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***Research Article** (Open Access)*

A REFINED AND COST-EFFECTIVE CASEIN ZYMOGRAPHY PROTOCOL FOR VISUALIZING CALPAIN ISOFORMS EXPRESSION DURING SKELETAL MUSCLE ATROPHY

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ABSTRACT
Cytosolic calpains hold a distinctive role in skeletal muscle development and homeostasis as being indulged in crucial cell survival strategies such as cellular motility, proliferation, and apoptosis. Literature has evidenced the over-expression of μ & m-calpain in muscle dystrophies while under-expression of calpain-3/p94 causes limb-muscle girdle atrophy 2A. The collusion of various calpain isoforms in different physiological events excludes the conventional photometry-based techniques to estimate individual calpain isoforms activity assessment within specific tissues. In casein-zymography, calpain isoforms were separated on a non-reducing native PAGE co-polymerized with identified calpain substrate; Casein. The gel was then subjected to calcium incubation for calpain activation and subsequent enzymatic activity. The gel was then negatively stained with Coomassie Brilliant Blue dye so as to attain translucent calpain activity bands against a white background. All three isoforms of skeletal muscle calpains were successfully separated and observed for activity in the gel in a comparatively short time span.

***Keywords:** Muscle atrophy, Calcium, Calpains, Casien, Zymography*

INTRODUCTION
Muscle wasting, the loss of both strength and function, is a complex process that involves the activation of various proteolytic systems (Furrer & Handschin, 2019; Lang *et al.,* 2017). Among these systems, calpains have been identified as key players in muscle protein degradation (Bartoli & Richard, 2005; Costelli *et al.,* 2005; Huang & Forsberg, 1998; Lang *et al.,* 2017). Calpains are calcium dependent cytosolic proteases involved in multiple cellular processes crucial to cell survival and tissue growth, muscle structure and function (Laszlo Kovacs, 2014). Up until 15 members have been identified in the family (Sorimachi *et al.,* 1994). Physiologically, Calpains depend upon calcium for their activation (Goll *et al.,* 2003). The proteolytic action of calpains is restricted to cleavage instead of end-processing to substituent amino acids (Goll *et al.,* 2003). During myogenesis, the key steps of myofiber generation; alignment and fusion of myoblasts are well reported for involvement of calpains (Brustis *et al.,* 1994; Dourdin *et al.,* 1997). It has been noted that m-calpain is localized to the fusing myofiber membrane, where it cleaves fibronectin and causes the myoblasts to fuse (Brustis *et al.,* 1994; Dourdin *et al.,* 1997). Moreover, *CAPN3* null mutation leads to LMG2A (Kramerova *et al.,* 2016). Also, calpains are not only limited to physiological assistance in myofiber generation but also in their degradation. Over-expression of u-&m- calpain is well reported in various muscle dystrophies also (Bartoli & Richard, 2005). Literature have evidenced the over expression of μ & m-calpain in muscle dystrophies while under-expression of calpain-3/p94 to cause limb-muscle girdle atrophy 2A (Duguez *et al.,* 2006; Ono *et al.,* 2016). In addition to a diminished rate of survival, the degeneration of muscles is also associated with diminished functional abilities and reduced quality of life. Therefore, it is imperative to promptly identify the presence of calpain, at an early stage to facilitate timely administration of targeted therapies or antagonists. Several techniques can be utilised to assess calpain activity, including zymography, qPCR, and immunoblotting. However, it is important to note that zymography is the sole method capable of specifically detecting isoform-specific calpain activity. In the current investigation, a refined zymography

protocol has been developed to effectively identify all three skeletal muscle-specific calpain isoforms. The findings indicate that the protocol is capable of detecting calpain activity in skeletal muscle tissue, even at low protein concentrations (10 µg), within a single gel and in a relatively short period of time. In summary, the primary objective of this study is to establish a relatively straightforward and thoroughly documented experimental protocol that can be employed for investigations pertaining to calpain activity in muscle-specific calpain investigations.

MATERIALS AND METHODS

Chemicals and Reagents:

Tris Base (Himedia-TC072M), HC (SRL-7647-01-0), Sodium Chloride (Himedia-TC046), Ethylene-di-amine-tetra-acetic acid (Himedia-TC038M), β-mercaptoethanol (SRL- 60-24-2), Triton-X100 (MERCK-9036-19-5), Sodium fluoride (Himedia-GRM7502), Sodium orthovanadate (13721-39-6), Acrylamide (GRM-1110), Bis-acrylamide (MERCK-A2792), Casein (Himedia-RM10675), Ammonium sulfate (Himedia-TC553U), Tetramethyl ethylenediamine (MERCK- 110-18-9), Glycine (Himedia-PCT0310), Dithiothreitol (Himedia-TC540G), Glycerol (SRL- 56-81-5), Bromophenol Blue (Himedia-GRM914), Calcium Chloride anhydrous (Himedia-TC-097), Methanol (SRL- 67-56-1), Acetic acid (SRL- 64-19-7).

