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Research Article

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High-Level Expression of Recombinant Saposin-Like Protein-2 of *Fasciola hepatica* and its Single Step Purification

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ABSTRACT

The importance of fascioliasis, as a zoonotic disease is severe damage to the liver and bile ducts. Thus, early diagnosis for prevention from irrecoverable damage is significance. The wide ranges of antigens for diagnosis of human and animal fascioliasis including native and recombinant proteins have been reported, but the latter forms showed more advantages than former ones. Saposin like protein 2 (SAP-2) is one of them that sounds useful for early detection of infection. In this study, the areas of signal peptides of Fasciola hepatica SAP-2 (FhSAP-2) gene are omitted in order to get higher expression. Truncated (t) FhSAP-2 gene was designed in the cloning vector pGH and then sub-cloned into pET-28b (+) expression vector. The recombinant plasmid pET28b (+)-tSAP-2 were transformed into different strains of Escherichia coli, such as BL21 (AI), BL21 (DE3) and origami (DE3) bacteria. The expression of recombinant FhtSAP-2 protein was induced with IPTG and arabinose. A Ni 2+ -NTA column was used for purification of the protein and analyzed by SDS-PAGE and Western blotting.The results showed that the highest level of expression of the protein was obtained in BL21 (AI) strain and a protein band of 10kDa in induced bacteria. The recombinant protein was purified by metal-affinity chromatography on Ni 2+ -NTA. FhrtSAP-2 protein was analyzed by western blot authentication.We concluded that Bl21 (AI) can be introduced as a suitable strain for expression of rtFhSAP-2.

Keywords: Fascioliasis; RecombinantSAP-2; Bl21 (AI) Expression

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INTRODUCTION

Fascioliasis is highly pathogenic caused by the liver fluke *Fasciola hepatica* and known as important disease in human and animals. Infection occur when hosts ingest the contaminated vegetables or water with metacercariae stage of *Fasciola* [1,2].The activity of this parasite in human body can cause acute or long term chronic disease. Based on WHO roadmap 2020 [3], fascioliasis have a specific place due to its emergence, worldwide distribution, and estimation of 17 million individuals infected throughout the world [4]. This infection also was reported as an important chronic disease, especially in the Guilan province, northern Iran (up to 10,000 infected patients) [5-7]. As a result prevention, early diagnosis and treatment are crucial. The coprological test is gold standard diagnostic method for human fascioliasis, with delay of 12 weeks after infection, when the parasite has migrated to the bile ducts and matured[1,8]. Reports indicated that, human immune response to *Fasciola* antigens occurs in early stages of the infection [1,9]. The antigens that generally used in serological tests are made from excretory/secretory (E/S) products of the live parasites that have shown cross reaction with other helminthes infections. Previous researches have reported that, using recombinant antigens increase the specificity of the diagnostic tests [8,10]. According to the reports, saposin like protein 2 (SAP-2) is one of them that can be used for diagnostic tests in early stages of chronic human fascioliasis [11-14].In this study, different strains of *E.coli* were evaluated in order to optimize the expression of FhSAP-2 protein. Over-expression of heterologous recombinant proteins in *E. coli* often leads to accumulation of insoluble aggregates, formation of inclusion bodies (IBs), and therefore expression of only about 30% of soluble form [15,16]. However, in recent years, reports indicated that IBs have many biotechnological

application and methods including isolation from cell, solubilization, purification and refolding, which are needed to obtain soluble bioactive proteins from IBs [17,18]. Thus the optimized rtFhSAP-2 can be applied in the next researches for detecting its usefulness in developing a serological test and/or its effectiveness in vaccine.

EXPERIMENTAL SECTION

Bacterial Strains and Vector

The *E. coli* DH5 strain (Invitrogen, Carlsbad, CA, USA), BL21 (DE3), BL21 (AI) and origami (DE3) (Promega, Madison, WI, USA) were used for cloning and expression of recombinant FhSAP-2 protein.

