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Application of DsbA Signal Peptide for Soluble Expression of *Leishmania infantum* P4 Nuclease in *E. coli*

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ABSTRACT

Drugs available for treatment of Visceral Leishmaniasis (VL) are toxic and drug resistance is increasing in many parts of the world, thus it seems that vaccine development is an ideal method for prevention and control of VL. P4 nuclease of *L. infantum*, an amastigote stage specific protein, is considered as a good candidate for VL. Previous efforts for recombinant expression of this protein in *E. coli* resulted in production as inclusion body form. In the present study, the effect of DsbA signal peptide on periplasmic expression and production of soluble recombinant P4 antigen were examined. DNA extracted from *L. infantum* was used for amplification of P4 nuclease gene (Li-P4) by PCR. The product was cloned, sequenced and expressed in *E. coli* under signal sequence DsbA. The results indicated that periplasmic expression of Li-P4 gene in *E. coli* leads to production of high levels of recombinant protein in soluble form.

Key words: *L. infantum*, DsbA signal peptide, P4 nuclease, vaccine candidate

INTRODUCTION

Protozoan parasites of the genus *Leishmania* causes a spectrum of clinical disease, including cutaneous, mucocutaneous and Visceral Leishmaniasis (VL). Approximately 12 million people are infected worldwide with 1.5-2 million new cases occurring each year (Desjeux, 2004). *Leishmania* parasites are dimorphic organisms which exist as promastigote in extracellular stage and in the sandfly midgut and as amastigote that lives intracellularly in the phagolysosomes of macrophages in the mammalian host cells (Campos-Neto *et al*., 2001; Campbell *et al*., 2003). Unfortunately, currently available treatment regimens are non-selective drugs with significant toxicity and limited efficacy (Jackson *et al*., 1990; Grogl *et al*., 1992). In the other hands, drug resistance is increasing in many parts of the world (Croft and Coombs, 2003), thus it is believed that vaccine development is an ideal method for prevention and control of *Leishmania* patients. P4 nuclease is an intracellular amastigote-specific protein that is initially identified in *L. amazonensis* (Soong *et al*., 1995) and then characterized in *L. pifanoi* (Kar *et al*., 2000), *L. major* (Farajnia *et al*., 2004) and *L. infantum* (Farajnia *et al*., 2011). Immunoblotting analysis of the purified Li-P4 with sera of VL patients indicated that Li-P4 protein is a highly immunogenic protein expressed in the amastigote-stage of *L. infantum* and could be considered as a potent vaccine candidate against VL caused by *L. infantum* (Farajnia *et al*., 2011). It has been reported that cytoplasmic expression of Li-P4 in *E. coli* resulted in accumulation as inclusion body due to disulfide bonded hydrophobic nature of the protein (Farajnia *et al*., 2004). Disulfide bonds are crucial for the folding, stability and

function of many extra-cytoplasmic proteins (Gilbert, 1997; Ritz and Beckwith, 2001). One of the methods used for production of soluble proteins is exportation of the protein to the periplasmic space (Schierle *et al*., 2003), where correct disulfide bonds are formed. This translocation is mediated by signal peptides that carried in the N-terminal of secretary proteins.

The objective of the present study was the evaluation of DsbA Signal peptide on soluble expression of Li-p4 protein in *E. coli*.

MATERIALS AND METHODS

Parasite and DNA extraction: In this study, Iranian strain of *L. infantum* was used. Promastigotes were cultured at 26°C in RPMI 1640 medium with glutamine (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich). Organisms were harvested in logarithmic phase and washed with phosphate buffer saline (PBS, pH 7.2). Parasites were disrupted in lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0) and incubated overnight with proteinase K (100 mg mL^{-1}, Sigma-Aldrich) at 37°C. DNA was then purified by phenol-chloroform extraction and ethanol precipitation.

