



Immunological characteristics of outer membrane protein omp31 of goat *Brucella* and its monoclonal antibody

W.Y. Zheng^{1*}, Y. Wang^{2*}, Z.C. Zhang¹ and F. Yan¹

¹Department of Infectious Disease, The 253th Hospital of P.L.A., Hohhot, China

²Department of Pharmacy, Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China

*These authors contributed equally to this study.

Corresponding authors: W.Y. Zheng / Y. Wang

E-mail: zhengwydid@126.com / wang_yi237@163.com

Genet. Mol. Res. 14 (4): 11965-11974 (2015)

Received January 14, 2015

Accepted June 9, 2015

Published October 5, 2015

DOI <http://dx.doi.org/10.4238/2015.October.5.10>

ABSTRACT. We examined the immunological characteristics of outer membrane protein omp31 of goat *Brucella* and its monoclonal antibody. Genomic DNA from the M5 strain of goat *Brucella* was amplified by polymerase chain reaction and cloned into the prokaryotic expression vector pGEX-4T-1. The expression and immunological characteristics of the fusion protein GST-omp31 were subjected to preliminary western blot detection with goat *Brucella* rabbit immune serum. The *Brucella* immunized BALB/c mouse serum was detected using purified protein. The high-potency mouse splenocytes and myeloma Sp2/0 cells were fused. Positive clones were screened by enzyme-linked immunosorbent assay to establish a hybridoma cell line. Mice were inoculated intraperitoneally with hybridoma cells to prepare ascites. The mAb was purified using the n-caprylic acid-ammonium sulfate method. The characteristics of mAb were examined using western blotting and enzyme-linked immunosorbent assay. A 680-base pair band was observed after polymerase chain reaction. Enzyme digestion identification and sequencing showed that the pGEX-4T-1-omp31

prokaryotic expression vector was successfully established; a target band of approximately 57 kDa with an apparent molecular weight consistent with the size of the target fusion protein. At 25°C, the expression of soluble expression increased significantly; the fusion protein GST-omp31 was detected by western blotting. Anti-omp31 protein mAb was obtained from 2 strains of *Brucella*. The antibody showed strong specificity and sensitivity and did not cross-react with *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, or *Bacillus pyocyaneus*. The pGEX-4T-1-omp31 prokaryotic expression vector was successfully established and showed good immunogenicity. The antibody also showed strong specificity and good sensitivity.

Key words: *Brucella*; Expression; omp31; Outer membrane protein; Purification

INTRODUCTION

Brucella is an intracellular parasitic Gram-negative bacterium that causes brucellosis in many types of animals and humans (Jahans et al., 1997). Brucellosis is one of the most prevalent animal-borne diseases worldwide (Mayfield and Betsy, 1988). Brucellosis not only severely harms human health but also strongly influences the development of animal husbandry. In recent years, with the combined application of rifampicin, streptomycin, and vibramycin, the cure rate of human brucellosis has improved significantly, but there are still reports regarding the prevalence and death cases of brucellosis (Doganay and Aygen, 2003).

The *Brucella* cell membrane (Checa et al., 2014) is comprised of 3 layers; the innermost layer is the cytoplasmic membrane, the intermediate layer is the peripheral cytoplasmic membrane, and the outermost layer is the outer membrane. The outer membrane closely binds to (Farshad et al., 2002; Turner et al., 2014) the peptidoglycan layer to constitute the cell wall containing lipopolysaccharide, protein, and the phospholipid layer. *Brucella* can be divided into 2 types, smooth (S) and rough (R), according to presence or absence of the O-chain in lipopolysaccharide (LPS) (Yuan et al., 2014). The S-type *Brucella* LPS contains O-chains, in which most of the antigenic sites on the *Brucella* surface are present; the R-type *Brucella* LPS lacks O-chains. Protein in the outer membrane is also referred to as outer membrane proteins (OMPs) (Sun et al., 2013; Guo et al., 2014). The OMP cannot be sufficiently exposed because the surface of the S-type *Brucella* is covered by LPS. The OMP can be exposed on the surface of the R-type *Brucella*, which lacks O-chains. *Brucella* OMPs show very strong immunogenicity, which may be associated with the survival of *Brucella* in macrophages (Bowden et al., 1998). Three families of *Brucella* OMPs have been identified: OMP2 family, OMP25/OMP31 family, and another consisting of all OMPs excluding those in the 2 families above (Schurig et al., 2002). The first 2 families have been widely examined and mainly include protective antibodies and antigens related to *Brucella* virulence. Research on these OMPs will reveal the mechanism by which the *Brucella* antigens induce and avoid the viability of macrophages mediated by T cells, and thus safe and effective *Brucella* vaccines can be developed.

