JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2011. Vol. **5**(2), p. 611-616

Cloning, Expression and Purification of Recombinant 31kDa Cell Surface Protein of *Brucella melitensis* 16M

Liela Azimi[1,2], Nima Khoramabadi[3], Ashraf Mohabati Mobarez[3], Haniyeh Aghababa[3], Amir Bakhtiari[1], Esmaeil Asli[1] and Abdolaziz Rastegar Lari[2]*

[1]Depatment of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran.
[2]Anti-Microbial Resistance Reaserch Center,
Tehran University of Medical Sciences, Tehran, Iran.
[3]Department of Bacteriology, Tarbiat Modares University, Tehran, Iran.

(Received: 13 May 2011; accepted: 02 August 2011)

Brucellosis is an important zoonotic disease of economic significance. *Brucella* species are gram-negative, facultative intracellular bacteria, and are capable of replicating in the phagosomes of macrophages. They cause infection in several animal species and humans. Prevention of the disease is necessary for eradication of human and cattle infection. Characterization and evaluation of different antigens of *Brucella* cells has a key role in progression of prevention programs. Here, we report the production and purification of recombinant 31kDa cell surface protein *Brucella melitensis* (BCSP31).

In Tarbiat Modares University Laboratory, *Brucella* 31kDa cell surface protein gene was amplified with PrimeSTAR® HS DNA polymerase, cloned in pJET1.2. The target gene was subcloned in pET28a (+). Recombinant pET28a vectors were transformed into *E coli* BL21 (DE3). Expression of recombinant protein was induced with 1mM IPTG and recombinant protein was purified by Ni-NTA agarose resins. Recombinant proteins were eluted with 250mM imidazol. Imidazol removed by dialysis. Proteins were assayed by Western-blotting and rBCSP31 was probed by *Brucella* rabbit anti serum. Purified protein conjugated to detoxify LPS of *Brucella*. This complex injected to BALB/c mice. Percentage of clearance and log unit protection in injected mice showed the significant protection against colonization of *Brucella melitensis* in spleen of mice. BCSP31 were successfully cloned, expressed and purified. The recombinant proteins were conjugated to detoxify LPS. Injections of this component can protection of mice against colonization in spleen of challenge strain.

Key words: Brucellosis, Recombinant 31kDa cell, PCR.

Brucellosis is one of important zoonotic disease and the incidence of brucellosis in livestock and wildlife is of great economic concern due to decreased productivity, increased numbers of abortions and weak offspring[1]. Human infections are commonly acquired through the consumption of untreated milk and another dairy products[1]. Microorganisms belonging to the genus *Brucella* are gram-negative, facultative intracellular, nonspore-forming cocobacilli, and are capable of replicating in the phagosomes of macrophages[2]. They cause infection in several animal species and humans[2,3]. Prevention of the disease and opportune diagnosis of new cases are essential for eradication of human and cattle infection. Characterization and evaluation of different antigens of *Brucella* cells has a key role in progression of this aim.

* To whom all correspondence should be addressed.
Tel.: 82943183; Fax: 82943183
E-mail: azizlari@gmail.com

Identifying the antigens of the etiological agent that are able to stimulate the host defensive is so important in infection diseases such as brucellosis[4]. Bacterial outer membranes are composed of different components though proteins have more important role to stimulate immune response[1]. Surface proteins of *Brucella* contains outer membrane proteins (OMPs) and Brucella cell surface proteins (BCSPs) are more attractive for researchers because of their immunogenic characterization[3]. Cloning, expression and evaluation of these Brucella proteins can help to find new methods in diagnosis and serum evaluation of immune response. This method may confirm the advantage of OMPs and BCSPs in eliciting the immune responses[5].

BCSP31 is one of the immunogenic proteins in *Brucella* cells. BCSP31 can induce immune responses that are detectable in immunogenic tests and therefore it would be useful to induct immune system for different aims[6].

