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Optimization of Expression and Purification of *Schistosoma mansoni* Antigens in Fusion with Rhizavidin

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Abstract

Schistosomiasis causes significant morbidity and mortality. Vaccine efforts to date indicate the need to increase the immunogenicity of Schistosoma antigens. The multiple antigen-presenting system, whereby proteins are genetically fused to rhizavidin and affinity linked to biotinylated templates, enables the generation of robust immune responses. The objective of this work was to express and purify the *S. mansoni* antigens, SmTSP-2 and SmCD59.2, in fusion with rhizavidin. The fusion with rhizavidin greatly decreased the expression level of rSmTSP-2, but not rSmCD59.2, and both were expressed in the insoluble fraction, requiring optimization of culture conditions. Evaluation of different *E. coli* strains and media showed that BL21-DE3 cultured in Terrific Broth provided the highest expression levels of both proteins. Investigation of a range of time and temperature of induction showed that *E. coli* strains expressing rRzv:SmTSP-2 and rRzv:SmCD59.2 showed the highest protein production at 23 °C for 15 h. Recombinant proteins were purified by a single step of affinity chromatography allowing isolation of these proteins in high concentration and purity. The optimization process increased final soluble protein yield of rRzv:SmTSP-2 by fourfold and rRzv:SmCD59.2 by tenfold, providing ~20 mg/L of each protein. Optimized fusion protein production will allow antigen use in biotin–rhizavidin affinity platforms.

Keywords Protein expression · Protein purification · Fusion proteins · Rhizavidin–biotin system · Schistosoma proteins

Introduction

Vaccines represent one of the greatest contributions to public health [1]. An enormous effort is invested in the development of new technologies for vaccines against important human diseases, such as schistosomiasis [2].

Subunit vaccine technologies such as, recombinant proteins or synthetic peptides, exploit immunogenic components of pathogens as antigens. However, many recombinant proteins are not sufficiently immunogenic to induce protective immunity and usually require potent adjuvants to ensure robust and long-lasting immune responses.

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Approaches with great potential to increase the immune responses are the new antigen presenting systems [1]. A recently developed strategy, multiple antigen-presenting system (MAPS), can incorporate recombinant proteins in fusion with rhizavidin into templates conjugated with biotin, generating a macromolecular complex. The integration of several antigen components, including polysaccharides and proteins in the same construct, has shown induction of a broad spectrum of immune responses, including specific antibodies, Th1 and Th17 responses [3]. The genetic fusion of the antigen to rhizavidin allows its affinity coupling to the biotinylated matrix [3].

The avidin–biotin interaction has been widely used as affinity probes and matrices for a wide variety of applications in biochemical assays, diagnostics, affinity purification, and drug administration [4]. In order to further expand the applications of the avidin–biotin technology, several developments have been investigated to link proteins or antigens to biotin-binding proteins [5–7]. Rhizavidin from *Rhizobium etli* is the first naturally occurring dimeric protein in the avidin protein family [8]. The novel rhizavidin–biotin-based MAPS has been studied in humans and found to be safe and immunogenic by Affinivax with ASP3772, its novel vaccine-targeting *Streptococcus pneumoniae* (ClinicalTrials.gov Identifier: NCT03803202).

The development of Schistosoma vaccines has gone through several phases. Initially, using irradiated cercarial immunization, both cell-mediated and humoral immune responses can be observed and contribute to protection in given model systems, ranging from rodents to non-human primates [9]. Following genome, transcriptome, and proteome studies [10–12], many potential vaccine antigens have been identified, cloned, expressed, purified, and evaluated for the induction of protective immune responses [13]. Overall, the results highlight the need to increase the immune response induced [14, 15].

This work aimed at the production of two recombinant vaccine antigens from *S. mansoni* in fusion with rhizavidin. The proteins produced in this study will be incorporated into the novel antigen-presenting system, MAPS, to be evaluated as vaccine. Thus, two surface antigens were selected. SmTSP-2 is an integral membrane Tetraspanin (TSP) that plays a structural role in establishing and maintaining the integrity of the parasite's tegument membranes. In *S. mansoni*, TSPs act as "support" proteins, providing a structure of homo and heterodimers, forming complexes with other proteins, such as Anexina B30, Sm29, dysferlin, and calpain. These complexes are known as Tetraspanin-enriched microdomains (TEMs) involved in plasma membrane fusion and fission activities [16, 17]. SmCD59.2 is essential for the development of the schistosomule, the circulating larva during the first days of infection, being immunogenic at the stage of intense host–pathogen interaction [18]. Orthologs

of this protein have recently been proposed as new vaccine targets for *Fasciola hepatica* and *S. japonicum* [18, 19].

The choice of expression system to produce an active biopharmaceutical substance is crucial in the development of manufacturing technologies [20]. Therefore, we explore the viability of *E. coli*-based expression systems which allow rapid bacterial growth, simple scaling of the process and are usually economically viable. In the present study, the coding sequences of SmTSP-2 and SmCD59.2 were genetically fused to the rhizavidin gene, and methods to optimize the production of these antigens in *E. coli* were explored in order to improve yields and quality of the final products.

