overall model quality) and energy plots were created using Prosa server. The Ramachandran Z-score (for calculating the quality of a Ramachandran plot) was calculated using SPDBV viewer.

DNA Extraction, Primers design and PCR amplification. Chromosomal DNA was prepared using DNA extraction kit (Roche, Germany) according to the manufacturer's recommendations. PCR was performed using primers 5'-CAT GCC ATG GCA GAA ACC AAG GTC-3' and 5'-CCG CTC GAG GAA CTT GTA GTT CAG ACC GAC G-3' with *NcoI* and *XhoI* restriction sites (underlined), respectively. Primers were synthesized by TAG (Denmark) and were based on the published sequence of Omp31 (GenBank accession No. AF076290). The amplification was performed in a 50 µl total volume containing 500 ngof template DNA, 1 µM of each primer, 200 µM dNTP-Mix, 1Xpfu buffer containing MgSO4 and 1 unit of pfu DNA polymerase (Thermo Scientific, EU). The following conditions were used for gene amplification: Hot start at 95˚C for 3 min, followed by 30 cycles ofdenaturation at 94˚C for 45 sec, annealing at 55˚C for 45 sec and extension at 72˚C for 1 min and a final extension at 72˚C for 7 min. The PCR product was analyzed by electrophoresis in 1% agarose gel and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified from the agarose gel by high pure PCR product purification kit (Roche, Germany) according to the manufacturer's recommendations. The purity of the eluted PCR product was assessed by electrophoresis in 1% agarose gel in 1X TBE buffer.

Cloning of TOmp31gene in bacterial expression vector. The purified PCR product was digested with *NcoI* and *XhoI* (Thermo Scientific, EU) and cloned intopET28a expression vector, digested by the same restriction enzymes, using T4 DNA ligase (Fermentas, USA) at 4˚C overnight. *E. coli* TOP10 and *E. coli* BL21 (DE3) competent cells were used for transformation of pET28a-TOmp31 plasmid. Transformed bacteria were cultured on LB agar containing 50 µg/ml of kanamycin and selected colonies were analyzed for the presence of the construct by PCR and restriction enzyme digestion and the sequencing of constructed plasmid was performed with specific T7 promoter and T7 terminator universal primers (GATC Biotech, Switzerland).

Expression and purification of recombinant TOmp31 protein. *E. coli* BL21 (DE3) transformed with pET28a-TOmp31 was grown in LB broth supplemented with kanamycin (50 µg/ml) at 37°C with agitation. Protein synthesis was induced with different concentration of IPTG (0.1, 0.2 and 0.4) when bacterial

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culture reached an OD600 of 0.6. Bacteria were incubated for 4 h at 37°C and harvested by centrifugation at 4000 rpm for 10 min at 4°C and stored at -20°C.The expressed protein was purified with Ni-NTA column (Genescript, USA) under denaturing conditions according to the manufacturer's instructions. The purified protein was dialyzed against 3M urea, 1M urea and PBS (pH 7.2) at 4°C overnight.

SDS-PAGE and Western blotting. Quality and quantity of the purified recombinant TOmp31 protein was analyzed by SDS-PAGE and Bradford assay, respectively [25]. The rTOmp31 protein was identified by Western blot for which the proteins were electroblotted onto nitrocellulose membrane at 100 V for 2 h. The blotted membrane was blocked with skim milk for 2 hat room temperature and incubated with 1:500 dilution of anti-His antibody (Roche, Germany) at room temperature for 2 h. Finally, blots were washed three times with TPBS and developed by Diaminobenzidine (DAB) Solution (Roche, Germany). The purified recombinant protein was stored at -20°C for further immunogenicity and protective efficacy analysis.