Here, we cloned, expressed, purified, and evaluated *Brucella* cell surface protein 31kDa (BCSP31), which is one of immunogenic *Brucella* proteins and it also is highly conserved with each known species and biovar. Then, recombinant proteins were conjugated to detoxify LPS and injected to BALB/c mice for surveillance of induction of protection.

Gene of BCSP31 was cloned and expressed in pET28a (+) bacterial expression system and conjugated to detoxify LPS.

MATERIAL AND METHODS

Bacterial strains, media, and growth conditions

B. melitensis 16M was routinely cultured on *Brucella* agar at 35°C for 72 hours. *E. coli* DH5á were used as non-expression host and *E. coli* BL21 (DE3) were selected as expression host. *E. coli* strains were cultured in Luria-Bertani broth (LB) (Liofilchem) or LB agar When antibiotic selection was needed Ampicillin (at 50 µg ml^{-1}) (Sigma) and Kanamycin was added to the agar and broth (at 30µg ml^{-1}) (Sigma).

PCR amplification

Bacterial DNA from cultures of *B. melitensis* 16M grown 72 hours was extracted *(AccuPrep*® DNA Extraction Kit, Bioneer). The region encoding the *Brucella* 31 kDa cell surface protein (BCSP31) (AE008917), consisting of 990bp. A primer complementary to the 5' end of BCSP31 gene 5' ACTGGATCCATGAAATTCGGAAGCAAAATCC 3' was designed to contain a *Bam*H1 restriction site (underlined). The reverse primer, 5'AATCTCGAGTTATTTCAGCACGCCCGCTTC 3' with *Xoh*1 restriction site (underlined). Primers were designed based on the nucleotides sequence of the BCSP31 gene from *B. melitensis* 16M (AE008917). BCSP31 gene was amplified with Primestar®HS DNA polymerase (TaKaRa) which has high fidelity. PCR was conducted in a final volume of 25 µl that contained 0.75 µl of [1]50µM dNTP, 5µl of 5x buffer, 0.1 µM of each forward and reverse primers, o.15 µl of Primestar®HS DNA polymerase, 1 µl of *B. melitensis* genome and 17.25 µl of PCR water. The PCR program was designed as 1 cycle of 98°C for 3-5 min; 30 cycles of 98°C for 10 s, 60°C for 10 s and 72°C for 1 min; and 1 cycle of 72°C for 5-10 min then refrigeration at 4°C.

Cloning of BCSP31 gene

The amplified blunt-ended BCSP31 gene from *B. melitensis* 16M was directly cloned into pJET1.2/blunt (clone JET® PCR cloning kit, Fermatase) as entry vector. Multiple cloning sit (MCS) exist in pJET1.2/blunt eco47IR region and allows better screening for cloned plasmid. Recombinant pJET1.2 was transformed chemically into competent *E. coli* DH5á. Recombinant plasmids were extracted by *AccuPrep*® Plasmin Mini Extraction Kit (Bioneer) and examined by restriction digestion and sequenced. One positive entry plasmid was selected for ligation into destination vector pET28a (+) (Novagen) which was digested by *Bam*H1 and *Xho*1.Prior to this step the fragment inserted between *Bam*H1 and *Xho*1 sites in pET28a (+) followed by conventional assays such as PCR and restriction digestion.

Expression of BCSP31 in *E. coli*

Recombinant pET28a (+) transformed into competent expression host *E.coli* BL21 (DE3). Expression was induced with 1mM IPTG. Expression was checked, at hourly intervals up to 4 hours after induction. Induced cells were analyzed on SDS-PAGE mini gels using slab gels of 12.5% polyacrylamid in a Bio-Rad. Samples were prepared by suspension of the bacterial pellet in sample buffer containing 10 ml of stacking gel buffer, 5 ml

Glycerol, 1 g SDS, 0.2 ml boromophenol solution (0.5% in ethanol), and 1 ml 2-mercaptoethanol (2-ME) in a final volume of 20 ml in distilled water. And heating for 5 min at 100°C, finally 20 µl of each sample was run. Followed by stains with Commassi Brilliant Blue G-250.

