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Original Article

Production of *Brucella lumazine* Synthase Recombinant Protein to Design a Subunit Vaccine against Undulant Fever

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ABSTRACT

Brucella bacterium causes Brucellosis, an infectious disease spreading from animals to human. *Brucella lumazine* synthase (BLS) is a highly immunogenic protein with adjuvant properties, which has been introduced as an effective protein carrier for vaccine development. This protein also plays a significant role in inducing immune system. This study aimed to clone, express, and purify the BLS gene from *Brucella melitensis* Rev1. The BLS gene was amplified by particular primers with the restriction enzyme sites as a linker and it was inserted into pTZ57R/T vector. Subsequently, it was ligated into pET32(a)+ expression vector. Recombinant expression vector containing coding sequence of BLS was transformed into *E. coli* BL21 (DE3) host gene expression and stimulated by 0.1mM IPTG. The results of sequencing showed that there were not any mutations in BLS encoding sequence. The expression results were set by sequencing and endorsed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses and western blotting that showed 35 kDa protein band appropriately.

Keywords: *Brucella melitensis* Rev1, BLS, Gene Expression, Recombinant Protein

Production de la Protéine Recombinante *Brucella lumazine* Synthase Afin de Concevoir un Vaccin Sous-unitaire contre la Fièvre Ondulante

Résumé: La bactérie *Brucella* est à l'origine de la Brucellose, une maladie infectieuse se transmettant de l'animal à l'homme. La *Brucella lumazine* synthase (BLS) est une protéine hautement immunogène dotée de propriétés adjuvantes, qui a été présentée comme un vecteur efficace de transport de protéines pour le développement de vaccins. Cette protéine joue également un rôle important dans l'induction d'une réponse immunitaire. Cette étude visait à cloner, exprimer et purifier le gène *BLS* de *Brucella melitensis* Rev1. Le gène BLS a été amplifié par des amorces spécifiques avec les sites d'enzyme de restriction servant de liant afin d'etre inséré dans le vecteur pTZ57R / T. Ensuite, il a été ligaturé dans le vecteur d'expression pET32(a)+. Le vecteur d'expression recombinant contenant la séquence codante de BLS a été transformé dans le système d'expression génique *E. coli* BL21 (DE3) et stimulé par 0.1 mM d'IPTG. Les résultats du séquençage ont montré qu'il n'y avait pas de mutations dans la séquence codant pour *BLS*. L'expression de la protéine recombinante a été vérifiée par séquençage ainsi qu'à l'aide d'analyses électrophorèse sur gel de dodécyl sulfate de sodium-polyacrylamide (SDS-PAGE) et d'un transfert de type western montrant une bande protéique de 35 kDa de manière appropriée.

Mots-clés: *Brucella melitensis* Rev1, BLS, Expression génique, Protéine recombinante

INTRODUCTION

Brucellosis is a commonly neglected zoonotic disease provoking abortion, reduced fertility, and undulant fever in animals as well as arthritis and osteomyelitis in humans (Zhao et al., 2009; Sekhavati et al., 2015; Yousefi et al., 2016). In fact, *B. abortus*, *B. melitensis*, and *B. suis* are responsible for most cases of Brucellosis (Pappas et al., 2006; Franco et al., 2007). Currently, immunization against Brucella infections is usually accomplished using live attenuated Brucella bacteria strains, such as *B. abortus* S19, *B. melitensis* Rev.1, and *B. abortus* RB51 (Corbel, 2006). However, live attenuated vaccines have some problems for both animals and humans, including abortion in immunized pregnant animals, response to human pathogen, and interference with the lipopolysaccharides-based serological tests (Cassataro et al., 2005; Yousefi et al., 2016). Over recent years, various types of subunit vaccines, including Om25, Omp31, GroEL, and Omp19 recombinant antigens with protective immunity against infectious organisms have been developed although most of them induced poor immunity (Vahedi et al., 2011; Azimi et al., 2012; Ghasemi et al., 2013). The *Brucella lumazine* synthase (BLS) is a highly immunogenic protein with adjuvant properties that has been introduced as an effective protein carrier to enhance cellular response for vaccine development (Mejias et al., 2013). In addition, this protein is effective in stimulating immune response, and it has been extensively used as a carrier for antigen presentation to the immune system for vaccine development, such as BLS-OMP31 antigen (Rossi et al., 2015). In this regards, the present study aimed to conduct cloning, expression, and purification of BLS antigen from *B. melitentis* Rev1 to develop a subunit vaccine.

