Title: Leishmania metacaspase: an arginine-specific peptidase.

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Journal: Methods in molecular biology (Clifton, N.J.)

Year: 2014

Volume: 1133

Pages: 189-202

DOI: 10.1007/978-1-4939-0357-3_12

Metadata of the chapter that will be visualized online

Chapter Title	Leishmania Metacaspase: An Arginine-Specific Peptidase	
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Keywords (separated by "-")	*Leishmania* - Cysteine peptidase - Arginine-specific peptidase - Metacaspase - Enzymatic assay - Protease inhibitors	

Leishmania Metacaspase: An Arginine-Specific Peptidase

Ricardo Martin, Iveth Gonzalez, and Nicolas Fasel

Abstract

The purpose of this chapter is to give insights into metacaspase of *Leishmania* protozoan parasites as arginine-specific cysteine peptidase. The physiological role of metacaspase in *Leishmania* is still a matter of debate, whereas its peptidase enzymatic activity has been well characterized. Among the different possible expression systems, metacaspase-deficient yeast cells (*Δyca1*) have been instrumental in studying the activity of *Leishmania major* metacaspase (LmjMCA). Here, we describe techniques for purification of LmjMCA and its activity measurement, providing a platform for further identification of LmjMCA substrates.

Key words *Leishmania*, Cysteine peptidase, Arginine-specific peptidase, Metacaspase, Enzymatic assay, Protease inhibitors

1 Introduction

In 2000, Uren et al. described a group of cysteine proteases orthologous to caspases but absent in mammals, which was named metacaspases [1]. Metacaspases belong to the C14 family of CD clan of cysteine proteases [2] and possess caspase-like domain with a highly conserved catalytic dyad of histidine and cysteine. They are divided into two structurally different types: type I metacaspases with an additional N-terminal extension that is similar to initiator/inflammatory caspases and type-II metacaspases with an insertion of around 200 amino acids between two caspase-like subunits. Interestingly, genomic analysis reveals the presence of a large pool of metacaspases in unicellular and filamentous cyanobacteria that are still poorly studied [3], whereas metacaspases from plants, *Saccharomyces cerevisiae*, and protozoan parasites have rather been extensively investigated.

Depending on the species, *Leishmania* protozoan parasites induce different forms of diseases ranging from cutaneous, mucocutaneous or visceral leishmaniasis. All the *Leishmania* species express a unique type of metacaspase harboring a central catalytic

Peter V. Bozhkov and Guy Salvesen (eds.), *Caspases, Paracaspases, and Metacaspases: Methods and Protocols*,
Methods in Molecular Biology, vol. 1133, DOI 10.1007/978-1-4939-0357-3_12,

domain (containing the conserved catalytic dyad histidine and cysteine) flanked by an N-terminal domain containing a mitochondrial localization signal and a less conserved proline-rich C-terminal domain (61.4–100 % homology), which probably plays a role in protein–protein interactions. Interestingly, although the N-terminal mitochondrial localization signal is functional, most of *Leishmania major* metacaspase (LmjMCA) is detected in the cytoplasm either in a full length or in a processed form corresponding to the central catalytic domain lacking the N- and the C-terminal domains [4].

Due to the mitochondrial localization signal and the proline-rich sequences, LmjMCA N- and C-terminal domains could preclude expression and activity measurement of metacaspase. Therefore, it is necessary to limit expression and activity measurement of LmjMCA to the 251 amino acids (amino-acid residues 63–314 of LmjF35.1580) predictive of the catalytic domain (cd-LmjMCA). To do so, the DNA sequence encoding the catalytic domain was amplified and the PCR product was inserted into the pESC-His vector (Stratagene) using appropriate cloning sites [5]. This vector contains a galactose inducible promoter and N-terminally 6× His and C-terminal FLAG epitope encoding sequences respectively allowing purification with Ni-NTA resin or with murine monoclonal antibodies against the Penta-His-epitope (α-His5; Qiagen) or the FLAG epitope (α-FLAG; Stratagene). A single step was sufficient to enrich for enough material for specific enzymatic activity tests (Subheading 3.6; Fig. 1).

In contrast to caspases that have strict substrate specificity towards aspartic acid, metacaspases rather cleave arginines or lysines at the substrate P1 position [5–8].

