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MOLECULAR CLONING AND EXPRESSION OF THE *LEISHMANIA TROPICA* KMP-11 GENE

By
MOUAYAD MERIEE[1], CHADI SOUKKARIEH[1]* AND ABDUL QADER A. ABBADY[2]
Department of Animal Biology[1], Faculty of Sciences, Damascus University, and Department of Molecular Biology and Biotechnology[2], Atomic Energy Commission of Syria (AECS), Damascus, Syria (*Correspondence:soukkarieh@gmail.com)

Abstract

Kinetoplastid membrane protein-11 (KMP-11) is a small protein of 11 kDa present in all kinetoplastid protozoa studded so far. This protein which is highly expressed in all stages of the *Leishmania* life cycle is considered a potential candidate for a leishmaniasis vaccine against many *leishmania* species. KMP-11 has been recently described in *Leishmania tropica*. In the present study, the KMP-11 gene was extracted from *L. tropica* by PCR using two oligonucleotide primers designed to amplify the entire coding region of this gene. Then, the purified PCR products were successfully ligated into a high expression vector the pRSET-GFP. This expression vector provides the opportunity to clone the desired insert as a fusion protein with a GFP and a tag, polyhistidine region. The GFP use as a carrier to improve immune response and the polyhistidine tag facilitates detection of the expressed protein with anti-His antibodies and also purification of the protein using affinity purification. After wards KMP-11 coding region was sequenced and the recombinant protein was induced and purified from Escherichia coli cultures. The results of the present study will increase our knowledge about molecular cloning and expression of the *L. tropica* KMP-11 gene, and this may be used as an effective target for controlling cutenous leishmaniasis.

Key words: *Leishmania tropica,* Molecular cloning, Expression.

Introduction

Leishmaniasis caused by the most genetically diverse intracellular protozoan parasite is exemplified by its diversity and complexity (Croft *et al*, 2006; Kumar*et al*, 2013). Because of their extremely diverse epidemiology (Desjeux 2004), an effective vaccine would be the most comprehensive means of prevention. Many candidate vaccines have been proposed, which can be broadly classified into first and second generations. First generation vaccine candidates consist of whole-cell extracts or fractions of killed *Leishmania* promastigotes, whereas second generation vaccine candidates constitute chemically defined preparations, such as recombinant proteins or DNA (Khamesipour *et al,* 2006). Various parasite molecules have been identified as potential candidates for second-generation vaccines, of these candidates was kinetoplastid membrane protein-11 or KMP-11 (Basu *et al*, 2005).

KMP-11 was discovered as a protein complex tightly associated with lipophosphoglycan, the major cell surface glycoconjugate of *Leishmania* promastigotes (King *et al,* 1987). After the demonstration that T cell responses to lipophosphoglycan preparations were in fact due to protein contaminants (Mendonça *et al,* 1991), these contaminants were identified and termed lipophosphoglycan-associated protein (Jardim *et al,* 1991). Its present denomination, KMP-11, is due to the presence of this 11 kDa protein on the membrane of all kinetoplastid protozoa (Stebeck *et al,* 1995).

Leishmania spp. is digenetic, existing as amastigotes within phagocytic cells while in the vertebrate host, and as extracellular promastigotes within the gut of the sand fly vector (Pulvertaft and Hoyle, 1960). Therefore, a suitable vaccine candidate antigen must be expressed in amastigotes, the infective stage for humans. KMP-11 has been shown to be expressed in promastigotes and amastigotesof all *Leishmania* species studied (Stebeck *et al,* 1995; Zhang *et al,* 2009). Besides, the immunoprotective capacity of

vaccine prototypes against visceral leishmaniasis (VL) based on the KMP-11-coding gene (Ramírez *et al,* 2001, Basub *et al,* 2005), as well as the significantly reducing of parasite load in vivo after immunization with polyester poly (lactide-co-glycolide acid) (PLGA) nanoparticles loaded with the 11-kDa (Diego *et al.* 2013) were the basis of the selection of KMP-11 as a vaccine candidateantigen against CL.