PCR amplification: A pair of primers was designed based on P4 gene sequence previously reported for Cutaneous Leishmaniasis (CL) strains: Lip4-F:5'-TAGAGCTCGTGGGGCTGCGTGGGT CACAT-3', Lip4-R:5'-ATGTCGACCGCACCTCGCTTCGGACGTG-3'. Each PCR reaction contained 200 ng DNA, 10 p mol each of forward and reverse primers, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1x PCR buffer, 2 unit of Pfu DNA polymerase (Fermentas) and up to 25 µL dH_2O. PCR amplification was carried out in 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63.0°C for 60 sec and extension at 72°C for 60 sec with a final extension cycle at 72°C for 5 min. PCR products were electrophoresed on 1.5% agarose gel and stained by ethidium bromide. The DNA bands were visualized under an ultraviolet light (UV transilluminator) and documented.

Gene cloning: The PCR product was purified by PCR product purification kit (Roche) and ligated into the pGEM-T easy (Promega) vector. The ligation reaction was transformed into DH5α (Promega) competent cells and plated on Luria-Bertani agar (LB Agar) containing ampicillin (50 mg mL^{-1}), 5 bromo-4 chloro-3-indolyl-**β**-D-galactoside (X-gal: 20 mM) and isopropyl thio-**β**-D-galactoside (IPTG: 200 mg mL^{-1}). The white colonies containing recombinant plasmid were selected (Bothwell *et al*., 1990) for plasmid extraction and PCR screening (Feliciello and Chinali, 1993). Then cloning was verified by restriction digestion and sequencing.

Expression and solubility analysis: The pGEM-T easy vector containing Li-P4 gene was digested with SalI and Sac I and the insert was purified, subcloned into the SalI-SacI digested pAES30 (Athena system) expression vector and transformed into the *E. coli* DH5α. The bacteria containing pAES30 Li-P4 was cultured in LB broth medium and grown until OD = 0.5. Expression of recombinant Li-P4 was induced by addition of 1 mM isopropyl-**β**-D-thiogalactoside (IPTG) then incubated for further 4 h at 37°C and analyzed by sodium dodecyl sulfate-poly acryl amide gel electrophoresis (SDS-PAGE).

For solubility analysis of recombinant Li-P4, *E. coli* DH5α containing expression vector was cultured in 1 L LB broth medium and following induction with IPTG, the cells was collected by centrifugation (10000 rpm for 15 min). The bacterial sediment was then disrupted in 10 mL lysis buffer (pH = 8, 50 mM NaH_2PO_4, 300 mM NaCl) by sonication (45 sec pulses interrupted with

cooling on ice). The soluble and insoluble fraction was separated by centrifugation of cell sonicate at 12,000 g for 15 min at 4°C and analyzed by SDS-PAGE.

SDS-PAGE analysis: SDS-PAGE was performed using the Laemmli buffer system (Laemmli, 1970). Prior to electrophoresis, the samples (cell lysates and fractions) were heated at 100°C for 10 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol and separated by a 15% SDS-PAGE and stained by coomassie blue G-250. Protein markers used were phosphorylase (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), lactate dehydrigenae 35.0 kDa), RE Bsp981 (25.0), beta-lactoglobolin (18.4) and lysozyme (14.4 kDa).

RESULTS

Gene cloning: After culture, the *L. infantum* was subjected to DNA extraction and Li-P4 gene amplification. PCR amplification of P4 nuclease gene from *L. infantum* resulted in a 862 bp PCR product that was in expected size (Fig. 1). The PCR product was cloned into the pGEM-T easy vector using T-A cloning method and confirmed by restriction digestion (Fig. 2).

Expression and purification of recombinant P4 nuclease: For expression of recombinant Li-P4, the PCR product was subcloned in the SalI-SacI site of pAES30 (Athena) and transformed into the *E. coli* DH5α (Fig. 3). Induction of recombinant protein expression by IPTG resulted in high level of expression that appeared as a 33 kDa band in SDS-PAGE analysis of cell lysates (Fig. 4).

