Expression and Purification of *Brucella spp.* Lumazine Synthase Decameric Carrier in Fusion to Extracellular Domain of Influenza M2E Protein

Majidi, Behjat; Fathi Najafi, Mohsen*+
Education and Extension Organization (AREEO), Agricultural Research, Razi Vaccine and Serum Research Institute, Mashhad Branch, Mashhad, I.R. IRAN

Saeedian, Shahriar
Department of biology, Payam Noor university, Tehran, I.R. IRAN

ABSTRACT: *Brucella spp. Lumazine synthase enzyme is a decameric protein carrier that displays foreign antigens effectively in a polyvalent manner. The applied strategy using this molecule results in a higher density of antigens and enhances the immunogenicity of peptide vaccines. In the current study, Brucella lumazine synthase (BLS) was applied for fusion with influenza matrix protein 2 ectodomain (M2E) as a foreign peptide. The primary studies were based on bioinformatics tools and the fusion was expressed and purified in the following levels. Forming of the decamer was confirmed by electrophoresis and western blotting techniques. Influenza matrix protein 2 was stably expressed at the 10 amino terminals of lumazine synthase. The purified fusion was injected into mice and immune responses were evaluated with the indirect enzyme-linked immunosorbent assay (ELISA) technique. According to ELISA results yield of the purification process was 41% with the ion-exchange method and the protein was as a single band in Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). The titer of immunized mice serum with a decameric fusion of lumazine synthase and matrix protein (M2BL) was determined to be more than 1:32000 by indirect ELISA. The level of responses against matrix protein in the decameric state of M2BL, was about 20% higher than monomer M2BL. Anti M2BL was cross-reacted effectively with influenza M2E and in comparison with samples injected with adjuvant, the level of antiM2E was similar. The results in this study confirm the role of multi-copy presentation systems and the applicability of BLS as an antigen carrier and adjuvant in designing peptide vaccines.*

KEYWORDS: *Lumazine synthase; Decamer; Purification; ELISA.*

INTRODUCTION

Vaccines are one of the most effective achievements in human medical products. Vaccines universally save millions of human lives and are good protection tools against cancer and viral and bacterial infections. Among these vaccines, the use of subunit and peptide vaccines which are able to stimulate long and effective immune responses against pathogens becomes a tendency in vaccine designing and development. Production of peptide vaccines

* *To whom correspondence should be addressed.*
+ *E-mail*: *najafiii99@gmail.com*
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is simple, easy, safe, and cost-effective [1]. Besides all these benefits, unfortunately, these vaccines are weak immunogens and require using adjuvants and multi-dose injections [2].

In the creation of efficient immune responses, there is a close relationship between the organization of an antigen and its repetitiveness. Viruses and self-assembling proteins have a highly organized surface and display epitopes on the surface so that they (epitopes) have an efficient crosslink with specific membrane immunoglobins on the B cells. The advantage of using these particles is the induction of antigen distribution and density. These properties create a molecular scenario so that multiple binding events occur. Viral proteins and virus-like particles (VLPs), bacterial platforms, and micellar nanoparticles are among the group of self-assembling proteins [3].

Lumazine Synthase (LS), represents an example of a bacterial platform for designing and optimizing novel vaccine candidates on the basis of self-assembling proteins. This enzyme catalyzes the penultimate step of riboflavin biosynthesis. One of it's most distinctive features is the structural quaternary divergence in different species. Protein forms a pentameric or icosahedral structure from monomers depending on the origin. *Brucella spp* Lumazine Synthase (BLS) is a highly stable,18-kDa decamer (consists of two pentamers) [4]. BLS can accept 10 amino acid sequences (up to 27aa residues) at its N terminals without any disturbing effect on the conformation of the decamer [5]. The chimera from this fusion is very efficient in the generation of immune responses even in the absence of adjuvants that their application is routine in the formulation of peptide and epitope-based vaccines [5].