Materials and Methods

DNA Manipulation

DNA cloning and gel electrophoresis were performed according to standard procedures [21]. Plasmid DNA was isolated using the Plasmid Mini Isolation Kit or the Illustra Plasmid Prep Mini Spin Kit (GE life science, US) according to the manufacturer's instructions. All restriction enzymes, ligase, and DNA ladder were purchased from New England BioLabs (US) and used according to the manufacturer's instructions. The amplification of DNA was conducted on a Veriti Thermal Cycler (Applied Biosystems, US) using GoTaq Flexi DNA (Promega, US) using standard conditions. The primers used in this study were synthesized by the Molecular Biotechnology Company (Brazil). PCR products were separated on agarose gels and purified using an Illustra GFX PCR DNA and Gel Band Purification (GE Healthcare, US) according to the manufacturer's instructions.

Genes and Vector Construction

The pET21b-*rhavi* plasmid containing the sequence encoding the rhizavidin gene (*rhavi*), the secretion signal and a flexible ligand in the 3' terminal region of the *rhavi* gene (Supplementary Figs. 1 and 2) was previously produced at Boston Children's Hospital [3]. The nucleotide sequences for the *smtsp-2* and *smcd59* genes were identified in GeneBank (Accession number: Af521091) and GeneDB (Accession number: Smp_105220), respectively. The signal peptide prediction was performed using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/) and excluded from the cloned sequence of each gene. The gene fragments corresponding to the protein sequences for SmTSP-2 (Gene Bank Af521091, aa E107-H184) and SmCD59.2 (GeneDB Smp_105220, aa C27-H101) were amplified from a *S. mansoni* cDNA library using the following primers: *smtsp-2* forward: 5'-TAC<u>GAGCTC</u>CGAAAAGCCCAAGGTCAAA-3' and; reverse: 3'- TAC<u>CTCGAG</u>GTGCGCTTTGCT

TAGATC-3'; and *smcd59* forward: 5'- TAC<u>GAGCTC</u>CTG TTATCGATGTTCCGATTGT-3'; and reverse: 5'- TAC<u>CTC GAG</u>ATGTTTAGGTGATGCATT-3'). The amplified gene fragments were purified and digested with Sac I and Xho I to generate inserts with overhanging ends. Inserts were purified and cloned into the previously digested pET21b-*rhavi* expression vector (Supplementary Figs. 1 and 2).

Bacterial Strains and Growth Conditions

The bacterial strains used for cloning and expression were *E. coli* DH5α, *E. coli* BL21 (DE3) (Thermo Scientific, US); *E. coli* One Shot® BL21 Star™ (DE3) pLysS (Agilent Technologies, US); *E. coli* BL21-CodonPlus (DE3) RIL (Thermo Scientific, US); and *E. coli* BL21-SI. The BL21 strains lack the OmpT outer membrane protease: cells deficient in these proteases accumulate recombinant proteins at a high rate and are less likely to degrade some proteins during purification [22] (Table 1). The standard culture conditions for growth of the different *E. coli* strains harboring the expression vectors were tested in 2YT medium (LBON for BL21–SI) supplemented with ampicillin (100 μg/mL), grown at 37 °C with 250 rpm shaking (Gyromax 737R, Amerex, Lafayette, CA, EUA). A pre-inoculum was grown overnight and used to inoculate 100 mL of medium under the same conditions until OD_{600} of 0.6–0.8. The temperature was adjusted to 16 °C and protein production induced with 1 mM IPTG (300 mM of NaCl for BL21–SI) and maintained for 18 h.

For investigation of the optimized culture conditions, transformed *E. coli* was grown in TB media supplemented with ampicillin (100 μg/mL). The pre-inoculum was incubated at 37 °C with shaking at 250 rpm for 3–4 h. The inoculum started at an initial OD_{600} of 0.15 in 100 ml media and the cultures were maintained under the same conditions, shaking at 250 rpm in Tunair Flask full baffled (Merck, US) until an OD_{600} of 3.0. The cultures were then acclimated for 30 min at the respective temperatures (16 °C, 23 °C or 30 °C) and induced with 0.6 mM IPTG for 12, 15, or 18 h.

The cells were harvested by centrifugation at $15,000 \times g$ for 15 min at 4 °C, resuspended in 10 mL of lysis buffer (150 mM Tris–HCl pH 7.5; 500 mM NaCl; 1 mM PMSF),

and lysed in a French Press and centrifuged at $22,100 \times g$ for 50 min. The supernatant was separated, and the pelleted inclusion bodies resuspended in 10 mL of solubilization buffer (150 mM Tris–HCl pH 7.5; 500 mM NaCl; 2% SDS). Aliquots (20 μL) from the soluble and insoluble fractions were separated to be evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Description of media and solutions are in Supplementary Table 1.