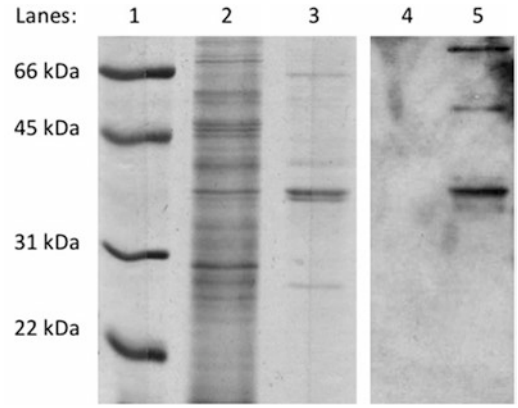

Fig. 1 cd-LmjMCA was purified from yeast expressing cells on an Ni-NTA resin and analyzed by 12 % SDS-PAGE and staining with Coomassie or by immunoblotting using the α-5His antibody. *Lanes 1–3*, Coomassie staining. *Lane 1*, molecular mass markers; *Lane 2*, whole cell lysate; *Lane 3*, cd-LmjMCA purified on Ni-NTA column. *Lanes 4* and *5*, immunoblotting with anti-5His antibody. *Lane 4*, whole cell lysate expressing cd-LmjMCA; *Lane 5*, cd-LmjMCA purified on Ni-NTA column [AU1]

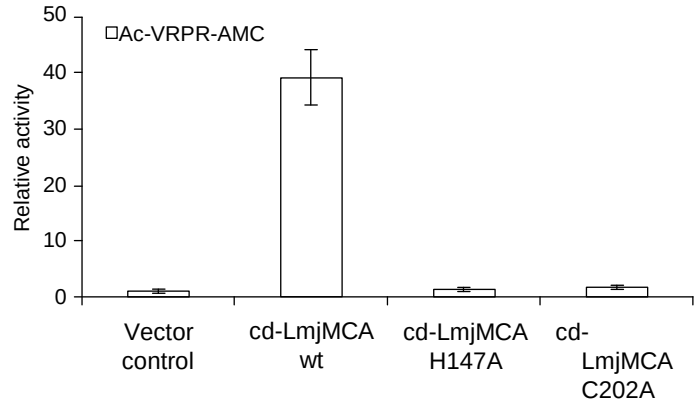

Fig. 2 Enzymatic activity of cd-LmjMCA with the peptidyl substrate Ac-VRPR-AMC. Protein extracts from Δ*yca1* yeast cells transformed with the pESC-His vector alone (vector control) and expressing the catalytic domain of LmjMCA (cd-LmjMCA) wild type (wt) and its respective H147A and C202A mutants, were evaluated for their activity towards Ac-VRPR-AMC substrate. The AMC release was measured every 15 min for 2 h to determine the activity as the slope of the resulting linear regression. Relative activity is expressed as the fold-increase relative to the activity of the vector control. Data show mean ± standard deviation

LmjMCA has been found to be an arginine-specific cysteine protease able to complement the yeast metacaspase (YCA1). In the evaluation of specific recognition of the *A. thaliana* metacaspase AtMC9 using a peptide library, amino acids valine, arginine, proline, and arginine were found to be important in positions P4, P3, P2, and P1, respectively, allowing the design of the optimized tetrapeptide substrate VRPR[9]. To examine the specificity of LmjMCA for this peptide, the catalytic domain of LmjMCA (cd-LmjMCA) can be expressed in *Δyca1* yeast cells and tested with the fluorogenic substrate (Subheading 3.9; Fig. 2).

Enzymatic activity of cd-LmjMCA can be tested in whole yeast cell lysate providing that specific substrates and inhibitors are available. Total protein extracts of *Δyca1* yeast cells expressing cd-LmjMCA were tested for their enzymatic activity with Boc-GRR-AMC, z-GGR-AMC, and Ac-VRPR-AMC substrates in the presence of different inhibitors such as a broad caspase inhibitor z-VAD-fmk, the cysteine protease inhibitor E64, and the serine protease inhibitors PMSF, leupeptin, and aprotinin (Subheading 3.5; Fig. 3). The caspase inhibitor z-VAD-fmk produced a low but significant inhibition of cd-LmjMCA activity with both Boc-GRR-AMC (p value = 0.0008) and z-GGR-AMC (p value < 0.0001) but not with the Ac-VRPR-AMC substrate. The cysteine protease inhibitor E64 had no significant effect on cd-LmjMCA activity with the three substrates. The serine protease inhibitors PMSF and aprotinin had no effect on cd-LmjMCA activity with both Boc-GRR-AMC