MATERIAL AND METHODS

DNA extraction. In order to extract bacterial DNA, B. melitensis strain Rev1 was obtained from the Brucella culture collection of Razi Vaccine and Serum Research Institute, Mashhad, Iran, and cultured as described in a study conducted by Delpino et al. (2007). Bacterial genome was, then, extracted using a commercial kit and visualized by gel electrophoresis (Bioneer, Korea). The quantity of the extracted DNA was determined by NanoDrop ND-100 spectrophotometer (Thermo, USA).

The polymerase chain reaction amplification. To perform polymerase chain reaction (PCR), genomic DNA of *B. melitensis* strain Rev1 was used as a template for amplification of BLS coding sequence, using EX-Taq DNA polymerase enzyme (Takara, Japan). The accessible nucleotide sequences on the NCBI GenBank database (KJ401344.1) was utilized to design the specific primers (Table 1) with restriction site as a linker (underlined) using Primer Premier5 software. The Personal Cycler™ thermocycler (Biometra, Germany) used for BLS amplification with the reaction mixture at a final volume of 25 µl, containing 2.5 µl of 10X PCR buffer, 2 µl MgCl2 (50 mM) and 2 µl dNTPs (20 mM), 1 µl of the DNA solution (50 to 100 ng/µl), 1.5 µl of mix primer (5 pmol/µl), 0.125 U/µl of EX-Taq DNA polymerase, and was complemented with some deionized water. The PCR program was conducted with an initial denaturation step at 94 °C for 10 min followed by 20 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 30 sec, and a final extension at 72 °C for 10 min.

Table 1. Primer sequences with restriction enzymes

Gene Symbol	Specific Primers	Restrictin enzyme	Size (bp)
BLS	F:5`-CCATGGCAATGAACCAAAGCTG-3` R:5`-AATTCTTATCAGACAAGCGCGG-3`	*NcoI* *EcoRI*	482

Cloning and bacterial strains. Amplicon was confirmed by agarose gel electrophoresis, and then PCR program was repeated in 100 µl volume. According to the instruction of Ron's Agarose Gel Mini prep Kit manufacturer (BioRon, Germany) the amplicon was purified from the 0.8% agarose gel, and ligated into pTZ57R/T cloning vector using T/A cloning strategy (PCR Product Cloning Kit, Mehrsun

Biotech, Iran). Competent cell preparation and transformation steps were followed as described in a study conducted by Maniatis et al. (1982). The recombinant vector was transformed into E. coli Top10 (F´) (Novagen, USA). Consequently, recombinant clones containing plasmid DNA with the insert were selected based on their ampicillin resistance. The fidelity of E.coli Top10 (F´) transformants harboring recombinant vector was endorsed using M13 universal primers. The plasmids were purified by the Ron's Plasmid Mini Kit (BioRon, Germany) and authenticated using NcoI and EcoRI (Thermo, USA) restriction enzyme digestion. The purified plasmids were subjected to sequencing (Bioneer, South Korea). The obtained nucleotide sequences were analyzed by homology search and BLS sequence alignment was conducted using Basic Local Alignment Search Tool (BLAST) and CLC Main Workbench 5.5 software, respectively.

Expression and purification. For the expression, BLS open reading frame was digested by NcoI and EcoRI restriction enzymes. The excised fragments were purified from agarose gel using commercial Kit (BioRon, Germany). The BLS fragments sub-cloned in pET32 (a) + vector (Novagen, USA), and subsequently was transformed into E.coli BL21 (DE3) expression host strain (Novagen, USA). The harboring transformed bacteria was spread on LB agar containing 50 µg/mL ampicillin (Sigma, Germany) cultivation and incubated at 37 °C overnight. One positive BL21 clone was selected using colony PCR with T7 universal primers. The presence of BLS coding sequence was confirmed by restriction endonuclease analysis. The positive colonies were cultured in selective LB medium at OD = ~ 0.6. Moreover, IPTG (isopropyl β -D-thiogalactoside) was added at the final concentration of 0.1mM to induce the expression of the BLS protein at 37°C. Harvest cells were suspended and lysed using lysis buffer and sonication (Yousefi et al., 2016). Cell lysate, was centrifuged at 9000 g for 15 min at 4 °C to separate the supernatant containing soluble materials from the pellet. To analyze the expression of recombinant BLS both the supernatant and the pellet were evaluated on SDS-PAGE 10% (upper gel was 8% and lower gel was 10%). The expressed protein was purified using Ni-agarose chromatography (Thermo, USA) from the insoluble phase of lysate using guanidine hydrochloride 6 M to dissolve the pellet. The quality and identity of the recombinant BLS protein was analyzed using SDS-PAGE (10%) and western blotting assay, respectively. For western blotting, the SDS-PAGE gels were electroblotted onto nitrocellulose. Afterwards, the blotted nitrocellulose was blocked with skim milk for 3 h. The membranes were washed 3 times and then Anti Poly-Histidine-HRP (Sigma, 1:2000 diluted in BSA 1%) was added. For visualization, the incubation was at room temperature, and membranes were washed with diaminobenzidine (DAB) as chromogen an hour later. Finally, the quantity of the recombinant protein was estimated using Bradford assay (Yousefi et al., 2016). The purified recombinant BLS protein was stored at -20 °C for further studies.