Members of the *Brucella* OMP25/OMP31 family show strong antigenicity and can be used to diagnose brucellosis. In addition, the family members are associated with bacterial virulence and are potential protective antigens (Gomez et al., 2013). A recent study indicated that proteins in the OMP25/OMP31 family are highly conserved as an immunodominant antigen and are related to the

Brucella virulence (Minhas-Ramneek et al., 2013). Hence, in this study, we established an OMP31 prokaryotic expression vector, prepared monoclonal antibodies, optimized the conditions for its expression in *Escherichia coli*, preliminarily identified its immunogenicity and the characteristics of anti-*Brucella* OMP31 mAb by western blot analysis, and laid a foundation for studies of the biological characteristics of OMP31.

MATERIAL AND METHODS

Main material and reagents

In this study, we used female BALB/c mice aged 6-12 weeks (HFK Bioscience Co., Ltd., Beijing, China), mouse myeloma Sp2/0 cell line (preserved in the laboratory), goat *Brucella* M5 vaccine strains, *E. coli* DH5 α , anti-rabbit *Brucella*-infected serum (preserved in the laboratory), pGEX-4T-1 (Pharmacia, Stockholm, Sweden), horseradish peroxidase-marked goat anti-rabbit IgG (Sunshine Biotechnology (Nanjing) Co., Ltd., Nanjing, China), Taq DNA polymerase, restriction endonuclease, T4 DNA ligase (Takara, Shiga, Japan), plasmid extraction kits, kits for polymerase chain reaction (PCR) product purification and gel recycling and purification (Axygen, Union City, CA, USA), glutathione-S-transferase (GST) affinity chromatography protein purification columns (GE Healthcare, Little Chalfont, UK), thrombin, HT, HAT, and mouse monoclonal antibody subclass detection kits (Sigma, St. Louis, MO, USA).

Design of primers

Primers were designed according to the gene sequences after excision of the 19 amino acid signal peptides at the N-terminus of OMP31 (Vizcaíno et al., 2001). *Nde*I and *Xho*I enzyme digestion sites were included. The primer sequences were as follows (underline indicates enzyme digestion sites): upstream primer: 5'-GGAATTCCATATGGCCGACGTGGTTG-3', downstream primer: 5'-CCGCTCGAGGAACTTGTAGTTCAGAC-3'.

PCR amplification and establishment of the expression vector for the OMP31 gene

Single colonies (Singh et al., 2014) were selected from the culture plate for *Brucella* M5 strain rabbit serum, inoculated into 5 mL 2xYT culture medium, shaken at 37°C, and cultured overnight. Next, 1 mL colony solution was centrifuged. The colonies were collected, re-suspended in 50 μ L water, boiled for 10 min, and centrifuged. The supernatant served as a template. The primers described above were used for PCR amplification of the target gene fragments. The reaction conditions were as follows: 95°C for 10 min followed by 80°C for 5 min for denaturation; 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for a total of 30 cycles; extension at 72°C for 10 min. PCR products were subject to double-enzyme digestion with *Nde*I and *Xho*I after extraction using the phenol-chloroform method and precipitation with ethanol. Next, the samples were purified after agarose gel electrophoresis and conjugated to the pGEX-4T-1 vector digested using the same enzyme. *E. coli* DH5 α cells were transformed by the electroporation method, cultured on an ampicillin-resistant agar plate, and selected for cloning the following day. Finally, the plasmids were extracted. *Nde*I and *Xho*I were used for double-enzyme digestion identification. Positive clones were selected for sequencing. Positive clones were referred to as pGEX-4T-1-OMP31.