Construction of the Recombinant Expression Vector pET28b-FhSAP-2

A 270bp of SAP-2 fragment encoding amino acid 16 to 101(accession no. AF286903.1) excluding the hydrophobic signal peptide, in pGH vector by Generay Biotech co. (Shanghai, China). The truncated fragment of SAP-2 was cleaved with XhoI and NdeI enzymes, then was sub-cloned in pET-28b (+). The presence of the tSAP-2 fragment in the expression vector was appraised by colony-PCR, digestion enzymes, and finally confirmed with sequence determination by Macrogen co. (Seoul, Korea).

Expression of Recombinant tFhSAP-2 (rtFhSAP-2)

E. coli strains such as origami (DE3), BL21(DE3) and BL21 (AI) were transformed by pET28_tSAP-2 and grown in Luria Bertani (LB) broth, in addition to 25 μg/ml kanamycin and 5 mg/ml tetracycline. The culture was grown with sever shaking at 28ºC. Protein production was induced by 0.2mM isopropyl-β-D-thiogalactoside (IPTG), 0.05, 0.1, 0.2% arabinose and OD600 of 0.6-1nm. Bacteria were incubated by sever shaking for 2, 4 and 16 hr to abtained the best expression situations. The expression of rtFhSAP-2 was analyzed by 12% SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) and densitometry.

Analysis of Protein Solubility

The optimized expression strain was cultured in final volume of 50 ml media and induced by IPTG and arabinose. Bacterial pellet was harvested by centrifugation at 5000rpm for 15 min and re-suspended in (diluted 1/10) Solubility buffer (300 mM Tris-Base, 5 M Nacl, pH 9.6). Cells were disrupted by intermittent sonication (Hielscher) for 4 min on ice using 20 s pulses and 30 s breaks and then incubated on ice for 20 min. Insoluble materials were separated by centrifugation at 5000rpm for 15 min and soluble parts were analyzed on 12% SDS-PAGE.

Isolation and Washing of Inclusion Bodies

The pellet from a 250 ml bacterial culture were suspended in 14 ml washing buffer1 (Tris–Cl 50 mM, EDTA 4 mM, 2ME 14M, pH 8) then was vortex and centrifuged at 8000 rpm for 20 min at 4°C to obtain Inclusion bodies (IBs). After that 14ml lysis buffer (the wash buffer containing Triton X-100 1.0%) were used to remove bacterial impurities and cell residue. The pellet were Disrupted for 10 min with sonication on ice (Hielscher, Germany at 80% power, 40 s intervals), then incubated for 20 min at room temperature (gentle shaking). Once more centrifugedin the same condition as mentioned above. Finally, 14 ml washing buffer 2 (Tris–Cl 50 mM, EDTA 4 mM, pH 8) were added, and after vortexing the cells were centrifuged as before.

Purification of rtSAP-2 by Immobilized Metal Affinity Chromatography (IMAC)

The obtained pellet from washing of IBs, were suspended in 25 ml of binding buffer (Tris–Cl 30 mM, NaCl 500 mM, Urea 8 M and imidazole 2 mM, pH 8.0), containing dithiothreitol (DTT) 10mM and phenyl methyl sulfonyl fluoride (PMSF) 5mM. The cells suspension were sonicated for 6 min, and then centrifuged at 8000 rpm at 4°C for 20 min. The Supernatent of rtSAP-2 protein was purified by affinity chromatography using a 5 ml column His-Trap™ FF (GE), which previously equilibrated with binding buffer. The column was washed (the binding buffer containing 30 mM imidazole) and rtSAP-2 was then eluted with the elution buffer, containing 500 mM imidazole and 5 M urea at the flow rate of 2.0 ml/min. The concentration of purified rtSAP-2 protein was determined by Bradford method and refolding was done by dialysis method based on report by Mirzadeh et al [13].

