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Optimizing of a protein extraction method for *Mycobacterium tuberculosis* proteome analysis using mass spectrometry

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ABSTRACT

A critical step in proteomic analyses comprises the implementation of a reliable cell lysis method with high yields of qualitative proteins. In Mycobacteria, the protein extraction step is often hampered by the thick waxy cell wall which is rich in mycolic acids. Harsh disruption techniques to release proteins from the cells are thus required. Here, we demonstrate an optimized protein extraction procedure for *Mycobacterium tuberculosis* (Mbt) that results in protein extracts that are useful for all currently used proteomics platforms, including gel and LC-MS based strategies. We compared the effectiveness of using both thiourea and urea and/or SDS and DTT in the solubilization buffer, in combination or not with sonication and/or bead beating. After some preliminary optimization steps on fast-growing Mbt-like organisms, namely *Mycobacterium smegmatis* and *Mycobacterium fortuitum*, the final protein extraction protocol was tested on *M. tuberculosis*. Based on the concentrations of the proteins recovered from each of the tested methods and on the quality of the extracted proteins as evaluated by SDS PAGE, we propose a lysis buffer that contains both thiourea and urea, in combination with two mechanical cell disruption methods: sonication and bead beating. The optimized protocol results in protein extracts that are useful in *M. tuberculosis* proteomics studies based on any proteomics strategy or platform.

1. Introduction

Tuberculosis (TB) remains a global health problem worldwide, in particular in developing countries. Annually, there are an estimated 9.6 million new TB cases and 1.5 million TB deaths (WHO report, 2015). In addition, the TB situation is aggravated by the emergence of drug resistant strains, multidrug resistant (MDR) and extensively drug resistant (XDR).

As alternative to molecular detection of drug resistance, proteomics analysis, based on mass spectral analyses, has become recognized in systems biology studies and as an emerging tool for rapidly assessing drug resistance. New high-throughput platforms based on liquid chromatography hyphenated with tandem mass spectrometry (LC-MS/MS) have been developed and improved the accuracy of protein identification as their quantification, opening new ways for TB drug resistance studies (Silva et al., 2006; de Souza et al., 2010; Gillet et al., 2012).

A unique characteristic of mycobacteria is their thick waxy outer cell wall, consisting of mycolic acids and arabinogalactans associated with the peptidoglycan envelope (Brennan, 1995). Mycolic acids are extremely hydrophobic molecules that affect the permeability at the cell surface. The cell envelope helps mycobacteria to survive in extreme environmental conditions and the presence of antibiotics, thus playing an important role in drug resistance. The lipid shell around the organism renders mycobacteria highly resistant to common lysis techniques and, consequently, harsh disruption methods are required to release proteins from these cells. As such, proteomics analysis is often hampered by the complexity of the mycobacterial cell wall: thick, waxy, hydrophobic and rich in mycolic acids, requiring an optimized sample preparation. Sample preparation is a crucial step in proteomics studies, especially in comparative proteomics, where minor differences between experimental and control samples are searched. Currently, few protocols for protein extraction and solubilization of mycobacterial proteomes are available. Cell lysis of mycobacteria remains challenging: several cell lysis techniques including chemicals, sonication, French press disruption, bead beating and combinations thereof, have been employed in mycobacterial genomics, immunological and proteomics studies (Monahan et al., 2001; Betts et al., 2000; Odumeru et al., 2001; Hunter et al., 1990; Hurley et al., 1987; Mutharia et al., 1997; Jungblut et al., 1999; Rosenkrands et al., 2000a; Rosenkrands et al., 2000b; Lee et al., 1992; Yoshimura et al., 1987; Rabilloud et al., 1997; Herbert, 1999; Gorg et al., 2004). In this study, we compared and optimized different lysis buffers in combination with sonication and bead beating to

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Table 1
Compositions of tested Lysis buffers.

Lysis buffer initial (Lbi)	Lb_thUr	Lb_thUr_DTT	Lb_thUr_DTT_SDS
50 mM NH_4HCO_3, pH 7.4 10 mM $MgCl_2$	Lbi **+7 M urea**	Lbi **+7 M urea**	Lbi **+7 M urea**
0.1% NaN_3	**+2 M thiourea**	**+2 M thiourea**	**+2 M thiourea**
1 mM EGTA +MSSafe protease inhibitor cocktail (Sigma-Aldrich, 1× final concentration)		**+5 mM DTT**	**+1% SDS** **+5 mM DTT**

Lb_thUr: Lysis buffer with combination of Thiourea and urea; Lb_ thUr_DTT: Lb_thUr plus DTT; Lb_thUr_DTT_SDS: Lb_thUr plus DTT and SDS.
Components that make the difference between each tested lysis buffer is indicated in bold.

extract the proteomes of several *Mycobacterium* species as a model for *M. tuberculosis*.

2. Methods

2.1. Mycobacterial strains, growth and cell extraction

For safety and convenience reasons, fast growing mycobacteria under Biosafety Level 2 (BSL-2) *Mycobacterium smegmatis CCUG 28063* and *M. fortuitum CCUG* 31556, obtained from the CCUG collection (http://www.ccug.se), were initially used. After optimization of the protein extraction method, the optimized protocol was used with the H37Rv *M. tuberculosis* strain. Bacteria were grown in 40 mL Middlebrook 7H9 liquid culture (BD Difco™ Middlebrook 7H9 Broth) medium supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC) and 0.5% glycerol at 37 °C until exponential growth phase. To ensure safety prior to protein extraction, bacterial cells were inactivated by heat treatment at 90 °C for 30 min. After inactivation, the culture was harvested by centrifugation at 10,000*g* for 10 min at 4 °C and washed twice with 100 mL sterile ice-cold phosphate buffered saline (PBS). The pelleted cells were weighted and stored at −80 °C until needed. Three biological replicates were used for *M. tuberculosis*.

