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RESEARCH PAPER

Recovery of recombinant proteins CFP10 and ESAT6 from *Escherichia coli* inclusion bodies for tuberculosis diagnosis: a statistical optimization approach

Ludmilla Dela Coletta Troiano Araujo[a], Daniel Ernesto Rodriguez-Fernández[b], Márcia Wibrantz[a], Susan Grace Karp[a], Gilberto Delinski Junior[a], Emanuel Maltempi de Souza[c], Carlos Ricardo Soccol[a], Vanete Thomaz-Soccol[a,*]

[a] *Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná (UFPR), Cel. Francisco H. dos Santos Avenue, 100. Zip Code 81530-000, Curitiba, Brazil*
[b] *School of Pharmacy, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil*
[c] *Department of Biochemistry and Molecular Biology, Federal University of Paraná (UFPR), Curitiba, Brazil*

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Abstract Tuberculosis (TB) is among the top ten causes of mortality worldwide and has high prevalence in developing countries. The dissemination of efficient and low-cost diagnosis tools able to identify its latent form, *e.g.* the delayed hypersensitivity reaction, is of great importance to accomplish the target of TB eradication. Recent studies have shown the potential of specific *Mycobacterium tuberculosis* immunodominant antigens, CFP10 and ESAT6, as substitutes of tuberculin skin test. The purpose of this study was to optimize the recovery and purification of recombinant CFP10 and ESAT6 from *Escherichia coli* inclusion bodies using central composite design. The production of CFP10 and ESAT6 in bioreactor presented yields of 233 and 121 mg L^{-1}, respectively, after extraction under optimized conditions: biomass concentration of 15 g per 150 mL of sonication buffer, using 12 cycles of disruption and 7 cycles of solubilization, followed by affinity chromatography purification and removal of endotoxins by the micellar method. The identity of the antigens was confirmed by mass spectrometry and their immunoreactivity after recovery and purification was confirmed by Western blot. These results demonstrate the suitability of the preparation methods in the development of a TB diagnosis kit with potential to be diffused in high TB burden countries.

Introduction

Tuberculosis is among the top ten leading causes of mortality worldwide, and the leading cause from a single infectious

* Corresponding author.
E-mail: vanetesoccol@gmail.com (V. Thomaz-Soccol).

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Table 1 Experimental design and levels of independent variables studied using a 2^3 factorial design on the recovery of recombinant protein (CFP10).

Factors	Low level (−1)	Intermediate level (0)	High level (+1)
Biomass concentration (g[a]/150 mL) (X_1)	5	10	15
Number of disruption cycles (X_2)	8	12	16
Number of solubilization cycles (X_3)	4	6	8

[a] wet basis.

agent, accounting for about 1.5 million deaths and 10 million new cases annually (Raviglione & Sulis, 2016; WHO, 2018). TB containment and eradication has been progressively reliant on the diagnosis and treatment of latent TB infection (LTBI). According to the World Health Organization (WHO) - Global Tuberculosis Report 2018 (WHO, 2018), latent infection is a massive encumbrance worldwide, affecting 1.7 billion people, from which 5–15% will suffer from reactivation of TB during their lifetime (Schluger, 2017).

There are two major tests available to confirm a diagnosis of LTBI: the tuberculin skin test (TST) and the *in vitro* blood test interferon gamma (IFN-γ) release assay (IGRA). Either tests evaluate cell-mediated immunity. The first uses an antigen purified protein derivative (PPD), and the second uses two recombinant proteins, ESAT6 (6-kDa - early secretory antigenic target) and CFP10 (10 kDa - culture filtrate protein) encoded by *M. tuberculosis*-specific RD1 region (Hemmati et al., 2011), that induce strong T-cell IFN-γ responses, mainly activating Th1 cells (Renshaw et al., 2005). These proteins do not elicit cross-reaction with the Bacillus Calmette-Guérin (BCG) used in TB vaccination or with environmental mycobacteria. Recent studies have shown the potential of CFP10 and ESAT6 as substitutes of tuberculin in TST.

Escherichia coli has been a widely preferred organism to produce recombinant proteins for over two decades. However, heterologous gene expression in prokaryotic expression systems often results in the production of proteins in insoluble aggregates or inclusion bodies (IBs). The recovery of these proteins without affecting the native-like structure and retaining the product's biological activity still represents one of the major bottlenecks for the development of biopharmaceutical products. Several novel approaches have been developed over the last few years to enhance protein recovery for use in diagnosis and to design a high-quality commercially-viable process. In particular, the regulation of factors like temperature, pH, aeration and plasmid stability, and the effective solubilization of the IBs and refolding of proteins into native conformation are fundamental steps to recover proteins from insoluble aggregates (Junker, 2004; Singh, Upadhyay, Upadhyay, Singh, & Panda, 2015). A careful study for optimization at bench scale is therefore necessary, not only to obtain a large quantity of target proteins, but also to verify the diversity of factors that can affect a process considering the lack of standard methodologies.