Electrophoresis and Western Blot Analysis for rtSAP-2

The protein samples were analyzed by SDS PAGE, and transferred onto PVDF membrane. Membrane was blocked with 2% BSA in PBS containing 0.1% Tween20, pH 7.2 and probed with pooled human sera diluted 1:100 in blocking buffer andgoat anti-human IgG antibody conjugated to horseradish peroxidase (HRP) with 1:10000

dilution. At the end, the protein bands were revealed by the peroxidase activity on 3, 3′-diaminobenzidine tetrahydrocholoride (DAB) as the chromogenic substrate (Kem-En-Tec, Taastrup, Denmark).

RESULTS AND DISCUSSION

Cloning of FhSAP-2 Gene Fragment

After sub cloning the FhSAP-2 gene fragment in pET28b(+)plasmid, the recombinant plasmid of pET-28b-FhtSAP-2 confirmed by double digested with Nde1 and Xho1 restriction enzymes as shown in Figure 1. Sequence analysis of the insert of tSAP-2 displayed 100% similarity. Between sequences of SAP-2 of *F.hepatica* cloned gene and published data in GenBank (AF286903.1) (data not shown).

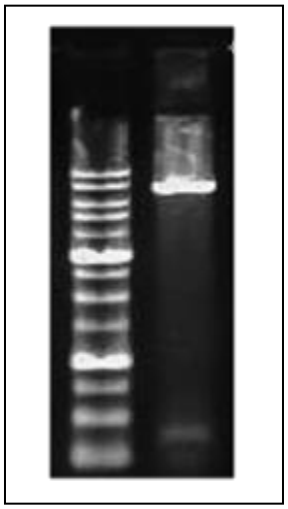

Figure 1: Identification of recombinant plasmid pET28b-tSAP-2 by enzymatic digestion. Lane1: 1 kb DNA size marker, lane 2: Nde1/Xho1 digested pET28b-tFhSAP-2

Optimized Expression of FhtSAP-2

Expression of rtFhSAP-2 protein was investigated in 3 different *E. coli* strains including origami (DE3), BL21 (DE3) and BL21 (AI).The optimum expression was attained, in Bl21(AI) cell, induced by IPTG at concentration of 0.2mM and 0.05% arabinose, cell density (OD600) of 0.6 and induction time 16hr with temperature of 28°C, (Figures 2-4) respectively. Results showed a protein band at 10 kDa in induced bacteria by analyzed SDS-PAGE. The recombinant protein solubility was studied at the optimized expression condition. Due to the fact, the analysis illustrated that most of the expressed rtSAP-2 was detected in the inclusion bodies (IBs) form, In this study we attempted to isolate IBs by washing it, in order to dispel bacterial impurities and cell remaining.

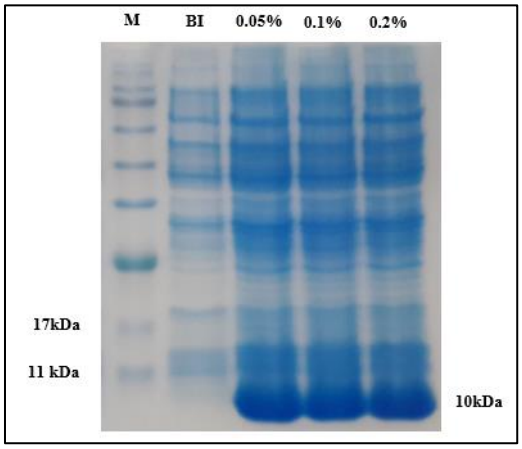

Figure 2: Expression of rtFhSAP-2 at concentration 0.2mM of IPTG and differentpercent'sof arabinose. Bl21 (AI) bacteria were induced with arabinose at Percents 0.05% to 0.2%.Analysisof rtFhSAP-2 protein band showed no difference in protein expression when arabinose increased from 0.05% to 0.2%. BI: before induction. M: protein ladder.

