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In silico analysis of *Brucella abortus* Omp2b and *in vitro* expression of SOmp2b

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Purpose: At present, there is no vaccine available for the prevention of human brucellosis. *Brucella* outer membrane protein 2b (Omp2b) is a 36 kD porin existed in common *Brucella* pathogens and it is considered as priority antigen for designing a new subunit vaccine.
Materials and Methods: In the current study, we aimed to predict and analyze the secondary and tertiary structures of the *Brucella abortus* Omp2b protein, and to predict T-cell and B-cell epitopes with the help of bioinformatics tools. Subsequently, cloning and expression of the short form of Omp2b (SOmp2b) was performed using pET28a expression vector and *Escherichia coli* BL21 host, respectively. The recombinant SOmp2b (rSOmp2b) was purified with Ni-NTA column.
Results: The recombinant protein was successfully expressed in *E. coli* host and purified under denaturation conditions. The yield of the purified rSOmp2b was estimated by Bradford method and found to be 220 μg/mL of the culture.
Conclusion: Our results indicate that Omp2b protein has a potential to induce both B-cell– and T-cell–mediated immune responses and it can be evaluated as a new subunit vaccine candidate against brucellosis.

Keywords: *Brucella*, Omp2b, *In silico* approach, Epitope prediction, Protein expression

Introduction

Brucellosis is an important zoonotic disease caused by gram-negative facultative intracellular *Brucella* [1]. According to zoonotic potential and primary host preference, the genus of *Brucella* is classified into nine species: *Brucella melitensis* (sheep and goats), *Brucella suis* (hogs), *Brucella abortus* (cattle), *Brucella ovis* (sheep), *Brucella canis* (dogs), *Brucella neotomae* (rats), *Brucella ceti* (cetaceans) and *Brucella pinnipedialis* (seals), and *Brucella microti* (common vales) [2]. The zoonotic potential of *B. melitensis, B. abortus*, and *B. suis* are considered high and they are most human pathogens [3]. Annually, worldwide incidence of brucellosis is more than 500,000 human infections. Although the disease has a restricted geographic distribution, but it still remains major public health challenge in the Mediterranean region, Asia, Africa, and Latin America. Animal brucellosis causes vast economic losses due to abortion, reproductive failure, and decreased milk production [4,5]. Among different control measures for the eradication of brucellosis, vaccination is indicated to be the most economic measure for control of the disease in endemic areas [6]. For the development of an effective vaccine, it is nec-

essary to select the best antigen that elicits adequate immune responses (biased towards a Th1) and induces protection against *Brucella* infection [6,7].

Bacterial surface exposed antigens are prime vaccine candidates as they represent the initial point of contact between the pathogen and the host immune system. Several *Brucella* immunogenic antigens have been identified in the outer membrane of this pathogen [6,8,9]. According to their molecular mass, the major outer membrane proteins (Omps) of *Brucella* are classified to group 2 and group 3 proteins representing 36-38 and 25-31 kDa Omps, respectively [8,9]. The gene products of *Brucella* major Omps have been designated as Omp2a and Omp2b for the 36-38 kDa porin proteins, Omp25 for the 25-27 kDa Omp, and Omp31 for the 31-34 kDa Omp [9]. Two closely related *omp2a* and *omp2b* genes encode and potentially express the 36-38 kDa porin. The genes sequences share about 85% identity and are encoded in the same genetic locus but in opposite directions. The *B. abortus* biovar1 *omp2b* gene encodes a 36 kDa protein while *omp2a* would encode a protein of 33 kDa [9]. The pore-forming activities of Omp2a and Omp2b are different in which *B. melitensis* 16M Omp2a demonstrates characteristics of a larger pore than Omp2b. Only Omp2b has been detected in *Brucella abortus* biovar 1, indicating the absence of Omp2a protein at least in this biovar [9,10].