In this paper, the expression and the experimental design to obtain the highest yield of two recombinant proteins, ESAT6 and CFP10, solubilized and purified from inclusion bodies, is presented. These proteins are important skin test immunogens for diagnosis of *M. tuberculosis* infection that could compensate the specificity disadvantages presented by PPD. In developing and high TB burden countries, TST still remains the most widely used test for LTBI, due to the low reagent cost, applicability, and no requirement of standardized labs or venipuncture skills. Also, in low and middle-income countries WHO strongly recommends TST for diagnosis of LTBI (Sharma, Vashishtha, Chauhan, Sreenivas, & Seth, 2017).

Material and methods

Plasmid and strains construction and protein expression in bioreactor

The strains were characterized by DNA fingerprinting (mixed-linker PCR) as *M. tuberculosis* (Malaghini, Brockelt, Burger, Kritski, & Thomaz-Soccol, 2009; Malaghini et al., 2011). The genes were cloned using Invitrogen's Gateway® System. The CFP10 and ESAT6 recombinant proteins from H37Rv strains of *M. tuberculosis* were expressed in *E. coli* BL21pLysS cells, and single bacterial colonies were cultured with shaking at 150 rpm for 12 h at 37 °C in 700 mL Luria-Bertani (LB) medium containing antibiotics (25 mg L^{-1} chloramphenicol and 100 mg L^{-1} ampicillin). The optimal conditions for heterologous protein expression in a 7.5-L bioreactor were optimized previously and can be found at Dela Coletta Troiano Araujo et al. (2019).

Recovery of proteins from *E. coli* biomass

The experimental design for protein recovery optimization was conducted with the recombinant strain *E. coli* BL21-CFP10, and samples were processed in two steps. First, the biomass produced in bioreactor was separated by centrifugation (10,000 g, 4 °C, 15 min) and resuspended in a sonication buffer (Tris-HCl 50 mM, pH 8), at different proportions (Tables 1 and 2). Sonication for cell disruption was operated with acoustic power of 85 W, duty cycle of 70% and 19 kHz. Each cycle encompassed a short burst of 30 s followed by a 30 s cooling interval. The samples were then centrifuged (10,000 *g*, 4 °C, 15 min) and the supernatant (soluble fraction) was separated. Fifty milliliters of a solubilization buffer (urea 5 M, imidazole 10 mM, NaCl 300 mM, pH 7.4) were added to the sediment, and the suspension was subjected to a series of ultrasound-assisted solubilization cycles as described above. The sample was centrifuged again (10,000 *g*, 4 °C, 15 min) and the supernatant (insoluble fraction, solubilized from inclusion bodies) was separated. The effects of biomass concentration, number of sonication cycles for cell disruption and number of sonication cycles for solubilization were evaluated in a factorial design 2^3

Table 2 Real and coded values of the Central Composite Rotatable Design (CCRD) to optimize biomass concentration, number of disruption cycles and number of solubilization cycles for the release of recombinant protein (CFP10).

Factors	$-\alpha$	-1	0	+1	$+\alpha$
Biomass concentration (g[a]/150 mL) (X_1)	7	10	15	20	23
Number of disruption cycles (X_2)	5	8	12	16	19
Number of solubilization cycles (X_3)	3	4	6	8	9

[a] wet basis.

with replicates of the central point (Table 1). Based on the results, the design was complemented to a Central Composite Rotatable Design (CCRD) for the optimization of the recovery conditions (Table 2). All experiments were performed in random order, and the protein concentration was considered as response factor. Experimental results were analyzed considering 0.05 as significance level and employing the software Statistica 7.0. The recovery conditions optimized for CFP10 were applied to ESAT6.

Purification of CFP10 and ESAT6

The recombinant protein purification was achieved by affinity chromatography (HisTrap FF) according to the manufacturer's protocol (GE Healthcare) and as described by Dela Coletta Troiano Araujo et al. (2019). The endotoxins were removed using the micellar method of separation. Triton X-114 was added to the purified protein fraction (2.5 mg mL^{-1}) to a final concentration of 1%, at 4 °C. The solution was incubated for 10 min in a water bath at 37 °C, and centrifuged (5000 g, 15 min) at 25 °C. The top aqueous phase containing the protein was precisely transferred to another tube. This cycle was repeated two more times. For protein precipitation, four volumes of acetone were used at 4 °C to remove the residual detergent. The pellet was then solubilized in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with 10 M urea, following dialysis against HEPES medium. The solution of proteins was stored at −70 °C and then lyophilized in a ModulyoD freeze dryer (Thermo Fisher Scientific) for 24 h. The quantification of endotoxins was conducted according to the manufacturer's instructions (Chromogenic Limulus Amebocyte Lysate - LAL assay QCL). The concentrations of the purified proteins were determined after sonication (for cell disruption and solubilization), purification by affinity chromatography and removal of endotoxins, using the Bradford's method, and were related to the initial concentration of biomass as described previously (Dela Coletta Troiano Araujo et al., 2019).

