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Refolding of the recombinant protein *Sm*29, a step toward the production of the vaccine candidate against schistosomiasis

Rosa M. Chura-Chambi[a], Erika Nakajima[b], Roberta R. de Carvalho[b], Patricia A. Miyasato[c], Sergio C. Oliveira[d,e], Ligia Morganti[a], Elizabeth A.L. Martins[b,*]

[a] *Centro de Biotecnologia, Instituto Pesquisas Energéticas e Nucleares, São Paulo, SP, Brazil*
[b] *Centro de Biotecnologia, Instituto Butantan, São Paulo, SP, Brazil*
[c] *Laboratório de Parasitologia, Instituto Butantan, São Paulo, SP, Brazil*
[d] *Departamento de Imunologia e Bioquímica, UFMG, Belo Horizonte, MG, Brazil*
[e] *Instituto Nacional Ciência e Tecnologia-Doenças Tropicais, CNPq, MCT, Salvador, BA, Brazil*

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ABSTRACT

Schistosomiasis is an important parasitic disease, with about 240 million people infected worldwide. Humans and animals can be infected, imposing an enormous social and economic burden. The only drug available for chemotherapy, praziquantel, does not control reinfections, and an efficient vaccine for prophylaxis is still missing. However, the tegumental protein *Sm*29 of *Schistosoma mansoni* was shown to be a promising antigen to compose an anti-schistosomiasis vaccine. Though, recombinant *Sm*29 is expressed in *Escherichia coli* as insoluble inclusion bodies requiring an efficient process of refolding, thus, hampering its production in large scale. We present in this work studies to refold the recombinant *Sm*29 using high hydrostatic pressure, a mild condition to dissociate aggregated proteins, leading to refolding on a soluble conformation. Our studies resulted in high yield of r*Sm*29 (73%) as a stably soluble and structured protein. The refolded antigen presented protective effect against *S. mansoni* development in immunized mice. We concluded that the refolding process by application of high hydrostatic pressure succeeded, and the procedure can be scaled-up, allowing industrial production *of Sm*29.

1. Introduction

Schistosomiasis is an important neglected tropical disease caused by parasites of the genus *Schistosoma*. More than 240 million people are infected in 78 countries and more than 700 million live in endemic areas (http://www.who.int/wer/2013/wer8808.pdf). Infection of man by *Schistosoma* larvae may occur in water sources, frequently accessed by the population, mainly in rural areas (Ross et al., 2002). The disease can present acute or chronic forms and it can be seriously debilitating, leading to morbidity and death (Gryseels et al., 2006; Hotez et al., 2010; McManus et al., 2010).

Governmental multifaceted health programs for schistosomiasis control and prevention have been used in different countries (Amaral et al., 2006; Gray et al., 2010; McManus et al., 2010; Uneke, 2010; Wang et al., 2009). However, in some places re-emerging transmission has occurred, requiring new strategies for a full control of the disease, denoting that an effective vaccine to complement health programs have not been yet made available (Bergquist et al., 2008; Gray et al., 2010; Hotez, 2011; Seto et al., 2011; Spear et al., 2011).

A multitude of *Schistosoma* antigens with prophylactic potential are being studied, selected by immunological data (Bickle, 2009; McManus et al., 2010; Oliveira et al., 2008), or using bioinformatics tools over the transcriptome and genome data of the parasite (Fitzpatrick et al., 2009). After tests in laboratorial scale, using immunization and challenge assays in animal models, a few antigens were selected as protective. The best results of protection achieved were 50–65%, measured by inhibition of worm development. Mathematical models indicate that this partial protection may result in a high impact on schistosomiasis control by reducing the transmission rates (Williams et al., 2002).

Although the efforts to develop a vaccine against schistosomiasis have experienced many failures, nowadays there is only one antigen-based vaccine in the clinical trial phase, the *Sm*-GST (Capron et al., 2002; Riveau et al., 2012). A few other antigens, like *Sm*-p80 are in preclinical phase (Ahmad et al., 2011) or in process of development for scaling up the production, such as *Sm*14 (Ramos et al., 2009; Tendler and Simpson, 2008), paramyiosin (Gobert et al.,

Abbreviations: HHP, high hydrostatic pressure; GSSG and GSH, oxidized and reduced form of glutathione* respectively; IB, inclusion bodies; CD, circular dichroism; IMAC, immobilized metal affinity chromatography; LS, light scattering; GdnHCl, guanidine hydrochloride.

* Corresponding author. Tel.: +55 11 26279822.
E-mail address: elizabeth.martins@butantan.gov.br (E.A.L. Martins).

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1997; Jiz et al., 2008) and *Sm*TSP-2 (Loukas et al., 2007; Tran et al., 2006).

The *Sm*29 protein was identified by immunolocalization as one of the most exposed and expressed antigens in the outer tegument of *Schistosoma mansoni*, being present in the surface of lung-stage schistosomula and adult worms (Cardoso et al., 2006b, 2008). Antibodies IgG1 and IgG3 specific against *Sm*29 were detected in sera of patients living in endemic areas in Brazil, and significantly higher levels of these antibodies were found in individuals who presented resistance to re-infection (Cardoso et al., 2006a). A recombinant *Sm*29 (r*Sm*29) was shown to be a good immune-protective antigen in animal model. The protection was associated with a typical Th1 immune response and reduction of worm burden, liver granulomas and in intestinal eggs. These results demonstrated that r*Sm*29 is an important antigen with potential to compose a vaccine against schistosomiasis (Cardoso et al., 2008).

An important step toward making a vaccine available is to scale up the production of the antigen, requiring an efficient and reproductive downstream process (Bergquist, 2002). The large-scale production of an antigen is often limited by difficulties, such as the yield of expression, achieving the desired purity, the action of proteases or insolubility. In fact, the need to refold insoluble proteins is a major bottleneck for recombinant protein production.

The expression of r*Sm*29 in *Escherichia coli* occurs in insoluble form and the solubilization and refolding of the protein by traditional methods resulted in low yields, only suitable for studies in laboratory scale. For clinical application, reasonable levels of the protein should be recovered, in stably soluble condition, presenting native epitopes. Therefore, efficient refolding of r*Sm*29 from IB is an issue of paramount interest.

The application of high hydrostatic pressure (HHP), in levels up to 3 kbar, in the presence of low concentration of guanidine hydrochloride (GdnHCL) was described as a non-denaturing solubilization process of aggregated proteins and IB, which may result in high yields of refolded proteins (Chura-Chambi et al., 2008; Fraga et al., 2010; Malavasi et al., 2011; Qoronfleh et al., 2007; St John et al., 1999).