RESULTS

The candidate gene was successfully amplified and its accuracy and purity were visualized on agarose gel electrophoresis (Figure 1). Consequently, the amplified products were successfully ligated into pTZ57R/T cloning vector and transformed into *E. coli* Top10 (F´) competent cells. The integrity of the positive colonies was also confirmed by restriction digestion, after the selection of positive colonies harboring recombinant plasmids using Colony-PCR, (Figure 2). As can be seen in Figure 1, the recombinant plasmids sequence was confirmed by both specific and universal M13 primers and analyzed using BLAST and CLC Main Workbench 5.5 software for controlling the mutations. There were no mutations in the amplified gene. The TA vector containing BLS coding sequence (pTZ-BLS) and pET32(a)+ expression vector was digested with the same restriction enzymes for producing sticky ends

(Figure 2). After gel extraction, purified BLS coding sequence fragment was correctly ligated into pET32 (a)+ plasmid and transformed into BL21 (DE3).

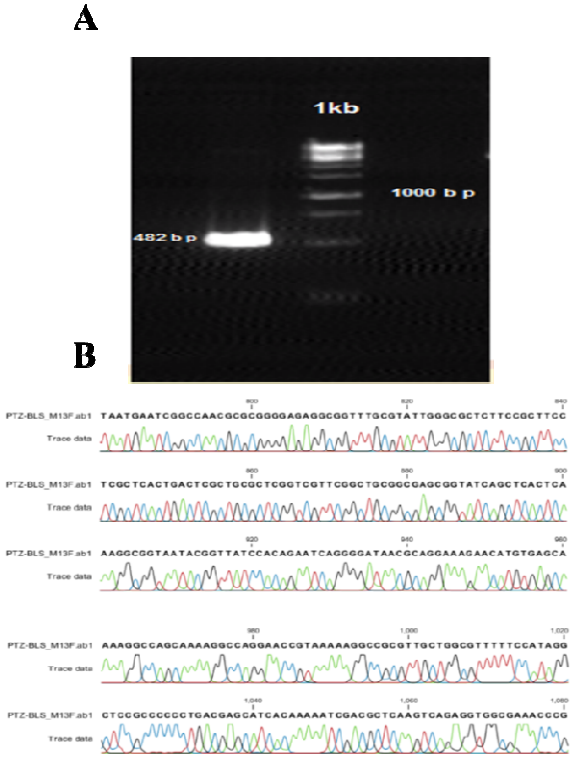

Figure 1. (A) PCR amplification of BLS coding sequence on agarose gel 1%. (B) The result of partial sequencing for BLS gene.

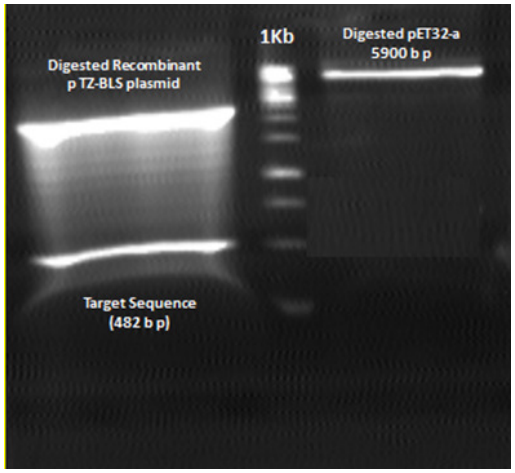

Figure 2. Result of digestion for pTZ – BLS (left column of 1Kb ladder) and pET32 vectors (right column of 1Kb ladder) using NcoI and EcoRI restriction enzymes.