Expression of OMP31

The pGEX-4T-1-OMP31 was transformed to *E. coli* DH5 α , transferred into 5 mL 2xYT containing 100 μ g/mL ampicillin (Zhao et al., 2014), mixed, and cultured at 37°C overnight, and then transferred into 20 mL 2xYT culture solution at 1:100 the following day. When the culture solution reached OD₆₀₀ = 0.4-0.6, isopropyl β -D-1-thiogalactopyranoside was added at a final concentration of 1 mM to induce expression. The solution was cultured for 4 h, with 1 mL culture solution was centrifuged before and after induction. Cells were collected and re-suspended in 50 μ L dH₂O. Next, 50 μ L buffer solution was added to the sample before induction. The sample was subjected to ultrasound pyrolysis and centrifuged after induction. The supernatant and precipitate were collected, and 50 μ L buffer solution was added. The sample was boiled for 10 min, and 15 μ L were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with Coomassie brilliant blue R250 solution used for staining. The pGEX-4T-1 empty vector served as a negative control for expression.

Purification of OMP31

Single colonies were selected from the agar culture plate and cultured in 10 mL 2xYT culture solution containing 100 μ g/mL ampicillin, shaken at 37°C overnight, induced for expression, incubated on ice for 30 min, and centrifuged at 11,000 *g*. The colonies were collected, re-suspended, mixed well, and incubated for 20 min at room temperature. The protease inhibitor phenylmethylsulfonyl fluoride at a final concentration of 25 μ g/mL was added (Baihao Biotechnology Co., Ltd., Beijing, China). Samples were subjected to mild ultrasound for 20 min, placed in on ice, and centrifuged at 13,700 rpm for 30 min (Gupta et al., 2007).

The GSTrap 1-mL column (GE Healthcare) was used for affinity chromatography to purify OMP31 according to the manufacturer instructions. The main steps were as follows: the sample passed through the column at a flow rate of 0.125 mL/min, washed with 10 mL phosphate-buffered saline, and eluted with 50 mM reduced glutathione eluant (Xi'an Zhoudingguo Co., Ltd., Xi'an, China). The eluent and eluant were collected with 0.5-1 mL/tube, and 15 μ L liquid from each collection tube and subject to electrophoresis after sample preparation. The protein concentration was determined using the Bradford method.

Western-blot identification for OMP31

The purification products were semi-dried and transferred to a polyvinylidene fluoride membrane for 40 min at 15 V after electrophoresis. The membrane was blocked for 3 h in 5% skim milk, washed 3 times with Tris-buffered saline (Taizhou Jinghao Chemical Co., Ltd., Taizhou, China) (Gyuranecz et al., 2013) for 15 min each time, incubated at 4°C overnight after addition of rabbit anti-*Brucella* serum diluted at 1:1000, washed with Tris-buffered saline, incubated for 1 h at room temperature after addition of horseradish peroxidase goat anti-rabbit IgG diluted at 1:3000 (Xi'an Zhoudingguo Co., Ltd.), washed with Tris-buffered saline containing Tween 20, and developed using 3,3'-diaminobenzidine. Development was terminated by adding deionized water.

Preparation and identification of OMP31 mAb

Female BALB/c mice aged 6 weeks were immunized by inactivation (Ghasemi et al., 2013)

(HFK Bioscience Co., Ltd., Beijing, China) once every 2 weeks, 3 times in total, an immunized once before fusion. The valence of the mouse serum antibody was detected by indirect ELISA (Liu et al., 2014). The splenocytes and myeloma cells Sp2/0 of the immunized mice were fused using the polyethylene glycol method. Screened positive hybridoma cells were cloned using the limiting dilution method (Vicente et al., 2014). The specific resistance of the hybridoma cell strains obtained was detected by ELISA. The hybridoma cells were mixed with serum-free culture medium and the number of cells was adjusted to 2×10^6 /mL. Each BALB/c mouse was intraperitoneally injected with 0.5 mL cell solution. The ascites were drained and centrifuged at 3000 rpm for 20 min, after which the supernatant was collected. The valence of the antibody in the mouse ascites was detected using ELISA. The ascites were purified using the n-caprylic acid-ammonium sulfate method (Ridler et al., 2014).

The prepared mAb was identified using the mouse Ig subclass detection kit (Sigma) (Ridler et al., 2014) according to the manufacturer instructions. The specificity of the monoclonal antibody was detected by western blotting. The sensitivity, relative affinity, and cross-reactivity were detected by ELISA.