Influenza virus is the causative agent of a pandemic or seasonal influenza infections. The host displays symptoms such as fever, headache and etc. Commercially produced vaccines against influenza are egg-based and have limitations such as supplying high-quality eggs for vaccines, time-consuming in the production process that can be lead to lack or reduction of cross-reactivity and vaccine shortage, allergic responses in some people, and the probability of occurring additional mutations while production. As an alternative, the epitope-based approach focuses on conserved sequences of an antigen with minimal components. Conserved proteins of influenza type "A" virus, are the target antigens for the development of universal vaccines against influenza. Influenza M2 is a 97 amino acid protein with the ability to form ion channels in viral membranes[6]. The M2 protein ectodomain (M2E) is a short 24 amino acid peptide that is highly conserved and thus is an appropriate target for "universal" influenza vaccine design. Previous studies have shown that the immunogenicity of native M2E is poor, but it can be increased by using multimeric forms of M2E, the fusion of M2E to highly immunogenic carriers, or application with adjuvants [7]. In the present study, the ability of lumazine synthase as a strong carrier, to present a foreign peptide-like Influenza M2e peptide was evaluated using bioinformatics tools, and then the fusion expressed in BL21 DE3 and purified.

EXPERIMENTAL SECTION

Bioinformatics study

Brucella melitensis Lumazine synthase (BLS) coding sequence was obtained from National Center for Biotechnology Information (NCBI) (accession number: KJ401344.1). The primers were designed according to flanking regions in the gene. The amino acid sequence of M2E according to its surface-exposed region was selected and the sequence was: MSLLTEVETPIRNEWGCRCNDSSD. The DNA sequence of BLS was translated to amino acid sequence and after confirming by BLAST, it was designed to express in pET28 expression vector. In the bioinformatics program fusion of M2E to BLS was studied.

The structure of fusion (BLS/M2E) and BLS alone, was first designed by submitting amino acid sequences in I-Tasser online program. The outputs consisted of secondary structure and tertiary structure (in PDB format) of both sequences. PDB structure of decameric BLS was obtained from PDB online databank (PDB code: 1XN1). The structure of fusion was compared structurally with decameric BLS and single-chain BLS by using UCSF Chimera software.

Cloning and expression of BLS and M2BL (fusion of BLS and M2E)

BLS and M2BL were cloned in pET28a+ expression vector. After confirming positive colonies by PCR (polymerase chain reaction), selected clones were cultured in 5mL 2YT medium (IPTG 100uM and *Kanamycin* 100 ug/mL) and after centrifuge according to *Majidi et al* [8], pellet (after freeze and thaw in 1mL D.W) and supernatant were subjected to ELISA (Enzyme-linked immunosorbent assay) against anti polyhistidine, tagged on recombinant

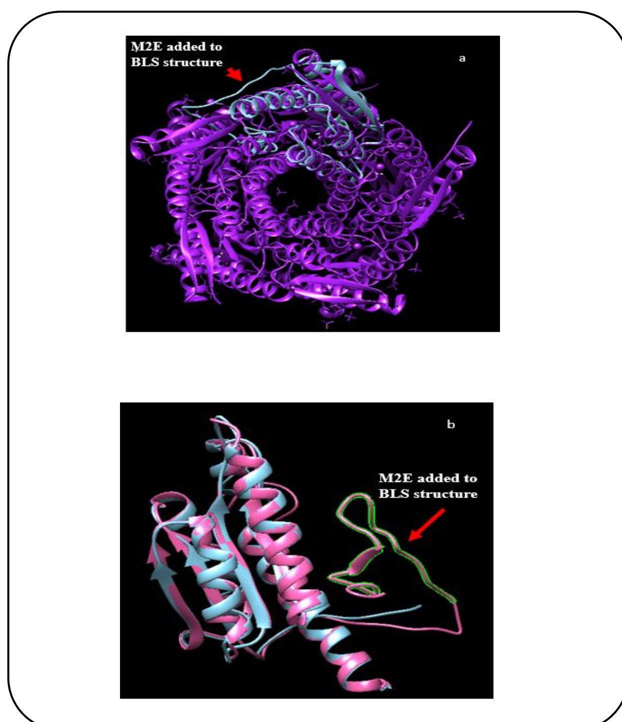

Fig. 1: Structural alignment of lumazine synthase. a) Structural alignment of M2BL (blue) with the decameric construct of BLS (purple) (1XN1 PDB ID). b) Structural alignment of M2BL (pink) with monomeric BLS (blue). The added amino acid sequence (M2E) is shown in green. As is clear here, the M2E had no disruption effect on the secondary and tertiary structure of BLS.

