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Efficient and modified protocol for zymography to detect muscle specific calpain activity

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ABSTRACT

Calpain, a proteolytic enzyme plays critical role in the skeletal muscle physiology by maintaining the protein metabolism. Up-regulation in the activity of such enzyme under diverse clinical settings (i.e. diabetes, cancer, AIDS, chronic heart failure, immobilization, aging etc) leads to loss in muscle proteins and causes atrophy/cachexia/sarcopenia. Beyond a reduced survival rate, atrophy is also linked to poor functional status and quality of life. Thus, easy detection of calpain at very early stage is highly desired under such settings so that specific therapy or antagonist could be given in time. Multiple methods are available (zymography, qPCR and immunoblotting) and among these zymography is the only approach which actually detect calpain activity. In the present manuscript, we have improvised two zymography protocols which are able to detect calpain in all muscle cells and tissues. Result shows that modified protocols can detect calpain activity even at low proteins concentration (< 10 μg) in muscle cells (C2C12 myoblasts, proliferating state; myotubes, differentiating state) and tissues (cardiac and skeletal) in a single gel in comparatively short time span. Overall, purpose of the present study is to provide relatively simple and well described experimental protocol which can be used for muscle-specific calpain study.

1. Introduction

Growth and maintenance of tissue requires a fine tuning between synthesis and degradation of cellular proteins. Any alteration in such tuning is likely to result in pathophysiological changes in the body. Protein catabolism is of crucial importance for muscle homeostasis, and responsible for the clearance of damaged or aged proteins. However, up-regulation of protein breakdown exceeding protein synthesis rate may result in skeletal muscle atrophy/wasting, contractile dysfunction and cardiac cachexia in wide range of disease/pathophysiological states including diabetes, cancer, chronic obstructive pulmonary disease, chronic heart failure, and also in sarcopenia (aging-induced decrease in muscle mass). A large number of reports are available which have illustrated the existence of different proteolytic machineries including Ca^{2+}-dependent calpain in muscles whose level gets up-regulated under these clinical settings. Involvement of calpain (specifically calpain 1, μ-calpain; calpain 2, m-calpain) as a key player in such settings have made this enzyme a potential therapeutic target in treatment of muscle specific disorders (Dutt et al., 2015; Iorio et al., 2013; Pomponio et al., 2008; Tidball and Spencer, 2002). Besides its negative role in muscle disorders, calpain contributes in meat industry by improving the quality of meat through enhancing its tenderization process. Thus, a fine balance of calpain activity is highly essential for regulating muscle growth and meat quality. In spite of such importance, there is lack of clear and specific methodology to study calpain activity in muscles.

According to published data qPCR and immunoblotting are the preferred approaches used by scientific communities to study calpain. Zymography, another approach to study the calpain activity, is quite apparent and tells in detail about the active nature of this enzyme that is not possible by other available techniques. This technique was applied for the first time by Raser et al., 1995 in cerebrocortical cells, rat brain, Molt-4 (a leukemic cell line) and thereafter by other researchers for detection of calpain activity by casein zymography (Arthur and Mykles, 2000; Pomponio et al., 2008; Raser et al., 1995). Though this tool is standardized and being used by researcher globally

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in different tissues including muscles even than application of zymography is quite low. This is probably due to requirement of high protein concentrations, being time consuming and lack of clearly defined protocol. These shortcomings have placed such a powerful and potential approach on least priority among the scientific community for the study of calpain. Thus there is a need to provide new or modified protocol of zymography which can be used to study calpain activity in any muscle tissue/cells as other approaches are insufficient and far away from the natural behavior of the calpain. In the present article, we are providing the modified version of casein zymography protocol in detail and compared the muscle calpain data with other published protocols.

2. Materials and methodology

2.1. Chemicals

Tris-base, Tris-Cl, Casein, Glycine, EDTA, Calcium chloride, Glycerol, Bromophenol blue, DTT, Phosphoric acid (88%), Ammonium persulphate (APS), Ammonium sulphate, Sodium fluoride, 2-mercaptoethanol, HEPES, Sodium chloride, Phosphate buffer saline, Trypsin EDTA solution were purchased from Himedia chemicals (India). Sodium orthovandate and Protease inhibitor cocktail, set-I were procured from Calbiochem and CBB-G250 from S.D. Fine, Chem (India). DMEM medium, Heat Inactivated Horse serum and Penicillin-streptomycin were purchased from Gibco. Fetal bovine serum was purchased from Sigma. C2C12 skeletal muscle mouse cell lines obtained from Dr. Jyotsana Dhawan, CCMB, Hyderabad, India.