Recent study focusing on identification of the *Brucella* protective antigens demonstrated that Omp2b is a priority antigen for designing a recombinant protein vaccine [1,11]. Moreover, the results of study done by Sung et al. [3] showed that Om2b may be a potential lipopolysaccharide-free protein for developing diagnostic tests. Production of cytokines and nitric oxide (NO) was investigated in RAW 264.7 cells and mouse splenocytes after stimulation with the protein. The production of tumor necrosis factor α, interleukin (IL)-6, and NO were significantly induced in RAW 264.7 cells. Stimulation by Omp2b resulted in significant increase in levels of interferon γ and IL-4 produced by mice splenocytes [3]. Additionally, *B. melitensis* Omp2b has been identified as anti-apoptotic effector candidate that essentially prevents Bax-induced cell death in *Saccharomyces cerevisiae* [12].

The aim of the present study was to *in silico* analysis of Omp2b of the *B. abortus* 544 as a new vaccine candidate and *in vitro* expression of the short form of Omp2b (SOmp2b) lacking the signal peptide sequence.

Materials and Methods

Omp2b gene sequence alignment

The full-length sequences of Om2b from several *B. melitensis* and *B. abortus* strains were aligned using multiple sequence alignment software (http://workbench. sdsc.edu/) [13]. The identical regions between Omp2b sequences were considered as conserved areas.

Omp31 protein structure analysis

The location of signal peptide and transmembrane regions within the Omp2b sequence were determined using SignalP [14] and TMAP [15] servers, respectively. Surface accessibility, hydrophilicity and antigenicity of the Omp2b protein were determined using Immune Epitope Database (IEDB) analysis resource (http://www.iedb.org). The molecular weight, theoretical isoelectric point (pI), extinction coefficient, total number of positive and negative residues, half-life, instability index, aliphatic index, and grand average hydropathy (GRAVY) of the Omp2b protein were computed using ProtParam server (http://us.expasy.org/tools/ protparam.html) [16].

Secondary and tertiary structure prediction

The secondary and tertiary structures of the Omp2b protein were predicted using GOR4 (https://npsa-prabi.ibcp.fr/cgi-bin/npsa) [17] and I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) servers, respectively [18].

Validation and analysis of the three dimensional models

The confidence score (C-score), template modeling score (TM-score) and root-mean-square deviation (RMSD) were calculated by I-TASSER server. Energy minimization for the three dimensional (3D) models was performed using Swiss-PDB Viewer 4.1 software. Analysis of the 3D model was made using protein structure analysis (ProSa) server (https://prosa.services.came.sbg.ac.at/prosa.php) [19] and Ramachandran Plot Analysis resource (RAMPAGE) [20]. The Z-score (overall model quality) and energy plots were created by ProSa server.

T-cell epitope prediction

IEDB T-cell epitope prediction tools were used for identification of MHC II epitopes of *B. abortus* Omp2b protein [21].

Prediction of antigenic B-cell epitopes

BCPred server was used for prediction of continuous B-cell epitopes (20mers) of the Omp2b [22]. Prediction of discon-

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tinuous B-cell epitopes from 3D protein structure was performed using ElliPro antibody epitope prediction tool [23].

Polymerase chain reaction amplification of SOmp2b

Chromosomal DNA of *B. abortus* strain 544 (Pasteur Institute of Iran) was prepared according to the manufacturer's protocol of DNA extraction kit (Roche, Mannheim, Germany). Polymerase chain reaction (PCR) was performed using forward 5′-CTAGCTAGCATGGCCGACG CAATCGTCGC-3′ and reverse 5′-CCGCTCGAGTTAGAACGAACGCTGGAAGCG-3′ primers with *Nhe*I and *Xho*I restriction sites (underlined), respectively. The amplification was carried out in a 50 μL total volume component of 500 ng template DNA, 1 μM each primer, 200 μM dNTP-mix, 1× pfu buffer containing $MgSO_4$, and 1 unit of pfu DNA polymerase. PCR conditions used for gene amplification were as follows: hot start at 95°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. High pure PCR product purification kit (Roche) was used for purification of the PCR products.

Cloning of SOmp2b gene in pET28a expression vector

The purified SOmp2b PCR product and pET28a expression vector (Novagen, Madison, WI, USA) were digested with *Nhe*I and *Xho*I (Thermo Scientific, Waltahm, MA, USA) restriction enzymes. The ligation reaction has been carried out using T4 DNA ligase (Thermo Scientific) at 4°C, overnight. Transformed *E. coli* BL21 (DE3) (Stratagene, Kirkland, WA, USA) competent cells were grown on Luria-Bertani agar containing 50 μg/mL of kanamycin (Gibco, Grand Island, NY, USA). Finally, pET28a-SOmp2b positive clones were identified by clone-PCR, restriction enzyme digestion, and sequencing.

