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# Cloning, expression and purification of *Leishmania major* PSA-*sf*GFP fusion protein

Aisha Al-jaghasi[1], Abdul-Qader Abbady[2,4], Sahar Al-Khatib[1] and Chadi Soukkarieh[1,3,*]

[1]*Dept. of Animal Biology, Faculty of Sciences, Damascus University, Damascus, Syria;*[2]*Dept. of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria (AECS),* [3]*Damascus, Syria;*[4]*Faculty of Pharmacy, Syrian Private University, Damascus, Syria ;*[5]*Faculty of Pharmacy, International University for Science and Technology, Damascus, Syria*

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**Abstract**

PSA (Promastigote Surface Antigen) is one of the immunogenic antigens in *L. major*. Protection depends on the source of PSA antigen, whereas native protein and plasmid DNA encoding PSA, but not recombinant PSA purified from *E. coli* provided significant protection. The green fluorescent protein (GFP) is commonly used as an excellent expression tag for fusion proteins, which can improve their expression while retaining their function and native-like structure. Trying to develop anti cutaneous leishmaniasis vaccine based on PSA (as recombinant protein or DNA vaccine), this study evaluated the cloning, expression, of the secreted PSA protein from *L. major* as a fusion partner to the superfolder form of green fluorescent protein (*sf*GFP) in both *E. coli* and HEK-293T cells. This included constructing protein expression plasmids pRSET-*sf*GFP-PSA and pcDNA-*sf*GFP-PSA, then producing the recombinant of 6× His tagged *sf*GFP-PSA protein (65 kDa) that was confirmed by SDS-PAGE and western blotting. Although *sf*GFP-PSA fusion protein was expressed with full length in both types of cells, a partial separation of *sf*GFP protein was observed when the fusion protein was expressed in *E. coli*. Here we presented an efficient method to produce the full length of PSA as a fusion protein with *sf*GFP, which could greatly facilitate its uses as a vaccine against cutaneous leishmaniasis.

**Keywords:** PSA, *sf*GFP, Gene cloning, Protein expression

## 1. Introduction

Cutaneous leishmaniasis is endemic in more than 70 countries worldwide, and 90% of cases happen in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, and Syria (Reithinger *et al*., 2007; Von Stebut, 2015). Leishmaniasis remains one of the promising parasitic diseases for vaccine development since defective cellular immune responses are restored after effective chemotherapy and hosts who recover from leishmaniasis are typically insusceptible to further infection (Handman, 2001).

Leishmania secreted factors and surface proteins are key players in the pathogenesis of the disease; they mainly facilitate the parasite's initial contact with the host cells, and interfere with immune cells' functions such as antigen presentation, cytokine production and cell activation (Soto *et al*., 2009). The interest in Leishmania excreted molecules was confirmed by past studies showing interesting properties in terms of protection, which is likely to be special targets for the immune system (Holzmuller *et al*., 2005; Lemesre *et al*., 2005; Tonui *et al*., 2004). Promastigote Surface Antigen (PSA) is an abundant glycolipid-anchored protein on the surface of the promastigote form of most *Leishmania* species (Devault and Bañuls, 2008; Jiménez-Ruiz *et al*., 1998). It belongs to a unique family of membrane-bound and secreted proteins, and has a specific Leucine Rich Repeats signature, which is involved in protein-protein interactions and pathogen recognition (Devault and Bañuls, 2008; Kędzierski *et al*., 2004). PSA protein is involved in Leishmania attachment and invasion of host macrophages via interactions with the complement receptor 3 (CR3), and in resistance to complement lysis (Kedzierski *et al*., 2004; Lincoln *et al*., 2004). It has been proven that this protein is a major immunogenic component of secreted antigens (Bras-Gonçalves *et al*., 2014). The development of vaccines based on the PSA protein was evaluated in several experimental models. PSA isolated from *L. amazonensis* promastigotes, as well as recombinant vaccine viruses expressing this protein, have been demonstrated to induce a protective immune response against infection with *L. amazonensis* in BALB/c mice (Champsi and McMahon-Pratt, 1988; McMahon-Pratt *et al*., 1993). With the recently available whole genome sequences of some *Leishmania* species, thirty-two PSA genes were described in *L. major* genome (Devault and Bañuls, 2008). Moreover, the presence of Th1–type memory to PSA in humans and the ability of the antigen to protect injected mice against *L. major* infection make PSA an attractive candidate to be used as vaccine against human cutaneous leishmaniasis (Kemp *et al*., 1998). Protection depends on the source of PSA antigen, whereas protection was