RESULTS

***In silico* design.** The Genbank Omp31 gene sequences of *B. melitensis* M5, *B. melitensis* ATCC23457 were 100 percent identical to that of *B. melitensis* 16M (accession No.AF076290). The *B. abortus* species 544 and 2308 showed 100 identities. Multiple sequencing alignment of Omp31 gene between *B. melitensis* and *B. abortus* showed 66% homology. Mouse MHC-I binding predictions results showed that peptides with high binding affinity for MHC-I were located in the highly conserved area. Based on the alignment and epitope prediction results, the amino acids 127 to 240 formed the truncated form (Fig. 1A). The C-score, structure Z-score, TM score and Ramachandran Z-score of the truncated Omp31 protein structure were -0.53, -0.72, 0.65 and -0.98, respectively. The Z-score indicated that it was in the range of native conformations and the plot showed that the structure was within the range of scores typically found for native proteins of a similar size (Fig. 1B). With regard to the plot of residue scores, overall the residue energies were largely negative for the model with the exception of some peaks in the N-terminal part (Fig. 1C).

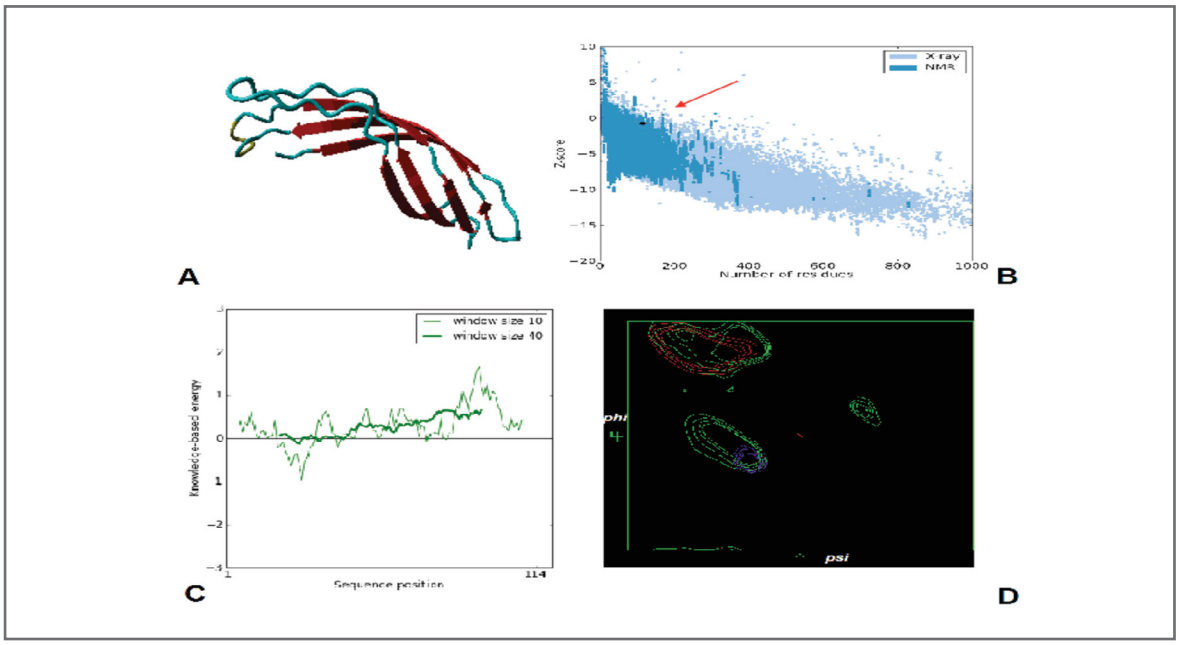

Fig.1. Results of *in silico* studies showing **(A)** TOmp31 protein (aa 127-240) structure prediction by I-TASSER server. **(B)** The Z-score plot containing the Z-scores of all experimentally determined protein chains in current PDB. In this plot, groups of structures from different sources (X-ray, NMR) are distinguished by different colors. The Z-score plot of TOmp31 protein shows that the structure is within the range of scores typically found for native proteins of similar size. The arrow shows the location of the truncated protein. **(C)** The plot of the residues scores showing the quality of local model by plotting energies as a function of amino acid sequence position. In general, positive values correspond to problematic or erroneous parts of the input structure. A plot of single residue energies usually contains large fluctuations and is of limited value for model evaluation. Hence the plot is smoothed by calculating the average energy over each 40-residue fragment s*(i,i+39)*, which is then assigned to the 'central' residue of the fragment at position *i+19* (thick line). A second line with a smaller window size of 10 residues is shown in the background of the plot (thin line). **(D)** The Ramachandran plot of the TOmp31 protein which shows that the protein structure has 100% of its residues in most favored areas resulting in Z-scores of -0.98. Blue is used for helix, red for strand and green for turn and loop according to DSSP.