In this work, we have studied the effect of high pressure for IB dissociation and for refolding the recombinant r*Sm*29. The refolding of the antigen using HHP is a suitable process for large scale production, thus, representing an interesting strategy for obtaining high yield of r*Sm*29, in stably soluble condition.

2. Material and methods

2.1. Cloning Sm29 and expression in E. coli

The partial *Sm*29 gene (GenBank: AF029222.1) for expression of the recombinant antigen r*Sm*29 (40–169 amino acid fragment, without the signal peptide and the transmembrane domain), fused with a C-terminal 6× histidine tag cloned into pET21a vector (Novagen, NJ, USA) (Cardoso et al., 2006b) was provided by Dr. Sergio C. Oliveira. The gene was transferred into pAEsox, a vector constructed in our laboratory, derived from pAE vector (Ramos et al., 2004). Using pAEsox the protein expression was induced by paraquat, an oxidative stress agent. The correct cloning was confirmed by DNA sequencing. *E. coli* strain BL21 (DE3) Star [pLyss] (Novagen) transformed with pAEsox-Sm29 was cultured in 2YT media with ampicilin (100 g/mL) at 37 °C and stirring (180 rpm). Expression of *Sm*29 was induced by addition of paraquat (150 M) to the culture when optical density (OD) at 600 nm reached 0.5. The incubation at 20, 30 or 37 °C was performed for further 16 h.

2.2. Cells lysis and inclusion bodies preparation

Bacteria from 500 mL culture were recovered by centrifugation (8500×*g* for 10 min at 4 °C) and were suspended in 30 mL of Lysis Buffer (100 mM Tris–HCl pH 8.0, 5 mM EDTA, 0, 1% [w/v] sodium deoxycholate and lysozyme (50 g/mL)). After 15 min incubation at room temperature (RT), cells were lyzed by sonication (Sonifier 450—Branson) and the suspension centrifuged (8500×*g* for 10 min at 4 °C) to separate soluble and insoluble fractions. Inclusion bodies (IB) were prepared by washing the pellets three times by suspension in 30 mL of Lysis Buffer without lysozyme and centrifugation. IB preparations were dissolved in Tris Buffer (Tris–HCl 50 mM, pH 8.0) or Tris Buffer with NaCl (150 mM NaCl) and turbidity of the suspensions were measured by OD at 350 nm to estimate protein recovery. Each liter of culture yielded about 50 mL suspension with turbidity 10.

2.3. Analysis of the solubilization of rSm29 containing IB

IB generated by expression of r*Sm*29 at 20, 30 or 37 °C, prepared as described above, were suspended in Tris Buffer containing 0 to 6 M guanidine hydrochloride (GdnHCl) and suspensions were incubated 72 h under 180° vertical rotation at 35 rpm. Turbidity was measured by OD at 350 nm.

2.4. rSm29 refolding at atmospheric pressure

The IB generated by expression of r*Sm*29 at 30 °C, equivalent to 500 mL culture, were re-suspended in 50 mL Tris Buffer with NaCl, 8 M urea and 5 mM imidazole. Suspension was kept 16 h under stirring at RT and clarified by centrifugation (8500×*g* for 10 min at 4 °C). Denatured protein solution was used to test different conditions for refolding of r*Sm*29. Protein purification was performed by immobilized metal affinity chromatography (IMAC) in 5 mL Ni-Sepharose column (HisTrap HP, GE Healthcare). Bound r*Sm*29 was eluted from IMAC with Tris Buffer with NaCl containing 300 mM imidazole in the presence of urea when indicated.

Conditions tested for r*Sm*29 refolding are described below.

2.4.1. Refolding by dilution

A volume of 10 mL protein solution (8 M urea) was slowly diluted in 2000 mL of Tris Buffer with 5 mM imidazole, before applying for IMAC.

2.4.2. Refolding in the IMAC column

Ten milliliters of protein solution (8 M urea) was applied for IMAC, followed by a series of washes with 50 mL of Tris Buffer with NaCl containing 20 mM imidazole and decreasing concentrations of urea (8, 6, 4, 2, 1, 0.5 and 0 M). A test was performed by adding Glutathione (5 mM GSSG and 5 mM GSH) to the column washing solutions (1 to 0 M urea), and other test was performed by adding L-Arginine (0.2 M) to the column washing solutions (1 to 0 M urea).

2.4.3. Refolding by dialysis

A volume of 10 mL protein solution was applied to IMAC and the eluted solution (8 M urea) was dialyzed sequentially against Tris Buffer or PBS containing decreasing concentrations of urea (6, 4, 2, 1, 0.75, 0.325 and 0 M), using dialysis membrane Snake-Skin (Pleated Dialysis Tubing 3.5 MWCO, Thermo Scientific). A test was performed by adding glycerol (to 10%) to the dialysis solutions (from 1 to 0 M urea), and other test was done by adding proline (to 500 mM) to the dialysis solutions. Proline then was removed by dialysis against Tris buffer with decreasing concentration (250, 125, 50 and 0 mM) of the amino acid.

2.4.4. Refolding by desalting chromatography

Two milliliters of protein solution eluted from IMAC in presence of 8 M urea was applied to a 10 mL G25 sephadex™ (GE-Healthcare) column at a 1 mL/min flow rate with PBS as equilibrium solution and running buffer.

The final protein solutions were centrifuged (8500×*g* for 10 min at 4 °C) to remove eventual precipitates, and stored at 4–8 °C.

2.5. Refolding of rSm29 at HHP

2.5.1. Effect of different agents on HHP induced refolding of rSm29

The contribution of different reagents for pressure-induced refolding of r*Sm*29 was tested by adding to the IB suspension in Tris Buffer with 5 mM EDTA: the redox pair oxidized (GSSG) and reduced (GSH) glutathione, GdnHCl, L-Arginine and the agents Triton X-100 (0.5 M), glucose (1 M), sucrose (1 M), NaCl (0.15 M), Tween 20 (1 mM) or 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonate (bis-ANS) (8.5 mM). One milliliter samples of IB suspensions with the different agents were placed into plastic bags and positioned in a pressure vessel (model R4-6-40, High Pressure Equipment Company). Pressure was applied with oil as pressure-transmitting fluid.