Purification

Recombinant protein was purified via denaturation procedure using 8M urea to dissolve proteins which are present as inclusion bodies. Urea-soluble N-terminal 6His-taged proteins were absorbed to Ni-NTA agarose resins and eluted with 250mM imidazol. Imidazol removed by dialysis. Proteins were assayed by western-blotting and rBCSP31 was probed by *Brucella* rabbit anyi serum.

Western blot analysis of the expressed recombinant proteins

The tank electroblotting transfer of proteins from the polyacrylamide gel to a nitrocellulose membrane was carried out using Tris/glycine buffer with 20% methanol. Non –specific sites were blocked with tween 20 (0.05-0.1%). The membranes were washed three times with TBS-T, and then suspend 1-2 hours in antibody (anti-6-His conjugated with peroxidase). The membranes were washed three times with TBS-T and the reaction was developed with DAB/H_2O_2 (5 mg ml^{-1}) substrate solution and hydrogenperoxide 1% in TBS (pH 5.7) . On appearance of a golden brown band at the site of reaction, rinsed the membranes in distilled water stopped the enzymic reaction, and the results were read visually after the nitrocellulose membranes dried completely.

RESULTS

Genome DNA prepared from *B. melitensis* was use as template in the PCR by using Primestar®HS DNA polymerase. Results show that single band with correct molecular weight has been amplified for BCSP31 gene. [2]

Cloning of the *B. melitensis* 16M BCSP31 gene

Screening of the recombinant pJET1.2 plasmids were carried out with restriction digestion and sequenced (Fig. 1)[3].

Insertions were excised from recombinant pJET1.2 with positive results and subcloned into pET28a (+). Cloned plasmids were examined with restriction digestion and colony PCR.

Expression of BCSP31 in *E. coli*

Expression of induced cells was checked, at hourly intervals up to 4 hours after induction with 1mM IPTG (Fig 2).

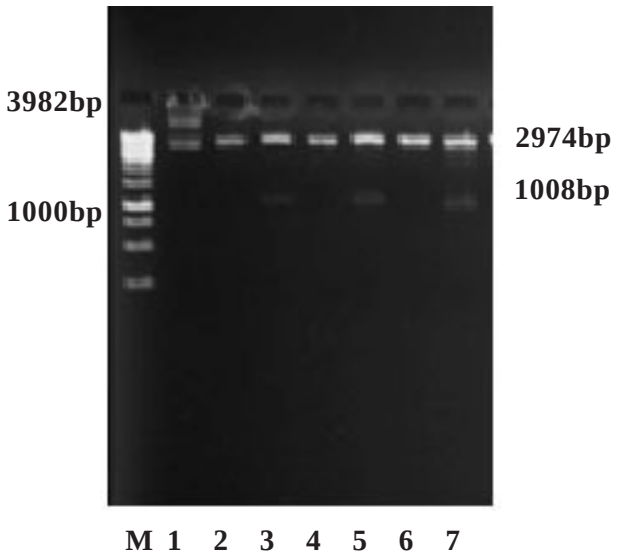

Fig. 1. Double digestion and mono digestion of recombinant pJET1.2
(M: 1kb DNA ladder, 1: undigested recombinant pJET1.2, 2-4-6:
mono digested recombinant pJET1.2, 3-5-7: double digested recombinant pJET1.2