Amplification, cloning and sequencing. Amplification of TOmp31 produced a single fragment of approximately 345 bp (Fig. 2A). The PCR product was cloned into pET28a expression vector and the integrity of the constructed vector pET28-TOmp31 was confirmed by PCR (Fig. 2B) and restriction digestion analysis (Fig. 2C). The sequencing result was confirmed by comparison with the original sequences using basic local alignment search tool (BLAST) resources.

Expression and purification of the TOmp31 recombinant protein. The TOmp31 protein was expressed after induction with 0.1, 0.2 and 0.4 mM IPTG with similar results.

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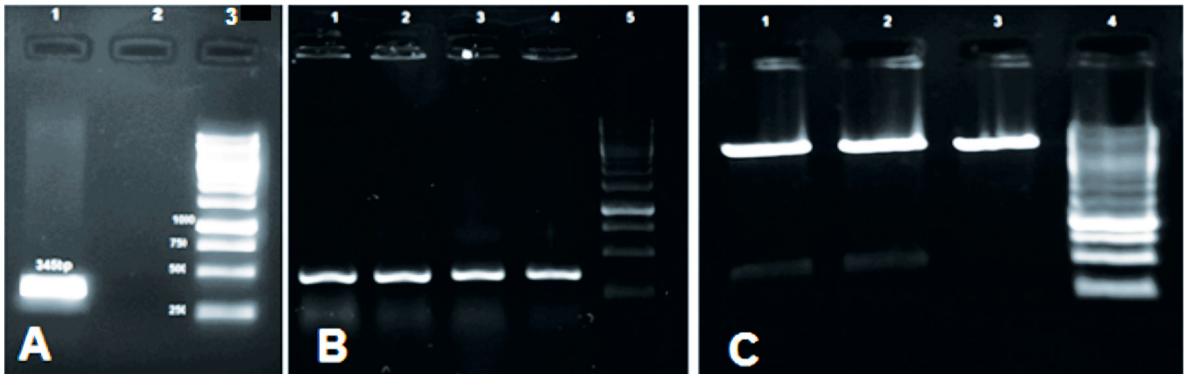

Fig. 2. Amplification results of TOmp31, transformed clones and restriction enzyme digestion of the construct.
(A) Lane1: TOmp31, Lane2: Negative control, Lane3: 1 Kb DNA ladder. **(B)** Lanes1-4: positive clones, Lane 5: 1 Kb DNA ladder. **(C)** Lane1-2: Positive clones, Lane3: Negative clone, Lane4: 1 Kb DNA ladder.

Therefore, further protein expressions were performed with 0.2 mM IPTG at OD 600=0.6 for 4 h at 37°C and the expected recombinant protein of 12 kDa was detected.
The high level expression of the protein led to the formation of inclusion bodies; consequently purification of the rTOmp31 by Ni-NTA affinity chromatography was performed using denaturing method. SDS-PAGE (15%) analyses of the lysate from the induced and the uninduced *E. coli* carrying the TOmp31 construct and the purified protein showed the expected recombinant protein with the molecular mass of approximately 12 kDa (Fig. 3A). The yield of the purified protein was estimated at approximately 250 µg/ml. Western blot with anti-His antibody confirmed the expression of TOmp31 in the prokaryotic system (Fig. 3B).

