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Skeletal Muscle Lysosomal Function via Cathepsin Activity Measurement

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Abstract

Muscle wasting or cachexia is commonly associated with aging and many diseases such as cancer, infection, autoimmune disorders, and trauma. Decrease in muscle mass, or muscle atrophy, is often caused by dysfunction of protein proteolytic systems, such as lysosomes, which regulate protein turnover and homeostasis. Lysosomes contain many hydrolases and proteases and, thus, represent the major organelle that control protein turnover. Recently, lysosomes have emerged as a signaling hub to integrate cellular functions of nutrient sensing and metabolism, autophagy, phagocytosis, and endocytosis, which are all related to tissue homeostasis. In this chapter, we describe the protocol used to measure lysosomal proteinase (cathepsins) activity in the skeletal muscle. A better understanding of lysosomal function in muscle homeostasis is critical in developing new therapeutic approaches to prevent muscle wasting.

Keywords

Atrophy; Autophagy; Enzyme kinetics; Fluorimeter; Muscle acid lysates (MAL); Protein degradation; Skeletal muscle function

1 Introduction

Skeletal muscle is integral to human health for both movement and metabolism, and is maintained by a balance of protein synthesis and degradation [1, 2]. Upon disuse, such as during bed rest, aging, or due to diseases like diabetes mellitus or cancer, disruption of protein homeostasis and subsequent atrophy of tissue cause skeletal muscle to lose between 6 and 24% of its mass and strength [3, 4]. Atrophy is the removal of proteins via degradation without subsequent protein synthesis. In skeletal muscle, atrophy is often characterized as a loss of muscle mass caused by the increase in one or more of the four canonical protein degradation pathways: calcium-dependent cysteine proteases (calpains), cysteine-aspartic acid proteases (caspases), lysosomal cysteine proteases (cathepsins), and ubiquitin mediated proteasomal degradation [5]. For the purpose of this protocol, we will focus on the lysosomal cysteine protease (cathepsins) protein degradation pathway for studying skeletal muscle atrophy.

In its conventional role, lysosomes are viewed as digestive organelles for macromolecule turnover. Lysosomes are membrane bound acidic organelles that contain a plethora of enzymes which breakdown molecules that are no longer needed by the cell via hydrolysis.

Recently, lysosomes have emerged as a signaling center that integrate many cellular processes, such as nutrient sensing, metabolism, and autophagy, which are essential to cell and tissue homeostasis [6, 7]. Moreover, lysosomal membranes contain many channels and transporters that maintain luminal ion homeostasis and are essential to regulate digestive enzyme activity, vesicular trafficking, and response to cellular metabolic stress. Impaired lysosomal function is often associated with cancer, neurodegenerative disorders, and lysosomal storage diseases [6–8].

Autophagy is a nonselective process by which cytoplasmic contents, such as damaged organelles and aged protein aggregates, are degraded. Targeted cytoplasmic content is engulfed by double membrane bound vesicles, known as autophagosomes, and are subsequently delivered to lysosomes for bulk degradation within the cell. Lysosomes fuse with autophagosomes to form autolysosomes, allowing the acidic environment and enzymes within the lysosome to interact specifically with the biomolecules targeted for degradation [9, 10]. Recent studies of autophagy in skeletal muscle of cancer patients with and without cachexia have examined the expression of proteins and their mRNA upstream of lysosomal formation [9]. Other studies have used fluorescent dyes such as LysoTracker Green to monitor the movement of lysosomes in conjunction with other fluorescent dyes during different stages of autophagy. However, although these studies provide detailed information on lysosome biogenesis and trafficking, they do not directly assess lysosome function.