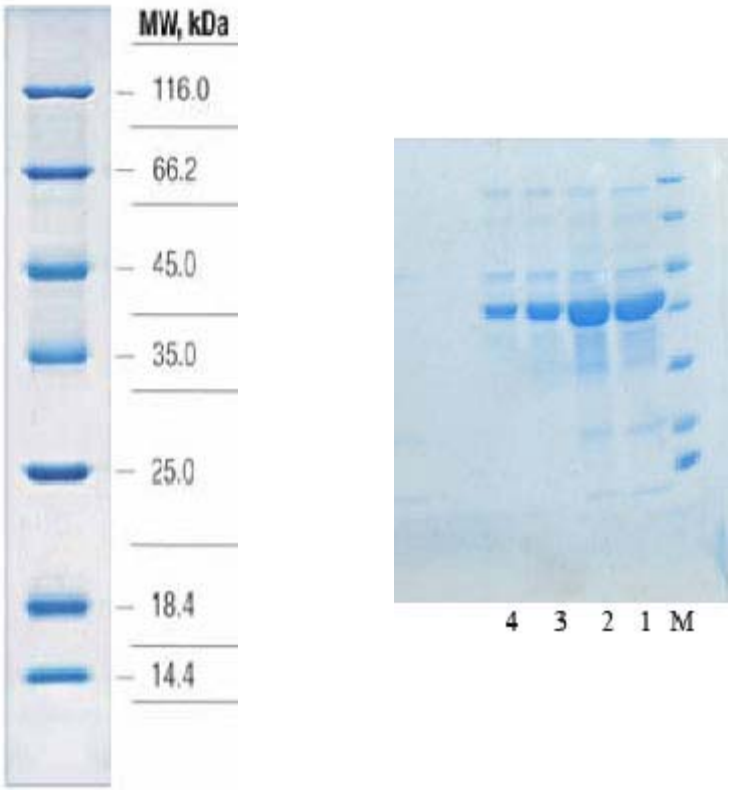

Fig. 2. Expression was checked, at hourly intervals up to 4 hours after induction. BCSP31 molecular weight has been changed because additional nucleotides were designed in primers for enzyme restriction sites. (M: marker, 1: 1 hour, 2: 2 hours, 3: 3 hours, 4: 4 hours) SDS-PAGE showed best expression after 3-4 hours

Fig. 3. Western blotting of BCSP31[1]

Western blotting

Purified recombinant proteins were analyzed with Western blotting after dialysis (Fig 3).[4]

DISCUSSION

Brucellosis is a widespread zoonotic disease that can infect variety of livestock and wildlife[10]. This disease is endemic in many countries and it leads to economic losses[10]. Producing of an effective human vaccine and improved animal vaccines and diagnostic tests would significantly assist prevention of brucellosis[3].

Brucella standard vaccines which used generally are *Rev*1 and *S*19.They are live attenuated bacteria strains. These vaccines have some problems, such as weak induced immunity and have potential of human infection[11-13]. Based

on this data, the discovery of immunogenic bacterial constituent is vital component of subunit vaccine and diagnostic development process[3]. Surface proteins of the bacteria have been reported to play important role during *Brucella* infection and inducing immune response. Generally, successful vaccines and diagnostic test against their bacteria primarily induce antibodies against surface structure. These surface proteins of the bacteria have been thought of as useful antigens for development of both diagnostic reagent and vaccine candidates[3].

Naturally preparation proteins of *Brucella* need high amount of bacterial culture and purification of them costs a lot. Producing process is difficult and dangerous. For these reasons, we obtained BCSP 31 from recombinant technique that is able to prepare amount of target protein.

In the preset study, we cloned and expressed BCSP 31. This protein has potential to stimulate immune responses and can be assayed in serum evaluation to detect disease.

The gene of BCSP31 was PCR synthesized and cloned into the pET28a (+) expression system. The system provides a benefit for optimal expression of this expression of *Brucella melitensis* 16M membrane proteins using alternative destination vectors for different purposes. For expression of BCSP31 gene pGEX-4T-1 vector was used in another study and the results as well as pET28a (+) that was used in our research has better expression[14] . Another researcher used pETDEST42 as a expression vector (Invitrogen) for *Brucella* Omps [3] and the results were satisfied. These expressed recombinant proteins have been designed to be fused with 6-His tags at their N terminal. In this study, all expressed proteins were confirmed by SDS-PAGE. Purify recombinant proteins with Ni-NTA agarose resins prepared us truth folding proteins. Purified proteins checked by western blotting. This recombinant surface protein will be further examined in vivo model to test their immune stimulation.

