Original Article

Cloning, expression and purification of *Brucella* lumazine synthase protein in *E. coli BL21*

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ABSTRACT

Brucella sp. Lumazine synthase, the enzyme involved in riboflavin biosynthesis composed of 10 identical subunits. According to extended applications of LS, it is necessary to set up a high yield expression and purification method for this enzyme.in current study, Lumazine synthase primary structure was achieved from NCBI and it was expressed by pET28a in BL21 *E. coli*. The optimum concentrations of IPTG and Kanamycin was evaluated and applied for high yield expression of rBLS. For LPS removal and purification of protein, ammonium precipitation and ion exchange chromatography were performed and no background in ELISA (against Brucella) was observed. Purification of protein with DEAE sephadex resulted in a single band purified rBLS. The results of rBLS based ELISA in comparison with c-ELISA (SVANOVIR kit) indicates that lumazine synthase cannot completely and efficiently detect *Brucella*. The approach applied in this study can be used in generation a relatively pure rBLS as a valuable recombinant product in vaccine industries.

Keywords: Lumazine synthase, Brucella, recombinant, purification.

Introduction

Lumazine synthase, the enzyme involved in riboflavin biosynthesis, is composed of homooligomers with variable subunit number and size in different species. This oligomeric feature of lumazine synthase made it suitable for different biomedical applications in the fields of antigen presentation (in vaccine researches), drug delivery systems, and bioengineering.

As mentioned before, lumazine synthase, depending on the origin, varies in its oligomeric state of assemblies.It can compose of 5, 10 or 60 monomers [1]. In *Brucella spp*. (specially abortus and melitensis) lumazine synthase encoded by RibH2 gene, located on chromosome II [2] is a decamer (10 monomers),so that, Two pentameric blocks bind together to form this decamer. Brucella lumazine synthase (BLS), was the first lumazine synthase that be identified and studied and is the most studied one between the others.

One of the most important features of BLS, is its stability against chemical and thermal shocks. It was found that BLS do not show significant change in its decameric structure in response to urea (8M) or guanidine HCl [3]. Additionally, thermal structural stability of BLS has been studied and shown that is resulted from its hydrophobic, hydrogen bonds and ionic interactions in capsid structure [4].

BLS and other lumazine synthases, form virus like particles (VLPs) with the ability to efficiently present foreign immunogenic epitopes on their surfaces. They present multi copies of those epitopes with high density to immune system and can interact strongly with B cell receptors. In comparison to single presentation of epitopes, this type of stimulation in VLP based vaccines can efficiently elicit immune system and responses [5]. BLS can accept 10 amino acid sequences (up to 27aa residues) at its N terminals without any disturbing effect on conformation of decamer [6]. In different studies, foreign epitopes of shiga toxin [7], Tenia solium [6] and influenza [8] was added to N terminal of BLS and their immunogenisity was evaluated and all confirm the ability of BLS in immune responses stimulation.

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Studies on BLS has shown that this protein can be used in Brucella serological diagnostic methods as a merker [9]. Because of similar epitopes on brucella lipopolysaccharide (LPS) and some another gram negative bacteria, the presence of LPSs in purified fractions of cytoplasmic diagnostic proteins can cause false positive results [10].

The goal of this study is to generate an empty construct to accept different foreign and high yield expression optimization. Setting up a cost effective method for LPS removal and BLS purification is valuable in recombinant protein field which is evaluated in current study.

Materials and Methods

Bacterial strain and DNA extraction

Brucella melitensis strain was purchased (ATCC 23456; 16 M) and cultured in appropriate medium. The extraction of DNA was performed using phenol/Chloroform method [11]. Obtained amount of DNA was measured with Nanodrop ™ instrument.

Polymerase chain reactions (PCR)

Brucella melitensis lumazine synthase gene was amplified with two designed primers (FBLS: 5'-CCGGAATTCATGAACCAAAGCTGTCCGAAC-3') and (RBLS: 5'-CGGGTCGACGTCAGACAAGCGCGGCGATGC-3') .EcoRI and SalI restriction sites are shown as underlind regions in both revers and forward primers. the length of product was 489 bp.