Cysteine cathepsins are a group of 15 proteins, representing the most abundant enzymes present in the lysosome that mediate proteome homeostasis [8, 11]. Cathepsins are ubiquitously expressed with the exception of cathepsin K (osteoclasts, epithelial cells, and synovial fibroblasts), cathepsin S (antigen presenting cells such as dendritic cells and B-cells), cathepsin V (thymus and testis), and cathepsin W (CD8⁺ lymphocytes and NK cells) [8, 12]. Since cathepsins regulate protein turnover within the lysosome, they provide an excellent readout for lysosomal function. Cathepsins function by binding target proteins in their active site cleft and hydrolyzing target peptides preferentially after basic or hydrophobic residues [13]. Early studies of lysosomal degradation kinetics were performed via radioactive labeling of peptides or proteins with [¹⁴C] acetylated hemoglobin, a substrate for cathepsins D and L [14]. Alternative methods to quantify cathepsin activity also exist, such as using fluorogenic substrates in conjunction with immunofluorescent microscopy [15], measuring the fluorescence extinction coefficient of trichloroacetic acid (TCA) extracts [16], or zymography [17]. To study individual cathepsin activity, subtype specific substrates, such as Ac-RR-AFC (Arg-Arg-7-amino-4-trifluoro-methylcoumarin) for cathepsin B, could be applied [18].

In this protocol, we demonstrate extraction of lysosomes from mouse skeletal muscle and the use of a sensitive omnicathepsin fluorogenic substrate assay to measure cathepsin activities. This cathepsin activity assay protocol measures the total activities of cathepsins B, C, F, K, L, O, S, and X and is not specific for a specific cathepsin subtype. The substrate Z-FR-AMC (Z-Phe-Arg-AMC) is a small peptide attached to an AMC group (7-amino-4-methylcoumarin) that fluoresces at 465 nm when cleaved via hydrolysis from the peptide and excited at 360 nm. To ensure that the activity of cathepsins is assayed via AMC fluorescence, a parallel set of samples are treated with the cathepsin inhibitor, E-64-D (L-

trans-Epoxy-succinyl-leucylamido(4-guanidino)butane), as a negative control. It is important to note that all cathepsins can be inhibited by E-64-D, with the exception of cathepsin C which is only partially inhibited. Rates of cathepsin activity are calculated based on the amount of Z-FR-AMC cleavage, derived from a standard curve of free AMC. Additionally, cathepsin specific activity can be measured as V_{max} (RFU/time) kinetics. Compared to the inherent risk of working with radioactive chemicals and the semi-quantitative nature of fluorescent microscopy and zymography, this protocol accurately and consistently measures cathepsin activity and is readily adaptable for specific cathepsin activity [19].

2 Materials

1. Tissue homogenizer with plastic Omni tips (OMNI International).
2. Centrifuge (either temperature controlled or placed in a refrigerator at 4 °C).
3. Sonicator (Branson Digital Sonifier).
4. 96-Well plate, opaque-walled, flat-bottom plates (available from several suppliers).
5. Fluorimeter or microplate reader capable of reading fluorescence and temperature control (e.g., FlexStation 3 from Molecular Device): Pre-warmed to 37 °C.
6. Cytosol lysis buffer: 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% glycerol, and 2 mM dithiothreitol (DTT, freshly prepared) (pH 7.8) (*see* Notes 1 and 2).
7. Acid lysis buffer: 200 mM Na-acetate, 50 mM NaCl, and 0.1% Triton X-100 (pH 5.0) (*see* Note 3).
8. Cathepsin assay buffer: 100 mM Na-acetate, 120 mM NaCl (pH 5.0), and 1 mM EDTA (pH 5.5).
9. E-64-D, Enzo Life Sciences (BML-PI107): 10 mM stock in DMSO stored at −20 °C. Working solution made fresh at 325 μM by diluting the 10 mM stock 1:30.8 in the Cathepsin Assay Buffer. The working concentration during preincubation period is 50 μM.
10. Z-FR-AMC (benzyloxycarbonyl-Phe-Arg-(7-amino-4-methylcoumarin)), Enzo Life Sciences (BML-P139): 50 mM substrate stock in DMSO stored at −20 °C in 2.4 μL aliquot in 0.5 mL amber tubes to protect from light (*see* Note 4).
11. AMC (7-amino-4-methylcoumarin) fluorescence reference standard (ThermoFisher Scientific, A191, mol. weight 175): 32 mM stock in DMSO stored at −20 °C, to be used for free AMC standard curves (*see* Sect. 3.2).