ACKNOWLEDGEMENTS

Authors thank Mr. M. Nejati for extraction of LPS of Brucella.

REFERENCES

1. Thavaselvam, D., *et al.*, Cloning and expression of the immunoreactive Brucella melitensis 28 kDa outer-membrane protein (Omp28) encoding gene and evaluation of the potential of Omp28 for clinical diagnosis of brucellosis. *J Med Microbiol*. **59**(Pt 4): p. 421-8.
2. Vizcaino, N., *et al.*, Cloning, nucleotide sequence, and expression of the Brucella melitensis omp31 gene coding for an immunogenic major outer membrane protein. *Infect Immun*,. 1996; **64**(9): p. 3744-51.
3. Ding, X.Z., *et al.*, Cloning, expression, and purification of Brucella suis outer membrane proteins. *Protein Expr Purif*,. 2005; **40**(1): p. 134-41.
4. Gonzalez, M., *et al.*, Cloning, expression and immunogenicity of the translation initiation factor 3 homologue of Brucella abortus. *Immunobiology*, 2009; **214**(2): p. 113-20.
5. Cloeckaert, A., *et al.*, Major outer membrane proteins of Brucella spp.: past, present and future. *Vet Microbiol*,. 2002; **90**(1-4): p. 229-47.
6. Halling, S.M., *et al.*, Deletion of the BCSP31 gene of Brucella abortus by replacement. *Infect Immun*, 1991. **59**(11): p. 3863-8.
7. Bowden, R.A., *et al.*, Outer-membrane protein- and rough lipopolysaccharide-specific monoclonal antibodies protect mice against *Brucella ovis*. *J Med Microbiol*,. 1995; **43**(5): p. 344-7.
8. Cloeckaert, A., *et al.*, Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of Brucella. *J Med Microbiol*,. 1991; **34**(3): p. 175-80.
9. Jacques Dornand, A.G., Virgine Lafont,Janny Liautard, Jane Oliaro, Jean-Pierre Liautard. The innate immune response against Brucella in humans *Veterinar Microbiology*, 2002; **90**: p. 383–394.
10. Araceli Contreras-Rodriguez1, Mohamed Naguieb Seleem, Gerhardt G. Schurig, Nammalwar and S.M.B.A.L.-M. Sriranganathan, Cloning, expressionand characterization of immunogenic aminopeptidaseN fromBrucellamelitensis. *FEMS Immunol Med Microbiol*,: 2006; p. 252-256.
11. J.M.Blasco, A review of the use of B.melitensis Rev 1 vaccine in adult sheep and goats. *Prev. Vet . Med*,: 2003; p. 275-283.
12. Meeting, R.o.W., The Development of New / Improved Brucellosis vaccines. Report of WHO

Meeting, WHO / EMC/ZDI/ Geneva, Switzerland, 1997.
13. Wilson, T., *Brucella SPP.*, in Topley & wilson Microbiology & Microbiali Nfections. 2005; p. 1719-1745.
14. CAO Xiao-an, Q.C.-q., ZHOU Ji-zhang,LIN Guo-zhen, High-Dissolved Expression of BCSP31 Gene of Brucella abortus in *E. coli Journal of Jilin Agricultural University*, 2007.
15. Fang, C.M., Cloning, expression, and purification of recombinant protein from a single synthetic multivalent construct of Mycobacterium tuberculosis. *Protein Expr Purif*,. 2006; **47**(2): p. 341-7.
16. Grover, A., Expression and purification of the Mycobacterium tuberculosis complex-restricted antigen CFP21 to study its immunoprophylactic potential in mouse model. *Protein Expr Purif*,. 2006; **48**(2): p. 274-80.