RESULTS

Results of PCR amplification and identification for the OMP31 gene

A target band of approximately 680 base pairs was amplified as expected using the goat *Brucella* M5 strain genome DNA as a template (Figure 1a). These fragments were cloned into pGEX-4T-1 and the positive clones were selected for sequencing and BLAST analysis after double-enzyme digestion identification (Figure 1b). The results indicated that the homologies of the fragments obtained and the gene sequence of goat *Brucella* OMP31 were 100% concordant, suggesting that the vector was correctly established. The positive clones were referred to as pGEX-4T-1-OMP31.

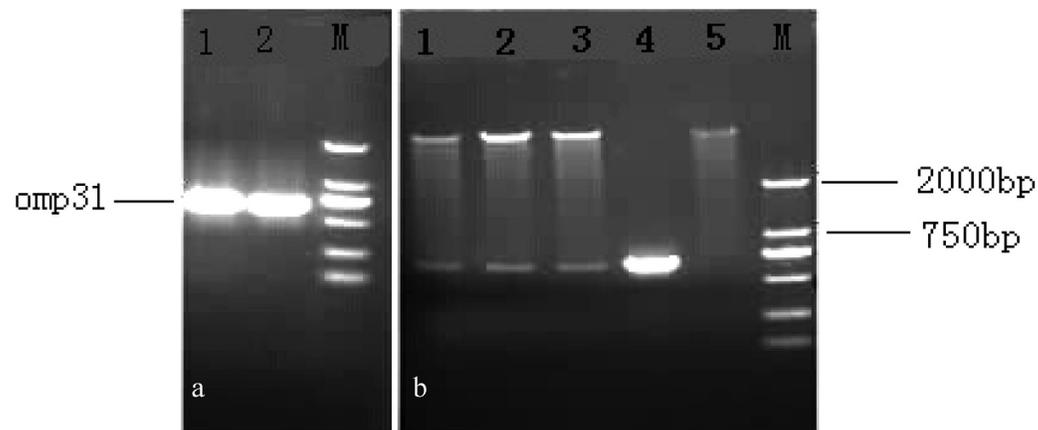


Figure 1. OMP31 gene identification: M-DL2000 DNA Marker. **a.** PCR amplification result. *Lanes 1 and 2* = PCR amplification products of the target genes. **b.** Double-enzyme digestion identification for vector; *lanes 1-3* = results of *XhoI* and *NdeI* double-enzyme digestion for the plasmid extracted after conjugation and transformation; *lane 4* = target gene fragment; *lane 5* = recycled band after pGEX-4T-1 double-enzyme digestion and before conjugation. *Lane M* = marker.

Expression, purification, and identification of OMP31 fusion protein

The pGEX-4T-1-omp31 recombinant bacterium was induced at 37°C for 4 h. As shown in Figure 2a, a target band of approximately 57 kDa was observed. The apparent molecular weight was consistent with the size of the target fusion protein. The protein was present in inclusion bodies. The recombinant bacteria were induced with 0.7 mM IPTG at 25°C for 6 h. Soluble protein expression increased significantly (Figure 2b, Lane 5). GST-OMP31 (Figure 2b, Lane 7) and OMP31 (Figure 2b, Lane 8) were obtained by affinity chromatography. The concentrations were 3.09 and 1.78 mg/mL, respectively, based on the Bradford method. The purified protein was transferred onto a polyvinylidene fluoride membrane and detected by the goat *Brucella* rabbit immune serum. There was a clear positive band (Figure 2c). There was no band for the negative control of *E. coli* lysis solution. This indicated that the purified OMP31 could be identified by the rabbit anti-*Brucella* serum, which was the target *Brucella* OMP31.