2.5.2. Effect of different strategies of compression/decompression on HHP induced refolding of rSm29

Suspensions of IB in refolding buffer were pressurized according to the following strategies: (a) 2.4 kbar for 16 h; (b) 2.4 kbar for 16 h followed by decompression at the decreasing rate of 0.4 kbar every 30 min; (c) 2.4 kbar for 2 h plus 0.4 kbar for 16 h; (d) 2.4 kbar for 2 h plus 0.8 kbar for 16 h; (e) 2.4 kbar for 2 h plus 1.2 kbar for 16 h. The samples were centrifuged (12,000×*g* for 15 min) to remove insoluble aggregates. The supernatant was dialyzed against Tris Buffer with NaCl and re-centrifuged.

2.6. Quantification of rSm29 by SDS-PAGE

Samples of IB suspensions and soluble fractions obtained by the different methods of solubilization were applied for SDS-PAGE (15% polyacrylamide) as described by Laemmli (1970). Digital images of the gels stained with Coomassie Blue G-250 were analyzed using Image J software (http://rsbweb.nih.gov/ij/) to determine the percentage of r*Sm*29 solubilization. Total protein was measured by Bradford assay (Bradford, 1976).

2.7. Fluorescence and light-scattering (LS) measurements

LS and fluorescence measurements were recorded on a Cary Eclipse spectrofluorometer (Varian). LS was measured at 320 nm with an angle of 90° relative to the incident light, and the emission was recorded at 315–325 nm. Fluorescence measurements of Tryptophan emissions were carried out with 288 nm excitation wavelength. The emission fluorescence spectra were collected between 300 and 400 m with response time 1 s and scan speed 240 nm/min.

At atmospheric pressure, data was collected using a 1 cm-path length cuvette. For measurements under pressure, a high-pressure cell equipped with optical sapphire windows (ISS), was connected to a manual pressure generator (high pressure equipment) using ethanol as pressure-transmitting fluid. Round quartz cuvettes filled with the samples and sealed with flexible polyethylene caps were placed into the high-pressure cell and subjected to HHP.

2.8. Circular dichroism measurements

CD spectra were obtained using a Jasco-J810 spectropolarimeter equipped with a temperature-controlled liquid system and a 0.1 cm-light path cuvette. The reported curve of ellipticity is the average of five measurements collected over 3 min period at 20 °C. The *in silico* analysis of the structure of the r*Sm*29 .based on the amino acid sequence was performed using Psipred service (http://bioinf.cs.ucl.ac.uk).

2.9. Immunization of mice with refolded rSm29 and challenge with cercariae

Groups of five female BALB/c mice were immunized by three subcutaneous doses, within 15 days intervals, with 10 g of r*Sm*29 in 200 L PBS and Alhydrogel (0.2 mg Al^{3+}/dose) as adjuvant. Control animals received PBS/Alhydrogel.

Ten days after the last dose, the animals were anesthetized with 200 L of a 1% ketamine and 0.2% xylasine solution and challenged with *S. mansoni* by exposing their tails during 30 min to a suspension of 150 cercariae in 1 mL. Precise number of penetrating cercariae was confirmed by counting eventual remaining ones in the suspensions. Forty five days after infection mice were perfused and adult worms recovered from the mesenteric veins were counted. Percentage of protection was calculated as $[(AC - AI)/AC] \times 100$, where AC is the average number of worms per animal from the control group and AI is the average number of worms recovered from the immunized animals.

2.10. Measurement of antibodies in the sera of the animals

Blood of the animals were collected fourteen days after the second (B1) or third (B2) doses of immunization, and forty days after infection (B3). Serum was separated to measure total and r*Sm*29 specific IgE, IgG and the isotypes IgG1, IgG2a.

To measure specific antibodies, ELISA plates (Corning, NY) were coated with 500 ng/well of rSm29 (5 g/mL in carbonate buffer, pH 9.6, 16 h, 4 °C), then blocked (for 2 h at RT) with 200 L/well PBST (PBS plus 0.05% Tween-20) plus 10% fat free milk protein. Serial dilution of sera from control or immunized animals in PBST were added (100 L per well) and the plates were incubated 1 h at RT. After wash, specific goat anti-mouse antibodies were introduced at dilution 1:1000 in PBST, to detect total IgG or the isotypes IgG1 or IgG2a. After one hour incubation, the plates were washed and received anti-goat antibodies conjugated with peroxidase (1:5000) (all specific antibodies from Sigma kit). Color was developed by addition of 100 L per well of 200 ρmol *o*-phenylenediamine (OPD, Sigma) in citrate buffer, pH 5.0 plus 0.04% H_2O_2. After 10 min, reaction was stopped with 50 L of 5% sulfuric acid and color was monitored by absorbance at 495 nm in an ELISA plate reader (Multiskan EX—Thermo Scientific). Results are presented in titers, meaning the dilution needed to decrease the absorbance to 0.1.

To measure total IgE, the plates were coated with capture antibodies (BD OptEIA™ Set Mouse IgE) as recommended by the manufacturer. For specific IgE, the plates were coated with r*Sm*29 as described above. Plates were blocked and the sera of control or immunized animals were introduced in serial dilution from 1:50 to 1:400. IgE bound to the plate were detected by biotinylated anti-mouse IgE followed by incubation with streptavidin-horseradish peroxidase conjugate. Color reaction was developed using Sure Blue™ TMB Microwell Peroxidase Substrate (KPL, USA). A standard curve of IgE was performed using capture antibodies and known masses of recombinant mouse IgE.