Positive colonies harboring recombinant expression vector were picked up using colony-PCR and were conferred successfully by restriction enzymes digestion. A selected colony was cultured and induced with 0.1 mM IPTG at 37 ºC and sampling was carried out several times. The products were visualized by SDS-PAGE gel staining and the expected purified BLS protein with the size of 35 kDa was observed after purification and western blotting analysis (Figure 3, A). The expression vector used in this study was pET32 (a)+, that adds Trx•Tag™ with about 17 kDa weight to the protein of interest. The band approximately 35 kDa band indicated that BLS protein with the weight of 17 kDa was expressed correctly (Figure 3, B). The concentration of protein was ~ 80 mg/ml.

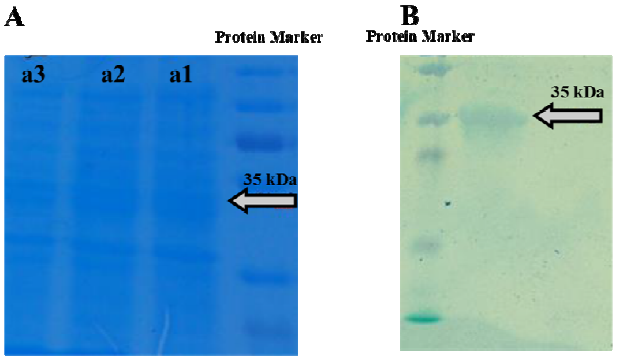

Figure 3. (A) SDS-PAGE analysis of the recombinant protein. Line a3: total cell lysate of E. coli BL21 (DE3) showing the expression of before induce; Lines a2 and a1: showing the expression after inducing by 0.1 mM IPTG (sampling after 1 and 2 hours). (B) Western blotting profile of the recombinant BLS protein with the molecular mass of around 35 kDa. Ladder is a pre-stained protein marker size (Thermo, USA).

DISCUSSION

Recently many efforts have been made to find new immunogens in Brucella using immune approaches. However, not all of these new targets showed in vivo protective efficacy (Yang et al., 2013). Selection and production of new vaccine candidates are the primary practical steps toward introducing new vaccines. Hemmen et al., 1995 introduced an 18-kDa protein as BLS and developed a capture ELISA based on the monoclonal antibody specific for this protein. Another

study by Goldbaum et al., 1993 showed the potential effectiveness of this protein for the serological diagnosis of Brucellosis. BLS as Brucella`s antigen has been shown to confer partial protection against B. abortus as a DNA vaccine or as recombinant protein, and it is also a potent activator of bone marrow dendritic cells (Rosas et al., 2006). When another antigen is covalently attached to BLS, it has adjuvant properties such as BLS-OMP31 antigen (Rosas et al., 2006). Furthermore, BLS has been defined to be an antigen delivery system to induce oral immunity (Rosas et al, 2006; Cassataro et al, 2007). Also, Velikovsky et al., (2003) found BLS stimulated Th1-Th2 immune response and improved protection against B. abortus 544. It also showed that confer potent immune responses as used in chimeric form with another antigen (Clausse et al., 2013). They showed recombinant chimeric BLSOmp31 could be a suitable candidate for developing of a subunit vaccine against B. canis, since it elicits antigen-specific immune responses and conferred protection in infected mice. Also, Estein et al., (2009) and Diaz et al., (2013) determined a sufficient protection against B. ovis using BLSOmp31. In this study, BLS antigen was designed and produced as a primary step toward introducing new vaccines in order to stimulate immune system against Brucella infectious disease. Sequencing of the cloning product confirmed the integrity of the cloning. The plasmid constructs pET32a- BLS was transferred into expression host containing T7 RNA polymerase. Successfully induced expression by different concentration of IPTG and high level production of the fusion demonstrated the high efficiency of our fusion construct. BLS antigen phylogenetic analysis, in our previous study, showed that selected gene was nearly similar in different Brucella species (Tahmoorespur et al., 2016). Moreover, Characterization of vaccine properties and the evaluation of immune responses of this recombinant antigen against *B. melitensis* infection in mice has already initiated in our laboratory.

In conclusion, the recombinant antigen produced in the present study could be feasible both as a candidate antigen with partial protection against *B. melitensis* and as proper adjuvants for recombinant subunit vaccine.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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