Confirmation of protein identity and immunoreactivity

The insoluble fractions from CFP10 and ESAT6 were subjected to denaturing 15% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). One square millimeter fragments corresponding to the bands of expected molecular mass were excised from the electrophoresis gel and submitted to mass spectrometry analysis as described previously (Dela Coletta Troiano Araujo et al., 2019). Experiments were performed using the Bruker Autoflex II MALDI-TOF mass spectrometer and the analysis was performed using the software FlexControl 2.0. Samples of the insoluble fractions from CFP10 and ESAT6 submitted to SDS-PAGE as described above were also transferred to polyvinylidene fluoride (PVDF) membrane for blotting; following, the membrane was blocked using 0.1 M phosphate buffered saline containing 0.3% Tween 20, pH 7.4 (PBST) at 37 °C for 1 h. The primary antibody was diluted with PBST (1:50) at 37 °C for 2 h and washed three times with PBST. The secondary monoclonal antibody, peroxidase-conjugated anti-rabbit IgG (Sigma), was diluted with blocking buffer (1:5000) and incubated for 1 h at 37 °C. The membrane was finally washed with PBST and revealed using 3,3′-diaminobenzidine (DAB) peroxidase substrate (Sigma).

Results

Protein expression and recovery from *E. coli* biomass

The production of *E. coli* biomass in bioreactor resulted in recombinant protein yields of 233 mg per liter of culture (CFP10) and 121 mg per liter of culture (ESAT6). These values were determined after recovery and purification under optimized conditions, which will be described subsequently.

The highest concentration of recombinant protein in the experimental design 2^3 for cell disruption and solubilization of inclusion bodies was obtained in the central point (approximately 18 mg mL^{-1}), while lower values were obtained in conditions 1, 2 and 4 (Table 3). This result indicates that 8 cycles of disruption and 4 cycles of solubilization are insufficient to recover the recombinant protein.

Analysis of variance (ANOVA) indicates that all the independent factors and the first order interactions for biomass concentration/number of disruption cycles and number of solubilization cycles/number of disruption cycles have a significant influence on the recovery of the protein at $\alpha = 0.05$ confidence level (Table 4).

The lack of fit of the linear model generated was significant. This lack of fit means that in the work interval chosen for the experiments there is a possible area of curvature that may contain an optimal working condition to produce the protein of interest. For optimization, the central composite rotatable design (CCRD) was chosen to obtain a model with quadratic terms, and results are shown in Table 5.

The statistical analysis of these results from the quadratic model was performed to obtain the regression coefficients (Table 6) and the response surfaces model (Fig. 1). Thereafter, a mathematical model was generated from the CCRD,