proteins expressed in pET28a+ vectors. The highest levels in O.D (optical density in nm) colonies were chosen to undergo other steps. Additionally by performing ELISA in this step, the location of expression can be detected. Here, because of the secretion of recombinant BLS and M2BL as inclusion bodies, the bacterial pellet will be used in the following steps. After colony selection, the best colonies with the highest expression levels were cultured in a 500mL broth medium (2YT, containing IPTG and Kanamycin). The culture was centrifuged and the pellet was subjected to freeze and thaw and release recombinant protein. The protein content was isolated by centrifuging again and separating cell debris from cell content released in water. And this crude extract was used for purifying BLS and M2BL. In each step, samples were controlled using Western Blotting, ELISA, and SDS-PAGE methods. According to Majidi et al(8) method for purification of BLS by ion exchange and because of the high similarity degree between BLS and M2BL, the same process was used for the purification step.

Serological studies on the decameric structure

To study the adjuvant effect of lumazine synthase, Balb/c mice were immunized with M2BL and M2BL with aluminum hydroxide adjuvant (200ug/injection). The injections were repeated every 2 weeks. Mice sera were collected and their reactivity and levels of anti M2BL were analyzed by ELISA.

To control anti M2E responses, Influenza H2N9 virus achieved from Razi vaccine and serum researches institute, Mashhad) treated with boiling and SDS 0.1% (to inactive and release M2E from virus envelope, as M2E native sample), M2BL (boiled and treated with SDS: as monomeric M2BL and non-treated M2BL as decameric sample), and BLS and PBS as negative control were injected to mice Balb/c (each sample with 3 repeats) and injections were repeated every 2 weeks. The levels of anti M2E were analyzed by ELISA against M2E protein.

RESULTS AND DISCUSSION

Designing of construct

The sequence of M2BL was designed so that M2E was upstream of Lumazine synthase. A restriction site for *EcoRI* enzyme was considered between two coding sequences. Amino acid sequence of lumazine synthase and M2Bl were submitted to I-Tasser online tertiary prediction site. The outputs in PDB format were structurally aligned with the decameric construct: 1XN1 (PDB ID) and monomeric form (output of I-Tasser). As is shown in Fig. 1, the M2E foreign fragment is located in the outer sites of decameric Lumazine synthase tertiary structure and about monomeric form, it has no effect on the secondary and tertiary structure and folding of monomer.

Generation and purification of Chimeras

According to the primary results of the bioinformatics study and alignment analysis in chimera software, an experimental phase was designed and performed. The BLS and M2BL were successfully expressed in the E.coli BL21 DE3 strain. The best colons according to ELISA results against His tag and SDS-PAGE protein pattern and were selected and grown. Figure 2A represents the protein pattern of 8 positive colons. M2BL band is clear in 8 selected colons in 21 kD area. The samples w by boiling and SDS treatment. As can be seen, in boiling treatment, monomers were separated (Figure 2A and 2B-third lane). In the presence of SDS without boiling,

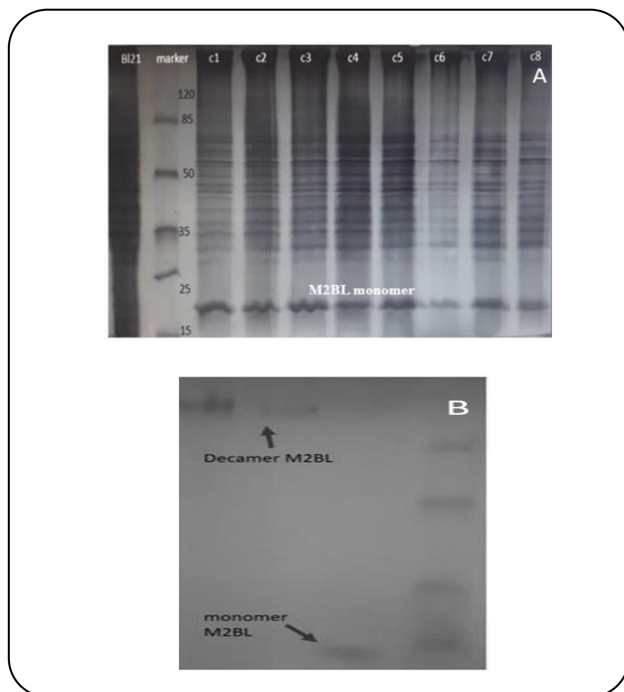

***Fig. 2:** SDS-PAGE performed for colony selection according to expression level(A), Western blot analysis to confirm decameric construct (B).*

