SHORT COMMUNICATION

Leishmania infantum: tuning digitonin fractionation for comparative proteomic of the mitochondrial protein content

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Abstract *Leishmania infantum* belongs to the *Kinetoplastidae* that is characterized by a specific mitochondrial DNA, the kinetoplast. This parasite is responsible for both benign cutaneous leishmaniasis and severe visceral leishmaniasis in humans. Molecular determinants of such differences in pathogenesis are not well understood, and the parasites as well as their hosts may contribute to the disease phenotype. Factors that help parasite to adapt its metabolism to nutritional conditions encountered in different location might play pivotal roles in controlling parasite development in these various host environments. Thus, we have decided to initiate studies aimed to compare the mitochondrial protein content of *L. infantum*. To avoid the drawback caused by the most abundant proteins such as tubulin and proteins of the cytoskeleton present in whole cell extract, we have decided to fractionate the subcellular components of the cells. Using both cytosolic and mitochondrial markers, we have improved a protein pre-fractionation protocol using digitonin that allowed us to generate an enriched mitochondrial fraction.

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Introduction

Leishmania parasites are responsible for veterinary and human diseases, called leishmaniasis, subdivided into three main categories: cutaneous, mucocutaneous, and visceral forms. *Leishmania infantum* is responsible not only for visceral leishmaniasis but also for benign cutaneous forms. *Leishmania* parasites belong to the *Kinetoplastidae* and have a specific DNA, the kinetoplast. This kinetoplast is a mass of circular DNA inside a large mitochondrion. *Leishmania* spp. have a dimorphic life cycle, the first form growing as free-living flagellated promastigotes in the midgut of the phlebotomine sand fly vector and the second one as rounded, non-motile amastigotes inside macrophages. *Leishmania* have to survive to widely differing host environment, and this adaptation is achieved by a combination of morphological and biochemical changes. The mechanisms by which *Leishmania* achieve this complex reshaping is currently poorly understood. Total proteome or transcriptome analysis demonstrates the existence of some differentially or stage-specific expressed proteins (McNicoll et al. 2006). At the subcellular level, mitochondria that is present in one specimen per cell and is localized at the flagellum basis is also deeply affected by the mechanisms that lead to the adaptation of *Leishmania* to their new environment (Brun and Krassner 1976). Accordingly, proteins belonging to the TIM and TOM transporter family are found to be differentially expressed between promastigotes and amastigotes of *Leishmania* (El Fakhry et al. 2002). However, repercussions of such variations on the mitochondrial proteome composition and/or activity during cytodifferentiation are currently unknown. Because the proteome coverage is often confined to the analysis of abundant proteins such as tubulin and proteins of the cytoskeleton, it is necessary to avoid whole cell extraction.

Mitochondrion protein purification requires gradient centrifugations and massive amount of materials. Alternative methodologies that use differential lysis by digitonin may represent a valuable approach for purification of mitochondrial proteins. The capacity of digitonin to bind to the membrane and to form pores depends on sterol composition that is variable between intracellular organella and the cytoplasmic membrane. Such differential lysis capacity of digitonin was used to increase the resolution and the depth of total cytosolic and intracellular proteome via sequential lysis of *Leishmania* parasites (Foucher et al. 2006). In this study, we improved the method for the isolation of an enriched mitochondrial fraction previously described by Castro et al. (2004). Purity fractions were estimated using luciferase activity as cytosolic marker and *LimTXNPx* as mitochondrial marker protein (Castro et al. 2004; Foucher et al. 2006).

Materials and methods

Cell culture

To monitor cell lysis by digitonin and to check for potential fractions contamination, *L. infantum* promastigotes expressing the luciferase gene (αNEOαLUC; Roy et al. 2000; Sereno et al. 2001, 2007) were used. *L. infantum* promastigotes (wild type: MHOM/MA/67/ITMAP263 and mutant: αNEOαLUC) were cultured at 26°C in RPMI 1640 medium (Lonza Ltd., Switzerland) supplemented with 10% heat-inactivated fetal calf serum (Lonza Ltd.), gentamycin 20 mM, and 1% L-glutamine 100X (Lonza Ltd.). They were harvested in exponential phase after counting (about 5×10^7 cells are required for each experiment). The pellet was washed twice in phosphate-buffered saline (PBS) by centrifugation (2,000×g, 20 min, 4°C) and immediately used for the subcellular fractionation.