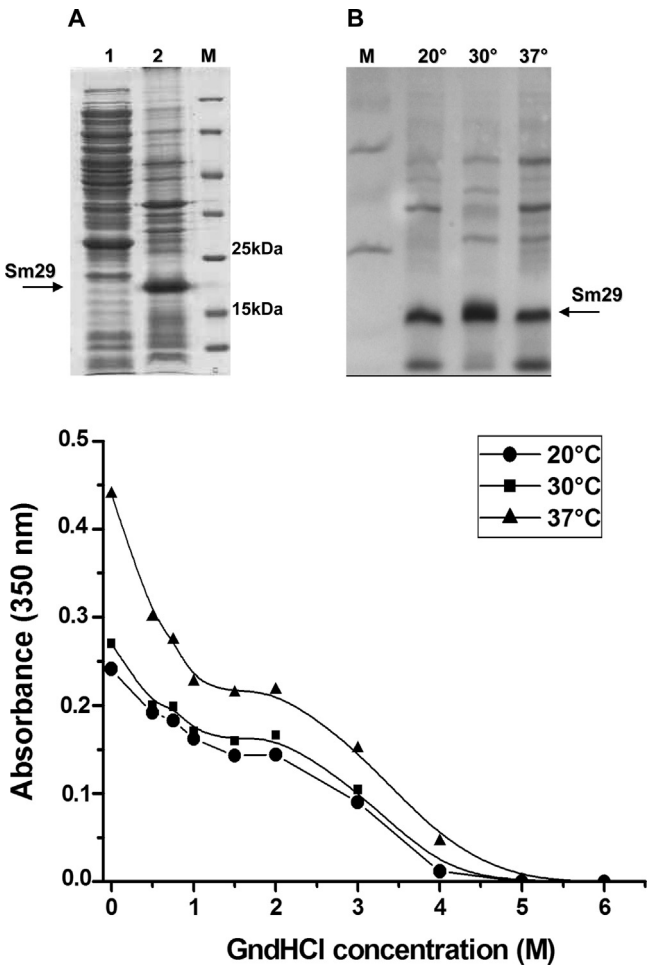

Fig. 1. Expression of rSm29 and solubilization of IB by GdnHCl. (A) Expression of r*Sm*29 in *E. coli* BL21-pAEsoxSm29 at 30 °C. Lanes 1 and 2: soluble and insoluble fractions of the bacterial extract, respectively. M—molecular marker. (B) Expression of r*Sm*29 induced at different temperatures. IB suspensions prepared from equal volumes of cultures were analyzed by SDS-PAGE. (C) The suspensions of IB containing r*Sm*29 expressed at different temperatures were incubated with GdnHCl and solubilization was monitored during 72 h by determination of the absorbance at 350 nm. The results are representative of two experiments.

3. Results and discussion

3.1. Expression of rSm29 and analysis of Inclusion Bodies (IB) solubilization

After expression of r*Sm*29 by BL21-pAEsox-Sm29, the cells were disrupted and soluble and insoluble fractions of the extract were analyzed by SDS-PAGE, confirming the recovery of r*Sm*29 as insoluble protein (Fig. 1A). Slightly higher expression of r*Sm*29 was verified by induction at 30 °C, when compared to 20 or 37 °C (Fig. 1B).

In order to verify if IB produced at different temperatures presented distinct resistance to solubilization, suspensions of IB were diluted to set equal absorbance at 600 nm and were subjected to 0 to 6 M GdnHCl. The turbidity of IB suspension generated at 20 and 30 °C decreased to nearly 50% when incubated 72 h under agitation, even in the absence of the denaturing reagent, while the IB produced at 37 °C were more resistant to dissolution (Fig. 1C). In addition, the IB produced at 20 and 30 °C were shown to be dissociated at lower GdnHCl concentration in comparison to the protein expressed at 37 °C. These results suggest that the aggregates produced at higher temperature present a superior degree of inter-molecular interactions at the expense of the intra-molecular native-like contacts. Therefore, in order to obtain enhanced expression of r*Sm*29 and to help the solubilization of the aggregates, the bacterial cultures were induced for expression at 30 °C.

3.2. Refolding of rSm29 IB at ambient pressure

The analysis of r*Sm*29 refolding was tested by conventional methods using 8 M urea for solubilization, and refolding by slow removal of the denaturing agent. Although GdnHCl 6 M was proven to be a better denaturing agent, urea was used in order to avoid the detrimental guanidine interference during protein purification by IMAC. The removal of the denaturing reagent to allow refolding of solubilized r*Sm*29 was performed using the strategies: 1-refolding by dilution, 2-refolding while protein was bound to the IMAC column, 3-refolding by dialysis and 4-refolding by desalting chromatography after IMAC. Samples of soluble r*Sm*29 obtained by these methods were applied to SDS-PAGE, and digital images of the gels were analyzed to estimate the yield of soluble protein. The effects of proline or glycerol on protein refolding were tested by using the additives in the dialysis solutions during urea removal. The effects of the presence of the glutathione redox pair (5 mM GSSG and 5 mM GSH) or L-Arginine (0.2 M) during the refolding of r*Sm*29 bound to IMAC resin were also analyzed. By using these methods, the yields of soluble r*Sm*29 recovery varied among 0.0% and 36.8%, with best recovery when using the redox pair. Moreover, except for samples from the glutathione assay, some protein precipitation was observed in all the other samples after storage at 4 °C, indicating instability of the protein in the solution.

Considering the difficulties for r*Sm*29 refolding by these conventional methodologies, we investigated the possibility of refolding the protein by application of high pressure to IB suspensions, in specific conditions.

3.3. Refolding of rSm29 by HHP

HHP was described as an efficient process for protein refolding from IB, even in cases of proteins that are difficult to obtain by using the conventional methods at ambient pressure (Chura-Chambi et al., 2013). In order to improve the yield of soluble r*Sm*29 to obtain a protein suitable for use as vaccine antigen a methodology using HHP was established.

3.3.1. Effects of the glutathione redox pair

The r*Sm*29 protein contains 15 cysteine residues, possibly involved in 7 intramolecular disulfide bonds. It is known that high pressure does not induce breakage of covalent bonds. Therefore, glutathione was used in order to gain insight on the conditions that would favor the breaking of non-native intra and intermolecular disulfide bridges covalently cross-linked in r*Sm*29, and favor the formation of more stable intramolecular native bonds. IB suspensions in refolding buffer (Tris–HCl 50 mM, pH 8.0, 1.5 M GdnHCl) were subjected to pressure-induced dissociation (2.4 kbar for 16 h) in presence of different proportions of reduced (GSH) and oxidized (GSSG) glutathiones. The rationale for this choice is that in case of proper r*Sm*29 refolding by formation of the intramolecular native contacts, the protein would stably remain soluble after dialysis. This assay was performed in the presence of 1.5 M GdnHCl, which usually helps to dissociate the aggregates and maintain the protein soluble.