* Corresponding author e-mail: soukkarieh@gmail.com..

observed with the protein purified from *L. major* membrane (native PSA) or expressed as a recombinant protein in *L. mexicana*, vaccination with the PSA expressed in bacteria did not give protection (Handman *et al*., 1995; Sjölander *et al*., 1998a). However, the immunization with PSA DNA vaccine induced an exclusive Th1 response and mice were protected against an *L. major* challenge (Sjölander *et al*., 1998b).

Several protocols are currently available to improve protein expression including fusion of the protein with a more soluble partner like GFP, where it dramatically aids in protein folding and increases its stability; furthermore, it rarely adversely affects biological activity (Waldo *et al*., 1999). A superfolder form of GFP was engineered by Waldo and colleagues (*sf*GFP), that is more resistant to denaturation and aggregation during refolding, and improved folding kinetics (Andrews *et al*., 2007; Pedelacq *et al*., 2006). The development of an efficient and low-cost technique to produce PSA protein could greatly facilitate its uses as a vaccine against leishmaniasis. In this study, we report the steps of producing *L. major* PSA protein as fusion with *sf*GFP (as recombinant protein and DNA vaccine); *sf*GFP-PSA fusion protein production was confirmed in *E. coli* and HEK-293T cells by SDS-PAGE and Western blotting.

## 2. Methods

### *2.1. Bacterial strains and plasmids:*

*E. coli* strains TOP10 (Invitrogen) and BL21 (DE3) Gold (Novagen) were used in cloning and protein expression. *E. coli* strain transformants were grown at 37C in Luria Broth (LB) (Bio Basic INC) that contained ampicillin (100 μg/ml) for selection of clones containing recombinant plasmid constructs. Plasmids used in this study were the plasmid pRSET (Invitrogen), pRSET-*sf*GFP (Al-Homsi *et al*., 2012), and the plasmid pcDNA-*sf*GFP which was kindly provided by Dr. Abbady, *Atomic Energy Commission of Syria (AECS*). Schematic diagrams of plasmid constructs were generated using Geneious pro 4.8.4.

### *2.2. Cloning of PSA*

*L. major* (Dermatology and Venereology Hospital in Damascus, Syria) was used as the parental strain for amplifying the PSA gene. *LMJF_31_1450* gene, which expresses the secreted PSA form, was amplified by polymerase chain reaction (PCR) technique. The full sequence of PSA gene was obtained from GenBank *Leishmania major freidlin strains*; Gene ID: 5654076, while the forward and reverse primers were designed based on it: the forward primer: (5′ATATATGTCGACGCAGGATCCATGCCCATGCTG CTGCTCCG3′) containing a *Bam*HI restriction site, and the reverse primer: (5′ATATATGGTACCGAATTCTTACTAGCACGCGTT GGCTGTCGAGC3′) containing an *Eco*RI restriction site. The Pfu DNA polymerase kit (Thermo) was used according to the manufacturers' instructions. PCR was performed by thermal cycler (Peqlap, Germany) under standard protocol of a 94 C for 2 min, 30 cycles of 94 C for 30 s, 58 C for 30 s, 72 C for 1 min and 72 C for 7 min as a final extension step. The obtained PCR product and the plasmid pRSET (Invitrogen) were digested with the restriction enzymes *Bam*HI and *Eco*RI (Fermentas). Digestion products were extracted from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen) and then ligated using T4 DNA ligase (Fermentas). The new plasmid construct pRSET-PSA was transformed into *E. coli* strain TOP10 (Invitrogen) by the heat shock method. Positive clones were confirmed by Colony PCR screening using PSA cloning primers, and then plasmid constructs were isolated from some positive clones by Plasmid Miniprep Kit (Qiagen). DNA sequencing and digestion with restriction enzymes were performed to confirm successful cloning.

### *2.3. DNA sequencing*

DNA sequencing for pRSET-PSA construct was performed on a Genetic 114 Analyzer system ABI-310 by the dideoxynucleotide chain-termination method, and the homology searches were performed using the BLAST program with NCBI Reference Sequence: XM_001685079.1.