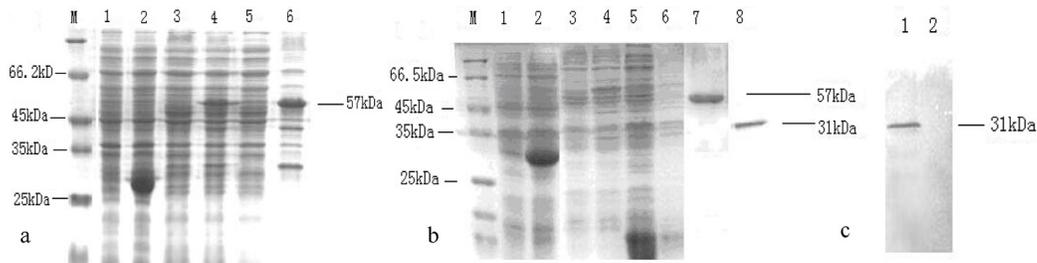


Figure 2. a. SDS-PAGE analysis for expression of OMP31 protein. Lane M: marker, lane 1 = pGEX-4T-1/DH5 α before induction, lane 2 = pGEX-4T-1/DH5 α after induction, lane 3 = pGEX-4T-1-omp31/DH5 α before induction, lane 4 = pGEX-4T-1-omp31/DH5 α after induction, lane 5 = supernatant after centrifugation, and lane 6 = precipitate after centrifugation. b. SDS-PAGE analysis for purification of OMP31 protein. Lane M: marker, lane 1 = pGEX-4T-1/DH5 α before induction, lane 2 = pGEX-4T-1/DH5 α after induction, lane 3 = pGEX-4T-1-OMP31/DH5 α before induction, lane 4 = pGEX-4T-1-OMP31/DH5 α after induction, lane 5 = supernatant after centrifugation, lane 6 = precipitate after centrifugation, lanes 7 and 8 = purified GST-OMP31 protein and OMP31 protein. c. Western blot identification of OMP31 protein, lane 1 = purified OMP31 protein and lane 2 = bovine serum albumin.

Identification result for anti-*Brucella* monoclonal antibodies

Two strains of hybridoma cell lines capable of stably secreting antibodies, including 1G3 and 2C5, were obtained after immunization, fusion, and screening. The valence of the ascites determined by ELISA and the concentrations of the purified monoclonal antibodies determined by the BCA method are shown in Table 1. Western blotting results indicated that the prepared mAb specifically reacted with the corresponding *Brucella* protein rather than with the *E. coli* cell lysis buffer (Figure 3).

Based on identification of the anti-*Brucella* mAb class and subclass, mAb 1G3 was mouse IgG2a and mAb 2C5 was mouse IgG1. ELISA was used to detect various strains of mAbs. *Brucella* was detected in 1×10^5 colony-forming units/mL for 1G3. *Brucella* was detected in 1×10^4 colony-forming units/mL for 2C5. ELISA was used to detect the relative affinity of various strains of mAbs to *Brucella*. A curve was plotted based on the detection results (Figure 4). The relative affinity of mAb was expressed with a 50% OD_{492nm} antibody concentration in the stage of antigen antibody combination. 1G3 was 1×10^{-5} μ g/mL and 2C5 was 1.5×10^{-5} μ g/mL. The ELISA was

used to detect the cross-reactivity of various strains of mAbs against several bacteria. The results indicated that all strains of mAbs specifically reacted with *Brucella* and did not cross-react with *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*, among others.

Table 1. Valence of ascites mAb and concentrations of purified antibodies.

Ascites	Valence	mAb concentration after purification
1G3	1:10 ⁵	19.98
2C5	1:10 ⁴	7.81

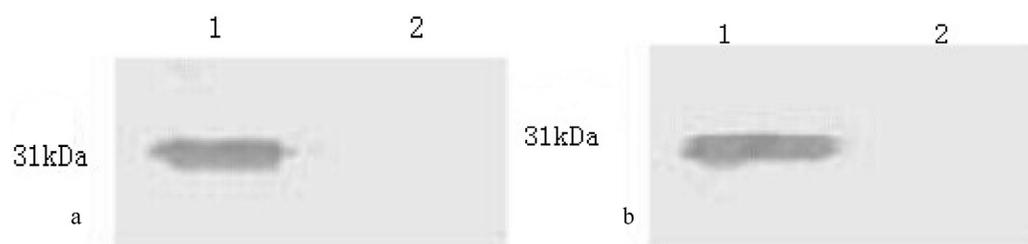


Figure 3. Analysis of mAb specificity using western blotting. Figure a shows 1G3 and Figure b shows 2C5. Both mAb 1G3 and mAb 2C5 bound with specificity to the OMP31 protein and neither could specifically react with the *Escherichia coli* cell lysis buffer.