The yield of refolded r*Sm*29 from the aggregates was clearly influenced by the presence of the redox pair during incubation at HHP, with maximum recovery (37%) in the presence of 4 mM GSH and 6 mM GSSG (Fig. 2). In the absence of the redox pair the yields of soluble protein were lower than 4%, which highlights the importance of the presence of these reagents, likely associated to the high

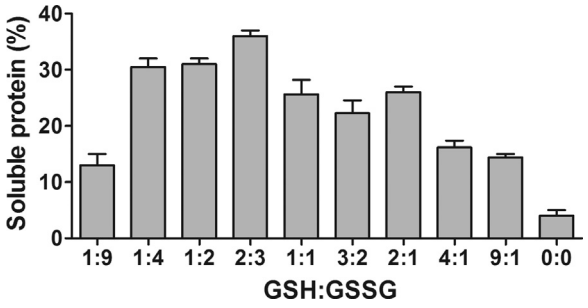

Fig. 2. Effect of the redox pair GSH and GSSG on the refolding of r*Sm*29 by HHP. Suspensions of IB in buffer Tris 50 mM, pH 8.0, 1.5 M GdnHCl, 1 mM EDTA were submitted to HHP (2.4 kbar, 16 h, decompression in steps of 0.4 kbar every 30 min) in presence of indicated ratios of GSH:GSSG (total concentration 10 mM) or in absence of glutathione (0:0). Soluble fractions were applied for SDS-PAGE and digital images of the gels were analyzed using Image J software to determine the intensity of the bands and the percentage of solublilizedr*Sm*29 in comparison to the total amount of r*Sm*29 in the IB suspension. The results are representative of three experiments. The error bars refers standard error means (SEM) of duplicate samples applied in the SDS-PAGE. The percentage of solubilization of all tested GSH:GSSG proportions were significantly different from the control in the absence of glutathiones (0:0) ($P < 0.05$, ANOVA one way with Dunnett's post-test).

number of cysteine residues in r*Sm*29 and their high propensity to form non-native intermolecular bonds.

3.3.2. *Effect of different agents on rSm29 refolding by HHP*

With the exception of Triton X-100 and L-Arginine, the presence of the other additives, such as salts, sugars, or detergents, which were shown to improve the refolding of other proteins under atmospheric pressure (Clark, 1998), and the fluorescent probe bis-ANS, that was previously described to prevent protein aggregation (Ferrao-Gonzales et al., 2005), presented no positive effect on r*Sm*29 refolding in our experiments or, in some cases, almost completely abolished the solubilization of the protein (Fig. 3A).

Interestingly, the recovery of soluble r*Sm*29 increased from 35% to 65% in presence of 0.75 M of L-Arginine (Fig. 3B). The effect of this amino acid on the protein refolding under HHP was previously observed (Fraga et al., 2010). The effect of L-Arginine on r*Sm*29 solubilization was more precisely analyzed by addition of increasing concentrations of the amino-acid (0 to 1 M) in the refolding buffer in substitution of GdnHCl. Higher yield of soluble r*Sm*29 (65.4%) was achieved when HHP was applied in the presence of 0.75 M L-Arginine, while practically no soluble protein was recovered in absence of the amino acid.

3.3.3. *Effects of different strategies of compression and decompression on rSm29 refolding*

The process of proteins refolding at atmospheric pressure present two stages: solubilization of the aggregates and proteins and then the refolding of the soluble protein. In the refolding process at HHP, pressures of 1.5–2.5 kbar are generally used for dissociation of the aggregates. Some authors claim that the protein refolding occurs at similar pressure levels (St John et al., 2001), however, other groups sustain that protein refolding occurs after the return to atmospheric pressure, specially for proteins presenting quaternary structure because association of the monomers is prevented at HHP (Foguel et al., 1999). In a recent article the refolding of the green fluorescent protein (GFP) under high pressure was determined by monitoring the green fluorescence (maximum at 509 nm) characteristic of the native protein. Higher yields of GFP in native conformation were found by IB dissociation at 2.4 kbar followed by incubation for extra 16 h under 0.35–0.70 kbar, before complete decompression (Malavasi et al., 2011). Also with the monomeric protein endostatin, the incubation at intermediate pressure (among the pressure utilized for dissociation of the IB and atmospheric pressure) was verified to be interesting, doubling the yields of the protein refolding (Chura-Chambi et al., 2013). It was also suggested that rapid depressurization rates could trap nonequilibrium and aggregation-prone species (Kim et al., 2006).Therefore, in order to verify if the rate of decompression could improve the yields of refolded r*Sm*29 from IB, the results of applying the following schemes of pressurization and decompression were compared: (a) 2.4 kbar for 16 h and direct decompression; (b) 2.4 kbar for 16 h followed by step-decompression of 0.4 kbar every 30 min; (c) 2.4 kbar for 2 h, direct decompression to 0.4 kbar, 0.8 kbar or 1.2 kbar and incubation for 16 h followed by direct decompression to 1 bar or (d) incubation for 16 h at atmospheric pressure (Fig. 4). Very low soluble r*Sm*29 is recovered by incubation at atmospheric pressure. The decompression rate did not substantially affect the recovery of soluble protein. The incubation of IB suspensions for 16 h at 0.8 kbar improved in 11.0% the r*Sm*29 recovery in comparison to the sample incubated

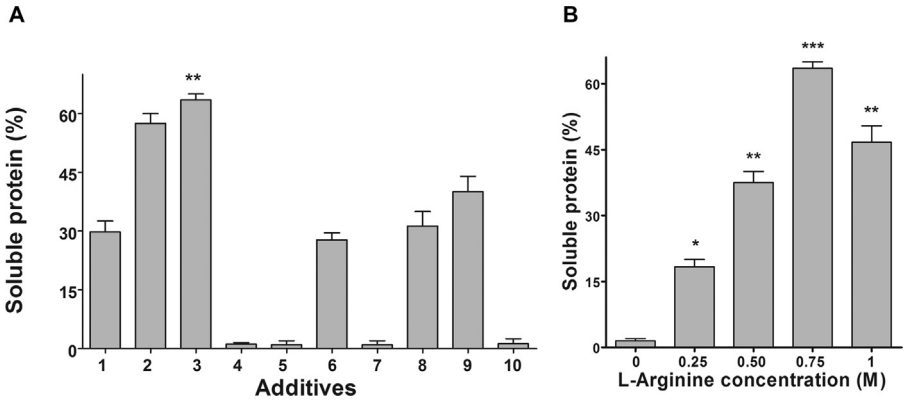

Fig. 3. Effect of the presence of additives on r*Sm*29 refolding by HHP. (A) Suspensions of IB in refolding buffer (50 mM Tris–HCl, 1.5 M GdnHCl, 1 mM EDTA, 4 mM GSH and 6 mM GSSG) were submitted to HHP (2.4 kbar 16 h, followed by decompression in steps of 0.4 kbar every 30 min) in presence of different additives. Lane 1—Absence of additives; lane 2—0.75 M L-Arginine; lane 3—0.75 M L-Arginine in absence of GdnHCl; lane 4—0.15 M NaCl; lane 5—1 M glicose; lane 6—1 M sucrose; lane 7—0.1%PEG; lane 8—1 mM Tween 20; lane 9—0.5 mM Triton X-100; lane 10—8.5 mM bis-ANS. (B) IB suspensions in refolding buffer without GdnHCl were submitted to HHP in presence of increasing concentrations of L-Arginine (0 to 1 M). Samples of the soluble fractions were applied for SDS-PAGE and the digital images of the gels were analyzed using Image J software to determine the intensity of the bands and the percentage of solublilizedr*Sm*29 in comparison to the total amount of r*Sm*29 in the IB suspension. The results are representative of three experiments. The error bars indicate SEM of duplicate of samples applied in the SDS-PAGE. The percentage of solubilization of the samples in the presence of L-Arginine are statistically different (***$P < 0.001$, **$P < 0.01$, *$P < 0.1$, Student t-test) to the controls (absence of Arginine).