Expression and purification of the recombinant SOmp2b

The pET28a-SOmp2b positive clone was cultured in LB broth supplemented with kanamycin (50 μg/mL) in shaker incubator at 37°C until reached OD at 600 nm of 0.5. Different concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1, 0.2, and 0.4) were added for induction of protein synthesis and the incubation resumed for an additional 4 hours at 37°C. Bacterial cell harvesting was performed by centrifugation at 4,500 rpm for 10 minutes at 4°C. The expressed recombinant protein was purified by Ni-NTA column (Genescript, Piscataway, NJ, USA) under denaturing conditions according to the manufacturer's instructions. Dialyzing of the purified protein was done against 5 M urea, 3 M urea, 1 M urea, and phosphate buffered saline (pH 7.2) at 4°C overnight.

Table 1. Protein structure analysis of Omp2b using IEDB

Parameter	Start-End	Peptide
Surface accessibility	40-48	YVRYDVKGG
	50-64	DVYSGTDRNGWDKSA
	90-107	DGKYGNETS
	150-157	GSYRTGKI
	175-181	QGGDNDG
	184-191	TGTTNYHI
	248-257	SAATPDQNYG
	269-277	LKYQATQKA
	284-289	AHDDWG
	310-316	EVSYTKF
	335-340	VRFQRSF
Hydrophilicity	5-14	VAPEPEAVEY
	13-19	EYVRVCD
	16-25	RVCDAYGAGY
	27-36	IPGTETCLR
	38-44	HGYVRYD
	68-74	LRVSTGS
	69-80	RVSTGSETELGT
	89-113	FNYAANNSGVDGKYGNETSSGTVME
	123-133	RVGIDESEFHT
	139-146	GDVINDDV
	143-150	NDDVISAGS
	147-160	ISAGSYRTTGKISYT
	158-165	SYTFTGGNG
	163-170	GGNGFSAV
	172-193	LEQGGDNDGGYTGTTNYHIDG
	192-198	DGYMPDV
	213-219	GVVAYDS
	225-237	AAKVRGDVNITDQ
	242-262	LQGAYSSAATPDQNYGQWGGD
	270-280	KYQATQKAAFN
	282-297	QAAHDDWGKTAVTANV
	294-300	TANVAYE
	308-315	TPEVSYTK
	313-319	SYTKFGGE
	317-332	GGEWKNTVAEDNAWGG
Antigenicity	4-29	IVAPEPEAVEYVRVCDAYGAGYFYIP
	31-44	TETCLRVHGYVRYD
	65-71	RFALRVS
	112-126	MEFAYIQLGGLRVGI
	134-141	FTGYLGDV
	143-150	NDDVISAG
	167-174	FSAVIALE
	195-205	MPDVVGGLKYA
	211-223	IAGVVAYDSVIEE
	225-231	AAKVRGD
	237-249	QFSVWLQGAYSSA
	266-276	WGGLKYQATQK
	278-284	AFNLQAA
	292-314	AVTANVAYELVPGFTVTPEVSYT

Omp2b, outer membrane protein 2b; IEDB, Immune Epitope Database.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting

Identity of the purified recombinant SOmp2b protein was confirmed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as previously described [24]. The quantity of the recombinant SOmp2b (rSOmp2b) was done by Bradford assay. The purified recombinant protein was stored at -20°C for further *in vivo* analysis of its immunogenicity and protective efficacy.

Results

Multiple sequence alignment

Multiple sequence alignment of Omp2b protein sequences demonstrated that the protein sequence is conserved among *B. abortus* and *B. melitensis* strains and the alignment score is 99%-100%.

Protein structure analysis

The cleavage site of signal peptidase was between aa22-aa23. Two transmembrane segments aa3-aa28 and aa225-aa253 have been predicted by TMAP tool. The results of surface accessibility, hydrophilicity and antigenicity prediction of the Omp2b protein were summarized in Table 1. The molecular weight and the theoretical pI of the SOmp2b were 36.68 kD and 4.53, respectively. Total numbers of negatively and positively charged residues were 42 and 24, respectively. The half-life of the protein is more than 10 hours in *E. coli* and *in vivo*. Aliphatic index, instability index, and GRAVY were 67.12, 24.12 (stable), and -0.294, respectively.