SDS-PAGE and Western Blotting

Soluble and insoluble fractions of the transformed *E. coli* cells were separated by SDS-PAGE (15%) and stained with Coomassie Blue [21]. The bacterial cultures were diluted to 1.0 OD_{600} and 25 μL loaded on the gels. Parallel gels were electro-transferred onto PVDF membranes (120 min, 30 V), which were then blocked overnight at 4 °C in 5% w/v skim milk powder in PBST (SM-PBST). After washing three times with PBST (PBS 1× and 0.05% Tween-20), the primary antibodies were added: polyclonal anti-rSmTSP-2 antibody serum (1:5000 in 1% SM-PBST) from mice immunized with rSmTSP-2, polyclonal anti-rSmCD59.2 antibody serum (1:5000 in 1% SM-PBST) from rats immunized with rSmCD59.2, or Mouse 6x-His Tag: HRP monoclonal antibody (Invitrogen) (1:1000 in 1% SM-PBST). After washing, anti-mouse IgG (H+L): HRP (1:1000 in 1% SM-PBST) was added for analysis of rSmTSP-2 samples, and anti-rat IgG (H+L): HRP (1:1000 in 1% SM-PBST) (Life Technologies) for analysis of rSmCD59.2 samples, incubating the membranes for 1 h at room temperature. The membranes were washed again three times with PBST, and the ECL substrate (GE Healthcare) was applied according to manufacturer's protocols; chemiluminescence was detected on ImageQuant LAS 4000 series (GE Healthcare).

Densitometry Analysis

The images from SDS-PAGE or Western blot of protein extracts were evaluated using the ImageJ program. The band area for each protein was calculated following the

Table 1 *Escherichia coli* strain attributes

Strain	Inducer	Features
DH5α	–	Used for routine cloning applications
BL21-SI	NaCl	Uses osmolarity to regulate expression (0.3 M NaCl). Lower production cost
BL21 (DE3)	IPTG	High-level of protein expression. Ideal for non-toxic proteins
BL21 Star (DE3) pLysS	IPTG	Offers enhanced mRNA stability. High-level of protein expression. Ideal for non-toxic and toxic proteins
BL21-CodonPlus (DE3) RIL	IPTG	Carries extra copies of the argU, ileY, and leuW tRNA genes, that most frequently limit translation of heterologous proteins in *E. coli*. Ideal for codon bias

BL21 strains lack the OmpT outer membrane protease

program's methodology. The areas were plotted in the GraphPad Prism program to compare the production of proteins with and without fusion with rhizavidin. Statistical differences were calculated using the non-parametric t test. For western blot, the band area for each protein was calculated following the program's methodology and the results were normalized in reference to the largest calculated area.

Protein Purification

The rRzv:SmTSP-2 fusion protein was purified from the soluble fraction and rRzv: SmCD59.2 purified from the inclusion bodies in the presence of urea (8 M). The recombinant proteins were then purified by metal-affinity chromatography using the AKTAprime system. Briefly, the sample was loaded onto a Ni^{2+}-NTA column (GE Healthcare) previously equilibrated with 25 mM Tris/HCl, 5 mM imidazole, pH 8.0. (Equilibration buffer). The column was then washed with 10 column volumes of the Equilibration buffer and then eluted with a 10–500 mM imidazole linear gradient. The fractions containing the main peak of rRzv: SmTSP-2 were pooled, and the proteins were dialyzed against PBS, pH 7.4. The pooled fractions containing the rRzv: SmCD59.2 protein were dialyzed against Equilibration buffer without imidazole, and the urea was gradually removed for refolding.

The purified recombinant proteins were detoxified to eliminate lipopolysaccharide (LPS) using the Triton X-114 wash method [23]. In brief, each 1 mL of purified protein was mixed with 10 μL of Triton X-114, shaken vigorously and incubated at 37 °C for 15 min for phase separation. After incubation, the samples were centrifuged at $15,000 \times g$ for 30 s, and the upper phase was recovered. This washing process was repeated three times to ensure the elimination of LPS. The protein was quantified by the Bradford colorimetric method (Merck), and the endotoxins was determined by LAL gel-clot reagent (Lonza, US).

Circular Dichroism Analysis

The circular dichroism (CD) spectra were obtained on a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) at 25 °C. The measurements were performed at wavelengths from 185 to 260 nm and intervals of 0.1 nm in a 0.1-cm-path cell. All samples were previously dialyzed against 10 mM sodium phosphate buffer, pH 7.5. The spectra presented are the averages of five scans, and the data obtained were reported as molar ellipticity (degrees·cm2·dmol-1). The secondary structure deconvolution analyses were performed with CAPITO (CD Analysis and Plotting Tool) [24], using the CAPITO algorithm.

Results

Expression Vectors

The *smtsp-2* and *smcd59.2* gene fragments were cloned into the pET21b-*rhavi* vector [3], generating the expression vectors, pET21b-*rzv-smtsp-2* and pET21b-*rzv-smcd59.2* (Supplementary Fig. 1). Constructs were confirmed by PCR using the T7 promoter forward primer with specific reverse primers for each gene and gene sequencing confirmed their identity (data not shown).