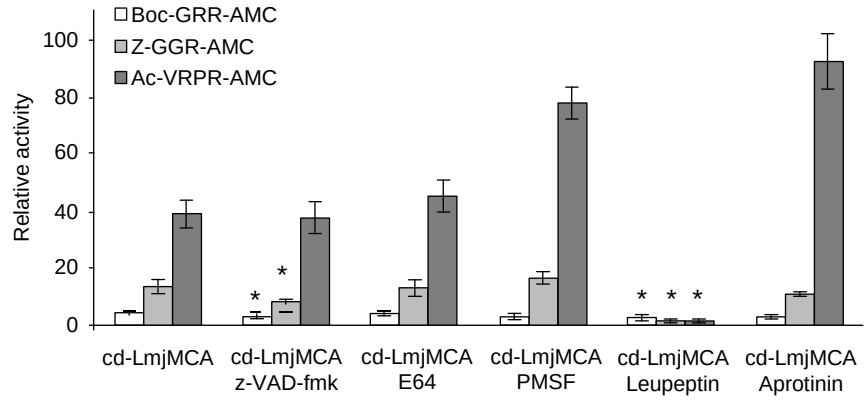

Fig. 3 Effect of protease inhibitors on cd-LmjMCA enzymatic activity. Protein extracts from Δ*yca1* yeast cells transformed with the pESC-His vector expressing the catalytic domain of LmjMCA (cd-LmjMCA) were tested for enzymatic activity with the Boc-GRR-AMC, Z-GGR-AMC, and Ac-VRPR-AMC substrates in absence or presence of 100 μM z-VAD-fmk, 100 μM E64, 10 mM PMSF, 1 mM leupeptin, and 100 μM aprotinin. The AMC release was measured every 15 min for 2 h to determine the activity as the slope of the resulting linear regression. Relative activity was calculated as the fold increase relative to the activity of the vector control (with and without protease inhibitors). Data show mean ± standard deviation. *$P < 0.05$

and z-GGR-AMC substrates, however, cd-LmjMCA activity with the Ac-VRPR-AMC substrate was increased when these two latter inhibitors were added. However, this increase was not always observed. The increase of activity of cd-LmjMCA with the Ac-VRPR-AMC substrate in the presence of PMSF and aprotinin could be due to a protective effect over cd-LmjMCA by inhibition of its degradation by other proteases. Since these experiments were done with total protein extracts, the influence of other yeast proteases cannot be excluded. Interestingly, leupeptin, a serine protease inhibitor, which can also inhibit some cysteine proteases such as calpains and cathepsins, completely abrogated cd-LmjMCA activity with all three substrates (Boc-GRR-AMC p-value < 0.0002; z-GGR-AMC p-value < 0.001; and Ac-VRPR-AMC p-value < 0.0001). Although the structural similarity of cd-LmjMCA with caspases could explain the slight inhibition found with z-VAD-fmk for Boc-GRR-AMC and z-GGR-AMC, this inhibitor was not able to affect the activity of cd-LmjMCA towards Ac-VRPR-AMC, the most preferred substrate of this metacaspase (Fig. 3).

2 Materials

All chemicals used are of Molecular Biology grade unless specified and solutions are prepared with deionized water. When not specified, incubations are performed at room temperature.

2.1 *Leishmania* Metacaspase Gene

1. *L. major* metacaspase gene: LmjF.35.1580 (Gene ID: 3684453).

2.2 YCA1 Disrupted Yeast Cells Expressing cd-LmjMCA

1. Metacaspase disrupted yeast cells: Euroscarf YCA1 disrupted strain (*yca1*Δ cells) Accession Number Y02453 (BY4741; MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR197w::kanMX4) transformed with the pESC-His vector (Stratagene, La Jolla) or with the pESC-His expressing LmjMCA or its inactive forms cd-LmjMCA H147A and cd-LmjMCA C202A [5].
2. Inactive forms of cd-LmjMCA obtained by using the QuikChange®multi site-directed mutagenesis kit (Stratagene).