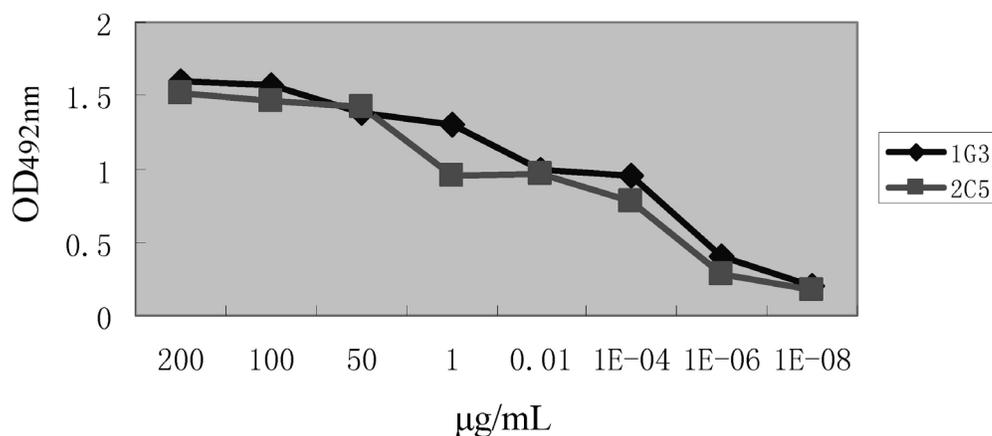


Figure 4. Detection of relative affinity of mAbs for *Brucella* using ELISA.

DISCUSSION

Brucella is a Class B pathogenic microorganism in China. It mainly results in brucellosis and further leads to spontaneous abortion and infertility in animals (Ghasemi et al., 2013). It can also infect humans and lead to a range of symptoms such as Malta fever. This disease typically progresses to chronic allergic diseases such as liver and spleen diseases, lymphadenectasis, and

arthritis. Microbiological detection is the standard for *Brucella* detection (Liu et al., 2014), but the method is characterized by strong subjectivity, low sensitivity and specificity, the presence of cross-reactions with other Gram-negative bacteria, requires a long period of time, poor universality, and existence of certain risks, thus making it difficult to test a larger number of samples. Vaccination is considered to be the most effective method for controlling the brucellosis epidemic, but it is difficult to distinguish natural infection from vaccine immunity. These factors make brucellosis an urgent problem. Research and development of molecular marked vaccines is an inevitable tendency of research and application of new *Brucella* vaccines (Vicente et al., 2014). The search for specific protein antigens has gained wide attention. Antibacterial immunity is dominated by cellular immunity, as *Brucella* is an intracellular bacterium (Nasruddin et al., 2014; Ridler et al., 2014). As an outer membrane protein in the *Brucella* OMP25/OMP31 family, OMP31 can not only play a role in protective humoral immunity but also induce specific cellular immunity (Snyder et al., 2013). It is a good candidate target site for a brucellosis subunit vaccine or DNA vaccine. International research has demonstrated that the deletion and mutation strains of the OMP31 gene of current attenuated vaccines, such as Rev. 1, will not change the immune protective result of their parent strains (Percin, 2013). Hence, the OMP31 antigen is considered to be one of the most appropriate markers for gene deletion vaccines. Serological testing involving a combination of OMP31 and other proteins with immunogenicity can be used to diagnose *Brucella* and to distinguish natural infection from vaccination.

Recombinant proteins are typically in the form of inclusion bodies in the *E. coli* prokaryotic expression system (Riquelme-Neira et al., 2013). Purified OMP31 was present in inclusion bodies after induction by IPTG for 4 h at 37°C. Inclusion bodies have such advantages as high expression, high purity, and difficult degradation, but also have disadvantages such as incorrect folding, poor biological activity, and complex renaturation procedures. Selecting purified inclusion bodies or changing induction conditions for purification of soluble proteins depends on the specific circumstance.

