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Expression, purification and *in vitro* refolding of the recombinant truncated Saposin-like protein 2 antigen for development of diagnosis of human fascioliasis

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ABSTRACT

Early diagnosis of fascioliasis is critical in prevention of injury to the liver and bile ducts. Saposin-like protein (FhSAP-2) is probably the most ideal antigen of *Fasciola hepatica* for development of ELISA kits. SAP-2 has a conserved tertiary structure containing three disulfide bonds and conformational epitopes. Therefore, antigenicity of SAP-2 is greatly depends on disulfide bond formation and proper folding. We produced the recombinant truncated SAP-2 (rtSAP-2) in the SHuffle® T7 and Rosetta strain of *Escherichia coli*, in soluble and insoluble forms, respectively and purified by immobilized metal affinity chromatography (IMAC). The refolding process of denatured rtSAP-2 was performed using dialysis and dilution methods in the presence of chemical additives, along with reduced/oxidized glutathione (*in vitro*). Physicochemical studies, including non-reducing gel electrophoresis, Ellman's assay, Western blotting and ELISA showed the most antigenicity and likely correct folding of rtSAP-2, which was obtained by dialysis method. An IgG ELISA test was developed using rtSAP-2 refolded by dialysis and compared with excretory/secretory products of parasite with 52 positive fascioliasis samples, 79 other parasitic samples and 70 negative controls samples. The results exhibited 100% sensitivity and 98% specificity for rtSAP-2, also, 100% and 95.3% for excretory/secretory (E/S) antigen, respectively. In conclusion, it is suggested that rtSAP-2 with the correct folding could be used as a candidate antigen for detection of human fascioliasis.

1. Introduction

Fascioliasis is an important human and animal parasitic disease, caused by liver flukes of the genus *Fasciola*. The chronic stage of infection lead to serious damage to the liver and bile ducts and introduced as a serious public health risk in human (World Health Organization, 2006). According to WHO reports, more than 2.4–17 million people are infected and also180 million people are at risk of the infection. In addition fascioliasis was reported in northern parts of Iran (Guilan province) and estimated up to 10,000 infected individuals (Massoud, 1989). Therefore, the use of an efficient tool with high sensitivity and specificity is required for early diagnosis of infection. The detection of anti-fluke antibodies in serum with complex antigens such as the excretory/secretory (E/S) products of the flukes by ELISA is considered as a sensitive and reliable mean for early detection of infection (Hillyer et al., 1992). However, E/S product can result in reduction of specificity of the assays due to share similar antigens by various helminthes (Hillyer et al., 1992; Rokni et al., 2002). Previous studies have shown that, recombinant antigens have great potential as diagnostic reagents (Figueroa-Santiago et al., 2011; Gottstein et al., 2014; Kuerpick et al., 2013). Amongst them, FhSAP-2 is one of the most ideal antigens for preparation of vaccine and diagnostic kit in chronic human fascioliasis (Espino and Hillyer, 2004; Espino et al., 2010). The basic principle for the improvement of sensitivity and specificity of serological tests is using the recombinant antigens with correct folding (Allahyari et al., 2015; Hwang et al., 2014). FhSAP-2 antigen belongs to saposin-like protein (SAPLIP) family and has six conserved cysteine residues in amphipathic α-helical domains (Espino

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and Hillyer, 2003). It has been shown that SAPLIPs family contains intermolecular disulfide bonds (Don et al., 2008). Disulfide bond (S–S) formation is essential for the correct folding and ultimately improving the antigenicity of proteins. The selection of expression host has a major impact on production of complex eukaryotic proteins in recombinant form with proper fold (Meyer et al., 2013). In previous studies, *Escherichia coli* was used to obtain recombinant SAP-2 protein (Espino and Hillyer, 2003; Figueroa-Santiago et al., 2011; Gottstein et al., 2014; however, a particular problem of *E. coli* expression system is the production of mis-folded proteins due to reducing cytoplasm and lacking essential enzymes for the creation of S–S bonds (Messens and Collet, 2006; Rosano and Ceccarelli, 2014). In addition, over-expression of heterologous recombinant proteins in *E. coli* often leads to accumulation of inclusion bodies (IBs), (Bednarska et al., 2013; Yang et al., 2011). However, in recent years, reports indicated that IBs have many biotechnological application and methods such as solubilization, purification and refolding are needed to obtain soluble proteins from IBs (Ramón et al., 2014; Singh et al., 2015). On the other hand, SHuffle is an engineered *E. coli* expression strain that is able to form the S–S bond in the cytoplasm and produce the soluble protein (Lobstein et al., 2012). Since presence of signal peptide region in recombinant protein results in reduction of expression, solubility as well as improper folding in *E. coli*, deletion of this region contributes to over expression and accurate folding (Gopal and Kumar, 2013).

Despite the importance of proper folding in upgrading the antigenicity of the recombinant truncated SAP-2 (rtSAP-2), there are no reports to achieve the appropriate folding. Thus in the present study, we produced the rtSAP-2 in various strains of *E. coli*, purified and refolded by different refolding methods. The formation of S–S bonds and so evaluation of correct folding of protein were investigated using the most effective methods such as non-reducing gel electrophoresis and Ellman's assay (Rudyk and Eaton, 2014). Then in order to evaluate the protein antigenicity, Western blotting and ELISA tests were used. The ELISA test was developed for antibody detection in sera using rtSAP-2 obtained with the most antigenicity and likely correct folding, and then compared with E/S antigens.