Generally, only large molecules, infectious agents, can elicit an immune response in an animal. However, KMP-11, which is a small protein, can be improving the immune response if itis coupled to a large carrier such as a GFP (green fluorescent protein).In addition, thefusion of KMP-11 protein and GFP in one single molecule would have theadvantage of targeting the antigen to the APCs (antigen presenting cells) for uptake, processing and presentation.

The aim of this study was to add knowledge concerning molecular cloning and expression of the KMP-11 gene of *L. tropica*. The KMP-11 gene of *L. tropica* encoding KMP-11 protein was cloned into a pRSET-GFP expression vector, and the fusion protein KMP-11-GFP was produced, besides, to provide an introduction for vaccine production against CL.

Materials and Methods

Promastigotes of *L. tropica* (MHOM/TN/80/IPI1) were cultured in a RPMI1640 medium supplemented with 10 % FCS (Fetal Calf Serum, Sigma-Aldrich). This culture was then incubated at 26^{o}C.

Genomic DNA was extracted from the promastigotes of Leishmania by a Wizard® Genomic DNA Purification Kit (Promega); this process was carried out according to the manufacturer's protocol. DNA concentration and quality was assessed by both UV absorbance and electrophoresis on the 1% agarose gel.

Plasmid, Bacterial strains and growth conditions: pRSET-GFP plasmid which is developed in our lab was used for expression. Thisexpression vector provides theopportunity to clone the desired insert as a fusionprotein with a GFP and a tag, polyhistidine region. Thesetags facilitate detection of the expressed proteinwith anti-His antibodies and also purification ofthe protein using the metal-binding site foraffinity purification of the recombinant protein. *E. coli* strains TOP10 (Invitrogen) and BL21-Gold (DE3) (Novagen) were used in cloning and protein expression after transformation by electroporation with the plasmid pRSET (Invitrogen). For general maintenance and protein expression, *E. coli* were grown in Luria Broth (LB; 1 % Tryptone, 0.5 % yeast extract, 171 mM NaCl) (Bio Basic INC) with ampicillin antibiotic 100 µg/ml (Sigma) in orbit-rotating 37°C incubator.

Plasmid construction: *L. tropica* genomic DNA was extracted using DNA purification kit (Promega). KMP-11 gene was amplified using High fidelity *pfu* polymerase (Promega). PCR was done by using KMP-11 specific primers (based on the *L. tropica* KMP-11 gene sequence, accession no. AJ000078.1): forward, 5'ATATATGCTAGCCTCGAGGC CATGGCCACCACGTAGAGGA-3' and reverse, 5'ATATATGAATTCTTAACGCG TTTAGGTACCCTGGACGGTACTGCGC CAG-3'. (XhoI and EcoRI site underlined) in a Thermo-cycler (PeQlab) under conditions at one cycle of 95°C for 2 min, 35 cycles of 95°C for 1 min, 60°C for 30 sec and 72°C for 1 min, and then one cycle of 72°C for 10 min. Primers were designed to amplified the full length of the gene without the start and stop codons, and to add XhoI and EcoRI restriction sites at the 5' & 3' ends, respectively. Amplified KMP-11 fragment was purified using Invisorb Fragment CleanUp kit (Invitek), then, purified KMP-11 fragment and pRSET-GFP plasmid were digested with XhoI and EcoRI restriction enzymes (Fermentas) and thenligated using T4 DNA ligase (Fermentas). Freshly prepared electro-competent *E. coli* TOP10 cells were transformed with the new plasmid construct pRSET-GFP-KMP11 by electroporation. Colony PCR screening for the

presence of recombinant plasmids was performed using pRSET specific primers (T7F/T7R) primers. Plasmid constructs were extracted from some positive clones by High pure plasmid extraction kit according to manufactures protocol (Fermentas) after being grown in LB/ampicillin medium. Successful cloning of these plasmid constructs was confirmed by digestion with restriction enzymes and by sequencing.