Solubility analysis of *E. coli* expressed Li-P4: The cells harboring pAES30-Li-P4 vector were cultured in 1 L volume and induced by IPTG induction. The cells were sonicated and sup and pellet fractions were separated by centrifugation. SDS-PAGE analysis of fractions after induction for

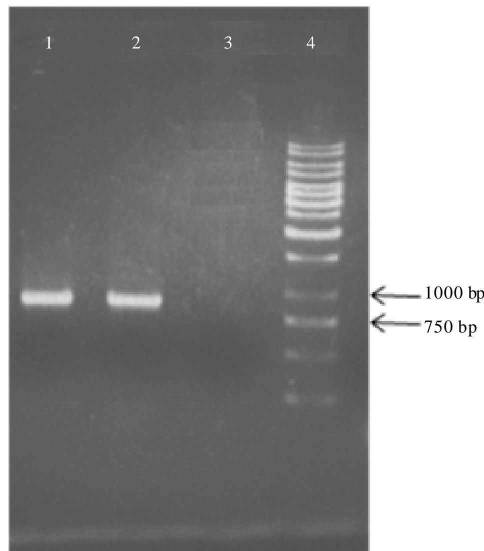

Fig. 1: Amplification of P4 gene of *L. infantum*. Lane 1 and Lane 2: 862 bp Li-P4 gene PCR product, Lane 3: No DNA and Lane 4: 1 kb DNA ladder

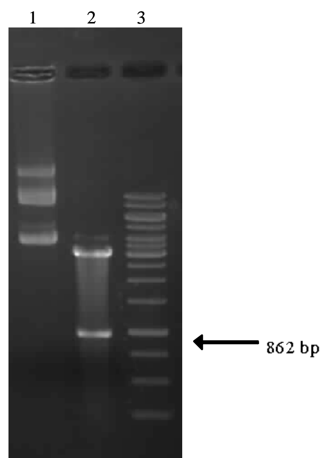

Fig. 2: Cloning of Li-P4 gene in pGEM-T vector. Lane 1: Undigested recombinant plasmid, Lane 2: SalI-SacI digested recombinant plasmid and Lane 3: 1 kb DNA ladder

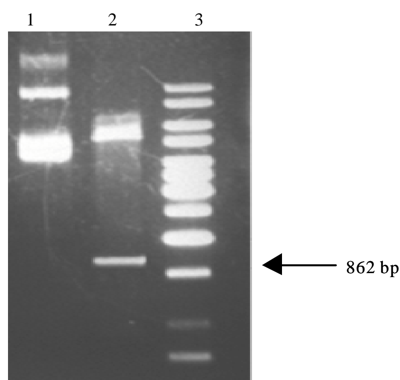

Fig. 3: Cloning Li-P4 gene in pAES30 vector. Lane 1: Recombinant plasmid, undigested, Lane 2: SalI-SacI digested recombinant plasmid and Lane 3: 1 kb DNA ladder

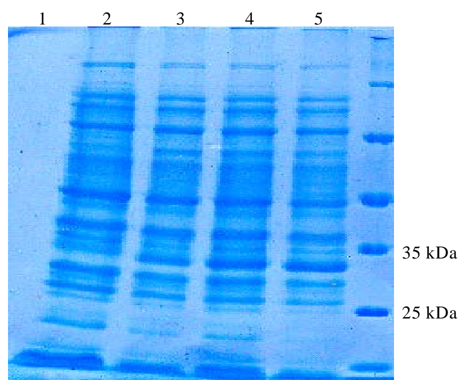

Fig. 4: SDS-PAGE analysis of recombinant Li-P4 produced in *E.coli* DH5 , Lane 1: Bacterial lysate before induction, Lane 2: Bacterial lysate1 h after induction with IPTG, Lane 3: Bacterial lysate 2 h after induction, Lane 4: Bacterial lysate 3 h after induction and Lane 5: Molecular weight marker

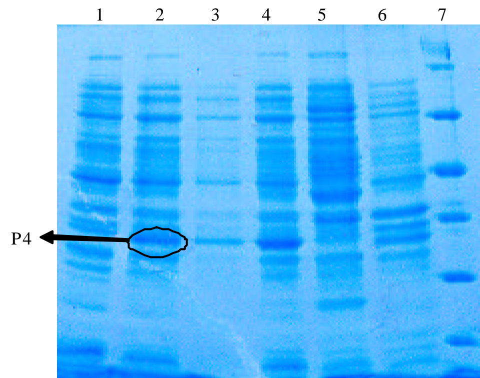

Fig. 5: Solubility analysis of recombinant Li-P4 produced in *E.coli* DH5α, Lane 1: Bacterial lysate before induction, Lane 2: Bacterial lysate after induction with IPTG, Lane 3: Cells pellet 3 h after induction with IPTG, Lane 4: Cells soup 3 h after induction, Lane 5: Cells soup 24 h after induction, Lane 6: Cells pellet 24 h after induction and Lane 7: Molecular weight marker

different hours revealed that Li-P4 was present mainly in soluble fraction after induction for 3 h whereas, expression for longer times increased protein in insoluble fraction with complete insoluble expression in 24 h samples (Fig. 5).