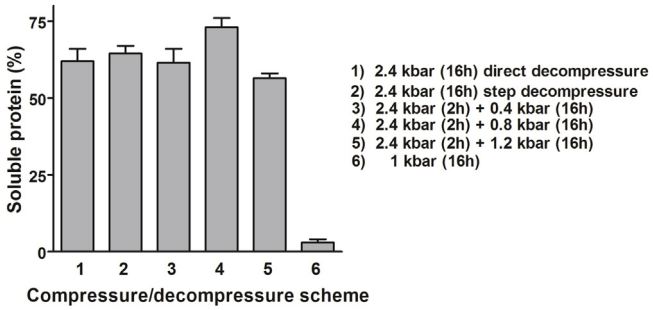

Fig. 4. Effect of different strategies of compression/decompression on the refolding of r*Sm*29. Suspensions of IB in refolding buffer (Tris–HCl 50 mM, 1 mM EDTA pH 8.0, 4 mM GSH, 6 mM GSSG) with 0.75 M L-Arginine were submitted to the indicated conditions of pressurization/depressurization. Samples of the soluble fractions were applied for SDS-PAGE and the digital images of the gels were analyzed using Image J software to determine the intensity of the bands and the percentage of solublilizedr*Sm*29 in comparison to the total amount of r*Sm*29 in the IB suspension. The results are representative of three experiments. The error bars indicate SEM of duplicate of samples applied in the SDS-PAGE. All the percentages of solubilization of the samples subjected to different compression/decompression schemes were significantly different from the sample maintained at 1 bar ($P < 0.01$, Student *t*-test).

for 16 h at 2.4 kbar, resulting in 73.0% solubilization. The results indicate that r*Sm*29 refolding was more efficient at 0.8 kbar than in other tested conditions. Interestingly, these results were similar to those obtained for endostatin and eGFP.

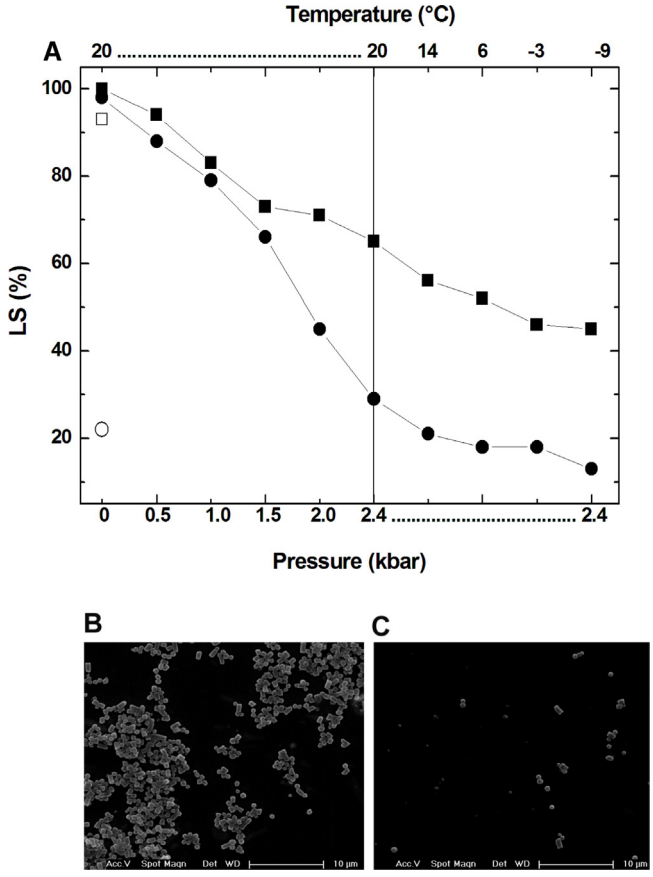

Fig. 5. Dissociation of r*Sm*29 IB under HHP and low temperatures. (A) Light scattering (LS) of samples in presence (●) or absence (■) of 0.75 M of L-Arginine were followed during pressure and temperature variation. The values were collected after pressure and temperature stabilization. Open symbols in the left represent the values of LS of the samples after return to 20 °C and 1 bar. (B) Scanning electron microscopy image of the dried r*Sm*29 IBs and (C) Scanning electron microscopy image of the structures that remain insoluble after HHP treatment. The results shown are representative of two experiments.

3.3.4. *Effect of negative temperatures in association to HHP on the dissociation of rSm29 aggregates*

HHP was used to work on subzero temperatures without freezing the samples, as it lowers the freezing point of water. The effect of the application of high pressure associated to low temperature on the dissociation of r*Sm*29 IB was monitored by the scattering of visible light at 350 nm (LS) (Fig. 5A). At high pressure (2.4 kbar) and 20 °C, an IB suspension in the absence of L-Arginine exhibited a decrease in LS to 65.8% of the initial value. Lowering the temperature to −9 °C further decreased the LS to 45.0%, indicating that the decrease of the temperature improves the dissociation of the aggregates at high pressure. However, returning the pressure level to 1 bar and the temperature to 20 °C, the LS was recovered to 93.0% of the initial intensity, showing that the dissociation of r*Sm*29 aggregates by HHP and low temperature is reversible. The application of high pressure (2.4 kbar) at 20 °C to the IB suspensions in the presence of 0.75 M L-Arginine induced a decrease in LS to 28.0% of the initial value. Lowering the temperature to −9 °C induced a further decrease of the LS to 13.8%, again indicating that at low temperature the effect of HHP on the dissociation of the aggregates is enhanced. Interestingly, in this case the return of the temperature to 20 °C and the pressure to 1 bar did not promote significant aggregation, bringing the final