2.3 Yeast Media and Transformation

1. YPD medium: 20 g/l Difco peptone, 10 g/l Yeast extract, H_2O to 950 ml, pH 6.5. Autoclave, let cool down to 55 °C, and add 50 ml of 40 % glucose filtered through a 0.22 μm size filter (Final concentration of glucose: 2 %).
2. YPD plates: same as YPD medium but supplemented with 20 g/l agar.
3. 10× Dropout [4] amino acid solution (without histidine when using the pESC-His vector): 200 mg/ml L-adenine hemisulfate salt, 200 mg/ml L-arginine HCl, 200 mg/ml L-histidine, 300 mg/ml L-isoleucine, 1,000 mg/ml L-leucine, 300 mg/ml L-lysine HCl, 200 mg/ml L-methionine, 500 mg/ml L-phenylalanine, 2,000 mg/ml L-threonine, 200 mg/ml L-tryptophan, 300 mg/ml L-tyrosine, 200 mg/ml L-uracil, 1,500 mg/ml L-valine. Pass the solution through a 0.22 μm size filter and aliquot in 50-ml tubes. Store at 4 °C.
4. SD/DO/Glucose medium: for 1 L weigh 6.7 g of Yeast nitrogen base without amino acids and add H_2O to 850 ml. Control the pH (pH 5.8). Autoclave and let cool down to 55 °C. Add 100 ml of the 10× DO solution (without histidine) and then 50 ml of 40 % glucose or galactose (filtered; final concentration 2 %).
5. SD/DO/Glucose plates: same as SD/DO medium (without histidine) supplemented with 20 g/l agar.
6. 10× TE: 0.1 M Tris–HCl, 10 mM EDTA, pH 7.5. Pass the solution through a 0.22 μm filter and store at −20 °C.
7. 10× LiAc: 1 M LiAc, pH 7.5. Pass the solution through a 0.22 μm filter and store at −20 °C.
8. 1× TE/1× LiAc solution: 500 μl 10× TE, 500 μl 10× LiAc, adjust to 4 ml with H_2O. Use freshly prepared solution.
9. PEG 1,000/Tris/LiAc solution: 4 ml of 50 % PEG 1,000 solution, 500 μl 10× TE, and 500 μl 10× LiAc.
10. 87 % glycerol.

2.4 Yeast Lysis (TCA Protocol) and Protein Extraction (Glass Beads) for SDS-PAGE Analysis

1. Solution B: 3.67 ml H_2O, 925 µl 10 M NaOH, 370 µl β-Mercaptoethanol, 50 µl 100 mM PMSF in isopropanol, 50 µl 0.5 M EDTA/KOH, pH 7.0.
2. Trichloroacetic acid (TCA).
3. Acetone.
4. Lysis buffer: 0.5 % NP40, 20 mM HEPES, pH 8.0, 84 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 5 µg/ml Aprotinin, 5 µg/ml Leupeptin, 1 µg/ml Pepstatin, 1 mM PMSF.
5. Pierce BCA protein assay kit (Thermo Scientific) with BSA as standard.

2.5 Enzymatic Activity Test in Whole or Purified Cell Lysates

1. Lysis buffer for whole cell lysate activity test: 50 mM KH_2PO_4, pH 7.5, 500 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 % CHAPS, 5 µg/ml Aprotinin.
2. Lysis buffer for purified cell lysate activity test: 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 % Triton X-100.
3. Washing Buffer for purified cell lysate activity test: 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole.
4. Elution Buffer for purified cell lysate activity test: 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole).
5. Amicon Ultra-4 centrifugal device (Millipore).
6. Base buffer: 150 mM NaCl, 25 mM HEPES, 10 % glycerol, pH 7.5. Store at 4 °C.
7. 10 % CHAPS solution: 1 g CHAPS in 10 ml H_2O. Store at 4 °C.
8. Activity buffer for whole cell lysate activity test: 3.92 ml Base buffer, 40 µl 10 % CHAPS, 20 µl 2 M DTT.
9. Activity buffer for purified cell lysate activity test: 3.52 ml Base buffer, 40 µl 10 % CHAPS, 20 µl 2 M DTT, 400 µl 1 M $CaCl_2$.
10. Fluorogenic substrates: Boc-Gly-Arg-Arg-7-amino-4-methylcoumarin (Boc-GRR-AMC), Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (Z-GGR-AMC) (both from Bachem AG, Switzerland), and *N*-acetyl-Val-Arg-Pro-Arg-7-amino-4-methylcoumarin (Ac-VRPR-AMC) (Sigma).
11. Protease inhibitors: Benzyloxycarbonyl-Val-Ala-Asp (*1010*) fluoromethylketone (z-VAD-fmk), E64, PMSF, leupeptin, and aprotinin.
12. Trypsin powder (Boehringer Manheim GmbH, Germany).
13. 96-well black plates (Optiplate-96 F, PerkinElmer).
14. Spectrophotometer.