In this study, the prokaryotic expression vector, pGEX-4T-1-OMP31, was established and induced to express in *E. coli*. The prokaryotic expression has simple requirements and high expression efficiency (Baldi and Giambartolomei, 2013) but some proteins, particularly those of high molecular weight, are likely to form inclusion bodies when they are highly expressed. Therefore, the induction conditions were optimized in this study. The soluble expression of proteins is associated with many factors (Doganay and Doganay, 2013), including increasing the ventilation volume, changing the induction conditions, and reducing the culture temperature. Increasing the ventilation volume helps increase the expression level and solubility of target proteins. Changes in the induction conditions include the time of inducer addition and inducer concentration, among other factors. Generally, higher expression of soluble protein was observed when the concentration of the bacterial solution reached OD_{600nm} 0.4-0.6. The inducer concentration requires additional study, and reducing the induction temperature can increase the expression of soluble proteins (Smith et al., 2013; Um et al., 2013). Temperature reduction was also used in this study. In this study, we achieved soluble expression of OMP31 by changing the inducer concentration and reducing the induction temperature. The solubility of the expressed fusion protein was increased with the use of a low temperature of 25°C and inducing the recombinant bacteria for 6 h with 0.7 mM IPTG. The OMP31 fusion protein exhibited soluble expression under low-temperature induction. Western blotting showed that the fusion protein specifically bound to the *Brucella* rabbit immune serum. The splenocytes of the *Brucella*-immunized mice were used for cellular fusion, and purified recombinant proteins were used for screening of hybridoma. The enabled mAb specific to have a native conformation antigenic epitope and reduce the number of laboratory animals used. In this

study, we obtained the OMP31 protein mAb for 2 strains of *Brucella*. The antibody showed strong specificity and sensitivity and no cross-reactions with *E. coli*, *S. aureus*, *B. subtilis*, *M. tuberculosis*, and *Bacillus pyocyaneus*, among others. The successful expression of the fusion protein lays a foundation for investigating the functions of the OMP31.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the 2010 Key Basic Research Project of Health Department of Inner Mongolia Autonomous Region (#2010045).

REFERENCES

- Baldi PC and Giambartolomei GH (2013). Immunopathology of *Brucella* Infection. *Recent Pat. Anti-Infect. Drug Discov.* 8:18-26.
- Bowden RA, Cloeckaert A, Zygmunt MS and Dubray G (1998). Evaluation of immunogenicity and protective activity in BALB/c mice of the 25 kDa major outer membrane protein of *Brucella melitensis* (OMP25) expressed in *Escherichia coli*. *J. Med. Microbiol.* 47: 39-48.
- Checa AG, Pina CM, Osuna-Mascaró AJ and Harper EM (2014). Crystalline organization of the fibrous prismatic calcitic layer of the Mediterranean mussel *Mytilus galloprovincialis*. *Eur. J. Mineral.* 26: 495-505.
- Doganay M and Aygen B (2003). Human brucellosis: an overview. *Int. J. Infect Dis.* 7: 173-182.
- Doganay GD and Doganay M (2013). *Brucella* as a potential agent of bioterrorism. *Recent Pat. Anti-Infect. Drug Discov.* 8: 27-33.
- Farshad S, Mehrabanpour MJ, Namavari MM, Hosseini SMH, et al. (2002). Production and characterization of monoclonal antibodies against *Brucella abortus* S (99) surface antigens. *Iran Biomed.* 6: 12-17.
- Ghasemi A, Salari MH, Zarnani AH, Pourmand MR, et al. (2013). Immune reactivity of *Brucella melitensis*-vaccinated rabbit serum with recombinant Omp31 and DnaK proteins. *Iran J. Microbiol.* 5: 19-23.
- Gomez G, Pei J, Mwangi W, Adams LG, et al. (2013). Immunogenic and invasive properties of *Brucella melitensis* 16M outer membrane protein vaccine candidates identified via a reverse vaccinology approach. *PLoS One* 8: e59751.
- Guo P, Wang N, Liu YJ and Lu CP (2014). Antimicrobial susceptibility and characterization of outer membrane proteins of *Aeromonas hydrophila* isolated in China. *J. Integr. Agr.* 13: 911-917.
- Gupta VK, Rout PK and Vihan VS (2007). Induction of immune response in mice with a DNA vaccine encoding outer membrane protein (omp31) of *Brucella melitensis* 16M. *Res. Vet. Sci.* 82: 305-313.
- Gyuranecz M, Kreizinger Z, Horváth G, Rónai Z, et al. (2013). Natural IS711 insertion causing omp31 gene inactivation in *Brucella ovis*. *J. Vet. Diagn. Invest.* 25: 234-236.
- Jahans KL, Foster G and Broughton ES (1997). The characterization of *Brucella* strains isolated from marine mammals. *Vet. Microbiol.* 57: 373-382.
- Liu WJ, Dong H, Peng XW and Wu QM (2014). The cyclic AMP-binding protein CbpB in *Brucella melitensis* and its role in cell envelope integrity, resistance to detergent and virulence. *FEMS Microbiol. Lett.* 356: 79-88.
- Mayfield JE, Bricker BJ, Godfrey H, Crosby RM, et al. (1988). The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. *Gene* 63: 1-9.
- Minhas-Ramneek P, Pawar HN, Kaur D, Deka D, et al. (2013). Development and evaluation of PCR assay based on outer membrane protein 22 gene for genus specific diagnosis of *Brucella*. *Proc. Natl. Acad. Sci. India, Sect. B Biol. Sci.* 83: 615-619.
- Nasruddin NS, Mazlan M, Saad MZ, Hamzah H, et al. (2014). Histopathology and immunohistochemistry assessments of acute experimental infection by *Brucella melitensis* in bucks. *Open J. Pathol.* 4: 54-63.
- Percin D (2013). Microbiology of *Brucella*. *Recent Pat. Antiinfect. Drug Discov.* 8: 13-17.
- Ridler AL, Smith SL and West DM (2014). Seroconversion and semen shedding in rams experimentally infected with *Brucella ovis*. *NZ Vet. J.* 62: 47-50.
- Riquelme-Neira R, Retamal-Díaz A, Acuña F, Riquelme P, et al. (2013). Protective effect of a DNA vaccine containing an open reading frame with homology to an ABC-type transporter present in the genomic island 3 of *Brucella abortus* in BALB/c mice. *Vaccine* 31: 3663-3667.