### *2.4. Subcloning of PSA*

The PSA gene was subcloned into pRSET-*sf*GFP and pcDNA-*sf*GFP (prokaryotic and eukaryotic expression plasmids respectively) as a fusion partner at the C-terminus of *sf*GFP gene. The new recombinant constructed plasmids pRSET-*sf*GFP-PSA and pcDNA-*sf*GFP-PSA were verified by restriction enzyme digestion. pRSET-*sf*GFP-PSA was digested by *Eco*RI and (*Bam*HI or *Xba*I) enzymes while pcDNA-*sf*GFP-PSA was digested by *Hind*III and *Eco*RI compared with the pcDNA-*sf*GFP plasmid.

### *2.5. Expression of PSA and sfGFP-PSA fusion protein in E. coli*

pRSET-PSA and pRSET-*sf*GFP-PSA constructions were transformed into *E. coli* strain BL21 (DE3) Gold (Novagen) competent cells by thermal shock. One positive clone of each construct was grown, and the expression of the recombinant proteins was induced in *E. coli* Gold cells by the addition IPTG (Isopropyl β-D-1-thiogalactopyranoside) to 0.5 mM final concentration, for 16. h. The induction of protein expression was done at 20 C for PSA, and 16 C for *sf*GFP-PSA fusion protein. Bacterial cells were harvested by centrifugation at 10000 rpm for 10 min at 4 C. The harvested cells were prepared for protein purification, so cell pellets were suspended in PBS 1X, then the lysates were loaded to the frenchpress (Constant systems), and pressure applied (Total processing time, 10 min; 1 Kbr), after that the disrupted extracts were centrifuged at 10000 rpm for 10 min at 4 C. Purification of PSA and *sf*GFP-PSA proteins from cytoplasmic extract was done on immobilized-metal affinity chromatography, using the Ni-NTA agarose beads; finally, eluted proteins were analyzed by 12% SDS-PAGE and western blotting.

### *2.6. Expression of sfGFP-PSA fusion protein in eukaryotic cells*

HEK-293T cells were transfected with pcDNA-*sf*GFP-PSA using polyethylenimine (PEI) Transfection Reagent (Roche, Germany). After two passages, the cells were seeded in four-well plate ($100\times10^3$ cells/well), and grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, 100 U/ml of penicillin, and

100 mg/ml streptomycin (all from Sigma Chemical) for 48 h. Transfection solution for each well contained 50 µl of serum-free RPMI medium, PEI (1:3 ratio) and 1.5 µg of plasmid either pcDNA-*sf*GFP-PSA or pcDNA-*sf*GFP (control). The previous mixture was incubated for 30 min at room temperature and then it was added dropwise to the 60-70% confluent cells with gentle shaking. The plate was incubated for 48 h and the production of *sf*GFP-PSA fusion protein was evaluated by fluorescence microscopy at 12, 24, and 48 hours after transfection; finally, it was confirmed in the supernatants medium by western blotting.

### *2.7. SDS-PAGE and Western Blotting*

Three protein samples (PSA and *sf*GFP-PSA) expressed in bacteria and HEK cells were separated on 12% SDS-PAGE followed by western blotting. The proteins were diluted in reducing SDS sample buffer and heated at 95 C for 5 min, and the electrophoresis (Bio-Rad) was performed at 140V. After electrophoresis, the gels were stained in coomassie blue buffer. Other SDS-PAGEs were performed, and the polypeptides from the gels were transferred to 0.45 µm nitrocellulose membranes using a wet blotting system (Bio-Rad). The membranes were blocked with PBS 1X containing 5% skim milk and 0.05% Tween 20 for overnight at 4 C. Then the blot of PSA protein was incubated for 1 h with (1:2000) dilution of mouse anti-6 × His antibodies (R&D Systems), while the blots of the fusion *sf*GFP-PSA proteins were incubated with (1:3000) dilution of a polyclonal rabbit anti-GFP (Al-Homsi *et al*., 2012). After washing, the membranes were incubated for 1 h with secondary antibody conjugated to Alkaline Phosphatase (AP), either Rabbit anti-mouse (1:2000; Sigma, Germany), or goat anti-rabbit (1:2000; Bio-Rad). The proteins were detected with NBT 1%, BCIP 0.5% (Bio-Rad, Germany) staining.