Secondary structure prediction

B. abortus strain 544 Omp2b protein Secondary structure prediction results showed that the protein consist of the percentages of alpha helix, extended strand, and random coil were 19.61, 30.39, and 50, respectively (Fig. 1).

Tertiary structure prediction

In silico prediction of 3D structure of Omp2b was performed using I-TASSER server (Fig. 2A). The C-score (-1.34), TM-score (0.55), and RMSD (9.5) of the model showed that the model has a high confidence value and it is with correct topology. Energy minimization of the best model was done using Swiss PDB Viewer (SPDBV). Structure analysis of the predicted model by Prosa indicated that the model is within the range of native proteins of the similar size (Fig. 2B). Stability of the model was evaluated by the Ranachandran plot (Fig. 2C).

T-Cell epitope prediction

The location of T-cell epitopes with strong affinity for human MHC-II alleles was predicted using IEDB resource. The list of the best human HLA-II epitopes was summarized in Table 2.

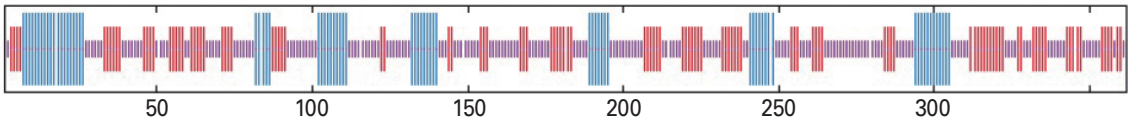

Fig. 1. Secondary structure prediction of SOmp2b. The purple, red, and blue lines are representative of random coil, extended strand and alpha helix, respectively. SOmp2b, short form of outer membrane protein 2b.

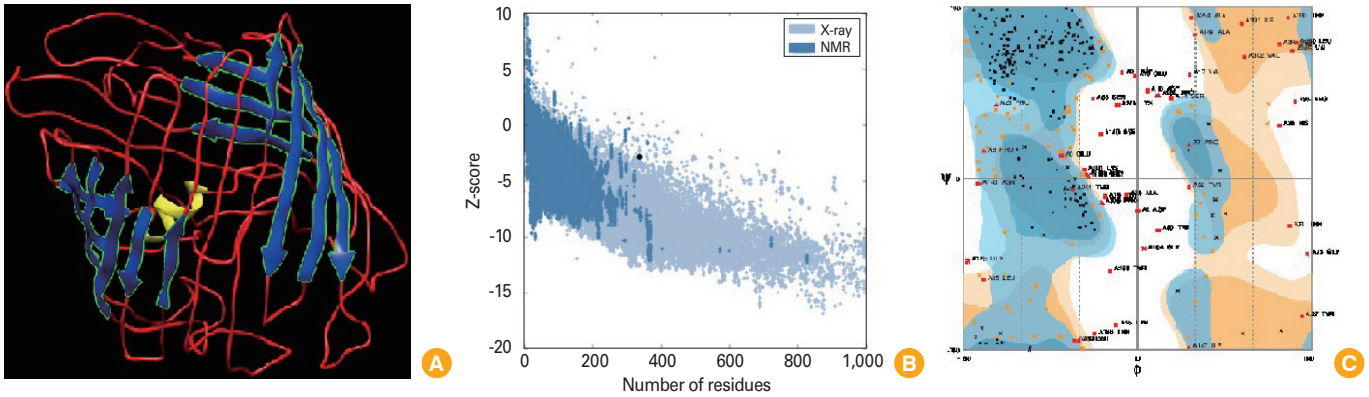

Fig. 2. *In silico* prediction and analysis of the tertiary structure of Omp2b. (A) The best model predicted by I-TASSER tool. (B) Z-score plot of the best model. (C) Ramachandran plot of the best model. Omp2b, outer membrane protein 2b; NMR, nuclear magnetic resonance.

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Antigenic B-cell epitope prediction

Identification of continuous was performed using BCPred tool (Table 3). Discontinuous B-cell epitopes were predicted using Elipro tool (Table 4). The best B-cell epitopes were selected according to the criteria based on cutoff values for BCPred and Elipro which were >0.8 and >0.5, respectively.