Muscle sample collection and preparation

Hind-limb Quadriceps skeletal muscles were obtained from freshly sacrificed Denervated male wistar rats from previous lab experimentation approved under ethical permission form Institutional Animal Ethics Committee, MDU Rohtak, vide letter no. 282-93 dated 11/12/2021. Following sacrifice, the muscle tissues were promptly transferred to a solution of ice-cold 0.9% saline. Fat tissue layer over the muscles was removed with sterile forceps and washed with ice-cold phosphate buffer saline. Washed tissues samples were used for further experimentation.

Reagents Setup

Calpains extraction Buffer: 100mM Tris-HCl buffer of pH 8.0 encompassing 5mM EDTA, 10mM mercaptoethanol, 10mM Dithiothreitol, 50µM Sodium fluoride, 50µM Sodium orthovanadate, 0.1% Triton-X100 was prepared in Millipore water and stored at 4° C. (Can be stored for one month at 4° C).

Acrylamide-Bisacrylamide Mixture: 30% acrylamide and 0.8% Bisacrylamide solution was prepared in millipore water. The solution was carefully filtered using Whatman filter paper and subsequently stored in a brown bottle at a temperature of 4°C. (Can be stored for one week at 4° C).

Separating gel buffer: 375mM Tris–HCl buffer of pH 8.8 was prepared in millipore water and stored at 4° C. (Can be stored for one month at 4° C).

Stacking gel buffer: 330mM Tris–HCl buffer of pH 6.8 was prepared in millipore water and stored at 4° C. (Can be stored for one month at 4° C).

0.5% Casien solution: 187.5 mM Tris–HCl buffer of pH 6.8 with 0.5% Casien (w/v) was prepared in millipore water and stored at 4° C. (Can be stored for one week at 4° C).

Ammonium persulfate: 10% Ammonium persulfate (w/v) solution was prepared in Millipore water and used fresh.

N, N, N', N'-Tetramethyl-ethylenediamine (TEMED): Used premade.

Running buffer: 25mM Tris–HCl, 192 mM glycine, 1mM EDTA buffer with pH 8.3 was prepared in millipore water and stored at 4° C. (Can be stored for one month at 4° C).

5X Sample loading buffer: 750 mM Tris-HCl buffer of pH 6.8 with 40% glycerol & 0.025% bromophenol blue was prepared in millipore water and stored at 4° C (Can be stored for one month at 4° C). 500mM DTT was added to the 5X sample loading buffer just before use.

Calpains Activation Buffer: 50mM Tris–HCl buffer of pH 7.5 with 4mM calcium chloride and 10mM β-mercaptoethanol was prepared in Millipore water and used fresh.

Staining solution: 0.1% CBB R-250 was dissolved in a solution of 50% methanol (v/v) & 10% acetic acid (v/v). The solution was filtered and stored at room temperature. (Can be stored for one year at room temperature).

De-staining solution: A solution of 50% methanol (v/v) & 10% acetic acid (v/v) was prepared in millipore water. The solution was filtered and stored at room temperature. (Can be stored for one year at room temperature).
Gel storage solution: 7% acetic acid (v/v) solution was prepared in millipore water.
Procedure
Calpains extraction: ~50mg of the skeletal muscle tissue was weighed and finely minced with surgical blade. The minced tissue was incubated for 15 minutes in 500 ul calpain extraction buffer at 4° C. Post-incubation the tissue was homogenized for 15 minutes with handheld motorized tissue grinder. The tissue homogenate was again incubated for 30 minutes at 4° C. The lysate was the centrifuged for 20 minutes at 12,000*g and 4° C. The resulting supernatant was collected, and the total protein content was determined using the Bradford method, utilizing Bovine Serum Albumin (BSA) as a standard for calibration.
Casien-PAGE preparation: 12% Separating and 6% stacking gels were casted using above prepared by mixing solutions in a composition as per Table-1. APS and TEMED were added in the last to ensure the controlled and uniform polymerization of Acrylamide.

Table-1: Casien PAGE composition for 12% separating and 6% stalking gel (80*70*.7 mm).

Sr. No.	Reagent	12% Separating gel (ul)	6% Stalking gel (ul)
1	Millipore water	2000	1750
2	Tris-HCl pH-8.8	1625	Nill
3	Tris-HCl pH-6.8	Nill	312.5
4	30% Acrylamide Solution	2500	412.5
5	0.5% Casien solution	1250	Nill
6	10% APS	50	20
7	TEMED	5	2