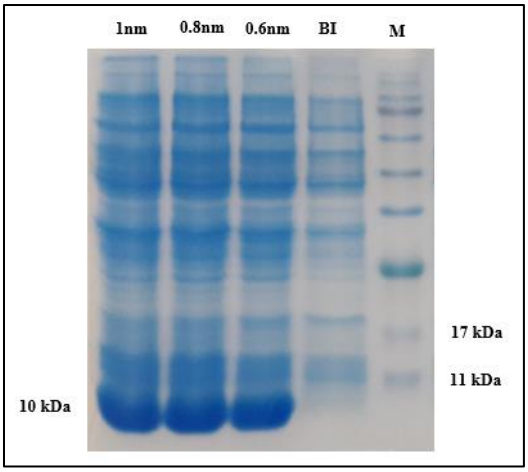

Figure 3: Expression of rtFhSAP-2 at different cell density. Bl21 (AI) bacteria were induced in OD6000.6, 0.8and 1nm.Densitometry analysis of rtFhSAP-2 protein band showed no difference in protein expression when cell density increased from 0.6nm to 1nm. BI: before induction. M: protein ladder

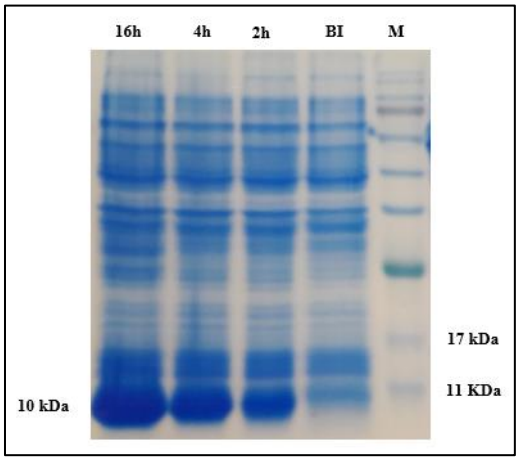

Figure 4: Expression of rtFhSAP-2 at different times of induction. Bl21 (AI) bacteria were incubated for 2, 4 and 16 hr. analysis of rtFhSAP-2 protein band showed highest expression of rtFhSAP-2 was achieved 16hrafter induction. BI: before induction M: protein ladder

Purification of Recombinant tFhSAP-2 Protein

The Soluble proteins rtSAP-2 was purified by a Ni-NTA column using immobilized affinity chromatography (IMAC). About 200 mg of rtSAP-2 was obtained with purity of 92.03% measured by Image J software (Figure 5).

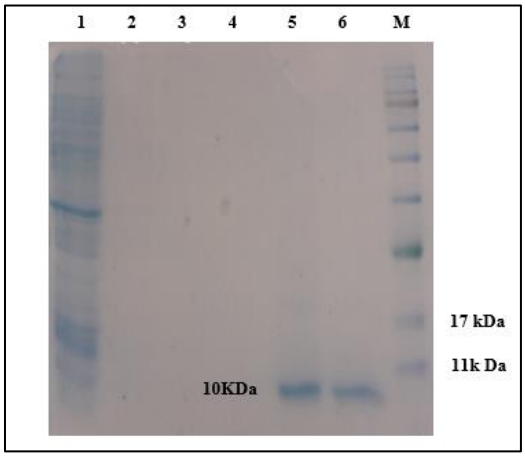

Figure 5: Purification of rtFhSAP-2. RtFhSAP-2 protein was purified by Ni2+-NTA affinity chromatography and analyzed by SDS-PAGE. Lane 1: flow through. lane 2:Wash1.lane 3: Wash2. Lane 4: Wash 3. Lane 5: Elution 1.lane6: Elution 2. M: protein ladder

Western Blot Analysis of rtFhSAP-2

To assay antigenicity of rtFhSAP-2, the purified protein was transferred onto a PVDF membrane and probed with pooled human sera representative of *Fasciola hepatica* infection. The reaction of the serum with the antigen showed the presence of antibody in the serum samples (Figure 6).