Expression and Purification of GFP-KMP-11 fusion protein in *E. coli:* Confirmed plasmid construct was used to transform by electroporation *E. coli* BL21-Gold (DE3) cells. One selected colony was inoculated into 5 ml test tube Luria-Bertani medium (LB) and allowed to grow at 37°C in a shaker at 220 rpm. Culturein logarithmic phase (at OD_{600} of ~0.5–0.6) was induced for 4 h with 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) (Sigma) at 37°C. After induction, 1 ml cells were lysed in 100 µl sample buffer (50 mMTris-HCl (pH 8), 10 % glycerol, 10 % SDS, and0.05 % bromophenol blue, with 100 mM DTT) and whole celllysates (WCL) was analyzed by 12 % SDS-PAGE. Uninducedcontrol culture was analyzed in parallel.For purification 200 mL of LB medium containing were inoculated with *E. coli* BL21-Gold (DE3) strain transformed with pRSET-GFP-KMP11, andgrown at 37°C to an $O.D._{.600}$ of ~0.6. Recombinant proteinexpression was induced by addition of 0.5 mM (IPTG) and the culture was incubated for an additional 4 h. The GFP-KMP-11 fusion protein was purified by affinity chromatography using $Ni2^{+}$ chelating resinto bind the His 6-tag fusion peptide derived from the pRSET-GFP vector. The cell pellet was resuspended in 4 mL of lysis buffer chilled STE buffer incubated for 30 min on ice and the suspension was sonicated for10×20 sec (with 30 sec intervals between each pulse) on ice.

The sonicated cells were centrifuged at 8,000 rpm and 4°C for 30 min, in order to obtain cleared lysate. Using fast protein liquid chromatography (FPLC) AKTA prime plus system (GE life science), fusion GFP-KMP-11 protein was purified from the cytoplasmic extract on a 5 ml column of Nickel charged Nitrilotriacetic acid (NTA) superflow Sepharose (Qiagen). After washing, the bound proteins were eluted from the column with a 500 mM Imidazole buffer. The eluted fraction was concentrated on Vivaspin concentrators with a molecular mass cutoff of 10 kDa (Vivascience). The concentration of the purified protein was determined by Bradford method using bovine serum albumin (BSA) as standard. The purity of the GFP-KMP-11 was evaluated in a Coomassie-stained SDS-PAGE. Protein samples were separated by SDS-PAGE using a Bio-Rad Mini-Protean Tetra Cell system following the manufacturer's instructions. Gels were prepared using stacking gel 5 % and running gel 12 %. After electrophoresis, the gel was stained in Coomassie brilliant blue buffer (45 % Methanol, 10 % Acetic acid, 0.25 % Coomassie R250) for 2 h and then washed several times in distaining buffer.

Results

Cloning of KMP-11 gene into pRSET-GFPplasmid: The full-length KMP-11 gene was amplified from *L. tropica* genomic DNA by using two specific primers. The amplified gene which is about 300 bp in length was identified following agarose gel electrophoresis of the PCR products (Fig. 1A). The PCR products were purified, digested with XhoI and EcoRI and ligated into linearized pRSET-GFPexpression vector (Fig. 1B). Ligated products were used to transform *E. coli* TOP10 cells and positive colonies on the selective plates were screened by PCR using pRSET-specific primers. This approach enabled distinguishing between empty pRSET-GFP containing colonies which gave a fragment of 911 bp and pRSET-GFP-KMP11 containing colonies that gave a longer fragment of 1189 bp due to the presence of the insert gene within (Fig. 1C). Plasmid constructs were prepared from positive PCR colonies by plasmid mini-prep Kit in order to obtain large quanti-

ty of the plasmids, enough for a digestion reaction to confirm the right insertion of the KMP-11 in pRSET-GFP-KMP-11construct. The extracted plasmids from were digested by XhoI or by XhoI and EcoRI enzymes and electrophoresis on 1% agarose gel (Fig. 1D). As shown in agarose gel, DNA fragment (3811), resulted from thedigestion of the plasmid construct with XhoI, was longer than fragment (3533bp) resulted from the control plasmid (Fig. 1D, lane 2 and 6). Although, XhoI/EcoRI double digestion of the plasmid construct showed two distinguished fragments;the linearized plasmid and the insert (KMP-11) (Fig. 1D, lane 7). Whereas, only linearized plasmid could be seen after thedigestion of the control plasmid with the same enzymes (Fig. 1D, lane 3). Finally, the recombinant plasmids were sequenced and compared with sequences in the GenBank. The KMP-11 gene sequence was 100 % identical with that of *L. tropica* (accession no. AJ000078.1; but not given).