2.6 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. The Bio-Rad Power Pac 3000 system or similar.
2. Separating gel (12 %): mix 1.625 ml H_2O, 2 ml 30 % acrylamide–Bis-acrylamide solution (29.2:0.8 acrylamide–Bis-acrylamide), 1.3 ml 1.5 M Tris/HCl buffer, pH 8.8, 25 μl 20 % SDS; 50 μl 10 % ammonium persulfate (APS) and 2 μl tetramethylethylenediamine (TEMED).
3. Stacking gel (3.9 %): mix 2.64 ml H_2O, 0.67 ml 30 % acrylamide–Bis-acrylamide solution, 0.5 ml 1 M Tris/HCl buffer, pH 6.8, 20 μl 20 % SDS; 40 μl 10 % APS, 8 μl TEMED, and 20 μl bromophenol blue.
4. 4× Tris/HCl/SDS solution: 91 g Tris base, 2 g SDS, 500 ml H_2O, pH 8.8.
5. 2× SDS sample loading buffer (100 ml final volume): 25 ml 4× Tris/HCl/SDS solution, 20 ml glycerol, 4 g SDS, 2 ml β-Mercaptoethanol (or 3.1 g DTT), 1 mg bromophenol blue, adjust to 100 ml with H_2O.
6. 10× SDS electrophoresis running buffer: 250 mM Tris base, 1.92 M glycine, 1 % SDS. Adjust pH to pH 8.3 when diluting to 1×.
7. Gel staining solution: 50 % methanol, 0.05 % Coomassie Brilliant Blue R-250, 10 % acetic acid.
8. Gel destaining solution: 5 % methanol, 7 % acetic acid.

2.7 Western Blotting

1. The Bio-Rad Power Pac 3000 system or similar.
2. 10× gel transfer buffer: 250 mM Tris base, 1.92 M glycine.
3. 1× gel transfer buffer solution: 10 ml 10× gel transfer buffer, 20 ml methanol, pH 8.3–8.4, adjust to 1 L with H_2O.
4. Nitrocellulose membrane (Whatman, GE Healthcare Life sciences or similar).
5. Filter paper, cut to the size of the gel.
6. Ponceau S Solution: 0.5 g Ponceau S, 1 ml glacial acetic acid, adjust to 100 ml with H_2O.
7. 1× Tris Buffered Saline supplemented with tween 20 (TBST): 25 mM Tris, 150 mM NaCl, 3 mM KCl, pH 7.5, 0.1 % Tween 20.
8. Blocking buffer: 1× TBST, 5 % nonfat dry milk (NFDM).
9. Primary antibody: 1/200 anti-Flag antibody or 1/1,000 anti-Histidine antibody in TBST with 1 % NFDM.
10. Secondary antibody: 1/2,500 horseradish peroxidase-conjugated antibody in TBST with 1 % NFDM.
11. Glass plate.
12. ECL Western Blotting Detection Reagent (GE Healthcare).