2.2. Muscle sample collection, preparation and zymographic assay

Muscle samples, collection/preparation.

A. C2C12 muscle cell lines culture protocol

i. Skeletal muscle myoblast:

- C2C12 (a myoblastic skeletal muscle cell line) cells were cultured as described by (Bhatnagar et al., 2012; Sachidanandan et al., 2002). In brief, C2C12 cells were maintained in DMEM medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 IU/ml penicillin, and 100 µg/ml streptomycin) until the 80% confluency was achieved.
- Thereafter, cells were washed twice with PBS and trypsinized with 0.25% Trypsin-EDTA solution and collected the cells named as skeletal muscle myoblast and stored at −80°C temperature for further use.

ii. Skeletal muscle myotubes:

- C2C12 cells will be differentiated into myotubes by incubation in differentiation medium (2% horse serum in DMEM) for 72 h.
- After then, fully developed myotubes were again trypsinized, washed with PBS, collected and stored at −80°C temperature for further use in future.

B. Chicken skeletal and cardiac muscle tissue

i. Freshly cut chicken skeletal muscle and cardiac muscle have been obtained from the local slaughter house. These tissues were immediately transferred to 0.9% ice cold saline solution.
ii. Fat tissue/components attached to tissue were removed using sterile forecep.
iii. Each tissue was individually perfused with ice cold 0.9% saline solution with the help of syringe.
iv. Perfusion was done until blood components were completely removed from tissue.
v. Perfused tissues were stored at −80 °C for further use.

1. **Lab Modification 1** (Pomponio et al., 2008 + Arthur and Mykles, 2000 + modification) - Protocol for zymographic assay

Solution:

A. Cell lysis buffer for myoblast and myotubes
   a. 50 mM HEPES pH 7.6
   b. 15 mM NaCl
   c. 5 mM EDTA
   d. 10 mM 2-Mercaptoethanol
   e. 10% glycerol
   f. 0.1% Triton X-100
   * For 100 µL of above cell lysis buffer, add
   a. 1 µL of 5 mM NaF
   b. 5 µL of 1 mM Na_3VO_4
   c. 1 µL protease inhibitor cocktail set-1 (AEBSF HCl, Aprotinin, E-64 protease inhibitor, EDTA disodium, Leupeptin, Hemisulphate)

B. Tissue homogenization buffer for skeletal muscle and cardiac muscle
   a. 100 mM Tris-base, pH 8.0
   b. 5 mM EDTA
   c. 10 mM 2-Mercaptoethanol
   d. 0.1% Triton X-100
   * For 100 µL of above cell lysis buffer, add
   a. 1 µL of 5 mM NaF
   b. 5 µL of 1 mM Na_3VO_4
   c. 1 µL protease inhibitor cocktail

C. Solutions for preparation of gel
   a. 30% Acrylamide solution
   b. Buffer A- 1.5 M Tris-Cl, pH 8.8
   c. Buffer B- 1.0 M Tris-Cl, pH 6.8
   d. Casein solution- 10 mg/ml casein in 0.75 M Tris-HCl, pH 8.8 (take 1:1 of buffer A and Millipore water for 0.75 M solution)
   e. 10% Ammonium persulphate (APS)

D. Sample loading buffer for electrophoresis
   a. 300 mM Tris-Cl pH-6.8
   b. 40% glycerol
   c. 0.02% bromophenol blue
   *Add 200 mM DTT (31 mg for 1 ml loading buffer) just before use.

E. Running buffer for electrophoresis
   a. 25 mM Tris-base pH 8.3
   b. 192 mM Glycine
   c. 1 mM EDTA
   d. Prepare fresh and keep it at 4 °C.

F. Ca^{2+} incubation buffer
   a. 50 mM Tris-Cl, pH 7.5
   b. 4 mM $CaCl_2$
   c. 10 mM 2-Mercaptoethanol.
   *Always prepare fresh before use.

G. Stop buffer
   a. 20 mM Tris-Cl pH 7.0
   b. 10 mM EDTA
   *Always prepare fresh before use.

H. Gel staining solution (Pomponio et al., 2008; Rabilloud, 2000)
   i. Equilibration buffer (50 ml)
   a. Phosphoric acid (88%).....1 ml
   b. Ammonium sulphate.....7.5gm
   c. MilliQ water.....40 ml
   d. 96% Ethanol.....9 ml