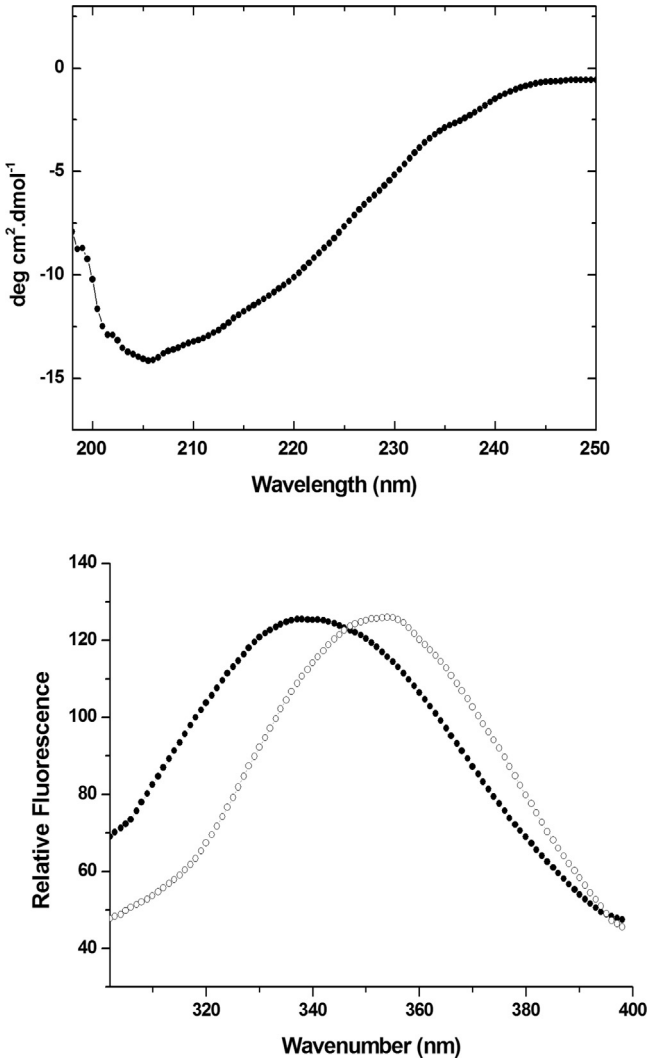

Fig. 6. Spectra of refolded r*Sm*29. (A) Spectrum of circular dichroism; (B)Spectrum of intrinsic fluorescence of refolded r*Sm*29 (●) and denatured r*Sm*29 (○) by incubation in the presence of 8 M urea for 2 h. Excitation 280 nm.

LS to 22.0%, suggesting a stabilization of the dissociated protein. The results indicate that the presence of L-Arginine is essential for enhancing the solubilization of r*Sm*29 from IB and maintenance of the dissociated states after the return to atmospheric pressure. These results are in agreement with the images on Fig. 5B and C that show the electronic microscopy scanning images of the dried r*Sm*29 IB structures and the remaining insoluble structures of the HHP-treated IB suspension, respectively. Although the HHP treated IB structures are similar to the non-treated ones, a lower amount of such structures is observed, in agreement with the results of LS (Fig. 5A).

About 72% of the expressed r*Sm*29 was recovered soluble after refolding under HHP in the presence of L-Arginine and glutathione, dialysis and centrifugation. The final yield, after purification by IMAC, dialysis and recentrifugation achieved 65%.

3.4. Structural characterization of refolded r*Sm*29 by HHP

The *in silico* analysis of the amino acid sequence of r*Sm*29 (http://bioinf.cs.ucl.ac.uk/psipred) indicates a predominance of random coil and β-sheets structures, with low α-helix content. These data are in agreement with the circular dichroism spectra of the HHP-refolded r*Sm*29 (Fig. 6A).

The r*Sm*29 protein has one tryptophan residue, which allows the evaluation of possible structural modifications by determination of the intrinsic fluorescence of the protein. The intrinsic fluorescence of the tertiary structure of HHP-refolded r*Sm*29 presents a fluorescence peak of 337 nm, which is shifted to 358 nm by the denaturation of protein with 8 M urea (Fig. 6B). The red-shift in fluorescence indicates that the tryptophan residue in the r*Sm*29 is in a relatively hydrophobic environment, and that the denaturation of the protein prompted the exposure of this residue to the aqueous environment.