PCR amplification and cloning

The SOmp2b single fragment of 1,044 bp was successfully produced upon PCR amplification (Fig. 3A). The integrity of pET28-SOmp2b positive clones were confirmed by PCR, restriction enzyme digestion (Fig. 3B) and sequencing.

Expression and purification of the SOmp2b recombinant protein

The expression of SOmp2b recombinant protein was induced with 0.1, 0.2, and 0.4 mM IPTG at OD_{600}=0.5 for 4 hours at 37°C. The expected recombinant protein of 36.6 kDa was detected. Purification of the rSOmp2b was done by Ni-NTA affinity chromatography using denaturing method. SDS-PAGE (15%) analyzing of the lysate from the induced *E. coli* BL21 (Fig. 4A) and the purified protein (Fig. 4B) revealed the expected recombinant protein with the molecular mass of approximately 36.6 kDa. The expression yield of the purified protein was estimated by Bradford assay as 220 μg/mL. The identity of the purified SOmp2b was confirmed by western blotting (Fig. 4C).

Table 2. The list of the best SOmp2b HLA-II epitopes

HLA subtype	Start-End	Epitope	Score
HLADRB3*01:01	188-202	NYHIDGYMPDVVGGL	0.01
HLADRB1*01:01	112-126	MEFAYIQLGGLRVGI	0.04
HLADPA1*02:01/DPB1*05:01	79-93	GTLKTFTELRFNYAA	0.28
HLADPA1*01:03/DPB1*02:01	80-94	TLKTFTELRFNYAAN	0.30
HLADQA1*05:01/DQB1*02:01	211-225	IAGVVAYDSVIEEWA	0.27
HLADQA1*05:01/DQB1*03:01	203-217	KYAGGWGSIAGVVAY	0.34
HLADQA1*04:01/DQB1*04:02	161-175	FTGGNGFSAVIALEQ	0.49

SOmp2b, short form of outer membrane protein 2b.

Table 3. Prediction of continuous B-cell epitopes

Start-End	Epitope	Score
174-194	EQGGDNDGGYTGTTNYHIDG	0.997
90-111	NYAANNSGVDGKYGNETSSG	0.996
251-271	TPDQNYGQWGGDWAVWGGLK	0.995
6-26	APEPEAVEYVRVCDAYGAGY	0.994
44-64	DVKGGDDVYSGTDRNGWDKS	0.977
149-169	AGSYRTGKISYTFTGGNGFS	0.965
302-322	VPGFTVTPEVSYTKFGGEWK	0.835
120-140	GGLRVGIDESEFHTFTGYLG	0.834

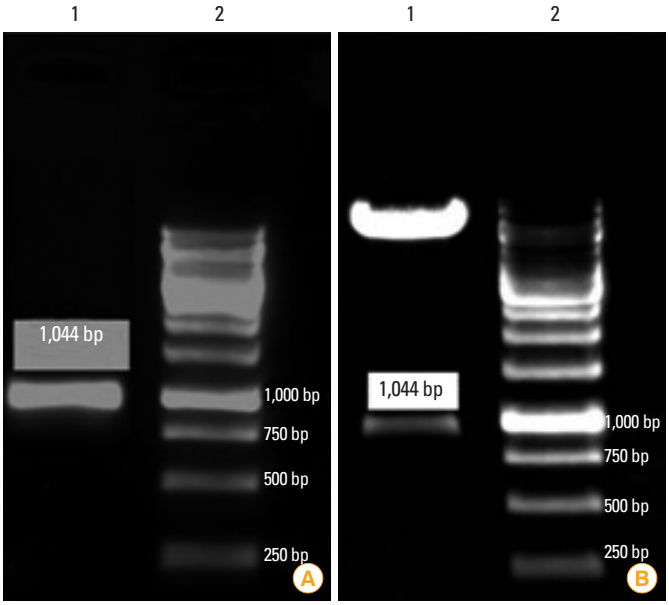

Fig. 3. (A) PCR amplification results of SOmp2b. Lane 1, PCR product; lane 2, 1 kb DNA ladder. (B) Restriction enzyme digestion of pET-SOmp2b positive clone. Lane 1, positive clone; lane 2, 1 kb DNA ladder. PCR, polymerase chain reaction; SOmp2b, short form of outer membrane protein 2b.