13. Filter papers.
14. Plastic wrap.
15. Cassette and X-Ray film.

3 Methods

3.1 Yeast Transformation

1. Plate *Δyca1* cells (*see* **Note 1**) from frozen stock onto YPD plates using a platinum loop, which has been previously sterilized by flaming and then cooled quickly on the plate.
2. Incubate at 30 °C for 4 days and then inoculate 1 ml of YPD medium with 1 colony and vortex for 2 min.
3. Transfer to 49 ml of YPD medium (total volume 50 ml) and place on a shaker at 30 °C overnight.
4. The next day, dilute the overnight culture to OD_{600} 0.2–0.3 in 300 ml (*see* **Note 2**) and further incubate at 30 °C with shaking for 2 h or until OD_{600} reaches 0.4–0.6.
5. Centrifuge at 1,000 × *g* for 5 min in 50 ml tubes, dilute and pool pellets in 50 ml H_2O, centrifuge at 1,000 × *g* for 5 min at room temperature.
6. Resuspend pellet in 1.5 ml of 1× TE/1× LiAc fresh solution.
7. Add 10 μl of 10 mg/ml herring sperm carrier DNA in a 1.5-ml vial, heat at 95 °C for 5 min and quick chill on ice.
8. Leave on ice and add 1 μg of cd-LmjMCA plasmid and mix.
9. Add 100 μl of yeast cell suspension and vortex.
10. Add 600 μl of PEG1000/Tris/LiAc fresh solution and vortex for 10 s.
11. Incubate at 30 °C with shaking for 30 min.
12. Add 70 μl of DMSO from stock solution and mix by inversion at 42 °C for 15 min (heat shock).
13. Leave on ice for 2 min, then microfuge at 10,000 × *g* for 5 s.
14. Resuspend the pellet in 500 μl of 1× TE.
15. Dilute with 1× TE and plate 100 μl of dilutions 1:1, 1:10, 1:100, and 1:1,000 on YPD plates and incubate at 30 °C for 3 days to obtain colonies.
16. Verify that the transformation was efficient and that your cells have the desired plasmid by using standard minilysate protocol.
17. Grow overnight culture: inoculate one transformed colony into 1 ml of SD/DO/Glucose medium, vortex, transfer to 9 ml of SD/DO/Glucose medium, and incubate at 30 °C with continous shaking overnight.
18. Prepare frozen stock of transformed yeast cells: mix 700 μl of the overnight culture and 300 μl of 87 % glycerol, mix and store at –70 °C.

3.2 Induction of the cd-LmjMCA Expression in Transformed Yeast Cells

1. Inoculate one transformed colony into 1 ml of SD/DO/Glucose medium and vortex.
2. Transfer to 9 ml of SD/DO/Glucose medium and incubate at 30 °C with continous shaking overnight.
3. Dilute overnight culture to OD_{600} 0.05–0.1 in 10 ml (*see* **Note 3**) in a 100-ml Erlenmeyer flask (ten times culture volume) and incubate at 30 °C with shaking for 6 h or until OD_{600} reaches 0.4–0.6.
4. Centrifuge 1 ml of culture at 10,000 × *g* for 1 min and store pellet at –70 °C (non induced control).
5. For the galactose induction, centrifuge the culture at 1,000 × *g* for 5 min and dilute the pellet with 10 ml of SD/DO/Galactose medium and then incubate at 30 °C with shaking overnight.
6. Measure OD_{600} after at least 16 h of induction.
7. Centrifuge the culture at 1,000 × *g* for 5 min and store pellet at –70 °C (galactose induced culture) until use.
8. The pellets are ready for lysis and analysis.

3.3 Yeast Lysis (TCA Protocol) for SDS-PAGE Analysis

1. Dilute frozen pellet of the 10 ml cultures (non-induced and galactose induced) with 500 µl of 1× TE and centrifuge at 10,000 × *g* for 1 min at 4 °C.
2. Resuspend the pellet with 500 µl of H_2O and add 75 µl of Solution B.
3. Shake 10 min at 4 °C and add 280 µl of 72 % TCA.
4. Put on ice for 5 min and then centrifuge at 10,000 × *g* for 10 min at 4 °C.
5. Add 700 µl of acetone to the pellet and centrifuge at 10,000 × *g* for 10 min at 4 °C.
6. Repeat the wash with acetone.
7. Let dry the pellet and then resuspend in 50 µl of 1× PBS (*see* **Note 4**).
8. Store at –70 °C until use.

3.4 Yeast Protein Extraction (Glass Beads)

1. Dilute frozen pellet with 50 µl of lysis buffer, transfer to a 1.5-ml vial with 0.08 g glass beads.
2. Vortex ten times, 1 min each, and collect supernatant.
3. Wash beads with 50 µl of lysis buffer and collect supernatant.
4. Pool supernatants and centrifuge at 10,000 × *g* for 1 h at 4 °C.
5. Collect and store supernatant at –70 °C in lysis buffer containing protease inhibitors.
6. Protein concentration in supernatant can be measured using a BCA protein assay reagent with BSA as standard.