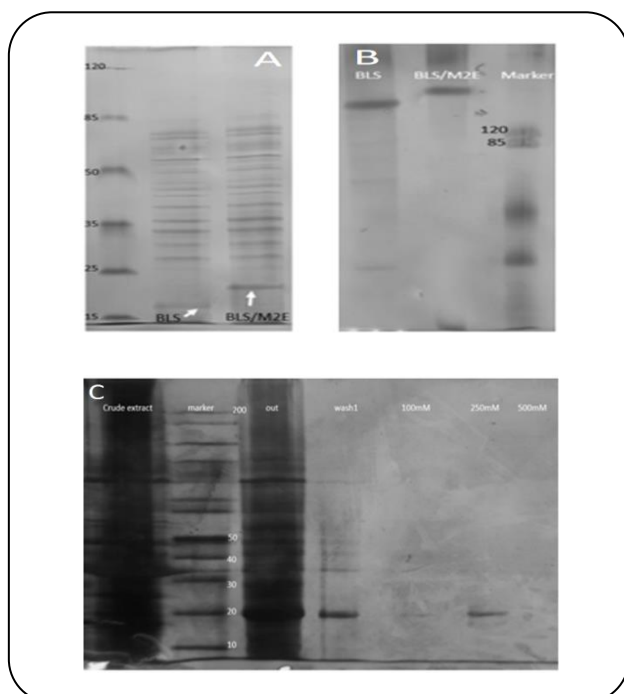

***Fig. 3:** A. monomeric state of M2BL and BLS comparing in size (the samples were prepared by SDS and boiling treatment), B. decameric state of M2BL and BLS comparing in size (prepared by SDS treatment without boiling). 3C. purification profile of M2BL, in 100mM NaCl fraction, the sample became purified.*

the decameric structure remains in complex shape (Fig. 2B). Results of cloning and expression of BLS were reported previously by *Majidi et al*. [8].

To compare and confirm BLS and M2BL size and oligomeric state, the samples were analyzed by SDS-PAGE and the result showed the size difference between the two recombinant proteins. Figure 3A shows both recombinants in the monomeric state and Figure 3B shows both in the decameric state. The results of the western blotting analysis represented in Figure 2B and 3B confirms the protein presence and oligomeric state of recombinant M2BL and BLS.

The results of BLS and M2BL purification are presented in figure 3. As can be seen in the picture, in 250mM concentration of salt (NaCl), the purified lumazine synthase was obtained. Fractions were analyzed with ELISA test against polyhistidine, tagged on recombinant protein as mentioned before. Table 1 summarized the purification results of the ion-exchange chromatography method. As can be seen in this table, M2BL was purified with 41% yield. For doing calculations, ELISA O.D against polyhistidine tag was used for obtaining specific activity.

Indirect ELISA and Western blot were performed to access the immunogenicity of the recombinant protein. After the mice received three times of immunization at 21 days intervals, these immunized mice were sacrificed and the sera were isolated. For the indirect ELISA, serum samples were diluted at 1/500, 1/1000, 1/2000, 1:4000, 1: 8000, 1:16000, 1:32000, 1:128000, 1:512000 respectively, and were used to react with the Influenza virus (boiled-SDS treated), with pre-immune serum served as the negative control. The titer of immunized mice serum was determined to be more than 1:32000 by indirect ELISA.

The reactivity and levels of anti M2BL (in different forms of the molecule: M2BL (decamer), M2BL-aluminium hydroxide (decamer), monomeric state of M2BL pretreated with boiling and SDS, with and without alum) detected by ELISA, are presented in figure 6 ($P<0.05$).