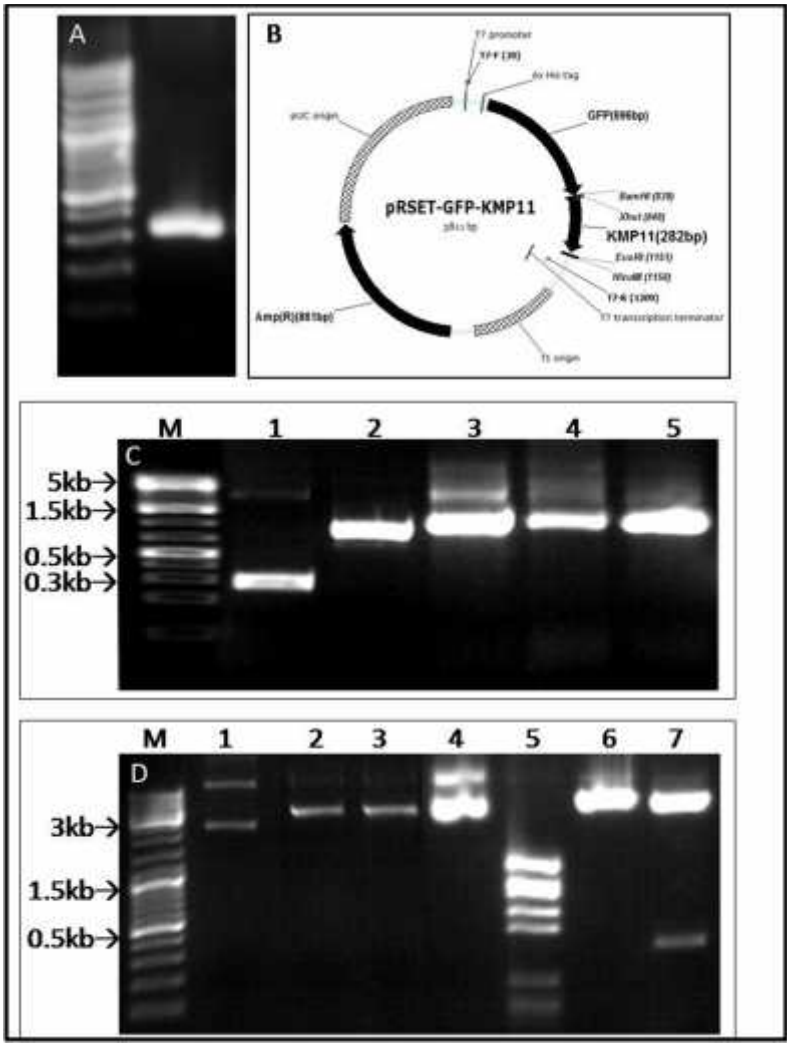

Fig. 1: Amplification of P36 gene and Cloning. (A) Electrophoresis of PCR Products for KMP-11 (~300bp) Fragment of *L. tropica* Amplification. (B) Map of resulted plasmid constructs (pRSET-GFP-KMP11) where position of inserted KMP-11 gene indicated. Most important elements of plasmids: included T7 promoter, His6 tag downstream the two restriction sites (XhoI & EcoRI) used for KMP-11gene ligation. Position of primers (T7-F & T7-R) used for PCR positive colonies screening and for sequencing given. (C) Colony PCR screening performed on 4 randomly selected colonies (lanes 3-5) after *E. coli* TOP10 transformation with ligation reaction products, whereas purified pRSET-P36 and pRSET-GFP used as a negative control (lane 1 &lane 2 respectively). (D) Confirmation of plasmid construct by digestion reaction. Agarose gel electrophoresis of pRSET-GFP (lanes 1) and plasmidconstruct pRSET-GFP-KMP11 (lanes 4) before digestion lane, and after digestion with XhoI (lanes 2 and 6respectively) or with XhoI/EcoRI (lanes 3 & 7 respectively), DNA molecular weight marker (M).