Table 4. Identification of conformational B-cell epitopes using Elipro tool

Residue	No. of residues	Score
A3, I4, V5, A6, P7, E8, P9, E10, A11, V12, E13, Y14, V15, R16, V17, C18, D19, A20, Y21, G22, A23, G24, Y25, F26, Y27, I28, P29, G30, T31, E32, T33, C34, L35, R36, V37, T72, G73, S74, E75, T76, E77, L78, G79, T80, L81, K82, T83, F84, Y116, I117, Q118, L119, G120, G121, L122, Y159, F161, T162, G163, G164, N165, G166, F167, Y204, A205, G206, G207, W208, G209, S210, I211, A212, G230, D231, V232, N233, I234, T235, D236, Q237, F238, S239, V240, W241, L269, Y271, Q272, A273, T274, Q275, K276, A277, F279, Y299, E300, L301, V302, P303, G304, F305, F336, F340	102	0.673
K46, G47, G48, D49, D50, V51, Y52, S53, G54, T55, N58, G59, W60, D61, Y91, A92, A93, N94, N95, S96, G97, V98, D99, G100, K101, Y102, G103, G155, L173, Q175, G176, G177, D178, N179, D180, G181, G182, Y183, T184, G185, T186, T187, N188, Y189, H190, D192, G193, Y194, M195, P196, V198, Y217, D218, S219, V220, I221, E222, E223, W224, A225, Y246, S247, S248, A249, A250, T251, D253, Q254, N255, Y256, G257, Q258, W259, G260, G261, D262, W263, A264, D286, D287, W288, G289, Y313, T314, K315, F316, G317, G318, E319, W320, N322, T323, V324, A325, E326, D327, N328, A329, W330, G331	100	0.649

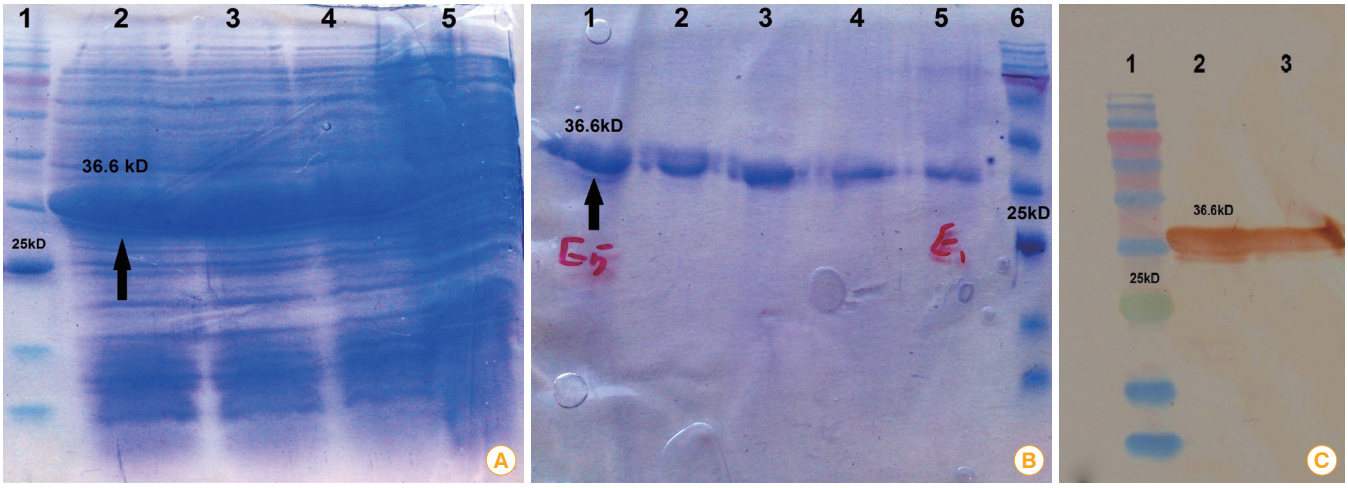

Fig. 4. (A) SDS-PAGE analysis of the SOmp2b protein expression. Lane 1, pre-stained protein marker (Vivantis); lanes 2-4, bacterial lysate of induction by different IPTG concentration (arrow shows rSOmp2b); lane 5, uninduced bacterial lysate. (B) SDS-PAGE analysis of the purified rSOmp2b protein. Lanes 1-5, purified rSOmp2b protein; lane 6, pre-stained protein marker. (C) Western blotting profile of the SOmp2b protein. Lane 1, pre-stained protein ladder; lanes 2 and 3, purified rSOmp31 protein. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-1-thiogalactopyranoside; rSOmp2b, recombinant short form of outer membrane protein 2b.