2.1. Optimization of the sample preparation

The sample preparation was performed initially according to the protocol of Brodie et al. (1979) with some modifications. For each extraction protocol, 25 mg of stored cell pellet was suspended in 1 mL of the respective lysis buffer and subjected to a cell wall disruption method. Four lysis buffers were evaluated: an initial lysis buffer (Lbi), this buffer supplemented with a combination of thiourea and urea (LbthUr), or additionally supplemented with dithiothreitol (DTT) (LbthUr_DTT), or with sodium dodecyl sulfate (SDS) (LbthUr_DTT_SDS) (Table 1).

2.1.1. Cell lysis method

Besides the buffer, several mechanic lysis strategies were tested: 5 or 15 min of sonication, bead beating with different proportion beads/buffer (v/v), (i;e. 1/1, 1/2 and 1/3- ratios. For sonication, cells suspended in lysis buffer were sonicated using a Sartorius Labsonic M sonicator at 0.6 cycles (100% amplitude), 4 °C for 15 min (LABSONIC M Sartorius). For the bead beating method, 0.1 mm zirconia beads (0.1 zirconium, BioSpec Products, Inc) were added to the cell pellet suspension and beaten for 30s at 30 Hz followed by 1 min cooling on ice. This procedure was repeated 5 times in total. The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C. Pellets were discarded and the proteins in the supernatant were precipitated by adding 25% trichloroacetic acid (TCA), (v/v) followed by an overnight incubation at 4 °C. The precipitated proteins were collected by centrifugation for 15 min at 16,000*g* at 4 °C and the TCA was removed after which the protein pellet was washed twice with 250 μL of cold water and 1 mL cold acetone. Next, the pellet was dissolved in 200 μL of a 50 mM NH_4HCO_3 and 1 M urea solution. The final protein yield was measured with the Bradford Protein Assay Kit (Thermo Scientific) according the manufacturer's guidelines. Protein quality was checked by SDS-PAGE. Fourteen μL of protein extract was mixed with 5 μL XT sample buffer (BioRad, Hercules, CA, USA). After reduction with 1 μL XT reducing agent (BioRad) for 5 min at 95 °C, samples and protein standards (Precision Plus protein standard, unstained (10-250KD), Bio. Rad Laboratories, Inc) were loaded on 12% Bis-Tris Criterion XT gels (BioRad) and separated in XT-MOPS buffer (BioRad) for 1 h at 200 V. Proteins were visualized by staining with Bio-Safe coomassie blue (BioRad) for 1 h and rinsed with ultrapure water for 30 min according to the manufacturer's instruction.

These methods were tested sequentially with *M. smegmatis*, *M. fortuitum* and finally *M. tuberculosis*. Triplicates were used when assessing the method with *M. tuberculosis*. Average value of protein yield obtained as well as the standard deviation (±SD) between replicates were calculated with excel program. SDS Page was performed in each test.

3. Results

In order to optimize the sample preparation method for *M. tuberculosis* prior to proteomics analysis, we assessed four lysis buffers in combination with sonication and bead beating. Preliminary experiments on *M. smegmatis* and *M fortuitum* showed that adding both urea and thiourea in the lysis buffer resulted in higher protein extraction yields (Table 2). Whereas the addition of SDS resulted in similar yields as adding (thio)urea, the DTT-buffer did not result in higher protein yields as compared to the control buffer. Therefore, a lysis buffer containing both thiourea and urea was used to evaluate the mechanical disruption methods. Concerning sonication, Table 2 shows that longer sonication times did not result in higher protein yields. Moreover, bead beating with a proportion beads/buffer equal to 1:3 resulted in a higher concentration of proteins when compared to the sonication method.

Table 2
Protein concentrations (μg/μL) for each strain obtained by different lysis buffer, sonication and bead beat methods.

	M. smegmatis (25 mg)				*M. fortuitum* (25 mg)	Mtb (25 mg)
	Lbi (μg/μL)	Lb_thUr (μg/μL)	Lb_thUr_DTT (μg/μL)	Lb_thUr_DTT_SDS (μg/μL)	Lb_thUr (μg/μL)	Lb_thUr (μg/μL) [Mean ± SD]
Sonic. 15 min	0.15	0.65				
Sonic. 5 min	0.11	0,63	0,15	0,51		
Bead beat. (v/v)		0,29	0,08	0,16	0.12	
Bead beat. (1/2)		0,53			0,91	
Bead beat. (1/3)		**0,81**			**1.17**	
Sonic. 5 min followed by bead beat. (1/3)						**1.42 ± 0.14**

Lbi = Lysis buffer initial; Lb_thUr = Lysis buffer (thio) urea (combination of thiourea and urea); Lb_thUr_DTT = Lysis buffer (thio) urea with DTT; Lb_thUr_DTT_SDS = Lysis buffer (thio) urea with DTT and SDS.
The highest protein extraction yeilds obtained for each strain after experiments are indicated in bold.