Expression and Purification of GFP-KMP11 protein in *E. coli*: Production of GFP-KMP11 as recombinant protein was obtained after transformation of *E. coli* BL21-Gold (DE3) cells with the confirmed pRSET-GFP-KMP11 plasmid construct after a mini preparation. Cells were grown in LB medium supplemented with antibiotic and protein expression was then induced by IPTG. Purification of GFP-KMP11 from cytoplasmic extract was done on immobilized-metal affinity chromatography using Nickel-charged NTA column installed on AKTA prime system. The UV-detector, supplemented with this system, enabled real-time monitoring of the different steps of GFP-KMP11 purification (Fig. 2A). The protein expression and purification procedures of recombinant GFP-KMP11 were followed by SDS-PAGE separation and coomassie brilliant blue staining (Fig. 2B). A remarkable expression of GFP-KMP11 could be observed after IPTG induction and incubation over night at 30°C. Although, expressed protein was totally purifiedfrom bacteria cytoplasmic extract by column purification-which yielded 90 % pure GFP-KMP11, yet the yield of purified recombinant protein estimably reached 100 mg/liter of bacteria culture.

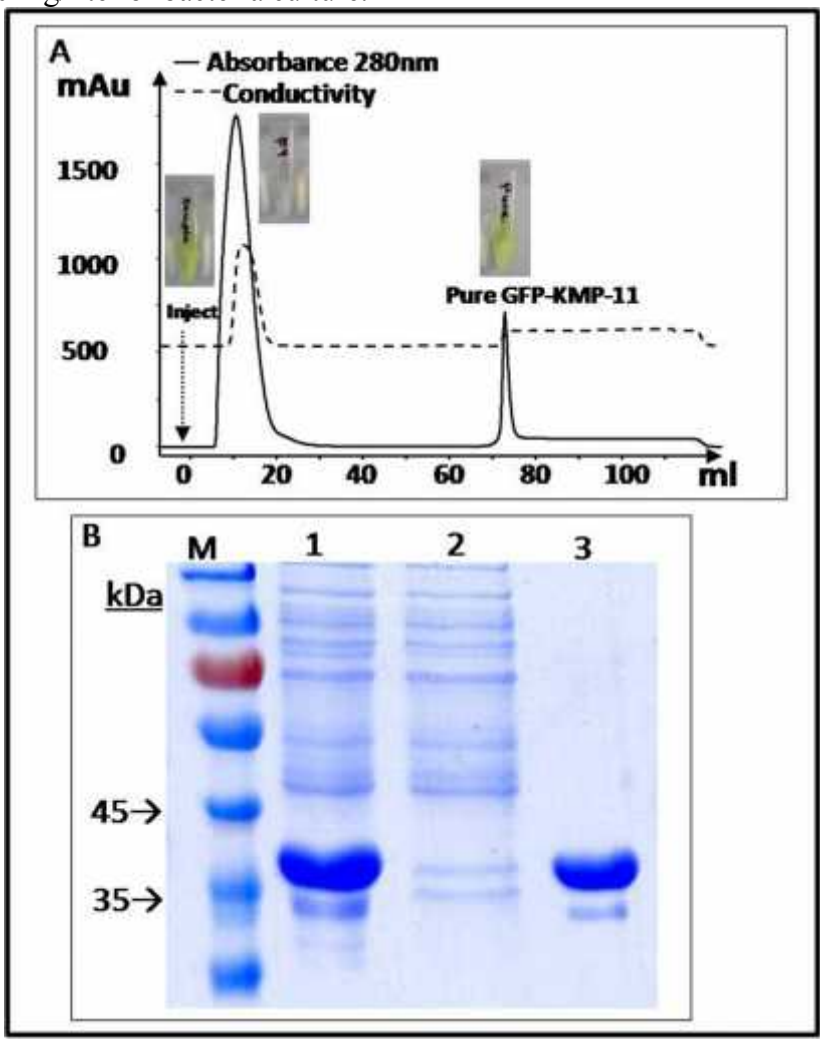

Figure 2: GFP-KMP11fusion proteinexpression and purification. (A) Diagram of purification procedures using Ni^{+}-NTA columninstalled on FPLC AKTA prime system. Continuous linerepresents absorbance of eluate, different purification steps shown below and peaks of flow-through sample and of purified GFP-KMP11 indicated. Extraction and purification accomplished using PBS. Dashed line represents conductivity of eluate. (B) SDS-PAGE (acrylamide 12 %) of protein samples from different steps of purification; total cytoplasmic extract after 4 h of IPTG induction (lane 1), flow-through sample from nickel-charged column (lane 2) and purified GFP-KMP11 fusion protein (lane 3).

Discussion

KMP-11 is found in all kinetoplastid protozoa (Stebeck *et al,* 1995) and is highly conserved (> 95 % homology) in all *Leishmania* species, suggesting an essential role for this protein in the biology of the parasite (Ramírez *et al,* 1998). KMP-11 Proteins in most of the kinetoplastids have common characteristics such as weight; 11 kDa molecular and expression at all stages of the parasite's life cycle (Medonca, 1991). Recently, it has been demonstrated that the surface expression of this protein is higher in the amastigotes than in the promastigotes of *L. amazonensis*. Moreover, in *L. amazonensis* promastigotes, the surface expression of KMP-11 increases during metacyclogenesis (Matos *et al,* 2010). The increased expression of KMP-11 in metacyclic promastigotes and especially in amastigotes indicates a role for this molecule in the infection of the mammalian host. In this connection, KMP-11 expression has been associated with virulence in *L. donovani* because the expression of this protein decreases in parallel with the loss of virulence associated with the repetition of subcultures promastigote cultures (Mukhopadhyay *et al,* 1998).

