Journal of Biotechnology 168 (2013) 511–519

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

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ARTICLE INFO

Article history:
Received 14 April 2013
Received in revised form
19 September 2013
Accepted 23 September 2013
Available online 29 September 2013

Keywords:
Protein refolding
Hydrostatic pressure
Schistosomiasis
Vaccine

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.

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http://dx.doi.org/10.1016/j.jbiotec.2013.09.017

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.