## 3. Results

### *3.1. Cloning of PSA*

Using the gene-specific primers with *L. major* genomic DNA as the template, a 909 bp long PSA gene was successfully amplified. Amplified PSA gene, as well as the pRSET plasmid, was digested with the restriction enzymes *Bam*HI and *Eco*RI, then they were purified from the gel and used in a ligation reaction in an appropriate molar ratio of 1:3 (plasmid: insert). The transformation with the ligated product was then performed in *E. coli* Top10 cells, positive clones were confirmed by colony PCR using PSA specific primers. Next, the recombinant plasmid pRSET-PSA was extracted from two of the positive colonies, and digested with *Bam*HI and *Eco*RI enzymes to confirm the presence of PSA insert. Furthermore, the integrity of the construct and the lack of deletions or mutations were verified by nucleotide sequencing (Fig. 1).

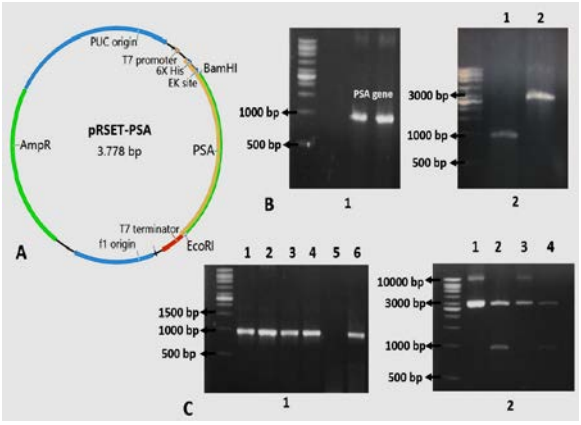


**Figure 1.** Cloning of PSA into pRSET plasmid.

**A**: Schematic diagram of pRSET-PSA construct. **B**: **B-1** Agarose gel electrophoresis of PCR products. The full-length of *L. major* PSA gene (909 bp) was separated on a 1% agarose gel. **B-2** DNA bands of pRSET plasmid and PSA PCR amplified gene after being digested with *Bam*HI and *Eco*RI restriction enzymes. Lane 1: PSA gene; Lane 2: pRSET plasmid. **C**: **C-1** Results of colony PCR screening performed on 6 randomly selected clones. Lanes 1, 2, 3, 4 and 6: positive clones, which contain full-length PSA gene (909bp); Lane 5: a negative colony that doesn't contain the PSA gene. **C-2** Identification of recombinant pRSET-PSA plasmid by restriction enzyme digestion. Lanes 1, 3: circular pRSET-PSA Plasmid; Lanes 2, 4: pRSET-PSA double-digested with *Bam*HI and *Eco*RI. DNA ladder (1 kb).

### *3.2. Sequence analysis of the PSA gene*

Sequencing analysis revealed that no changes were presented during the cloning process and the pRSET-PSA construct was exact. Open reading frame, which includes the full length of PSA gene and protein binding 6His tag followed by EK protease site, was composed of 1,032 nucleotides and the predicted molecular weight of the recombinant PSA protein was 38 kDa (Fig. 2).

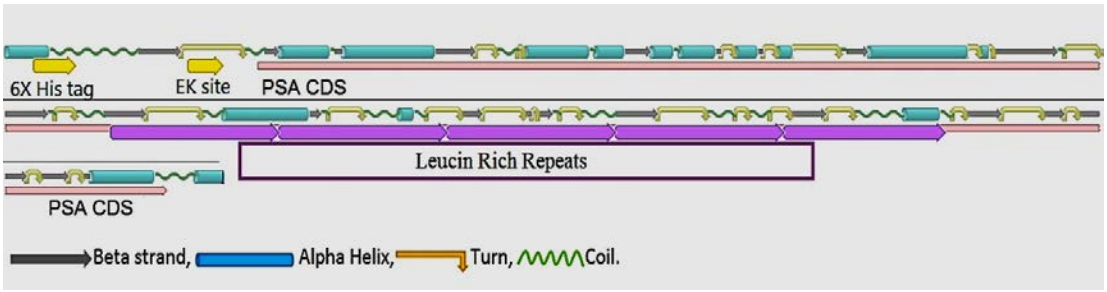


**Figure 2.** Diagrammatic representation of the Secondary structure of PSA protein, where LRR domains have been matched with NCBI Reference Sequence: XP_001685131.1. The model for PSA protein was generated using Geneious pro 4.8.4.