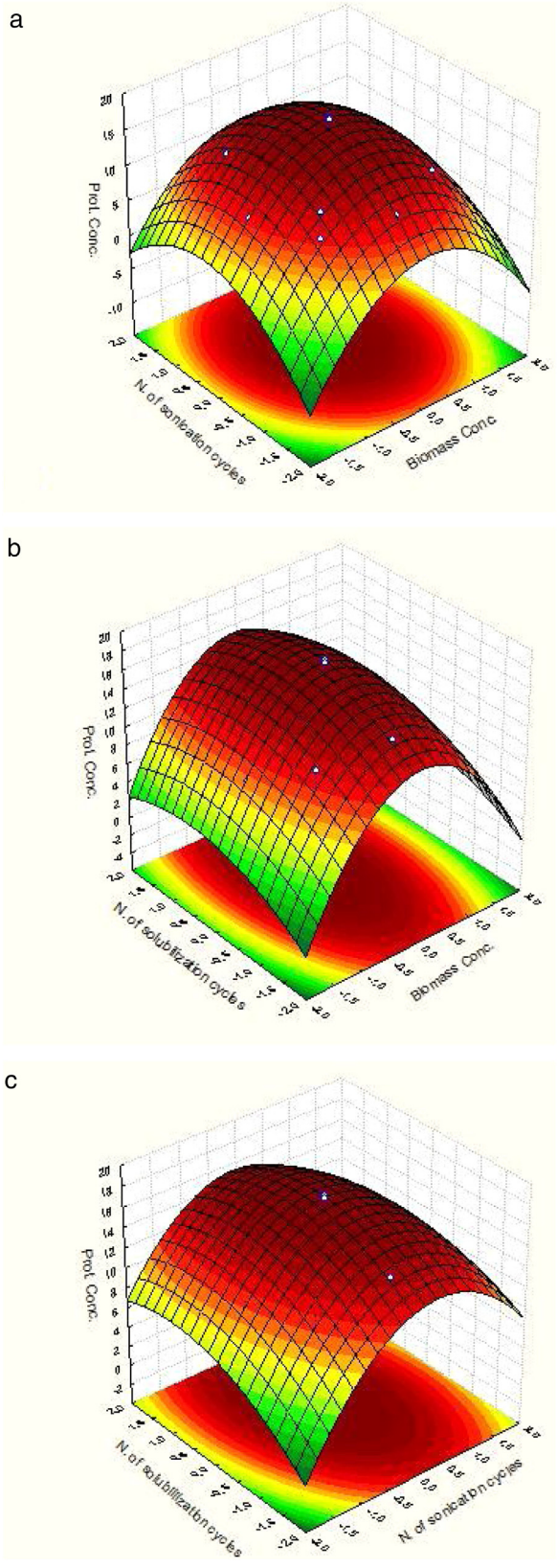

Figure 1 Surface responses generated from the central composite rotatable design (CCRD): (a) number of disruption cycles and biomass concentration *versus* protein concentration; (b) number of solubilization cycles and biomass concentration *versus* protein concentration and (c) number of solubilization cycles and number of disruption cycles *versus* protein concentration.

Table 3 Experimental design matrix for the 2^3 factorial design developed to evaluate recovery of recombinant protein (CFP10).

Run	Biomass concentration (g[a]/150 mL) (X_1)	Number of disruption cycles (X_2)	Number of solubilization cycles (X_3)	Protein concentration (mg g^{-1})[b]
1	−1	−1	−1	9.0
2	+1	−1	−1	10.2
3	−1	+1	−1	12.3
4	+1	+1	−1	10.6
5	−1	−1	+1	12.6
6	+1	−1	+1	11.5
7	−1	+1	+1	12.4
8	+1	+1	+1	11.9
9	0	0	0	17.7
10	0	0	0	18
11	0	0	0	17.8

[a] wet basis.
[b] mg protein per g biomass, wet basis.

Table 4 Effects of independent variables (factors) and second order interactions on recovery of recombinant protein (CFP10).

Factors	Regression coefficient	p-value
Mean/Intercept	13.09091	0.000012[a]
X_1	−0.26250	0.039817[a]
X_2	0.48750	0.012051[a]
X_3	0.78750	0.00467[a]
X_1 X_2	−0.28750	0.033522[a]
X_1 X_3	−0.13750	0.125809
X_2 X_3	−0.43750	0.014898[a]
Lack of Fit		0.000495[a]

[a] Independent factors and interactions with significant influence.

Table 5 Results of the central composite rotatable design (CCRD) for recovery of recombinant protein (CFP10).

Run	X_1	X_2	X_3	Protein concentration (mg g^{-1})[a]
1	−1	−1	−1	9.0
2	+1	−1	−1	10.2
3	−1	+1	−1	12.3
4	+1	+1	−1	10.6
5	−1	−1	+1	12.6
6	+1	−1	+1	11.5
7	−1	+1	+1	12.4
8	+1	+1	+1	11.9
9	0	0	0	17.9
10	0	0	0	18.1
11	0	0	0	17.8
12	−1.41	0	0	9.0
13	+1.41	0	0	10.1
14	0	−1.41	0	10.4
15	0	+1.41	0	13.3
16	0	0	−1.41	14.2
17	0	0	+1.41	15.1

[a] mg protein per g biomass, wet basis.

Table 6 Factor coefficients discrimination corresponding to the central composite rotatable design (CCRD).

Factors	Regression coefficient	p-value
Mean/Intercept[a]	17.27081	0.000022
X_1	−0.04584	0.408098
X_1^2[a]	−3.38953	0.000274
X_2[a]	0.66707	0.004350
X_2^2[a]	−2.23264	0.000631
X_3[a]	0.63200	0.004842
X_3^2*	−0.82427	0.004600
X_1 X_2[a]	−0.28750	0.033522
X_1 X_3	−0.13750	0.125809
X_2 X_3[a]	−0.43750	0.014898

[a] Independent factors and interactions with significant influence.

expressed in Eq. 1, and non-significant coefficients were then eliminated.

$$Y = 17.27 - 3.39X_1^2 + 0.67X_2 - 2.23X_2^2 + 0.63X_3 - 0.82X_3^2 - 0.29X_1X_2 - 0.44X_2X_3 \quad (1)$$