Comparison of rSmTSP-2 and rSmCD59.2 Expression with and without Fusion to Rhizavidin

Expression of rSmTSP-2 and rSmCD59.2 proteins has been obtained previously in reasonable levels (PhD theses by Kanno, A. I. and Tararam, C.A.). In order to compare protein expression levels with and without fusion to rhizavidin, *E. coli* BL21 (DE3) was transformed with the respective expression vectors (pET41a-*smtsp-2*, pET21b-*rzv-smtsp-2,* pAE-*smcd59.2* and pET21b-*rzv-smcd59.2*) and clones from each were grown in the standard culture conditions (Methods 2.3). The SDS-PAGE of cell extracts processed in parallel revealed production of the respective proteins (Fig. 1A) and densitometry analysis showed that fusion of the protein rSmTSP-2 to rhizavidin resulted in a significant reduction in production of soluble protein (Fig. 1B). After purification, quantification of final product showed a yield of 40 mg/L of rSmTSP-2 and 5 mg/mL of rRzv: SmTSP-2. Comparative densitometry of the rSmCD59.2 with or without fusion to rhizavidin showed that they were produced in comparable levels in the insoluble fraction of the initial extracts. However, after purification, quantification of the final product showed a yield of 33 mg/L of rSmCD59.2 and 2 mg/L of rRzv:SmCD59.2. The results suggest the need to further optimize of the purification step of the proteins in fusion with rhizavidin.

Evaluation of Different E. coli Strains for Protein Expression

The *E. coli* strains, BL21-SI, BL21 (DE3), BL21 Star™ (DE3) pLysS, and BL21-CodonPlus (DE3) RIL were transformed with the expression vectors pET21b-*rhavi-smtsp-2* and pET21b-*rhavi-smcd59.2* to evaluate the protein expression levels. Selected clones were grown until mid-log phase, induced with IPTG (NaCl for BL21-SI) and bacteria collected by centrifugation. SDS-PAGE analysis of extracts from induced and non-induced bacteria showed that the expected bands around 30 kDa were
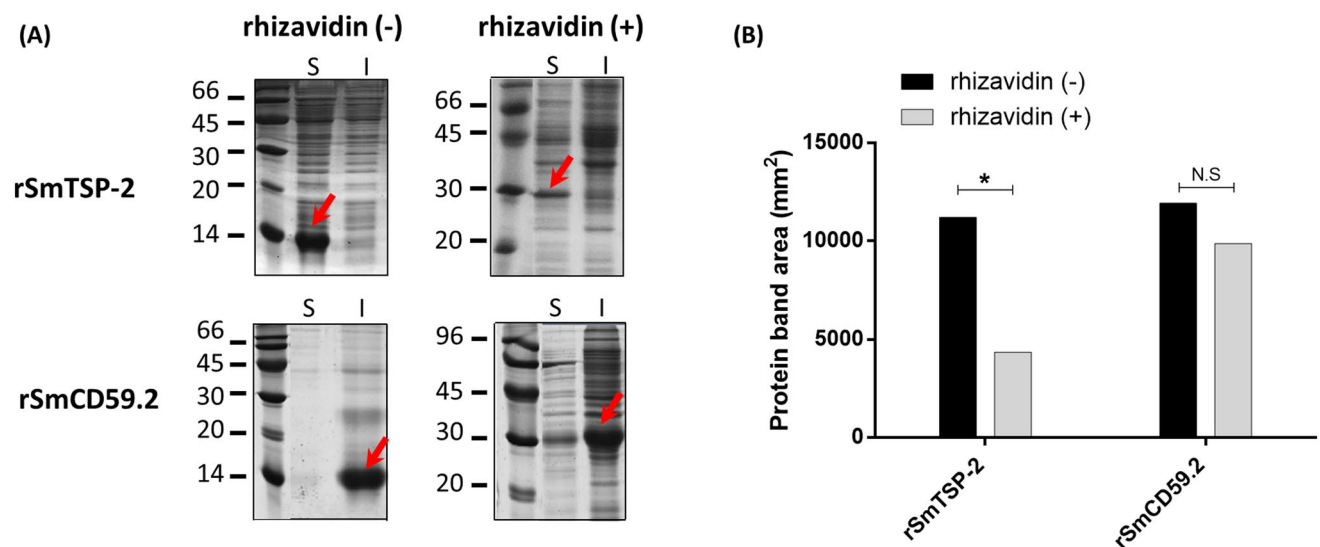
Fig. 1 Protein expression of rSmAg with and without fusion to rhizavidin. **A** SDS-PAGE analysis of the expression of rSmTSP-2, rSmCD59.2, rRzv:SmTSP-2, and rRzv:SmCD59.2 by *E. coli* BL21 (DE3) in 2YT medium. Proteins were separated in 15% SDS-PAGE and stained with Coomassie Blue. **B** Comparison between the band area values calculated by densitometry in the ImageJ program. Statistical analysis by "non-parametric t-test" in the Graph Prism program. *S* soluble fraction, *I* inclusion bodies fraction, *NS* no statistical difference; *P value > 0.05. Red arrows show the region of the protein band
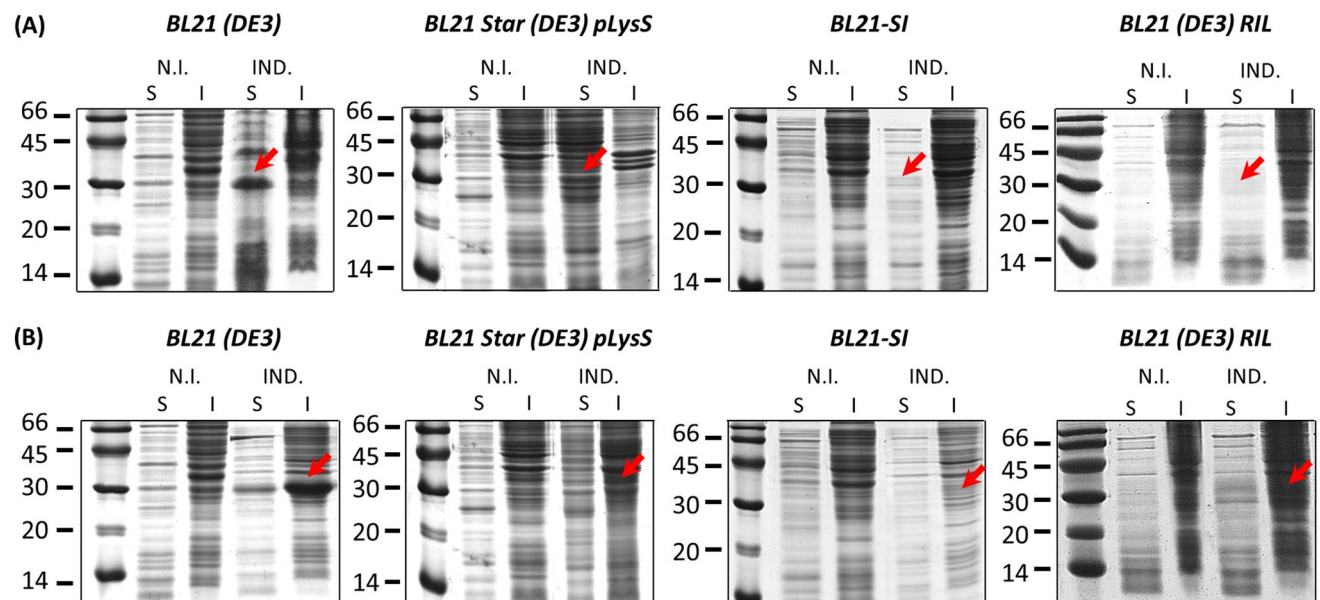