As Fig. 7 shows, M2BL antisera can react and recognize M2E protein in virus clearly and more than native M2E and the monomeric state of M2BL.

Discussion

In this work structural properties of the monomeric and decameric state of Brucella lumazine synthase were

Table1: Purification table for recombinant M2BL.

step	Total protein (mg)	Total ELISA O.D 490 nm *10^3	Specific activity*10^3	Fold purification	Yield (%)
Cell lysate	10.56	6.56	0.621	1	100
Ion exchange chromatography	1.496	2.69	1.798	2.895	41

Total protein (mg): based on Bradford protein quantification test
ELISA ODs: against anti-His-tag of recombinant protein measured at 490nm
Specific activity: ODs per mg of protein

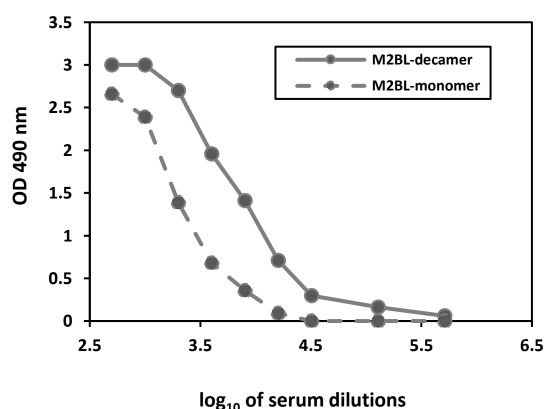

Fig. 5: Titer of anti-M2BL antibodies in the immunized mice sera determined by indirect ELISA. The pre-immune serum served as the negative control. Serum samples were diluted at 1/500, 1/1000, 1/2000, 1:4000, 1: 8000, 1:16000, 1:32000, and 1:128000, 1:512000. The titer and differences in answers show the effect of structure on the creation and stimulation of immune responses. The monomeric M2BL was prepared by boiling purified M2BL in the presence of SDS.

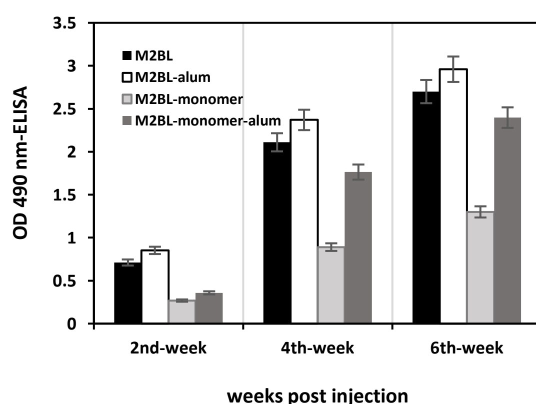

Fig. 6: Adjuvant effect of lumazine synthase on stimulation of immune responses against M2BL. The samples were treated with and without alum and with and without boiling (+SDS) to have different oligomeric states.

silica studied. According to the previous studies, lumazine synthase structure is stable even with adding a foreign oligopeptide in the N terminal side of each monomer (9). This claim was investigated in silico and by structural alignment of fusion monomer and native decamer and monomer. The target peptide of the current study was M2E peptide (matrix protein) of influenza A. The sequences of M2E are highly conserved among all influenza A virus strains. As can be seen in Fig. 1, the foreign M2E fragment is placed at the outside part of the monomers in decamer protein and has no spatial interference on it. In a monomeric state, this addition because of the location of secondary elements and the suitable selected size of M2E, has not affected the structure. The bioinformatics outputs were consonant with previous research results published before [10-14]. BLS folds as a highly stable dimer of pentamers which remains in the quaternary form in a range of hard conditions for other proteins (13). The structural stability of lumazine synthase in acceptance of a foreign peptide was shown in the current study by adding M2E structure at N terminal of protein (Figures 2 and 3). As can be seen in western blotting and SDS-PAGE patterns, the size of M2BL (and BLS) without boiling treatment (even in presence of denaturalizing reagent as SDS) was at decameric size order (~210 KDa), and this confirms decamer oligomerization and formation. In the presence of SDS and by boiling of protein the monomers release and it can be seen on a 21 KDa band in SDS-PAGE and western blotting results. Because of BLS industrial importance, optimization and setting up of cost-effective methods for purification and expression of BLS (and its fusion) could be valuable in future studies. Because of the high stability property of BLS (and M2BL), releasing of M2BL from inclusion bodies was done by SDS reagent that had no effect on the decamer [15]. By application of the ion exchange chromatography method, the recombinant