To determine the optimum protein recovery condition from inclusion bodies, a system of three equations was generated. The derivatives corresponding to each independent factor considered in the CCRD were equaled to zero. The equations are represented as follows:

$$\frac{dY}{dX_1} = -6.78X_1 - 0.29X_2 = 0$$

$$\frac{dY}{dX_2} = 0.67 - 4.46X_2 - 0.29X_1 - 0.44X_3 = 0$$

$$\frac{dY}{dX_3} = 0.63 - 1.64X_3 - 0.44X_2$$

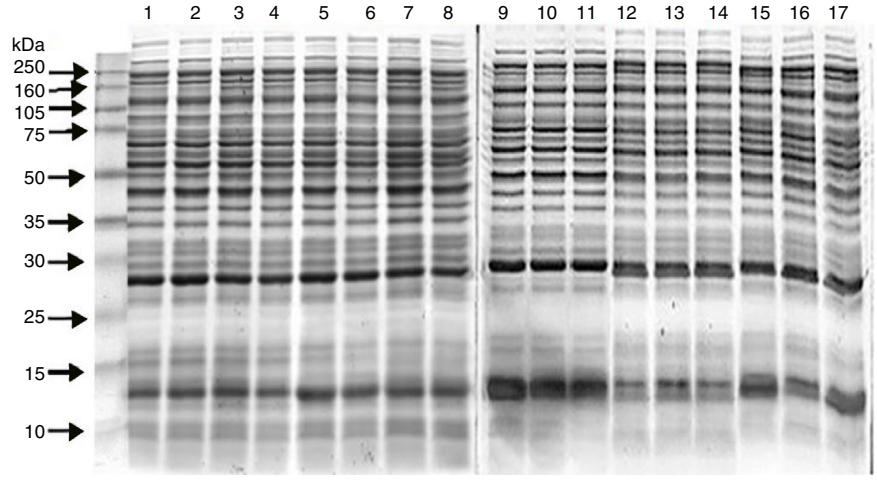

Figure 2 SDS-PAGE 15% analysis of the central composite rotatable design (CCRD) for optimization of recombinant protein recovery. Lanes 1–17 (runs) and M – Amersham Full-Range Rainbow marker (GE Healthcare) 250 kDa–10 kDa.

The values of the decoded independent variables, after solving the system of equations, were: biomass concentration of 15 g per 150 mL of buffer using 12 cycles of disruption and 7 cycles of solubilization. Fig. 2 shows the results of the 17 samples submitted to electrophoretic run (before purification), and it is visible that the highest concentrations were obtained at runs 9, 10 and 11 (central point). After optimizing the recovery of proteins, it was necessary to evaluate their integrity and immunological properties.

Characterization of the purified recombinant proteins

After treatment with Triton X-114, the endotoxin level was measured at 0.427 EU mL^{-1}, representing a 99% decrease of endotoxin concentration in the resulting protein solution. The CFP10 and ESAT6 purified recombinant proteins revealed a single band on SDS-PAGE with molecular weights corresponding to approximately 11 and 10 kDa, respectively (Fig. 3). The lower molecular mass band observed at lane 8 is a fragment of the same protein CFP10, as revealed by mass spectrometry. The MALDI-TOF mass spectrometry analysis determined that the amino acid sequence of the recombinant proteins produced in this study were homologous with the target proteins (CFP10 and ESAT6). Western blot analysis indicated that only recombinant CFP10 and ESAT6 proteins from insoluble fractions reacted with anti-serum against *M. tuberculosis*, which demonstrated specific and acceptable immunoreactivity (Fig. 4).

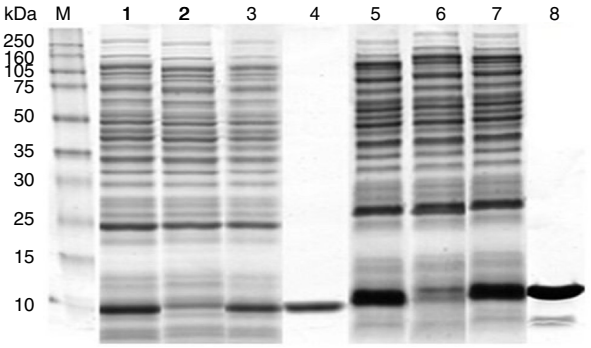

Figure 3 SDS-PAGE purification analysis of ESAT6 and CFP10, respectively. M, molecular marker; lanes 1 and 5, total protein after isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction; lanes 2 and 6, soluble fraction after cell disruption; lanes 3 and 7, insoluble fraction (inclusion bodies); lanes 4 and 8, fusion protein purified using a HisTrap FF affinity column (11 kDa and 10 kDa).

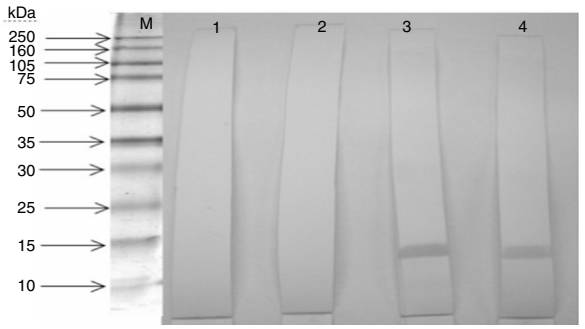

Figure 4 Western blot analysis of the recombinant proteins. Lane M, protein size marker; lanes 1 and 2, negative control; lanes 3 and 4, recombinant CFP10 and ESAT6 proteins recognized by polyclonal anti-*Mycobacterium tuberculosis*, respectively.