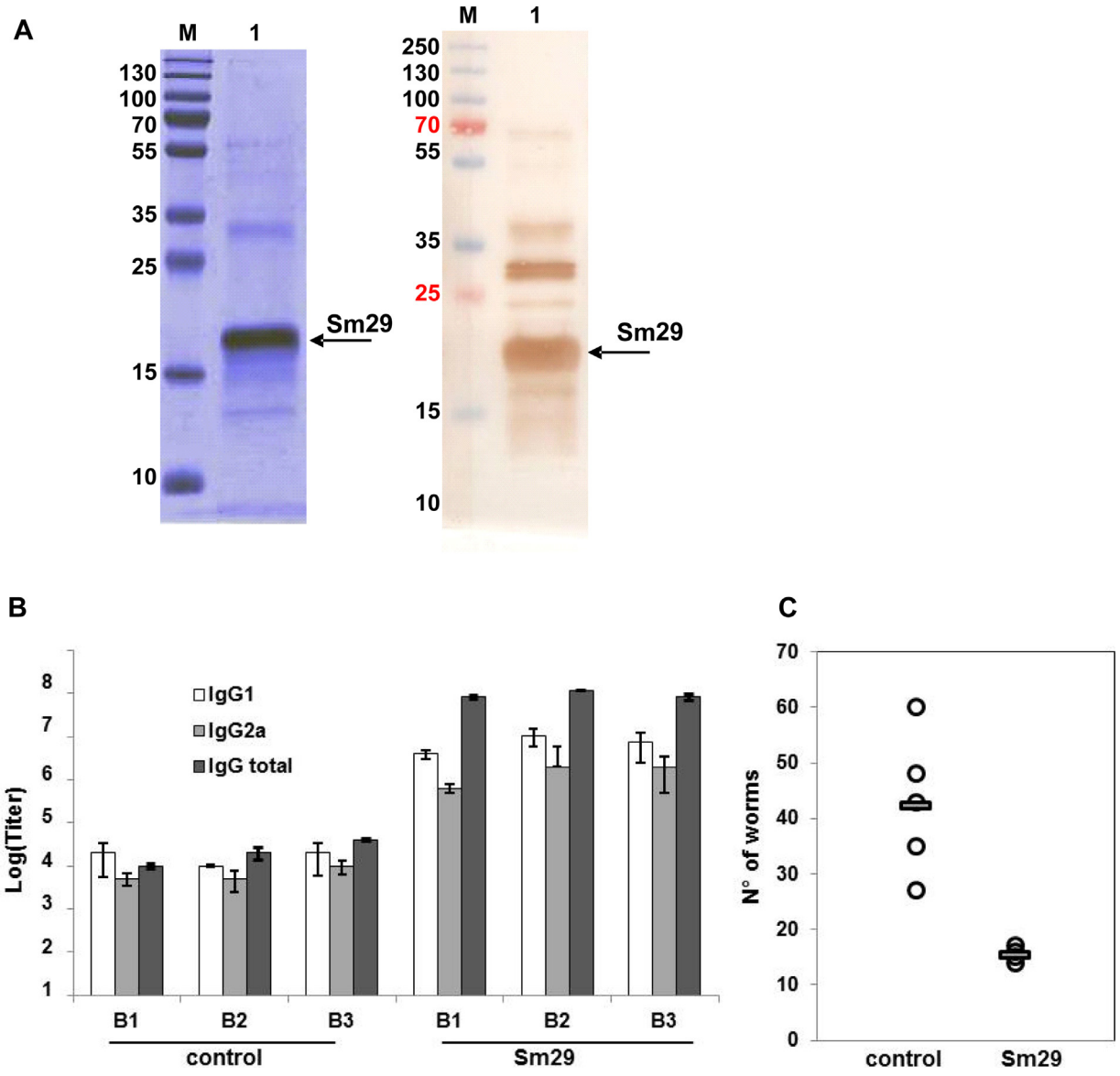

Fig. 7. Immune response to HHP refolded r*Sm*29 in mice and protection against *S. mansoni*. Groups of five animals were immunized with r*Sm*29 formulated with Alhydrogel as adjuvant. The sera of the animals were collected after second (B1) or third (B2) doses of immunization, and forty five days after infection with *S. mansoni* cercariae (B3). (A) SDS-PAGE of refolded and purified r*Sm*29 and Western blot with sera from immunized mice. (B) Specific anti-Sm29 IgG, IgG1 and IgG2a raised in the sera of immunized and infected mice were measured by ELISA. The titers are dilution of sera to render Abs 495 nm 0.1. (C) Protection against *S. mansoni* by immunization with r*Sm*29. Control and immunized mice were challenged with 150 cercariaes. After 45 days the animals were perfused for adult worms recover from the portal hepatic system and mesenteric veins. The circles indicate the number of worms recovered per animal and bars indicate the average of worms recovered in each group.

Due to the lack of a native *Sm*29 control, it cannot be stated that the refolded r*Sm*29 is in the native conformation. However, the CD spectra analysis and the shift on the peak of fluorescence of r*Sm*29 in presence of denaturing agent, together with the fact that the protein remains stably soluble, even after frozen storage, indicate that it is in a folded conformation.

3.5. Immunization of mice with rSm29 and S. mansoni challenge assay

The soluble r*Sm*29 antigen recovered by refolding under HHP in presence of L-Arginine and glutathione was dialyzed and purified by IMAC.

The purified, r*Sm*29 protein (Fig. 7A) was used to reproduce previous immunization and challenge assay in mice. After immunization, the animals were generally healthy, indicating no side effects of the vaccine formulation. The recognition of r*Sm*29 by antibodies present in the sera of immunized animals was tested by western blot (Fig. 7A). Measurements of total IgG and isotypes IgG1 and IgG2a specific to r*Sm*29 indicated that the protein is highly immunogenic and that both humoral and cellular immune responses were induced (Fig. 7B). In fact, it was reported previously that the immunization with r*Sm*29 and Freund's adjuvant induced immune response modulated toward Th1 type (Cardoso et al., 2008). In our work, the immunization was performed using Alhydrogel, an adjuvant suggested to induce more Th2 response, however, with r*Sm*29 a more complex immune response (Th1 and Th2) was observed.

After immunization, the mice were challenge with *S. mansoni* cercareae and protection was assessed by measuring the inhibition of larvae development into adult worms (Fig. 7C). The results indicated that immunization with HHP refolded r*Sm*29 conferred about 63% protection, (P-value < 0.008, according to Student's t-test), corroborating previous data (Cardoso et al., 2008).

Total and r*Sm*29-specific IgE were also measured in the sera of animals before and after infection. Total IgE induced by *S. mansoni* infection was lower (1/3) in the immunized group when compared to the non-immunized group, what might be related to the lower worm burden (total IgE content should be taken with restriction as the high content of IgG interferes with the IgE measurement). Higher level of IgE in the sera of the animals was observed, as expected by worm infection, however no specific r*Sm*29-IgE was induced, corroborating data from the literature (Cardoso et al., 2006a).

3.6. Considerations for large scale production of rSm29

The use of high pressure for protein solubilization and refolding presents a series of advantages: it is a mild method to solubilize the aggregates thus maintaining some native-like secondary structure of the recombinant proteins; concentrated IB suspensions can be used, which results in smaller process working volumes; lower amounts of additives can be used, which in some cases are expensive, such as Arginine and trehalose, and otherwise impeditive for industrial production.

However, further process development for large scale production of r*Sm*29 should include the different aspects of expression, refolding and purification steps. The platform and system chosen for industrial r*Sm*29 expression may interfere substantially with the refolding condition, considering that the produced IB can present different stabilities. In fact we observed that the expression of r*Sm*29 based on T7 promoter and IPTG as inducer, in specific conditions, result in IB that are easier to refolding by HHP, improving the yields here presented. However the expression of the antigen under the redox condition allowed to verifying that the use of HHP is a very interesting method for r*Sm*29 refolding.

4. Conclusion

After intensive studies, very few *Schistosoma* antigens are progressing toward large scale production to make a vaccine available. The previous studies on *Sm*29 protein showed that it holds very interesting characteristics as a potential vaccine antigen (Cardoso et al., 2006a,b, 2008).

The results here presented showed the possibility of using HHP to refold r*Sm*29. The application of HHP is feasible for large scale protein production and it can be easier and economically advantageous than the conventional methodologies, which use full protein denaturing condition and extensive dialysis. Therefore these results may contribute to the production of the antigen. The production of the soluble recombinant *Sm*29 under conditions suitable for formulation is quite exciting, whether to be proposed as a single antigen vaccine or to be tested as a combined multi antigen vaccine.

The HHP is relatively mild and can be used for other insoluble proteins, although requiring process development to find the better refolding condition for each specific protein.

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