¹The extraction buffer does not contain any detergent to ensure that membrane-bound lysosomes stay intact through the first few steps.

²Prepare DTT fresh by adding 3.1 mg to 10 mL buffer to a final concentration of 2 mM. DTT alkalizes over time and the reducing power of DTT is limited to pH 7.0 and above.

³Acid lysis buffer is acidic to match the acidic environment of the lysosome lumen, ideal for most cathepsin activities. The triton X-100 detergent along with sonication will break apart the lysosome membrane.

⁴Store Z-FR-AMC aliquots individually to reduce the number of freeze-and-thaw times. A 2.4 μL 50 mM Z-FR-AMC tube makes 3 mL of the 40 μM working substrate solution.

3 Methods

3.1 Lysate Extraction

Carry out all procedures on ice unless otherwise specified.

1. Weigh muscle and mince in 3 volumes of ice cold Cytosol Lysis Buffer.
2. Homogenize with a fresh Omni tip for each sample.
3. Centrifuge at $13,000 \times g$ for 30 min at 4 °C.
4. Remove the supernatant. Retain the pellet for lysosome extraction and cathepsin activity assay. The supernatant is mainly soluble cytoplasmic proteins and other soluble cellular debris, which can be stored in a separate tube at -20°C for other experiments. The pellet contains membrane-bound organelles, including lysosomes.
5. To break open the pellets containing lysosomes, add two volumes of acid lysis buffer to the pellet and sonicate on ice for 3 min with 10 s to 5 s “on-off” cycles.
6. Centrifuge again at $13,000 \times g$ for 30 min at 4 °C.
7. Collect this supernatant (muscle acid lysates, MAL) in a labeled 1.5 mL tube for the cathepsin activity assay.
8. Measure protein concentration of MAL with assays such as BCA, Lowry, or Bradford assays. Either use the samples immediately or store them at -80°C for up to 1 month (*see* Note 5).
9. (Optional) Confirm success of protein extraction in MAL via a 10% SDS-PAGE, with the gel stained by Colloidal Blue (*see* Note 6 and Fig. 1).

3.2 Standard Curve

Store Z-FR-AMC properly to prevent autolysis which could cause auto-fluorescence and interfere with the assay readout. To take into account of instability of Z-FR-AMC during storage, the standard curve should be derived from a calibration curve made from a 1:1 mixture of serial dilutions of free AMC (final 0–8 μM) and a constant concentration of Z-FR-AMC (final 20 μM).

1. Thaw a vial containing 32 mM free AMC stock solution. Dilute 1:1,000 with the cathepsin assay buffer to make a 32 μM free AMC solution.
2. Make serial dilutions of free AMC to final concentrations of 16, 8, 4, 2, 1, 0.5, 0.25, and 0 μM with a minimum final volume of 120 μL .
3. Thaw a vial containing 2.4 μL 50 mM Z-FR-AMC stock and dilute with the cathepsin assay buffer to make a 3 mL 40 μM Z-FR-AM solution of working substrate.

⁵A typical yield of acid/lysosome extraction is 0.7–1.6 $\mu\text{g}/\mu\text{L}$.

⁶A typical acid/lysosome extraction on a 10% SDS-PAGE gel is shown in Fig. 1.

4. Label 8 calibration tubes for AMC/Z-FR-AMC mixture for 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0 μ M free AMC.
5. Mix 120 μ L free AMC (16 μ M) with 120 μ L Z-FR-AMC (40 μ M) to make the highest calibration point of 8 μ M free AMC with 20 μ M Z-FR-AMC.
6. Follow the same dilution scheme to mix the next free AMC (8 μ M) with Z-FR-AMC (40 μ M) to make the next calibration point of 4 μ M free AMC with 20 μ M Z-FR-AMC.
7. Follow the same dilution scheme to make all calibration points with the lowest one being 0 μ M free AMC with 20 μ M Z-FR-AMC. This is the background reading to be subtracted from readouts from other calibration tubes.
8. Transfer 100 μ L of the free AMC/Z-FR-AMC mixture into an opaque wall flat bottom 96-well plate in duplicates from each calibration tube.
9. Include a buffer only sample as background reading (blank).
10. Incubate at 37 °C for 1 h and measure fluorescence with excitation 380 nm and emission 460 nm.
11. Generate an AMC standard calibration curve. A typical AMC standard curve is shown in Fig. 2.