Discussion

Currently, TST is the major immunological diagnosis tool to detect LTBI, because of the low cost, accessibility and easiness to use (Li et al., 2016a). However, the results are often inaccurate. The main drawback, as cited, is the low specificity, especially in BCG-vaccinated populations. Moreover, 10–25% of active tuberculosis individuals do not present a positive result to TST test (Scarpellini et al., 2004). During

the last decade, many efforts have been made towards the development of specific biomarkers capable of distinguishing between BCG vaccination, *M. tuberculosis* infection, and environmental mycobacteria using cost-effective and reproducible technologies (Bai, Liang, Yang, Zhang, & Wu, 2017; Li et al., 2016b; Li et al., 2017). For the improvement of delayed hypersensitivity reaction, specific *M. tuberculosis* antigens that are not encoded in the genome of any BCG vaccine strain, or in most species of non-tuberculous mycobacteria, could be potentially useful reagents for specific diagnosis (Pai et al., 2014; van Ingen, de Zwaan, Dekhuijzen, Boeree, & van Soolingen, 2009). In a prior study, seven secretory proteins of *M. tuberculosis* culture were produced by cloning technology. Among these, ESAT6 and CFP10, which are currently considered the most promising candidates for novel diagnosis in humans and animals (Mahmoudi, Mamishi, Ghazi, Sadeghi, & Pourakbari, 2013), were selected for further studies (Malaghini et al., 2009, 2011). The strategy of the present research focused on increasing the yield of the target proteins based on an experimental design to maximize bioactive proteins recovery after expression under optimized conditions (Dela Coletta Troiano Araujo et al., 2019).

The influence of ultrasonication conditions on recombinant protein recovery from *E. coli* BL21-CFP10 by varying cell concentration, cell disruption and solubilization cycles was investigated. The optimal conditions were tested for *E. coli* BL21-ESAT6, which expresses a protein with very similar molecular mass. The association of factors such as shear stress (resulting from ultrasonication conditions) and biomass concentration influences viscosity and heat amount inside the chamber, which in turn affect cell wall disintegration and stability of the product. Selecting the appropriate operation conditions often results in higher yield, lower denaturation and correctly folded product (Bystryak, Santockyte, & Peshkovsky, 2015). Typical yields of purified CFP10 and ESAT6 proteins varied between 20 and 40 mg L^{-1} respectively (Målen, Pathak, Søfteland, de Souza, & Wiker, 2010; Poulsen, Holton, Geerlof, Wilmanns, & Song, 2010; Refai et al., 2015; Renshaw et al., 2005). In this sense, the present study reports satisfactory results concerning the yields of the recombinant proteins CFP10 (233 mg L^{-1}) and ESAT6 (121 mg L^{-1}). The design of appropriate large-scale bioreactors together with the optimization of the recovery conditions have a significant impact on the overall viability of the process. High output and quality biomarkers can significantly improve the diagnostic attainability, what is especially important in developing countries.

The development of new antigens requires the application of efficient purification protocols that ensure the quality of the final product at reasonably low cost. Affinity chromatography is an efficient method to purify proteins based on biological properties and can be applied in industrial scale without the need of working with excessively large columns. There are different options of configurations to work at pilot and process scales which make of it a versatile method to separate and concentrate the antigens.

The presence of small amounts of polysaccharide components of bacterial lipopolysaccharides (LPS), also called endotoxins, in purified protein preparations for injectable use can present toxic effects (Lopes et al., 2010; Magalhães et al., 2007; Saraswat et al., 2013). The Food and Drug Administration (FDA) guidance provides the maximum allowable limit of five endotoxin units (EU) per kilogram body weight per hour, in intravenous applications of pharmaceutical and biological products (Daneshian, Guenther, Wendel, Hartung, & von Aulock, 2006). It was demonstrated that a simple phase separation method with Triton X-114 performed in three cycles resulted in low level of endotoxin, with a reduction in endotoxins concentration of 99% and associated protein loss below 10%. Triton X-114 extraction was found to be an appropriate and efficient strategy to eliminate endotoxin contamination from bioproducts, which is in agreement with other reports (Liu et al., 1997; Lopes et al., 2010; Mahmoudi, Pourakbari, & Mamishi, 2017; Teodorowicz et al., 2017; Xin et al., 2013).

Conclusions

The recombinant expression in bioreactor and the recovery of proteins from inclusion bodies by ultrasonication represents an efficient strategy to produce CFP10 and ESAT6 antigens for TB diagnosis. Optimized recovery conditions were determined as biomass concentration of 15 g per 150 mL of sonication buffer, 12 cycles of disruption and 7 cycles of solubilization. Affinity chromatography and application of the micellar method for removal of endotoxins resulted in purified and immunoreactive antigens. These results demonstrate the suitability of the preparation methods in the development of a TB diagnosis kit with potential to be diffused in high TB burden countries.

Declarations of interest

None.