Cloning and expression of PCR product

Because of presence of EcoRI and SalI enzymes in designed primers the amplified fragment has these sites before and after. So at the first, the purified PCR products and pET28a+ (as expression vector) were double digested directly with the mentioned enzymes. Digested fragments and vectors were subjected to agarose gel purification method (Roche agarose gel purification kit) and ligated with T4 DNA ligase enzyme (NEB-M0202). Ligation product was transformed into E.coli Bl21 DE3 (NEB-C2527). BL21 bacterial cells after transformation cloned on Kanamycin (100ug/ml) and IPTG (50ug/ml) plates and after overnight incubation, clones were selected according to the colony PCR with T7 and BLS specific primers. The confirmed colones were cultured in a 5ml IPTG and 100ug/ml kanamycin containing 2YT medium [12]. For selection of the best colones with highest expression levels, the supernatant and cell pellets (after freeze/thaw in liquid nitrogen and boiling water and centrifuge) and supernatant of freeze/thawed cells were analyzed with ELISA test against anti polyhistidin tagged at the end of the fragment (Monoclonal Anti-polyHistidine–Peroxidase antibody –Sigma A7058). Recombinant bacterial cells with the highest absorbance were selected for more analysis.

Kanamycin and IPTG concentration optimization

For culture medium optimization, the effect of IPTG and Kanamycin on celhel growth (O.D 600) and recombinant protein amount (ELISA against polyhistidin tag) was studied.

Protein solublization

According to expression of recombinant BLSs as inclusion bodies, to release and solubilize of them, treatment of cell pellets of culture (after centrifuge) was performed with 8M urea and SDS (1%-in Tris buffer, pH7.5). Selection of the best treatment was accomplished with ELISA as mentioned before.

Protein purification and Lipopolysaccharide (LPS) removal

After optimization procedure, culture was performed in a larger experimental scale (5 liter) and the optimized treatments on cell pellet was performed to obtain a large amount of recombinant protein for more analysis.

SDS treated cell pellets were subjected to salting out method using ammonium sulphate to concentrate and deletion of SDS from the samples. After centrifuge, the obtained pellet was loaded on to ion exchange chromatography (DEAE sephadex A25-Sigma) column, with Tris 50mM,pH 8 as equilibriation buffer and out fraction of the column was loaded on SDS-poly acrylamide gel (SDS-PAGE). For more removal of LPS and purification of lumazine synthase, Gel Filtration also called size-exclusion chromatography (Cephadex G50- Tris buffer 20mM pH 8) was used. The fractions were collected and their UV absorbance was measured in 280nm and applied in ELISA and SDS-PAGE for more analysis.

Characterization and confirming of the BLS decamer aggregation by Western Blotting (and SDS-PAGE)

Western blot analysis was done to characterize BLS and its decameric formation after expression. Transforming of protein from gel to nitrocellulose membrane (Amersham®) was performed and membrane was blocked with BSA 3% (1hr incubation time in 37°C). After washing with washing buffer (PBS/T) the membrane was incubated in Anti-his antibody (HRP conjugated- Sigma A7058,1/1000 dilution in PBS). Washing steps were repeated after passing incubation time (1hr,37°C) and finally the membrane was developed with Sigma DAB (Diaminobenzidine tetrahydrochloride) (Sigma D4293).

Evaluation of rBLS reactivity with different infected and non-infected sera samples

Indirect microtitre plate (IgG ELISA) using purified rBLS was designated. rBLS was diluted in PBS (pH 7.5) to 50 ug/ml from stock solution (1mg/ml) and was coated on ELISA immunolpates. Plates incubated at 37°C for 40 minutes and washed 3 times with PBS/Tween 20(0.01%) solution as washing buffer. The plates were blocked with 1.5% BSA (Bovine Serum Albumin - in PBS) and incubated for 20 minutes (37°C). A total 100 sheep sera samples from central veterinary organization of Mashhad (infected and non-infected to Brucellosis) were used to test reactivity and sensitivity of the test. Sera samples were diluted (1/20 in PBS) and used in the next step of the test and incubated for 40 minutes (37°C).

Plates were incubated with anti-sheep IgG HRP conjugated (1/1000 dilutions in PBS) for 40 minutes at 37°C. then washed 4 times and developed with sigma OPD (o-phenylenediamine dihydrochloride) powder in phosphate-citrate buffer (Sigma product information for P8287 sheet. Preparation of phosphate-citrate buffer) and kept in dark for 10 minutes. The reaction was stopped with H2SO4(0.5M) and read at 490nm (ELISA reader,Biotek,USA). The samples were run as triplicates and results analysis was performed by Excel program. The results were confirmed and compared with ELISA test (SVANOVIR® Brucella-Ab C-ELISA kit-according to kit manufacturers instruction). The O.D of the results was read and the PI (percent inhibition) was calculated. The positive and negative results were compared to the results of rBLS.