At present, the biological function of the KMP-11 proteins not yet fully understood, however, it clearly has three immunological roles; B-cell immunostimulatory, inducer lymphocyte proliferation and response cytotoxic and immunoprotective in animal models (Jensen *et al,* 1998). KMP-11 protein induced the proliferation of T lymphocytes $CD4^+$, $CD8^-$ in mice immunized with this protein (Yamanaka *et al,* 2012).

Besides, Basu *et al*. (2005) found that this protein is an excellent target for T cytotoxic lymphocytes ($CD8^+$) and natural killer cells (NK) when it used as DNA vaccine. It has been revealed that this vaccine induces significant production of IFN-γ cytokine from T-helper lymphocytes (Nakahara *et al*, 2003). KMP-11 was used previously for immunotherapy and immunization against leishmaniasis (Guha *et al*, 2013). However, KMP-11 is a small protein and could not be induce a significant immuneresponse when it used to immunize the models animals. Maranon *et al*. (2001) immunized transgenic mice with an A2/Kb fusion protein composed of the heat shock proteins HSP70 and KMP-11 in *T. cruzi* that induces a cytotoxic response against cells expressing the KMP-11 in the parasite. Planelles *et al*. (2002) found that the fusion protein HSP70/KMP-11, was capable of acting as a stimulator for mature mouse dendritic cells and the consequent production of interleukin 12 (IL-12) as well as tumor necrosis factor in human visceral and zoonotic cutaneous leishmaniasis (Morsy *et al*, 1994, 1995).

To the best of the present authors' knowledge, this is the first study to produce KMP-11 as a fusion protein with GFP. GFP originally isolated from the bioluminescent jellyfish *Aequoreavictoria* has become one of the most widely studied and exploited proteins in the biochemistry and the cell biology (Shimomura, 2000; Wiedenmann *et al,* 2006). GFP which is a 27 kDa protein and contains 238 amino acid residues has become well established as a marker of gene expression and protein targeting in intact cells and organisms (Roger, 1998). KMP-11-GFP fusion protein could improve the immune response and the increase the targeting of the antigen to the APCs for uptake, processing and presentation when immunized the animal models.

Conclusion

The recombinant KMP-11-GFP fusion protein was successfully produced in the present study, and could be used for future vaccine against leishmaniasis.

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