Acknowledgments

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References

Bai, X., Liang, Y., Yang, Y., Zhang, J., & Wu, X. (2017). A new method of screening for latent tuberculosis infection: Results from army recruits in Beijing in 2014. *Immunology Letters*, *186*, 28–32. http://dx.doi.org/10.1016/j.imlet.2017.03.014

Bystryak, S., Santockyte, R., & Peshkovsky, A. S. (2015). Cell disruption of *S. cerevisiae* by scalable high-intensity ultrasound. *Biochemical Engineering Journal*, *99*, 99–106. http://dx.doi.org/10.1016/j.bej.2015.03.014

Daneshian, M., Guenther, A., Wendel, A., Hartung, T., & von Aulock, S. (2006). In vitro pyrogen test for toxic or immunomodulatory drugs. *Journal of Immunological Methods*, *313*, 169–175. http://dx.doi.org/10.1016/j.jim.2006.04.009

Dela Coletta Troiano Araujo, L., Wibrantz, M., Rodríguez-Fernández, D. E., Karp, S. G., Talevi, A. C., Maltempi de Souza, E., et al. (2019). Process parameters optimization to produce the recombinant protein CFP10 for the diagnosis of

tuberculosis. *Protein Expression and Purification*, *154*, 118–125. http://dx.doi.org/10.1016/j.pep.2018.09.016

Hemmati, M., Seghatoleslam, A., Rasti, M., Ebadat, S., Mosavari, N., Habibagahi, M., et al. (2011). Expression and purification of recombinant *Mycobacterium tuberculosis* (TB) antigens, ESAT-6, CFP-10 and ESAT- 6/CFP-10 and their diagnosis potential for detection of TB patients. *Iranian Red Crescent Medical Journal*, *13*, 556–563.

Junker, B. H. (2004). Scale-up methodologies for *Escherichia coli* and yeast fermentation processes. *Journal of Bioscience and Bioengineering*, *97*, 347–364. http://dx.doi.org/10.1016/S1389-1723(04)70218-2

Li, F., Xu, M., Qin, C., Xia, L., Xiong, Y., Xi, X., et al. (2016). Recombinant fusion ESAT6-CFP10 immunogen as a skin test reagent for tuberculosis diagnosis: an open-label, randomized, two-centre phase 2a clinical trial. *Clinical Microbiology and Infection*, *22*, 889. http://dx.doi.org/10.1016/j.cmi.2016.07.015, e9-889.e16

Li, F., Xu, M., Zhou, L., Xiong, Y., Xia, L., Fan, X., et al. (2016). Safety of recombinant fusion protein ESAT6-CFP10 as a skin test reagent for tuberculosis diagnosis: an open-label, randomized, single-center phase I clinical trial. *Clinical and Vaccine Immunology*, *23*, 767–773. http://dx.doi.org/10.1128/CVI.00154-16

Li, G., Li, F., Zhao, H.-M., Wen, H.-L., Li, H.-C., Li, C.-L., et al. (2017). Evaluation of a new IFN-γ release assay for rapid diagnosis of active *Tuberculosis* in a high-incidence setting. *Frontiers in Cellular and Infection Microbiology*, *7* http://dx.doi.org/10.3389/fcimb.2017.00117

Liu, S., Tobias, R., McClure, S., Styba, G., Shi, Q., & Jackowski, G. (1997). Removal of endotoxin from recombinant protein preparations. *Clinical Biochemistry*, *30*, 455–463.

Lopes, A. M., Magalhães, P. O., Mazzola, P. G., Rangel-Yagui, C. O., de Carvalho, J. C. M., Penna, T. C. V., et al. (2010). LPS removal from an *E. coli* fermentation broth using aqueous two-phase micellar system. *Biotechnology Progress*, *26*, 1644–1653. http://dx.doi.org/10.1002/btpr.463

Magalhães, P. O., Lopes, A. M., Mazzola, P. G., Rangel-Yagui, C., Penna, T. C. V., & Pessoa, A. (2007). Methods of endotoxin removal from biological preparations: a review. *Journal of Pharmacy and Pharmaceutical Sciences*, *10*, 388–404.

Mahmoudi, S., Mamishi, S., Ghazi, M., Sadeghi, R. H., & Pourakbari, B. (2013). Cloning, expression and purification of *Mycobacterium tuberculosis* ESAT-6 and CFP-10 antigens. *Iranian Journal of Microbiology*, *5*, 374–378.