### *3.3. Subcloning of PSA*

PSA gene was subcloned into pRSET-*sf*GFP and pcDNA-*sf*GFP plasmids as a fusion partner to the *sf*GFP. PSA gene was introduced directly at the C-terminus of the *sf*GFP gene, and the two genes were in-frame cloning construct. The recombinant constructed plasmids pRSET-*sf*GFP-PSA and pcDNA-*sf*GFP-PSA were prepared from positive PCR colonies by plasmid miniprep Kit and the enzyme digestion was used to verify them (Figs. 3, 4).

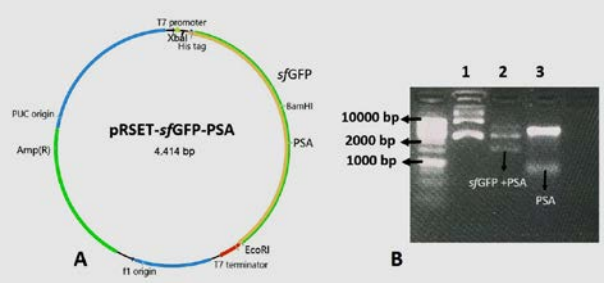


**Figure 3.** Cloning of PSA into pRSET-*sf*GFP plasmid.

**A**: Schematic diagram of pRSET-*sf*GFP-PSA construct. **B**: Identification of recombinant pRSET-*sf*GFP-PSA plasmid by restriction enzyme digestion. Lane 1: pRSET-*sf*GFP-PSA circular Plasmid; Lane 2: pRSET-*sf*GFP-PSA double-digested with *Xba*I and *Eco*RI (expected digested fragment: PSA and *sf*GFP genes); Lane 3: pRSET-*sf*GFP-PSA double-digested with *Bam*HI and *Eco*RI (expected digested fragment: PSA gene). DNA ladder (1 kb).

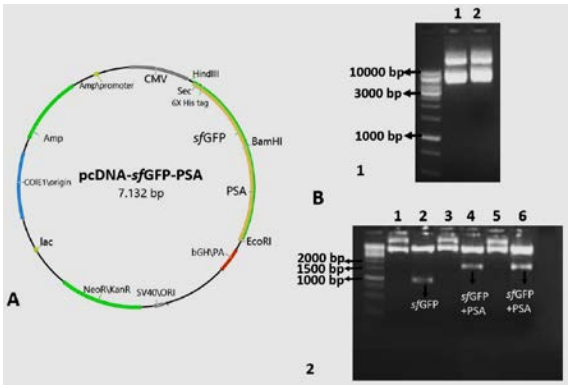


**Figure 4.** Cloning of PSA into pcDNA-*sf*GFP plasmid.

**A**: Schematic diagram of pcDNA-*sf*GFP-PSA construct. **B**: **B-1** Recombinant pcDNA-*sf*GFP-PSA plasmid was extracted from two of the positive colonies by miniprep. Lanes 1, 2: pcDNA-*sf*GFP-PSA Plasmid. **B-2** Identification of recombinant pcDNA-*sf*GFP-PSA plasmid by restriction enzyme digestion. Lane 1: pcDNA-*sf*GFP plasmid (control) without digestion; Lane 2: pcDNA-*sf*GFP double-digested with *Hind*III and *Eco*RI (expected digested fragment: *sf*GFP gene); Lanes 3, 5: pcDNA-*sf*GFP-PSA plasmid without digestion; Lanes 4, 6: pcDNA-*sf*GFP-PSA double-digested with *Hind*III and *Eco*RI (expected digested fragment: PSA and *sf*GFP genes). DNA ladder (1 kb).