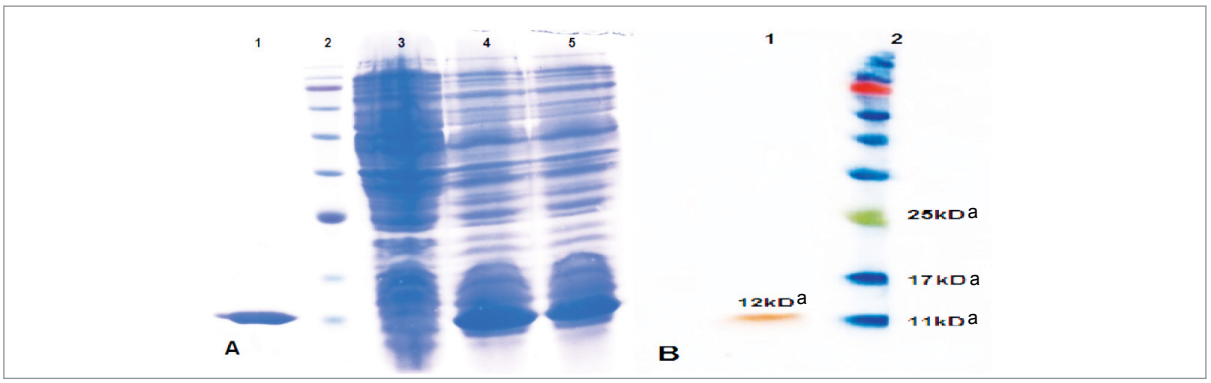

Fig. 3. SDS-PAGE and Western blotting profile of the TOmp31 protein expression and purification.
(A) Lane 1: Purified protein, Lane2: Pre-stained Protein ladder (Vivantis, USA), Lane3: uninduced bacterial lysate, Lane 4-5: induction results by IPTG. **(B)** Lane 1: Purified rTOmp31 protein, Lane2: Pre-stained protein ladder.

DISCUSSION

Identification of immunogenic proteins in *Brucella* has been facilitated considerably by recent immunoproteomic studies and although many immunogens have been identified, they have not always equated with protection. Evaluation of the immunogenicity and protective ability of these immunogens is helpful for developing effective vaccines [7]. The *B. melitensis* Omp31 protein is a potential candidate for vaccines design against *Brucella* infection and has been used as subunit and DNA vaccine [26]. Anti-Omp31 antibodies have also been detected in sera of cured humans and animals [27]. Some studies have evaluated the immunogenicity and protective efficacy of the recombinant Omp31 protein in combination with adjuvants and have shown that it could be protective against *B. melitensis* infection by eliciting a Th1 response mediated by CD4+ T cells [9, 10, 27-31].
In the present study, a truncated form of Omp31 was designed and its 3D structure was predicted using I-TASSER data base which generates the best 3D structure predictions among all the automated servers. Furthermore, since determination of the epitopes recognized by *Brucella*-specific CD8+ T cells will help to construct proteins critical for vaccine development, we have designed an Omp31 truncated form containing the epitopes recognized by CD8+ T cells [32-34]. Protein structure analysis indicated that the selected fragments formed a 3D structure similar to the native protein with a Z-score in the range of native conformations. Overall, the residue energies were largely negative for the model with the exception of some peaks in the N-terminal part (Fig. 2C). These peaks are assumed to correspond to the membrane spanning regions of the TOmp31 protein [35, 36].
For high level production of the recombinant protein, the sequence encoding the Omp31 truncated form was cloned in pET28a expression vector and the protein production was successfully induced by different concentrations of IPTG. The yield of TOmp31 (250 µg/ml) was significantly higher than the previous study performed by Vahedi et al. (37) who reported a yield of 0.4 µg/ml from their culture, demonstrating the efficiency of our construct in compar-

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ison to attempts made at expressing the full length gene [37]. Selection and production of new vaccine candidates are the initial practical steps toward making new vaccines. In this study, a new truncated protein vaccine candidate was constructed with the help of *in silico* studies and protein modeling. Future studies exploring the immunogenicity and cross-protectivity of this protein against *B. melitensis* and *B. abortus* are underway.

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CONFLICT OF INTEREST

The authors do not have any conflict of interests.

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