- Schurig GG, Sriranganathan N and Corbel MJ (2002). Brucellosis vaccines: past, present and future. *Vet. Microbiol.* 90: 479-496.
- Singh A, Gupta VK, Kumar A, Singh VK, et al. (2014). 16S rRNA and omp31 gene based molecular characterization of field strains of *B. melitensis* from aborted foetus of goats in India. *Sci. World J.* 2013: 160376.
- Smith JA, Khan M, Magnani DD, Harms JS, et al. (2013). *Brucella* induces an unfolded protein response via TcpB that supports intracellular replication in macrophages. *PLoS Pathog.* 9: e1003785.
- Snyder GA, Deredge D, Waldhuber A, Fresquez T, et al. (2013). Crystal structures of the Toll/Interleukin-1 receptor (TIR) domains from the *Brucella* protein TcpB and host adaptor TIRAP reveal mechanisms of molecular mimicry. *J. Biol. Chem.* 289: 669-679.
- Sun D, Zhang H, Lv S, Wang H, et al. (2013). Identification of a 43-kDa outer membrane protein of *Fusobacterium necrophorum* that exhibits similarity with pore-forming proteins of other *Fusobacterium* species. *Res. Vet. Sci.* 95: 27-33.
- Turner RD, Vollmer W and Foster SJ (2014). Different walls for rods and balls: the diversity of peptidoglycan. *Mol. Microbiol.* 91: 862-874.
- Um SH, Kim JS, Kim K, Kim N, et al. (2013). Structural basis for the inhibition of human lysozyme by PliC from *Brucella abortus*. *Biochemistry* 52: 9385-9393.
- Vicente AF, Antunes JM, Lara GH, Mioni MS, et al. (2014). Evaluation of three formulations of culture media for isolation of *Brucella* spp. regarding their ability to inhibit the growth of contaminating organisms. *BioMed Res. Int.* 2014: 702072.
- Vizcaíno N, Kittelberger R, Cloeckert A, Marin CM, et al. (2001). Minor nucleotide substitutions in the omp31 gene of *Brucella ovis* result in antigenic differences in the major outer membrane protein that it encodes compared to those of the other *Brucella* species. *Infect. Immun.* 69: 7020-7028.
- Yuan H, Liu L, Zheng S, Liu Z, et al. (2014). Experimental study of differentiation of umbilical cord mesenchymal stem cells into smooth muscle cells induced by bladder smooth muscle cells conditioned medium. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 27: 1506-1511.
- Zhao Q, Selomulya C, Xiong H, Chen XD, et al. (2014). Rice dreg protein as an alternative to soy protein isolate: comparison of nutritional properties. *Int. J. Food Prop.* 17: 1791-1804.