2. Materials and methods

2.1. Serum samples

A total of 52 confirmed positive serum samples from patients with chronic fascioliasis were provided kindly by Guilan University of Medical Sciences. To appraise potential cross-reactivity of available native parasites in Iran, serum samples were collected from confirmed cases of hydatidosis ($n = 34$), toxocariasis ($n = 21$), toxoplasmosis ($n = 12$), amebiasis ($n = 7$), and ascariasis ($n = 5$). Negative control sera were obtained from 70 healthy subjects in a nonendemic area of fascioliasis with no evidence of *Fasciola hepatica* eggs in their feces.

2.2. Preparation of parasite E/S antigens

Adult flukes were collected from infected cattle's liver from a local slaughterhouse, and *F. hepatica* E/S antigens were prepared as described by Guobadia and Fagbemi (1995).

2.3. Construction of the recombinant expression vector and Sequence analysis

A truncated fragment of 270 bp of SAP-2 encoding amino acid 16–101 excluding the hydrophobic signal peptide was produced according to the SAP-2 sequence on GenBank database (AF286903.1) in pGH vector by Generay Biotech co. (Shanghai, China). The tSAP-2 DNA fragment was cleaved with *XhoI* and *NdeI* enzymes, then was sub-cloned in pET-28b (+) expression plasmid (Novagen) which previously was cleaved by the same enzymes. The presence of the tSAP-2 fragment in the recombinant vector was confirmed with sequence determination by Macrogen co. (Seoul, Korea). The *E. coli* strain DH5α (Invitrogen, CA, USA) was used for plasmid maintenance and cloning.

2.4. Optimization of tSAP-2 protein expression

To produce the rtSAP-2, competent bacteria were transformed by pET28-tSAP-2 and grown in Luria Bertani (LB) broth and super optimal broth with catabolite (SOC) medium. LB medium was enriched with 25 μg/ml kanamycin and 35 μg/ml chloramphenicol and also SOC medium complicated with 100 μg/ml spectinomycin. The Rosetta (DE3) strain (Promega, Madison, WI) incubated at 37 °C with shaking 200 rpm, but the SHuffle® T7 (New England Biolabs, Inc, USA) incubated at two different temperatures, i.e. 30 °C and 22 °C. Expression of rtSAP-2 was induced with different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) (from 0.1 to 8 mM) at OD600 of 0.3–0.6, bacteria were incubated by various shaking for 2, 4, 6 and 16 h in order to find the best expression conditions based on SDS-PAGE analysis.

2.5. Solubility analysis

rtSAP-2 protein was expressed under the optimum conditions in Rosetta and SHuffle stranis. One ml of induced culture was pelleted and the crude cell extract (total protein) were obtained in 10 ml of extraction buffer (30 mM Tris–HCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), pH 8) afterward the mixture was shaken for 30 min in the cold room. The cells suspension was disintegrated by sonication for 5 times (15 s for each time). The supernatant (soluble protein) and the pellet (insoluble protein) were then separated by centrifugation at 8000 rpm, 4 °C for 20 min. Fractions were loaded onto SDS-PAGE and separated proteins were transferred onto polyvinyl difluoride (PVDF) (Millipore, Billerica, MA, USA). The samples were analyzed by western blotting using a HRP-conjugated anti-His tag antibody (Penta-His HRP conjugate, Qiagen, Germany) according to the manufacturer's protocols and percentages of solubility was determined by ImageJ software (Peterson and Clinic, 2016).

2.6. Purification of rtSAP-2 as soluble protein in the SHuffle strain

The cells in 250 ml culture media were centrifuged at 8000 rpm for 20 min and the induced SHuffle cells were collected and resuspended in 25 ml of binding buffer (30 mM Tris–HCl pH 8, 500 mM NaCl, 2 mM imidazole, 2 mM EDTA). The cells suspension was disintegrated by sonication for 10 min. The insoluble fractions were removed by centrifugation at 8000 rpm, 4 °C for 20 min. The BIO-RAD Biologic LP (low pressure) chromatographic system is applied for purification of soluble rtSAP-2 using a HiTrap IMAC FF 5 ml column (GE Healthcare Life Sciences, USA), then the column was washed with the binding buffer containing 30 mM imidazole and eluted with 500 mM imidazole at the flow rate of 2 ml/min. The concentration of purified protein was determined by Bradford method using bovine serum albumin (BSA) as a standard, and followed by western blotting.

2.7. Isolation of inclusion bodies and purification of rtSAP-2 from IBs under denaturing condition

The pellet from a 500 ml Rosseta strain culture suspended in 14 ml isolation buffer (50 mM Tris–HCl, 5 mM EDTA, 350 mM NaCl, 1 mM PMSF, pH 8). Isolation and washing of IBs were done similar to the work of Allahyari et al. (2015), except in the buffer pH, 8