Mahmoudi, S., Pourakbari, B., & Mamishi, S. (2017). Interferon Gamma Release Assay in response to PE35/PPE68 proteins: a promising diagnostic method for diagnosis of latent tuberculosis. *European Cytokine Network*, 36–40. http://dx.doi.org/10.1684/ecn.2017.0391

Malaghini, M., Brockelt, S. R., Burger, M., Kritski, A., & Thomaz-Soccol, V. (2009). Molecular characterization of *Mycobacterium tuberculosis* isolated in the State of Parana in southern Brazil. *Tuberculosis*, *89*, 101–105. http://dx.doi.org/10.1016/j.tube.2008.07.005

Malaghini, M., Thomaz-Soccol, V., Probst, C. M., Krieger, M. A., Preti, H., Kritski, A., et al. (2011). Recombinant antigen production for assays of intradermoreaction for diagnosis and surveillance of tuberculosis. *Journal of Biotechnology*, *156*, 56–58. http://dx.doi.org/10.1016/j.jbiotec.2011.07.015

Målen, H., Pathak, S., Søfteland, T., de Souza, G. A., & Wiker, H. G. (2010). Definition of novel cell envelope associated proteins in Triton X-114 extracts of *Mycobacterium tuberculosis* H37Rv. *BMC Microbiology*, *10*, 132. http://dx.doi.org/10.1186/1471-2180-10-132

Pai, M., Denkinger, C. M., Kik, S. V., Rangaka, M. X., Zwerling, A., Oxlade, O., et al. (2014). Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. *Clinical Microbiology Reviews*, *27*, 3–20. http://dx.doi.org/10.1128/CMR.00034-13

Poulsen, C., Holton, S., Geerlof, A., Wilmanns, M., & Song, Y.-H. (2010). Stoichiometric protein complex formation and over-expression using the prokaryotic native operon structure. *FEBS Letters*, *584*, 669–674. http://dx.doi.org/10.1016/j.febslet.2009.12.057

Raviglione, M., & Sulis, G. (2016). Tuberculosis 2015: burden, challenges and strategy for control and elimination. *Infectious Disease Reports*, *8* http://dx.doi.org/10.4081/idr.2016.6570

Schluger, N. W. (2017). Improving the diagnosis of latent TB infection. *Chest*, *151*, 1207–1208. http://dx.doi.org/10.1016/j.chest.2017.04.161

Refai, A., Haoues, M., Othman, H., Barbouche, M. R., Moua, P., Bondon, A., et al. (2015). Two distinct conformational states of *Mycobacterium tuberculosis* virulent factor early secreted antigenic target 6 kDa are behind the discrepancy around its biological functions. *FEBS Journal*, *282*, 4114–4129. http://dx.doi.org/10.1111/febs.13408

Renshaw, P. S., Lightbody, K. L., Veverka, V., Muskett, F. W., Kelly, G., Frenkiel, T. A., et al. (2005). Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *The EMBO Journal*, *24*, 2491–2498. http://dx.doi.org/10.1038/sj.emboj.7600732

Saraswat, M., Musante, L., Ravidá, A., Shortt, B., Byrne, B., & Holthofer, H. (2013). Preparative purification of recombinant proteins: current status and future trends. *BioMed Research International*, 1–18. http://dx.doi.org/10.1155/2013/312709

Scarpellini, P., Tasca, S., Galli, L., Beretta, A., Lazzarin, A., & Fortis, C. (2004). Selected pool of peptides from ESAT-6 and CFP-10 proteins for detection of *Mycobacterium tuberculosis* infection. *Journal of Clinical Microbiology*, *42*, 3469–3474. http://dx.doi.org/10.1128/JCM.42.8.3469-3474.2004

Sharma, S. K., Vashishtha, R., Chauhan, L. S., Sreenivas, V., & Seth, D. (2017). Comparison of TST and IGRA in diagnosis of latent tuberculosis infection in a high TB-burden setting. *Plos One*, *12*, e0169539 http://dx.doi.org/10.1371/journal.pone.0169539

Singh, A., Upadhyay, V., Upadhyay, A. K., Singh, S. M., & Panda, A. K. (2015). Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process. *Microbial Cell Factories*, *14* http://dx.doi.org/10.1186/s12934-015-0222-8

Teodorowicz, M., Perdijk, O., Verhoek, I., Govers, C., Savelkoul, H. F. J., Tang, Y., et al. (2017). Optimized Triton X-114 assisted lipopolysaccharide (LPS) removal method reveals the immunomodulatory effect of food proteins. *Plos One*, *12*, e0173778 http://dx.doi.org/10.1371/journal.pone.0173778

van Ingen, J., de Zwaan, R., Dekhuijzen, R., Boeree, M., & van Soolingen, D. (2009). Region of difference 1 in nontuberculous *Mycobacterium* species adds a phylogenetic and taxonomical character. *Journal of Bacteriology*, *191*, 5865–5867. http://dx.doi.org/10.1128/JB.00683-09

WHO - World Health Organization. https://www.who.int/tb/publications/global_report/en/, 2018. Accessed 15 March 2019

Xin, T., Jia, H., Ding, J., Li, P., Yang, H., Hou, S., et al. (2013). Assessment of a protein cocktail-based skin test for bovine tuberculosis in a double-blind field test in cattle. *Clinical and Vaccine Immunology*, *20*, 482–490. http://dx.doi.org/10.1128/CVI.00592-12