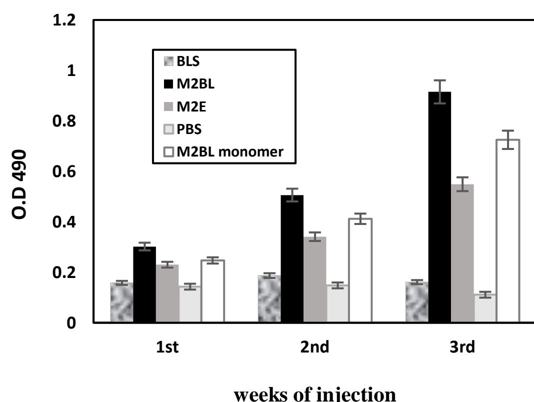

Fig. 7: Antibody responses developed in mice with BLS, M2BL, M2E, and monomeric M2BL determined by Indirect ELISA.

M2BL was purified as a single band with a 41% yield. This method was used before, by Majidi et al for BLS and it was successfully purified [8]. Purification of recombinant proteins (and BLS too) in many cases, is done by His Bind chromatography columns [16,17]. In some cases, BLS was purified by MonoQ column in HLPC apparatus [2,18,19].

According to previous research, BLS induces cross-presentation of attached peptides and creates strong and long-lasting humoral responses in the immune system without the addition of adjuvants [20]. The humoral responses elicited by M2BL were dependent on the oligomeric state of the injected protein and the dose of injection. At a similar amount of injection, the decameric state of M2BL has higher responses (Fig. 5 and 7). The monomeric state was prepared by pretreatment of M2BL (boiling + SDS) and confirmed on gel electrophoresis as mentioned before. In comparing of titer of obtained serums from the injection of decamer and monomer M2BL, the determined titer of decamer was 1:32000 and for monomer, it was 1:8000. This distance confirms the effect of the decameric state of lumazine synthase in eliciting immune responses.

The adjuvant effect of lumazine synthase as a carrier and stimulator of immune responses was studied and reported in different researches (2, 21-25). According to indirect ELISA reports presented in figure 6, anti M2BL level by injecting similar amounts of M2BL into mice was higher (50%) when it was injected in decamer form. By using alum as an adjuvant the anti M2BL level difference was just about 7% and this difference reveals the effect of lumazine synthase structure on the creation of adjuvant effects. In figure 6, this effect is shown on the clear difference between monomer and decamer responses. By formulation of monomer M2BL with alum adjuvant, the humoral responses increased significantly. The specific indirect ELISA titers were improved significantly compared with those injected at the monomeric level. When monomer M2BL inject into mice, it is not able to raise a specific antibody against M2E fragment. When both monomer and decamer have similar components, this high degree of difference arises from the structure of lumazine synthase. M2BL in decameric form and without using any adjuvant could interact and recognize influenza as an antigen. The level of responses against M2E in the decameric state, according to the results, was about 20% higher than monomer M2BL. The formation of decamer increases the stability of fusion and presents M2E fragment in form of a multi-copy to the immune system. According to *Lopez et al*, the key parameter in the elicitation of efficient immune responses is antigen density and distribution of antigen and epitopes on the surface [3].

CONCLUSIONS

In the current study, a flexible and stable system to develop new immunogens on the basis of remarkable multimeric order and stability of *Brucella* lumazine synthase was presented. Ease of manipulation and engineering of BLS, its flexibility in accepting a foreign peptide with no structural disruption, and simple production and purification processes, candidate this system as a novel tool in vaccine designing. The method presented here to create both monomeric and decameric states of an antigen-BLS fusion can be used in understanding the polymeric displaying of foreign peptides and the relationship between immunogenicity and repetition of them. Further studies are required to improve the efficacy of BLS, better understanding of protection mechanisms, and effectiveness and applicability of M2E-BLS fusion in vaccination against influenza.

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