Fig. 2 Expression of rRzv:SmTSP-2 and rRzv:SmCD59.2 in *E. coli* BL21 (DE3)*,* BL21 Star (DE3) pLysS, BL21–SI and BL21-Codon-Plus (DE3) RIL as determined by SDS-PAGE. **A** rRzv:SmTSP-2 and **B** rRzv:SmCD59.2; cell concentration was normalized by OD_{600}, separated in 15% SDS-PAGE and stained by Coomassie Blue. *N.I.* not induced, *IND.* induced, *S* soluble fraction, *I* inclusion bodies fraction; Red arrows show the region of the protein band

only clearly visible with the BL21 (DE3) strain (Fig. 2). Comparison of soluble and insoluble fractions showed that rRzv:SmTSP-2 was concentrated in the soluble fraction, and rRzv:SmCD59.2 was concentrated in the insoluble fraction. The calculated molecular weight for rRzv:SmTSP-2 was 27.2 kDa and for rRzv:SmCD59.2 was 26.9 kDa, but the electrophoretic pattern observed in the SDS-PAGE shows that the proteins bands were closer to 30 kDa. Altogether, the results show that the BL21 (DE3) strain allows higher expression of the recombinant proteins.

Evaluation of Different Growth Media for Protein Expression

Protein expression levels were evaluated using different culture media. The 2HKII and TB media allow induction at OD_{600} 3.0. Growth was performed under the standard culture conditions, performing the induction at OD_{600} 2.0–3.0. Soluble and insoluble fractions analyzed by SDS-PAGE and Western blotting showed that the TB media increased production of the rRzv: SmTSP-2 protein, despite more in the insoluble fraction (Fig. 3A and B). For production of rRzv:SmCD59.2, different media did not alter significantly the levels of protein expression. Therefore, further optimization was performed with the TB media.