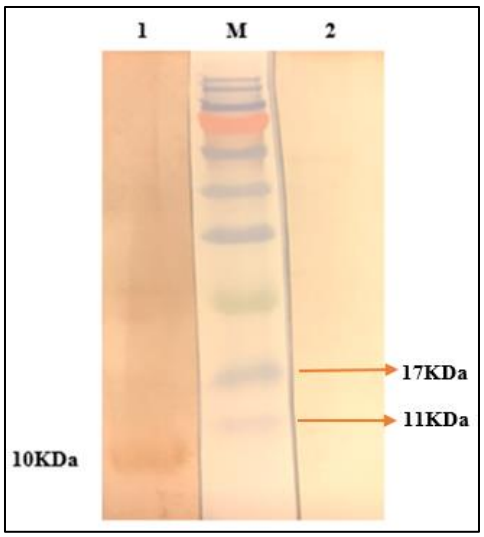

Figure 6: Western blot analysis of purified rtFhSAP-2 with human sera of fascioliasis -2. Lane 1: chronic phase, Lane 2: negative phase, M: protein ladder

The gold standard diagnostic method for F. hepatica identification is coprological inspection; however, the initial infection cannot be diagnosed due to lack of the eggs in the feces until 10-12 weeks after infection [19]. In this article we report expression and purification of *F.hepatica* saposin-like protein-2 in *E. coli* as candidate protein in detection of antibody in infected patient. Gene was cut and then sub-cloned into pET-28b (+) expression vector. In addition, expressions in various strains were assessed in order to achieve the proper one. The solubility analysis showed that most of the rtSAP-2 protein was detected in form of IBs. In this study to achieve optimized expression, recombinant plasmid pET-28b (+) tFhSAP-2 were surveyed in various conditions including different strains of *E. coli*, various concentrations of IPTG and arabinose, incubation times and different cell density. The highest expression was observed in Bl21 (AI) strain with 0.2 mM IPTG and 0.05% arabinose, OD (600) =0.6 and incubation time of 16 hr. Therefore, this finding indicated that rtFhSAP-2 is more likely to be a toxic protein as a result of obtaining a desirable expression in Bl21 (AI) [20]. This strain is specifically designed for recombinant protein expression from any T7-based expression vector. Because T7 RNA polymerase levels can be tightly regulated by L-arabinose [21]. previous reports represented using the full length of the protein that led to lower expression level [13,22]. In other report by Espino et al [23] they used the complete form of pBAD-SAP2. The great advantage of this assay is comparing of rtSAP-2 in three different host i.e. origami (DE3), BL21 (DE3) and BL21 (AI), which finally resulted in high protein expression in BL21 (AI). However in the study by Espino et al [23] only one host was used to expressed this protein. The rtFhSAP-2 protein purified by immobilized metal-affinity chromatography on Ni2+-NTA due to existence of six histidine tags, and finally eluted protein was refolded by dialysis method, according to the method by Espino et al [23] and Mirzadeh et al [13,24]. Disulfide bonds play a critical role in maintaining the overall fold of proteins and are important for the function of proteins [25]. Using of histidine tags significantly improves the purity of the protein [26]. Western blotting method identified IgG antibody of fasciola parasite in serums of infected patients. Optimization is the most effective way to obtain a protein which has a high ability for diagnosis. Despite the widespread use of recombinant proteins in serological tests, there are few studies about recombinant antigens and optimization of the proteins in diagnosis of fascioliasis especially about rtFhSAP-2. Due to importance of recombinant antigens in the diagnostic tests, further studies are needed in this field.

CONCLUSION

In this research we optimized expression and production of rtSAP-2 of *Fasciola hepatica* to obtain highly purified of this recombinant protein in *E.coli.* Our result showed that this purified protein has a high level cross reactivity with human serum in an immunoassay test. This purified protein can be used alone or in combination with other *Fasciola hepatica* antigen for developing serodiagnostic tests of human fascioliasis.

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Conflict of interest: The authors declare that there is no conflict of interest.

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