3.5 cd-LmjMCA Enzymatic Activity in Whole Yeast Cell Lysate

1. Harvest transformed yeast cells from a 50 ml culture following 24 h of induction. The pellet can be kept frozen at –70 °C.
2. Resuspend the frozen pellet in 100 µl of lysis buffer, transfer to a 1.5-ml vial and add 0.08 g of glass beads.
3. Vortex ten times, 1 min each.
4. Collect and save supernatant.
5. Wash the beads with 50 µl of lysis buffer, collect and save supernatant.
6. Pool supernatants and centrifuge at 10,000 × *g* for 1 h at 4 °C. Collect and store supernatant at –70 °C in lysis buffer containing protease inhibitors.
7. Measure protein concentration in the supernatant using a BCA protein assay reagent with BSA as standard.
8. For one black-plate well, add 196 µl of Activity buffer and 4 µl of 10 µg/µl total protein extract (40 µg total protein per well). Prepare duplicate or triplicate wells.
9. Dilute 50 mM of substrate-AMC to 5 mM with Activity buffer and add 2 µl of diluted substrate per well (final concentration 50 µM). Read fluorescence each 15 min for 2 h at 24 °C with 360 nm excitation and 460 nm emission wavelengths.
10. As a positive control use 10 ng of trypsin per well in the 200 µl reaction volume. As negative controls, use protein extracts from yeast cells transformed with the pESC-His vector or expressing cd-LmjMCA (H147A) and cd-LmjMCA (C202A).
11. Determine enzymatic activity by calculating the slope of the linear regression. Express results in arbitrary milli-fluorescence units per minute per µg of protein (mFU/min/µg), or as the fold increase relative to the activity of the vector control (*see* **Note 5**).
12. To test the effect of different protease inhibitors on the enzymatic activity, supplement activity reactions with the following concentrations of inhibitors: 100 µM z-VAD-fmk, 100 µM E64, 10 mM PMSF, 1 mM leupeptin, and 100 µM aprotinin.

3.6 Purification of Leishmania Metacaspase Catalytic Domain (cd-LmjMCA) from Yeast on Ni-NTA Resin

1. Resuspend frozen pellet from a 500 ml culture after induction with galactose for 18 h in 2.5 ml of lysis buffer.
2. Add 2.5 g of glass beads (0.25–0.5 mm) and vortex ten times, 1 min each (*see* **Note 6**).
3. Collect and save supernatant.
4. Wash the glass beads with 2.5 ml of lysis buffer, collect and save supernatant.
5. Pool the supernatants, centrifuge at 10,000 × *g* for 1 h at 4 °C, and save supernatant (contains soluble proteins).

6. Wash 1 ml 50 % Ni-NTA resin with 2 ml of lysis buffer and add the supernatant (soluble proteins) to the washed resin.
7. Incubate overnight at 4 °C on a wheel.
8. Centrifuge at 1,000 × *g* for 5 min at 4 °C, wash the resin twice with 500 μl of Washing Buffer.
9. Elute protein by adding three aliquots of 500 μl of Elution Buffer and then pool the eluates.
10. Centrifuge the three pooled elutions at 10,000 × *g* for 1 min at 4 °C.
11. Pool the supernatants and concentrate eluted proteins in 1× PBS with an Amicon Ultra-4 centrifugal device prior to protein concentration measurement.
12. Store at −80 °C until use for the activity test.

3.7 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Wash gel glass plates and mount the electrophoresis system according to manufacturer's protocol.
2. Prepare separating gel, fill to the three quarters the glass plate, add some isopropanol on the gel to obtain a flat surface and wait for the gel to polymerize.
3. Prepare stacking gel, fill the gel glass plate up to the edge, insert the comb and wait for the gel to polymerize.
4. Mix each sample (20 μg of total protein from yeast lysates) with 2× SDS sample loading buffer in a ratio 1:1 (v/v), boil samples for 5 min at 95 °C, spin in microfuge and load on the gel.
5. Run gel for 20 min at 80 V and then for 45 min at 180 V with chamber on ice.
6. Stain the gel with Coomassie Blue (*see* **Note 7**): Soak the gel in a staining solution and incubate with shaking at room temperature for 1 h to overnight.
7. Soak the gel in a destaining solution and incubate with shaking at room temperature for 30 min. Repeat until background disappears. Store the gel in water or dry (*see* **Notes 8** and **9**).