DISCUSSION

Different vaccine candidates have been tried for prevention of VL among them P4 nuclease antigen showed promising results in several studies. P4 protein is a single strand specific nuclease that has shown to be conserved in different species of *Leishmania* (Soong *et al.*, 1995; Kar *et al.*, 2000; Farajnia *et al.*, 2004). We recently characterized P4 nuclease in *L. infantum* and showed that sera from VL patients highly reacted with this protein indicating immunogenic nature of this protein (Farajnia *et al.*, 2011). These finding suggested Li-P4 as a promising vaccine candidate for VL.

Previous studies have shown that Li-P4 appears as inclusion body during recombinant expression in *E. coli*. Structural analysis shows that Li-P4 is a highly hydrophobic protein containing 2 disulfide bonds. Formation of disulfide bonds is critical for correct folding, stability and export of many secreted proteins by gram-negative bacteria (Missiakas and Raina, 1997; Rietsch and Beckwith, 1998). One of the methods for production of recombinant proteins in soluble form in the bacteria is exportation of proteins to the periplasmic space (Schierle *et al.*, 2003). Translocation to the periplasm of *E. coli* has several advantages over cytoplasmic expression including simplified downstream processing, higher product solubility, enhanced biological activity and N-terminal authenticity of the expressed proteins (Cornelis, 2000; Macrides, 1996; Mergulhao *et al.*, 2004). Furthermore, for proteins expressed in the periplasmic space, a simple osmotic shock or cell wall permeabilization can be used to obtain the product without contamination with cytoplasmic proteins (Mergulhao *et al.*, 2004; Shokri *et al.*, 2003).

Different signal peptides have been attempted for periplasmic expression of proteins, including OmpA (Chen *et al.*, 1980), DsbA (Schierle *et al.*, 2003), PelB (Guo *et al.*, 1995) and PhoA

(Inouye *et al*., 1982), among them DsbA signal peptide has shown good results in several studies (Schierle *et al*., 2003; Soares *et al*., 2003). DsbA is an oxidoreductase enzyme that are exported from the cytoplasm to the periplasmic space by its signal peptide (Jonda *et al*., 1999; Randall *et al*., 1998). Schierle *et al*. (2003) has shown that in comparison to PhoA, DsbA signal sequence exports the cytoplasmic protein thioredoxin 1 efficiently to the periplasmic space whereas, translocation by PhoA signal sequence had very low yields (Debarbieux and Beckwith, 1998). It has been suggested that the reason for this effect is related to the ability of DsbA signal sequence to direct the fused protein into the co-translational SRP pathway (Schierle *et al*., 2003). It has also shown that recombinant expression of human growth hormone by using DsbA signal peptides leads to higher expression level and solubility compared to expression without DsbA signal (Soares *et al*., 2003). In the present study we used the DsbA signal peptide for periplasmic soluble expression of Li-P4 in *E. coli*. The results showed that recombinant Li-P4 highly expressed using this signal peptide. This finding is consistent with previous reports about the potential of DsbA signal peptide (Schierle *et al*., 2003; Debarbieux and Beckwith, 1998). Analysis of time course of expression revealed that the recombinant protein appeared in soluble form 3 h after induction with IPTG whereas, continuation of incubation to 6 h and more result in gradual formation of inclusion body. The reason for such phenomena is not clear and may be related to the limited periplasmic export capacity of *E. coli*. By accumulation of recombinant protein expressed in cytoplasm of the cells, the proteins aggregate gradually results in inclusion body formation.

In conclusion, the results of present study indicated that expression of hydrophobic Li-P4 under DsbA signal peptide leads to soluble expression in *E. coli*. This finding could be exploited for soluble expression of other recombinant proteins that are expressed as inclusion body in bacteria.

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