Results:

After production of construct (pET28a containing BLS) the sequence of lumazine synthase (*Brucella melitensis* 16 M) was obtained. This sequence was aligned and compared with other lumazine synthases encoded in other *Brucella* strains before reported (Figure 1). The results of Blast and alignment of sequences show 100% similarity between amino acid sequences of other brucella lumazine synthases.

Figure1 - Nucleotide and Amino acid sequence of lumazine synthase (Brucella melitensis 16M)

The length of PCR product was 489bp which encodes a 158 amino acid sequence. The results of sequencing was subjcted to Protparam (https://web.expasy.org/protparam) online software. The output reveals that the molecular mass of this protein theoretically is 17.35 kDa and pI is 6.59. This product can assemble as a decamer (two pentamers) with a ~173.5kDa molecular mass.

Cloning and expression of BLS containing pET28a construct was successfully designed and generated and transformed into *E. coli* BL21 DE3 as mentioned before. The primary expression was identified by ELISA and western blot analysis (anti his tag and anti-brucella polyclonal antibody derived from patients)

Kanamycin and IPTG concentration optimization

According to the results of optimization tests (figure 2), 100 ug/ml of kanamycin and 100 uM concentration of IPTG were the best and most cost-effective amounts for them.

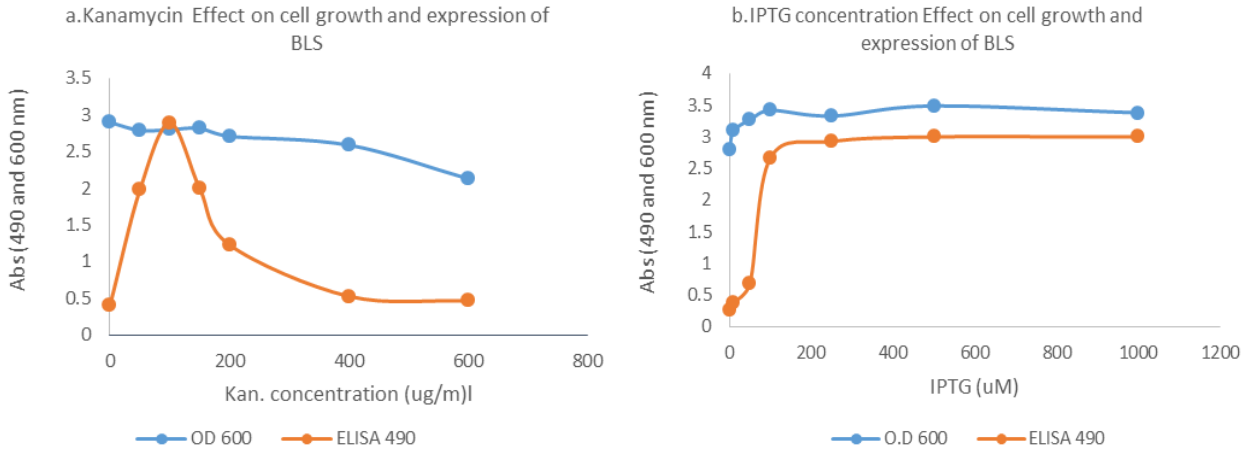

Figure 2. a and b: effect of IPTG and Kanamycin concentrations on bacterial cell growth and BLS expression level (identified as Abs in ELISA test against poly his tag).

Protein purification and Lipopolysaccharide (LPS) removal

Western blot analysis of culture samples (freeze/thawed bacterial cell pellets (CP), culture supernatant (sup), supernatant of lysated cell pellet (SCP)) revealed that the rBLS production is in inclusion body form and it was observed in CP and SCP samples.

According to western blotting and ELISA results, the samples treated with **SDS** had well been solubilized and released from inclusion bodies. The effect of urea on releasing proteins from inclusion bodies was ignorable and less than SDS treated samples. Urea and SDS treatments did not affect the decameric structure of the construct (size of decamer: ~173.5kDa and for monomer: ~17.35kDa) (figure2b).