3.3 Cathepsin Activity Assay

A pilot experiment is recommended using different amounts (0, 3, 7.5, 15, and 30 μ g) of muscle acid lysates (MAL) and 20 μ M Z-FR-AMC in 100 μ L cathepsin assay buffer in a 96-well format. We have obtained sensitive and reproducible cathepsin assay measurements with 15 μ g MAL and thus this is recommended accordingly [19].

1. Pre-warm the fluorimeter to 37 °C for optimal cathepsin activity assay.
2. Dilute 70 μ g MAL to 0.6 μ g/ μ L in the cathepsin assay buffer with a minimum final volume of 120 μ L per sample.
3. Place 55 μ L (equivalent to 33 μ g MAL) into two sets of tubes labeled “A”—without E-64-D, and “B”—with E-64-D for each sample.
4. Add 10 μ L cathepsin assay buffer to set A tubes and 10 μ L 325 μ M E-64-D to set B tubes (the final concentration of E-64-D is 50 μ M) and incubate at 37 °C for 15 min. This should inactivate all cathepsin activity in set B samples.
5. Place 30 μ L of the above solution (equivalent to 15 μ g MAL) into duplicated wells in a 96-well (opaque-walled, flat bottom) plate and add additional 20 μ L cathepsin assay buffer into each well.
6. Dilute Z-FR-AMC stock (50 mM) to a 40 μ M working solution. To do this, mix 2.4 μ L stock with 3 mL of the cathepsin assay buffer (*see* Note 4).
7. Add 50 μ L of the 40 μ M Z-FR-AMC working solution to each well. The final Z-FR-AMC substrate concentration is 20 μ M, with 100 μ L cathepsin assay buffer per well.

8. Incubate the plate in the fluorimeter at 37 °C. Read at excitation of 380 nm and emission of 460 nm every 3 min for 60 min. Record the intensity values, which will give a curve describing the kinetics of cathepsin activity (see Sect. 3.4 and Fig. 3).

3.4 Cathepsin Activity Calculation

The data obtained can be displayed in two ways.

1. Relative fluorescence units. The relative cathepsin activity is calculated based on the amount of free AMC [in relative fluorescence unit (RFU)] released from Z-FR-AMC by enzymatic cleavage, determined by comparing to the standard calibration curve of AMC (Fig. 2). The RFU values of all samples should be corrected by subtracting the value of the blank. The mean RFU value for each sample in duplicates is then calculated.
2. Relative enzyme kinetics (V_{max}). The rate of cathepsin enzyme kinetics is calculated as the change in free AMC (RFU) per second resulting from Z-FR-AMC cleavage in MAL for each sample treated with or without E-64-D (Fig. 3). This can also be displayed as RFU/ μ g total protein/min.