### *3.4. Expression of protein*

#### *3.4.1. Expression of PSA and sfGFP-PSA fusion protein in E. coli:*

The production of PSA and PSA-*sf*GFP proteins were obtained after the transformation of *E. coli* BL21 (DE3) Gold cells with the confirmed pRSET-PSA and pRSET-*sf*GFP-PSA constructs. Following positive bacterial cells growth, protein expression was induced overnight by IPTG and Low temperature. PSA protein was shown a significant expression with a molecular weight of around 38 kDa as observed in SDS-PAGE (acrylamide 12%). His-tagged PSA was not successfully purified as a single band through Ni–NTA affinity chromatography, and this recombinant protein appeared as lightly band at a smaller size on the western blotting (Fig. 5A).

The expected size of the *sf*GFP-PSA fusion protein is 65 kDa, but we observed two bands, one of them at the correct size and the other around 30 kDa (indicating separation product). We confirmed it was *sf*GFP through western blotting using polyclonal anti-GFP. To improve fusion product, we used protease inhibitors before its purification; however, that did not give any significant effect (Fig. 5B).

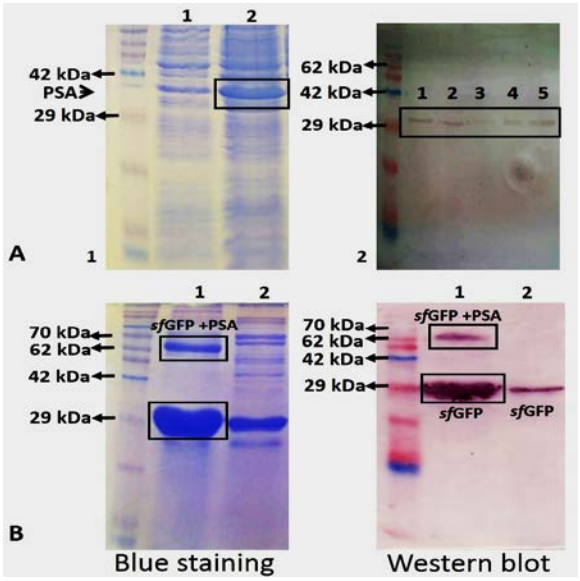


**Figure 5.** Expression of PSA and *sf*GFP-PSA fusion protein in *E. coli*.

**A: A-1** Detection of PSA expression with a molecular weight of 38 kDa in total cell cytoplasmic extract by SDS-PAGE separation (acrylamide 12 %) and coomassie blue staining. Lane 1: T0 expression; Lane 2: overnight expression. **A-2** Detection of the purified PSA protein, protein samples obtained after different steps purification were analyzed by western blotting, visualized using anti-6 × His tag antibodies. Lane 1: T0 expression; Lane 2: overnight expression; Lane 3: flowthrough sample; Lane 4: wash sample; Lane 5: Pure PSA protein.

**B:** Detection of the purified *sf*GFP-PSA (65 kDa) was done after SDS-PAGE separation (acrylamide 12 %), either by blue staining or by western blotting using anti-GFP antibodies. Lanes 1: Pure *sf*GFP-PSA fusion protein; Lanes 2: Pure *sf*GFP protein (control). Molecular mass standard (in kDa).

#### *3.4.2. Expression and detection of sfGFP-PSA fusion protein in eukaryotic cells:*

HEK-293T cells were transfected with pcDNA-*sf*GFP-PSA construct. Evaluation of fusion protein PSA-*sf*GFP expression by fluorescence microscopy at 12, 24, and 48 h after transfection showed that the construct was efficiently expressed in HEK cells. The expression of the *sf*GFP-PSA fusion protein with a molecular weight of 65 kDa was verified in the supernatants medium by western blotting using polyclonal anti-GFP, so that the correct length of the fusion protein has been demonstrated and there was no separation for *sf*GFP-PSA fusion product (Fig. 6).