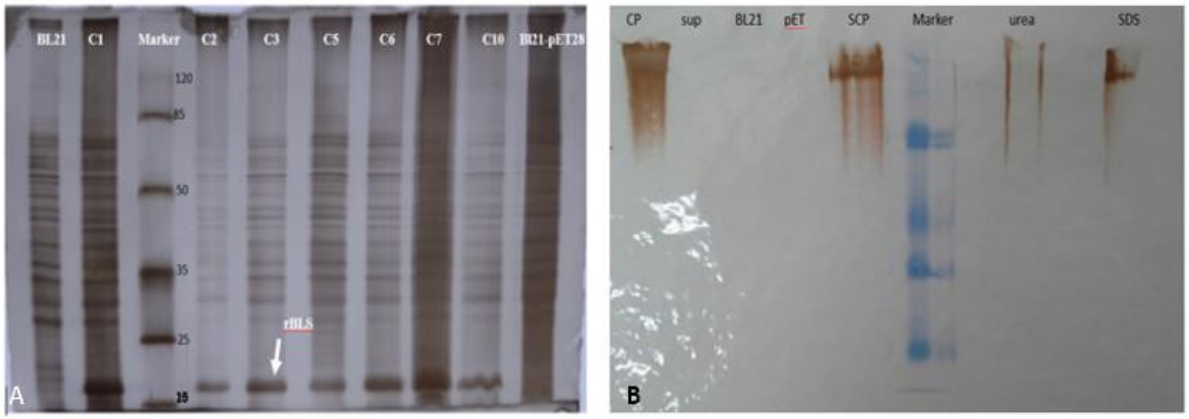

Figure 2. A. colony selection according to expression level of rBLS. B. western blot analysis of different samples for detection of rBLS in bacterial cells different parts. Analysis of the effect of SDS and urea on construct stability.

The results of DEAE ion exchange chrompatography are shown in figure 3. A separated single ~170 kD band was observed in 250mM NaCl (in NaCl gradient of column elutions).

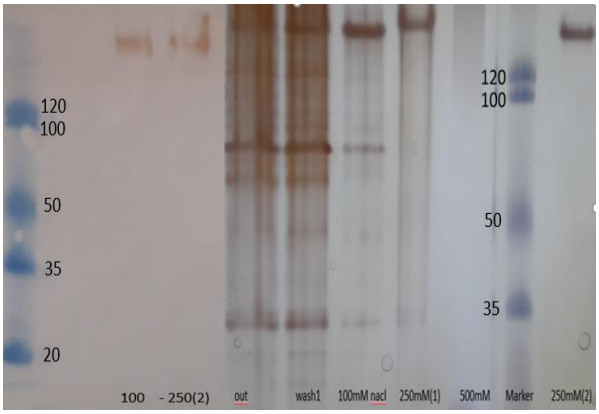

Figure 3. SDS PAGE and Western Blotting (detected with anti poly histidine antibody) results of ion exchange elutions. rBLS was purified in 250mM NaCl fraction.

ELISA results

The results of rBLS base ELISA and c-ELISA (SVANOVIR kit) are presented in table 1. As it can be seen the effectiveness of using rBLS as a diagnostic target for detection of Brucellose is low.

Table 1-a comparison between results of BLS base and c-ELISA (kit)

	Positive	Weak	Negative
BLS based iELISA	12	-	88
c-ELISA	37	23	40

Discussion

Brucella lumazine synthase is a highly immunogenic protein with a decameric structure. Different studies yet, confirmed its wide applications as delivery systems, adjuvants or carriers. Because of having a multimeric arrangement, lumazine synthase can show a repetitive presentation of foreign epitopes added in its N terminus without disruption of spatially order and complicated structure , hence, it can efficiently interact with antigen specific B cell receptors and stimulate immune responses to the target antigens [1].

In current study we produced lumazine synthase construct as an acceptor of foreign short sequences of different antigens according to excellent results on its capacity in foreign protein presentation to immune system. During optimization of expression, we found that its expression is in the form of inclusion body but releasing of protein from inclusion body was performed with SDS and its structure was not disrupted in 1% SDS. Onley boiling of lumazine synthase in SDS containing buffer could separate its monomers. These results confirm the structural stability of Brucella lumazine synthase reported in different papers [3, 13].The expression system and optimized conditions resulted in a high acceptable yield for rBLS. There are different effective methods to purify recombinant proteins. About rBLS, as other recombinant proteins we can use existing

methods but efficiency and cost effectiveness of some purification methods do not allow researcher to use them. In this study ion exchange with DEAE successfully applied that resulted in high yield, single banded BLS.

There are different recombinant proteins expressed for diagnosis of brucellosis. Outer membrane proteins and rBLS had been studied for this aim. About rBLS, the previous studies confirmed their effectiveness in detection of brucella. The results of rBLS based ELISA in comparison with SVANOVIR c-ELISA kit results revealed that rBLS although can interact with anti brucellosis antibodies, but its efficiency is not as enough as in diagnostic methods is needed. More extended studies is necessary for application of lumazine synthase as serological marker.

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