Optimization of Culture Conditions

In order to optimize the temperature and induction time of the cultures aiming at soluble recombinant protein production, an analysis was performed with several experiments in parallel varying the temperature between 16 and 30 °C and induction time between 12 and 18 h, using the previously selected TB culture medium. After the protein expression, OD_{600} was measured, and the cells were harvested. For evaluation of protein expression, samples were separated by SDS-PAGE, and proteins were confirmed by Western blot. The selected variables affected the cultures presenting the highest cell growth at 23 °C and 15 h induction in *E. coli*

transformed with pET21b-*rzv-smtsp-2* (Fig. 4A and Supplementary Table II). On the other hand, for *E. coli* transformed with pET21b-*rzv-smcd59.2,* the highest growth was obtained at a lower temperature (16 °C) and shorter time (12 h) (Fig. 4A and Supplementary Table II). Relative quantification of protein production was evaluated as the normalized densitometry of the bands obtained by Western blot in each condition (Fig. 4B, Supplementary Table II and Supplementary Fig. 3). The results show that the midpoint conditions induced greater protein production in both cases that is when incubating at 23 °C for 15 h (Fig. 4B, Supplementary Table II and Supplementary Fig. 3). However, rRzv:SmTSP-2 was produced mainly in the soluble fraction, while rRzv:SmCD59.2 was produced mainly as inclusion bodies (Supplementary Fig. 3).

Final Protein Production and Purification

BL21 (DE3) cells transformed with the expression vectors were grown in 1 L flasks under the established conditions, induced at 23 °C for 15 h. The rRzv:SmTSP-2 fusion protein was purified from the soluble fraction and rRzv:SmCD59.2 purified from the inclusion bodies in the presence of urea. The soluble proteins were submitted to metal-affinity chromatography and pooled fractions were dialyzed against PBS, to remove imidazole from rRzv:SmTSP-2. For rRzv:SmCD59.2, the protein was dialyzed to gradually remove imidazole and urea for refolding. Pooled fractions
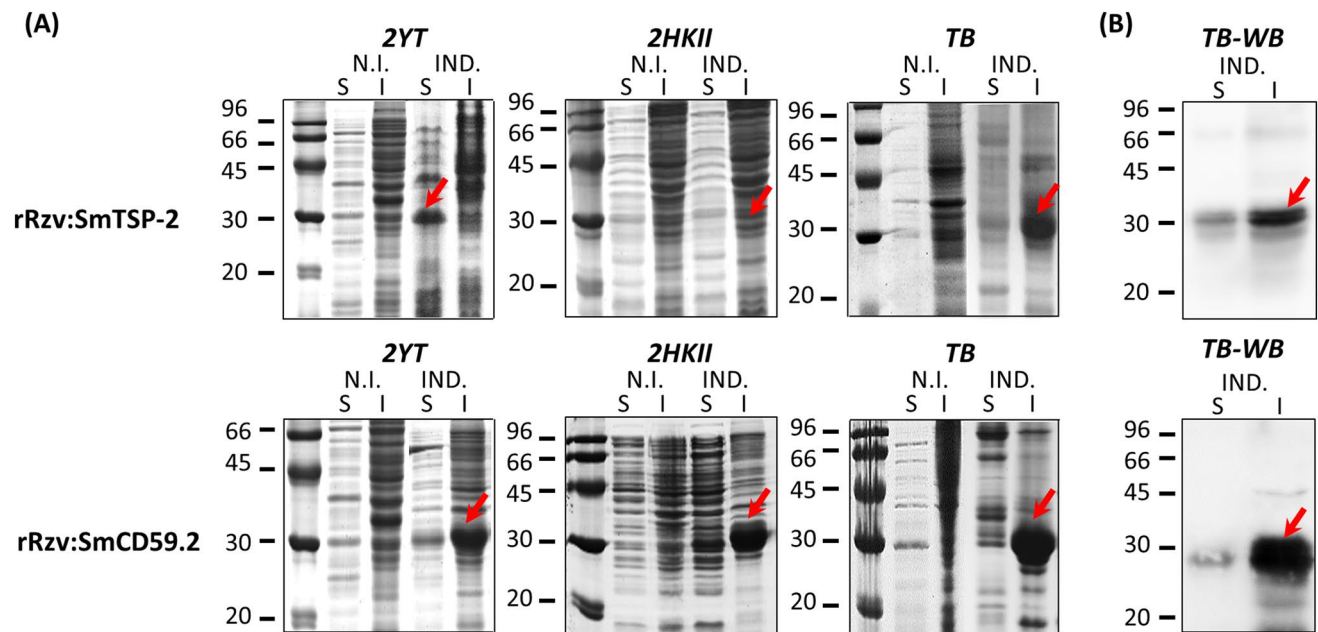

Fig. 3 Analysis of different culture media. **A** SDS-PAGE analysis of rRzv:SmTSP-2 and rRzv:SmCD59.2 expression by *E. coli* BL21 (DE3) in 2YT, 2HKII, and TB medium. Proteins were separated in 15% SDS-PAGE and stained by Coomassie Blue. **B** Western blot analysis of rRzv:SmTSP-2 and rRzv:SmCD59.2 expression by *E. coli* BL21 (DE3) in TB medium. *N.I.* not induced, *IND.* induced, *S* soluble fraction, *I* inclusion bodies fraction; Red arrows show the region protein band