3.8 Western Blotting

1. Equilibrate the gel, four filter papers, and sponges in 1× transfer buffer.
2. Mount a sandwich in the following way: white sponge, two filter papers, nitrocellulose membrane, gel, two filter papers, green sponge (white sponge oriented to the cathode—red face).
3. Remove bubbles by rolling a 15-ml tube over the sandwich.
4. Run in 1× transfer buffer for 1 h at 100 V with chamber on ice.
5. After protein transfer, incubate the nitrocellulose membrane on a shaker at room temperature for 5 min in Ponceau S Solution.

6. Incubate on the shaker at room temperature for 2 min in water to remove excess of Ponceau S red.
7. Take a picture and mark molecular weights with a pencil.
8. Incubate on a shaker at room temperature for 10 min in water to complete destaining.
9. Incubate the membrane on the shaker for 1 h at room temperature or overnight at 4 °C in the blocking buffer.
10. Incubate the membrane on a shaker overnight at 4 °C with the first antibody.
11. Wash four times for 15 min each with TBST.
12. Incubate the membrane on a shaker for 1 h at room temperature with the secondary antibody.
13. Wash four times for 15 min each with TBST (*see* **Note 10**).
14. For membrane development, deposit the membrane on a clean glass plate.
15. Dry quickly with a filter paper.
16. Overlay 1.5 ml of a developing solution (1:1 of ECL solutions A:B for a 0.125 ml/cm^2 membrane) and wait for 2 min.
17. Dry with a filter paper and cover the membrane with a plastic wrap.
18. Insert the membrane in a cassette and expose to an X-Ray film for different times (e.g. 2 s, 10 s, 2 min, 10 min); develop the film (*see* **Note 11**).

3.9 cd-LmjMCA Activity Measurement with the Ac-VRPR-AMC Substrate

1. Use 1 μg of purified protein in a total 200 μl volume per well of a 96-well black plate.
2. Add 196 μl of activity buffer and 1–4 μl of Ni-NTA purified cd-LmjMCA per well. Prepare duplicate or triplicate wells.
3. Dilute Ac-VRPR-AMC in Activity buffer to the final concentration 5 mM and add 2 μl of diluted substrate per well (final concentration 50 μM). Read fluorescence each 15 min for 2 h at 24 °C with 360 nm excitation and 460 nm emission wavelengths.
4. As a positive control use 10 ng of trypsin per well in the 200 μl reaction volume.
5. Determine enzymatic activity by calculating the slope of the linear regression. Express results in arbitrary milli-fluorescence units per minute per μg of protein (mFU/min/μg), or as the fold increase relative to the activity of the vector control (*see* **Note 5**).

4 Notes

1. *Δyca1* cells can be obtained from Euroscarf Accession Number Y02453.
2. ~10 ml overnight culture in 290 ml of YPD medium.
3. ~400 μl overnight culture in 9.6 ml of SD/DO/Glucose medium
4. Protein concentration can be measured using the BCA protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL) with BSA as standard.
5. Enzymatic activity tests must be performed at least three times and means and standard deviations must be calculated. The Student *t* test is used in statistical analysis and significance is considered when $p < 0.05$.
6. Use 1 g of beads per 1 ml of lysis buffer.
7. Detection limit is 0.3–1 μg/protein band.
8. First dilute Coomassie Blue in methanol.
9. If you want to keep your gel (after staining), we suggest to put a plastic sheet (candy wrap plastic) over a filter paper; Put the gel over the plastic sheet and soak with water; Put another plastic sheet over the gel; Perforate with a needle around the gel border; Put it on the desiccator at 70 °C under vacuum for 1 h.
10. For competitive blot, first incubate with the peptide at 10 μg/ml, then add antibody and incubate on wheel 60 for min at room temperature.
11. Stripping membranes: 15 min shaking in 0.1 M of glycine–HCl pH 2–3; rinse with 1 M of NaCl in 1× PBS; wash 2 × 5 min in 1× TBS–0.1 % Tween-20; rinse with H_2O; expose film for 10 min to detect former signal. If there is no signal the membrane is ready to be blocked and exposed to a new primary antibody.

Acknowledgements

The authors are grateful to the members of the Fasel's group and to Dr. Frank Madeo who provided the pFM21 construct, which served as a control. This work was funded by the grants FNRS N° 3100A0-116665/1 and IZ70Z0-131421 to NF.

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