Digitonin fractionation

The amount of protein present in a pellet of 5×10^7 promastigotes was determined spectrophotometrically (660 nm). Parasites were then resuspended in 1.125 ml of trypanosome homogenization buffer (THB: Tris–HCl 25 mM pH 7.8, EDTA 1 mM, sucrose 0.6 M, dithiothreitol (DTT) 1 mM, and one tablet of protease inhibitor cocktail). The amount of digitonin to be used and diluted in 125 μl of THB was then added to the homogenized sample. A large range of digitonin concentrations were tested: 0.0–0.1–0.2–0.25–0.3–0.4–0.5–1.0–1.5 mg of digitonin per milligram of total protein extract. After 2 min at 37°C, samples were centrifuged (10,000×g, 10 min, 4°C). At this step, the

efficiency of cell lysis and fractionation was checked according to different ways.

Luciferase activity

A gene coding the luciferase was previously inserted in the nuclear DNA of *L. infantum* ITMAP263 (αNEOαLUC; Sereno et al. 1998; Roy et al. 2000; Sereno et al. 2001). *Luciferase* is expressed in the cytosol and thus constitutes, in our study, the cytosolic marker. The activity of luciferase in the pellet and the supernatant of our different fractions was measured. Briefly, 1 μg of each fraction was deposited on a 96-well plate in a final volume of 20 μl (dilution in Glo lysis buffer; Promega) and 20 μl of Luc buffer [20 mM tricine, 1.07 mM $(MgCO_3)_4 \cdot Mg\ (OH)_2 \cdot 5\ H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 220 μM coenzyme A, 4.70 μM D-luciferin potassium salt, 530 μM ATP, 33.3 mM DTT] containing luciferin, substrate of the luciferase and the ATP necessary to the reaction. Measurements were carried out in triplicates. The emission of light in each well was measured, thanks to a luminometer (Viktor II, PerkinElmer), and the averages and standard deviations were estimated for each fraction and each point tested. Cytosolic contamination was
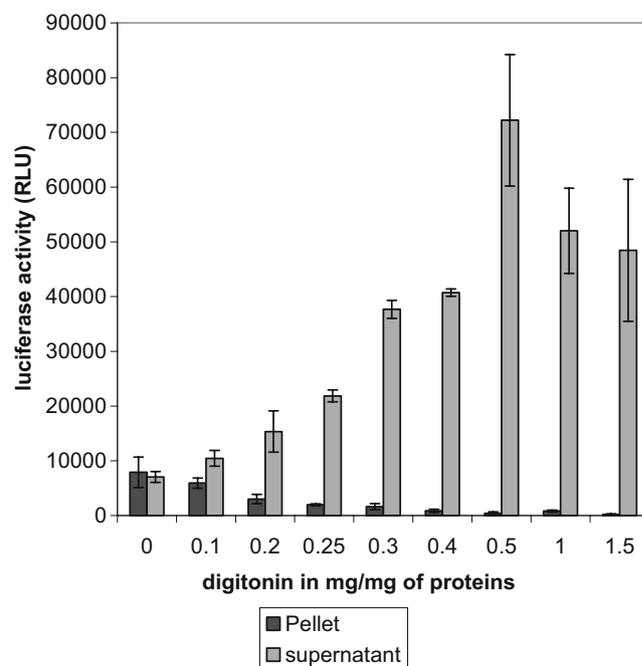

Fig. 1 Detection of the luciferase activity in parasites treated with various amount of digitonin. Pellets of 5×10^7 αNEOαLUC cells were harvested and lysed with various amount of digitonin; after centrifugation, luciferase activity (cytosolic marker) was detected in the different fractions. Results are expressed as a mean of a triplicate experiment
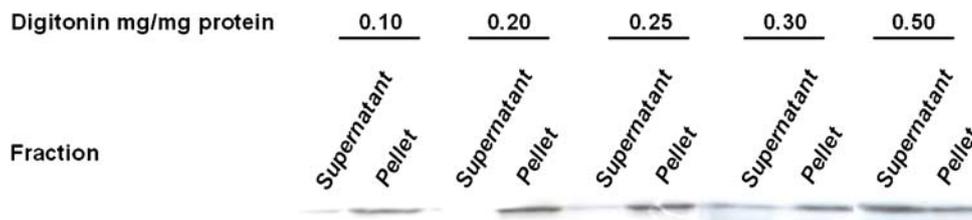
Fig. 2 Detection of the mitochondrial protein *LimTXNPx* after lysis with various amount of digitonin (0.10 to 0.50 mg digitonin per milligram total proteins). *Pellet* represents the enriched mitochondrial fraction and *supernatant* represents cytosolic fraction

then calculated using the following formula [100−(RLU of mitochondrial fraction/RLU of cytosolic fraction)]×100%.