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References

1. Bonaldo P, Sandri M. Cellular and molecular mechanisms of muscle atrophy. *Dis Model Mech*. 2013; 6:25–39. [PubMed: 23268536]
2. Sakuma K, Aoi W, Yamaguchi A. Molecular mechanism of sarcopenia and cachexia: recent research advances. *Pflugers Arch*. 2017; 469(5–6):573–591. [PubMed: 28101649]
3. Narici MV, de Boer MD. Disuse of the musculo-skeletal system in space and on earth. *Eur J Appl Physiol*. 2011; 111:403–420. [PubMed: 20617334]
4. Chopard A, Hillock S, Jasmin BJ. Molecular events and signalling pathways involved in skeletal muscle disuse-induced atrophy and the impact of countermeasures. *J Cell Mol Med*. 2009; 13:3032–3050. [PubMed: 19656243]
5. Bialek P, Morris C, Parkington J, St Andre M, Owens J, Yaworsky P, Seeherman H, Jelinsky SA. Distinct protein degradation profiles are induced by different disuse models of skeletal muscle atrophy. *Physiol Genomics*. 2011; 43:1075–1086. [PubMed: 21791639]
6. Xu H, Ren D. Lysosomal physiology. *Annu Rev Physiol*. 2015; 77:57–80. [PubMed: 25668017]
7. Xiong J, Zhu MX. Regulation of lysosomal ion homeostasis by channels and transporters. *Sci China Life Sci*. 2016; 59:777–791. [PubMed: 27430889]
8. Stoka V, Turk V, Turk B. Lysosomal cathepsins and their regulation in aging and neurodegeneration. *Ageing Res Rev*. 2016; 32:22–37. [PubMed: 27125852]
9. Aversa Z, Pin F, Lucia S, Penna F, Verzaro R, Fazi M, Colasante G, Tirone A, Rossi Fanelli F, Ramaccini C, Costelli P, Muscaritoli M. Autophagy is induced in the skeletal muscle of cachectic cancer patients. *Sci Rep*. 2016; 6:30340. [PubMed: 27459917]
10. Eskelinen EL, Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta*. 2009; 1793:664–673. [PubMed: 18706940]
11. Reiser J, Adair B, Reinheckel T. Specialized roles for cysteine cathepsins in health and disease. *J Clin Invest*. 2010; 120:3421–3431. [PubMed: 20921628]

12. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer*. 2006; 6:764–775. [PubMed: 16990854]
13. Choe Y, Leonetti F, Greenbaum DC, Lecaille F, Bogyo M, Bromme D, Ellman JA, Craik CS. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J Biol Chem*. 2006; 281:12824–12832. [PubMed: 16520377]
14. Schroter J, Schott KJ, Purtill MA, Neuheff V. Lysosomal protein degradation in experimental hyperphenylalaninaemia. *J Inherit Metab Dis*. 1986; 9:273–282. [PubMed: 3099071]
15. Creasy BM, Hartmann CB, White FK, McCoy KL. New assay using fluorogenic substrates and immunofluorescence staining to measure cysteine cathepsin activity in live cell subpopulations. *Cytometry A*. 2007; 71:114–123. [PubMed: 17200959]
16. Rossman MD, Maida BT, Douglas SD. Monocyte-derived macrophage and alveolar macrophage fibronectin production and cathepsin D activity. *Cell Immunol*. 1990; 126:268–277. [PubMed: 2107030]
17. Wilder CL, Park KY, Keegan PM, Platt MO. Manipulating substrate and pH in zymography protocols selectively distinguishes cathepsins K, L, S, and V activity in cells and tissues. *Arch Biochem Biophys*. 2011; 516:52–57. [PubMed: 21982919]
18. Jang BG, Choi BY, Kim JH, Kim MJ, Sohn M, Suh SW. Impairment of autophagic flux promotes glucose reperfusion-induced neuro2A cell death after glucose deprivation. *PLoS One*. 2013; 8:e76466. [PubMed: 24124562]
19. Lin PH, Duann P, Komazaki S, Park KH, Li H, Sun M, Sermersheim M, Gumpper K, Parrington J, Galione A, Evans AM, Zhu MX, Ma J. Lysosomal two-pore channel subtype 2 (TPC2) regulates skeletal muscle autophagic signaling. *J Biol Chem*. 2015; 290:3377–3389. [PubMed: 25480788]

Fig. 1.
The acid/lysosome extracts (3 μ g/lane) from murine gastrocnemius muscle were separated on a 10% SDS-PAGE and stained with Colloidal Blue. *Mw* molecular weight marker (*lane 1*)

Fig. 2.
A typical AMC standard calibration curve with relative fluorescence readout. *RFU* relative fluorescence units

Fig. 3. Acid protein extracts, derived from gastrocnemius muscles of wild type (wt) or mutant mice, were assayed simultaneously to measure cathepsin proteinase kinetics with 20 μ M Z-FR-AMC fluorogenic peptide substrate. Specific cathepsin activity was assessed using the cysteine protease inhibitor E-64-D (50 μ M). **(a)** A typical readout from a fluorimeter with plots of enzymatic kinetics (V_{max} , RFU/s) for the MALs. **(b)** A bar graph representation of the aggregated results of the enzyme kinetics expressed as RFU/s