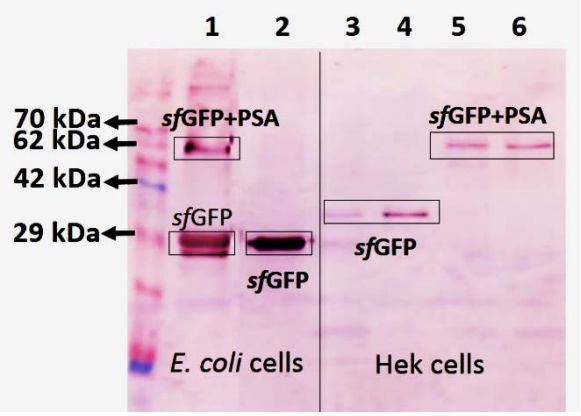


**Figure 6.** Expression of *sf*GFP-PSA fusion protein in HEK-293T cells.

Detection of *sf*GFP-PSA fusion protein (65 kDa) produced in HEK-293T cells. The Supernatants medium of transfected HEK-293T cells was analyzed by western blotting after 48 h of incubation, *sf*GFP-PSA fusion protein was detected by polyclonal rabbit anti-GFP antibodies. Lane 1: *sf*GFP-PSA fusion protein expressed in bacteria; Lane 2: *sf*GFP protein expressed in bacteria (control); Lanes 3, 4: *sf*GFP protein expressed in HEK-293T cells (control); Lanes 5, 6: *sf*GFP-PSA fusion protein expressed in HEK-293T cells. Molecular mass standard (in kDa).

## 4. Discussion

It is well-established that *L. major*-secreted molecules are highly immunogenic and can confer protective effects when introduced into experimental subjects (Tonui *et al*., 2004). Therefore, current efforts are directed towards assessing Leishmanial secreted proteins as future candidates for vaccines and targets for medications. Currently, the success of subunit vaccines based on recombinant proteins or peptides has been variable to poor, and more efforts are needed to reach optimal results (Kumar and Engwerda, 2014). Our data showed that prokaryotic-based systems succeeded in producing the full-length PSA when it was fused with *sf*GFP, which may be significant in the development of PSA-based vaccines.

In the context of developing vaccines based on proteins and peptides, several factors should be considered such as the properties of the proteins of interest, which are largely determined by its amino acid sequence, and the specific post-translational modifications taking place during or after synthesis of the polypeptide chain (Walsh *et al*., 2005). Some polypeptides such as PSA need to be in their native conformation to maintain its immunogenicity and *Escherichia coli* derived recombinant proteins may not achieve this requirement (Handman *et al*., 1995; Sjölander *et al*., 1998a). This problem could be solved by exploitation of the parasites themselves by overexpression of parasitic antigens in transfected nonpathogenic *Leishmania* strains and by the development of a DNA based vaccine (Chamakh-Ayari *et al*., 2014; Petitdidier *et al*., 2016; Sjölander *et al*., 1998b). DNA vaccine is attractive because they ensure appropriate folding of the polypeptide, and it is simple production technology (Alarcon *et al*., 1999; Handman, 2001; Hasan *et al*., 1999). Although many alternative organisms and expression systems are also used to produce PSA protein, *E. coli* rests the least expensive, easiest and quickest (Demain and Vaishnav, 2009). The GFP is commonly used as an excellent expression tag for many fusion protein, and it has been expressed in a variety of organisms, including bacteria, and mammals (Gerdes and Kaether, 1996; Waldo *et al*., 1999). An increased solubility was observed for several proteins when fused to *sf*GFP, proving the importance of this tag as a mean to improved protein expression, detection, and purification (Andrews *et al*., 2007). Here, due to the difficulty expressing and purifying PSA protein in *E. coli*, *sf*GFP was fused to PSA. We constructed a recombinant plasmids pRSET-*sf*GFP-PSA and pcDNA-*sf*GFP-PSA that can produce *sf*GFP-PSA fusion protein in *E. coli*, and eukaryotic cells respectively. In *E. coli*, the *sf*GFP separation may have resulted from overexpression of the fusion protein, and we probably need to change the whole expression system or one of its factors (promoter, cell strain, induction period) to decrease the overexpression rate and, therefore, separation. *sf*GFP protein as a fusion partner allowed us to monitor the existence of *sf*GFP-PSA fusion protein through expression and purification steps, as well as verify fusion protein production with the correct molecular weight by western blotting using anti-GFP.

## 5. Conclusion

In this study, we described an approach to expression of full-length PSA as a fusion protein with *sf*GFP. Our data showed that prokaryotic-based systems succeeded in producing the full-length PSA when it was fused with *sf*GFP; also, the plasmid DNA encoding *sf*GFP-PSA protein was expressed in eukaryotic cells indicating that the designing and cloning of the recombinant construct is correct and applicable. The potential protection by these vaccine candidates against experimental *L. major* infection will be evaluated in animal models.

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