Fig. 4 Production of rRzv:SmTSP-2 and rRzv:SmCD59.2 proteins varying the temperature and induction time. **A** The final OD_{600nm} values obtained in culture condition varying temperature (16, 23, and 30 °C) and induction time (12, 15, and 18 h). **B** Productivity as determined by protein expression levels (calculated area of Western blot bands by densitometry). Densitometry was performed using the program ImageJ and the results were normalized in relation to the largest calculated area. *h* hour, *Temp.* Temperature, *N* normalized values
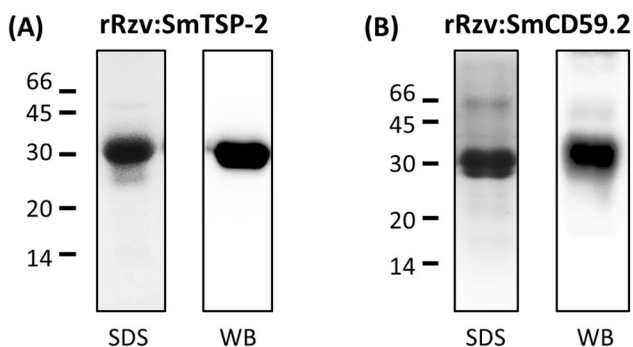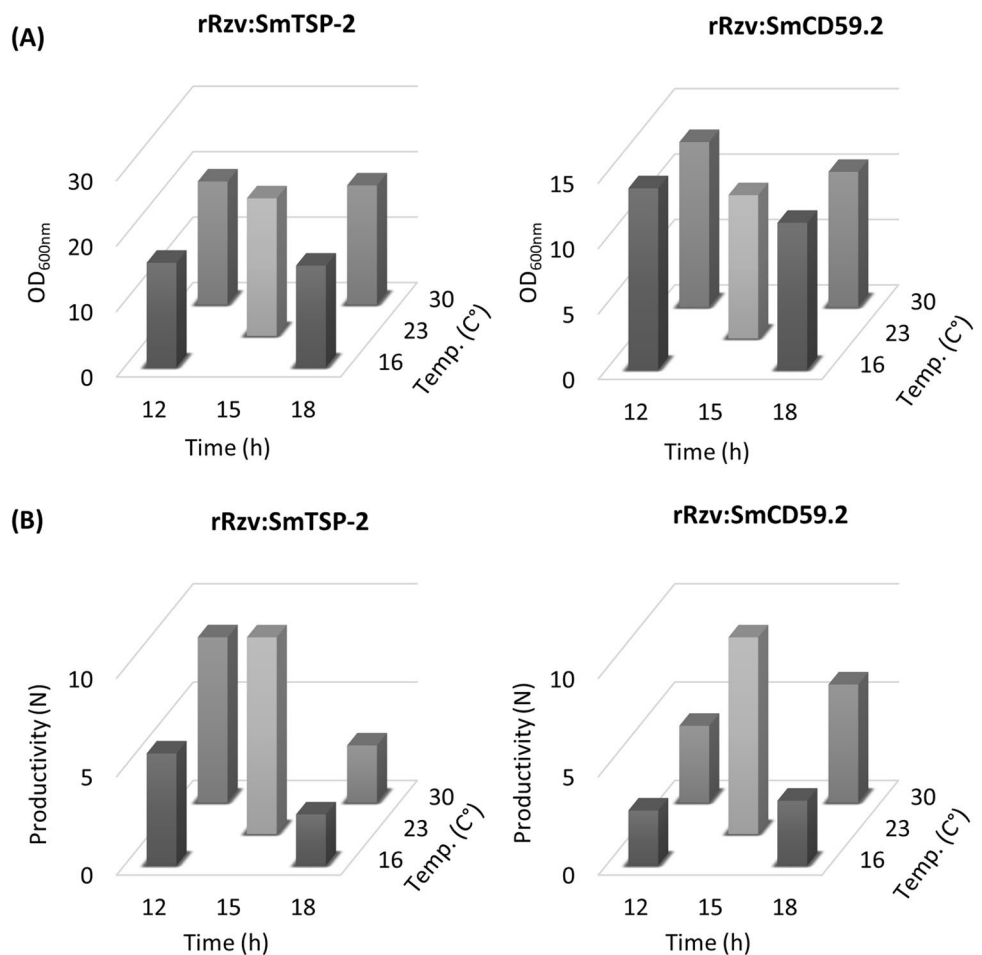

Fig. 5 SDS-PAGE and Western blot of purified rRzv: rSmTSP-2 and rRzv: SmCD59.2 proteins. **A** Purified rRzv: SmTSP-2 and **B** Purified rRzv: SmCD59.2; Left box contains SDS-PAGE of the purified proteins, and right box is the Western blot performed with specific antibody against each protein. *SDS* SDS-PAGE, *WB* Western blot

were treated with Triton X-114 and analyzed by SDS-PAGE and Western blot (Fig. 5). Purified proteins obtained with the optimized process showed a final yield of ~ 20 mg/L in soluble form for both proteins at a reasonable purity (> 80%). This yield was fourfold higher than that obtained with the standard culture conditions.