SDS-PAGE analysis using *LimTXNPx*, a mitochondrial marker

To test further the purity of the fractions, the presence of a mitochondrial marker in the various fractions treated with digitonin was checked (Castro et al. 2004). Before sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, supernatants were precipitated with trichloroacetic acid (TCA) and ice-cold methanol. To precipitate the sample, 1.250 ml cytosolic fraction was incubated 30 min on ice using 10% of TCA. TCA is eliminated by centrifugation (3,000×g, 15 min, 4°C) and washed twice with 1 ml of cold acetone [incubation 20 min in ice and centrifugation (3,000×g, 15 min, 4°C)]. The pellet was dried 10 min under hood with laminar flow and can be stored at −80°C for a later use. Fractions obtained using 0.1 to 0.5 mg of digitonin by total proteins per milligram were run on 12% polyacrylamide gels and then blotted onto nitrocellulose membranes (GE healthcare, Amersham). The blots were blocked 1 h under agitation in 5% dried milk in PBS (0.01 M pH 7.4). Polyclonal antibody directed against the mitochondrial protein *LimTXNPx* (kindly provided by Dr H. Castro diluted) was added (1:2,000), and membranes were incubated overnight under agitation at 4°C (Castro et al. 2004). Membranes were washed three times for 10 min in PBS-Tween (0.01 M pH 7.4, 0.1% Tween 20) and incubated with the goat anti-mouse peroxidase-conjugated IgG (1:500, Calbiochem). Membranes were then washed three times in PBS-Tween and revealed 5 min with Western blotting analysis system (GE healthcare).

Results and discussion

As illustrated in Fig. 1, an increase of luciferase activity in the supernatant was observed as a function of digitonin concentration; maximal luciferase activity was observed for a concentration of 0.5 mg digitonin per milligram of protein extracted. This corresponds to the lysis of the cytoplasmic

membrane that leads to a leakage of the luciferase. Concomitantly, a decrease in the luciferase activity was measured in the pellet fraction that corresponds to membrane and organelle not targeted by the detergent effect of digitonin. Minimum activity was observed when concentrations above 0.3 mg digitonin per milligram total protein were used. For 0.25 mg digitonin per milligram proteins, the contamination ratio calculated is about 8.96%. Using lower digitonin concentration increased dramatically the contamination ratio (19.63% and 56.85% for 0.2 and 0.1 mg digitonin, respectively), indicating therefore that either all the parasites were not lysed or that some luciferase contaminated the pellet fraction due to its binding to the external membrane of the mitochondria. Using higher amount of digitonin further decreased the luciferase activity present in the pellet fraction below 5%. However, a too large quantity of digitonin might damage mitochondria and should lead to a leakage of the mitochondrial protein content. To confirm the fractions purity, we have checked the presence of a mitochondrial marker, *LimTXNPx* [5]. As illustrated in Fig. 2, *LimTXNPx* is mainly present in the pellet fraction at concentration below 0.3 mg of digitonin; an equal amount of *LimTXNPx* is detected in supernatant and pellet when parasites were treated with 0.3 mg digitonin. When higher amount of digitonin, i.e., 0.5 mg, was used, *LimTXNPx* is mainly found in the supernatant fraction. These observations suggest that concentration superior to 0.25 mg digitonin might damage mitochondria. Thus, to enrich mitochondrial fraction with low cytosolic contamination, digitonin should be used at a concentration about 0.25 mg per milligram of total protein. The increase of digitonin concentration from 0.2 to 0.25 mg decreases cytosolic contamination by twofold (19.63% vs 8.96%). This simple, easily handled, and fast method will now allow us to investigate the mitochondrial content of *Leishmania* parasites isolated from different clinical forms (benign cutaneous form versus severe visceral form) using two-dimensional gel electrophoresis.

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