GenBank accession number

The sequence of SOmp2b has been submitted in GenBank under accession number KP071938.

Discussion

Selection and production of new vaccine candidates are the primary practical steps toward introducing new vaccines. According to studies published by Jain et al. [1] and He and Xiang [11], Omp2b is an outer membrane protein identified from stationary-phase exoproteome of *B. abortus* and considered as a potential vaccine candidate against *Brucella* infection. Bioinformatics analysis indicates that Omp2b is not present in live attenuated *B. abortus* vaccine strain 19, suggesting that Omp2b probably contributes to the attenuation of this mutant [11]. In the present study, the structure of Omp2b from *B. abortus* was analyzed using bioinformatics tools. Multiple sequence alignment of different *Brucella* Omp2b sequences shows the protein is highly conserved among *Brucella* pathogens (99%-100% sequence similarity) indicating that it could be a new vaccine target against *Brucella* infection.

Analyzing the physico-chemical parameters of the protein using protparm tools demonstrates that Omp2b is an acidic (pI, 4.53) and stable (with instability index less than 40) protein. The results of Omp2b structure analysis shows that it is a hydrophilic protein regarding its hydropathy values and hydrophilicity prediction of the protein using IEDB tools. Regarding protein secondary structure prediction, Omp2b consists of seven alpha helixes that could stabilize the protein structure. Prediction of the tertiary structure of Omp2b and evaluation of the predicted 3D model using Prosa and Ramachnadran plot indicate that the protein is with the correct topology and within the range of native proteins of the same size.

An effective vaccine candidate is one be able to induce strong B-cell and T-cell responses. Therefore, prediction of T-cell and B-cell epitopes are important approaches for designing an optimal vaccine. Prediction of human HLA-II epitopes was performed using IEDB tools and seven best epitopes were listed in Table 2. According to the prediction results, epitope ^{188}NYHIDG YMPDVVGGL^{202} is the best T-cell epitope with the highest binding affinity score for HLADRB3*01:01. Prediction of continuous B-cell epitopes using BCPred tool resulted in identification of eight epitopes among them ^{174}EQGGDNDGGYTGTTNYHIDG^{194} epitope was predicted as the best continuous B-cell epitope (score, 0.997). Parker hydrophilicity prediction tool (IEDB resource) also identified this epitope as the most hydrophilic peptide with the maximum score of 7.457. Moreover, all the predicted continuous B-cell epitopes were located in the surface accessible hydrophilic regions of the protein structure. Since strong T-cell and continuous B-cell epitopes confer protection against infection, they could be evaluated experimentally as new anti *Brucella* vaccine targets. Additionally, identification of conformational B-cell epitopes has been done using Elipro tool. Two potential discontinuous epitopes were predicted that can be used

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in designing *Brucella* novel immunodiagnostic tests and antibody production.

In order to *in vitro* express the SOmp2b, the cleavage site of signal peptidase was predicted SignalP Server and the SOmp2b coding sequence (KP071938) was amplified using specific primers and then cloned in pET28a expression vector. The recombinant protein was successfully expressed upon induction with different concentrations of IPTG and the yield of rSOmp2b was significantly high (220 μg/mL). The expected recombinant protein with the molecular mass of approximately 36.6 kDa was detected by SDA-PAGE and western blotting revealing the identity of the expressed protein.

Our data indicates that Omp2b protein has a potential to induce both B-cell– and T-cell–mediated immune responses and it can be evaluated as a new subunit vaccine candidate against brucellosis. Further studies evaluating the immunogenicity and protective efficacy of the SOmp2b against *Brucella* pathogens in mice model are underway in our lab.

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