In order to confirm that the refolding of the rRzv:SmCD59.2 protein recovered its structural properties, the protein preparation was analyzed through circular dichroism (CD). The results of the ellipticity data in Supplementary Fig. 4 indicated that the recombinant protein was composed mostly of alfa-helical structures (54%), confirming its structural integrity and indicating possible conservation of important conformational epitopes.

Discussion

The importance of coupling through biotin affinity is huge, with a wide range of applications [4]. Many of these systems require production of proteins in fusion with biotin-binding proteins, such as avidin or avidin derivatives. The recently developed MAPS delivery system requires higher yields of recombinant proteins in fusion with rhizavidin [3]. There are many challenges in the development of production systems for recombinant proteins in fusion with avidin derivatives or rhizavidin [6, 7]. The use of bacterial expression systems, such as *E. coli,* can lead to the accumulation of these proteins in inclusion bodies, requiring

downstream treatments for renaturation to obtain the active proteins [25, 26]. Even with these treatments, the final yields are generally low, ~ 1–2 mg of pure protein per liter of culture [7, 26]. As for vaccine development purposes, obtaining soluble protein at the expression level is usually preferable, since refolding may have serious implications in antigen presentation, e.g., in the preservation of conformational epitopes; this will affect subsequent generation of appropriate immune responses in the host.

In this study, two expression vectors comprising the rhizavidin gene in fusion with *S. mansoni* antigen-coding sequences (*smtsp*-2 and *smcd59*) were constructed and the conditions for efficient expression in *E. coli* were established. A series of improvements were necessary to obtain a satisfactory amount of protein at the end of the production process. First, the improvements of protein expression were carried out linearly, adjusting one factor at a time, thus, defining the working strain and culture medium. It is essential to optimize the cultivation medium composition in order to achieve the highest possible density of production in *E. coli* [27]. The BL21 (DE3) strain cultured in TB medium provided higher yields of growth and soluble protein production. We have previously observed the same result for production of the Zika virus ΔNS1 protein, a truncated form of NS1 [28]. The main difference of TB over 2YT or 2xHKSII is their buffering system, supporting a controlled pH during growth. The presence of glycerol as an extra carbon source also plays in important role in bacterial growth.

Time and temperature are two of the most important factors that affect the production and solubility of recombinant proteins [29, 30]. The time of induction and temperature were varied in a series of experiments to optimize cell growth and product formation. It was possible to obtain a correlation between the time of induction, temperature, and the amount of product formed, establishing the optimal culture conditions to produce the proteins [25]. Initially, the rRzv:SmTSP-2 protein was expressed at OD_{600} of ~ 3.0 and was mostly insoluble. Through these experiments, our best condition was 23 °C for 15 h; similar values were found for ΔNS1 (induction at 21 °C for 20 h). In agreement, a consensus protocol for *E. coli* expression provided by a multicentric study that compared a variety of targets, conditions, and strains recommends the use of *E. coli* BL21 (DE3) strain cultured between 15–25 °C [31]. Although it is possible to further optimize protein production using mathematical modeling, e.g., response surface methodology (RSM) experiments, in both upstream and downstream processes [28, 32], after optimization, this protein was purified from the soluble fraction at a concentration four times higher than the initial one. The rRzv:SmCD59.2 protein, after optimization was still directed to the insoluble fraction, but its recovery after refolding increased about tenfold. CD analysis confirmed

the structural integrity of the purified protein. The optimized culture conditions provided ~ 20 mg/L of each protein.

The recombinant proteins were purified from the total extracts in order to increase the yields, and the purity obtained was ~ 80%. For our purposes, this purity is reasonable, since the proteins will still pass through the coupling stage to the biotinylated matrix, which will bind specifically through biotin–rhizavidin affinity to the biotin moiety. The complex will then be purified by gel filtration, which will separate them from the non-bound proteins, thus, guaranteeing elimination of possible contaminants. Since the fusion proteins contain a secretion signal that should direct them to the periplasmic space, a strategy that could further increase purity or solubility would be to purify the proteins only from the periplasmic fraction. However, this could reduce the total yields and an evaluation should be performed.

Therefore, this study established the optimized production of the two Schistosoma proteins in fusion with rhizavidin in soluble form. It provides general conditions for the optimized expression of rhizavidin-fused proteins in *E. coli*. Specifically, this will allow the use of these proteins in biotin affinity systems, either in new antigen-presenting systems, such as MAPS, or for use in any platform that can benefit from biotin–rhizavidin affinity.

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