Zymogram running and development
Pre-running of the gel: The polymerized Casien-PAGE gel was assembled in the cassette and put into the electrophoresis tank. The tank was kept in ice bath to maintain a chilling temperature. The tank was filled with appropriate volume of ice-chilled running buffer, pH-8.3. The gel was pre-ran for 30 minutes at 80 V of constant power supply. The pre-run was performed to ensure the uniform distribution of casein within the polymerized gel and to run out any excessive casein.
Sample loading: Sample volume equivalent to 10ug of total protein content (as estimated by Bradfords method) was mixed with 5X Sample loading buffer in a ratio of 1:5 (Buffer: Sample). The mixture was loaded per well of pre-ran Casien-PAGE gel.
Electrophoretic run: The sample loaded gel was ran at 80V constant power supply for 3 hours. Post electrophoresis the gel was incubated in the ice-cold running buffer for 5 minutes at room temperature on a rocking shaker.
Zymogram Development/Calpain Activation: The Casien-PAGE was left in calcium incubation buffer for about 12 hours, for activation and caseinolytic activity of calpains.
Staining of the Zymogram: The calcium incubated Casien-PAGE was fixed in staining solution for 30 minutes at room temperature with continuous shaking.
Destaining and photography of the zymogram: The stained and fixed Casien-PAGE zymogram was washed in de-staining solution at room temperature with continuous shaking until visualization of translucent calpain activity bands. The images of zymogram were captured with a high-resolution digital camera against a white background.

RESULTS AND DISCUSSION

In the modified protocol, we used a tissue lysis recipe with 0.1% Triton X-100 and 10mM DTT followed by optimization of calpain extraction procedure. Tissue lysis is most critical phase in the process as it is the most-responsible cause behind loss of enzymatic activity. Thus, the tissue was incubated in ice-cold lysis buffer for 15 minutes prior to lysis. This pre-incubation made the plasma membrane porous and contributed to cellular proteins adaptation with the lysis environment. Post-incubation, the samples were grinded with a handheld-tissue grinder at low speed and on ice to avoid any loss of enzymatic activity. Similarly, the lysate was also kept on incubation in ice-cold lysis buffer for 30 minutes post-lysis followed by centrifugation. The lysis process ensured the successful structurally intact extraction of all three skeletal muscle calpain isoforms. At last, the lysate was subjected to Casien-PAGE separation and subsequent zymogram development. Interestingly, as shown in **Figure 1**, all three skeletal muscle isoforms of calpains were observed with caseinolytic activity in a single gel.

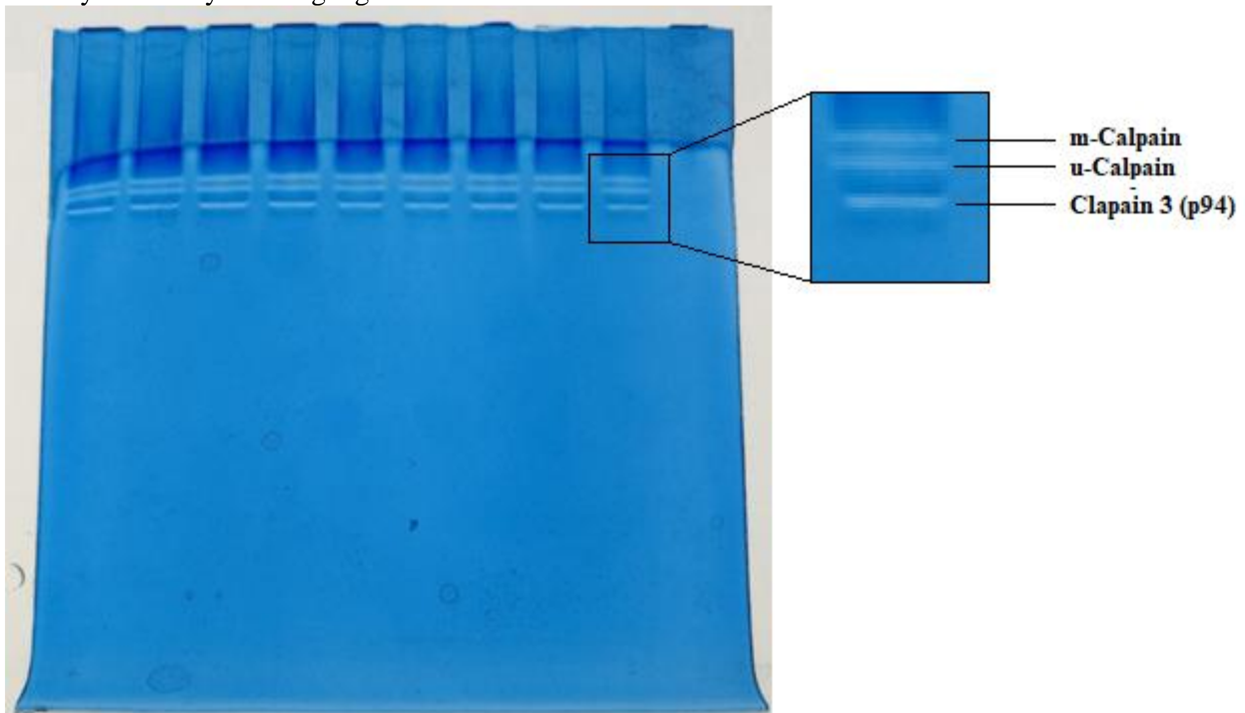

Figure 1: Casien-PAGE Zymogram showing translucent band represent in-gel caseinolytic activity of m-calpain, µ-calpain & calpain-3.

CONCLUSION

Since the first reported study on calpain in-gel activity assay by Raser (Raser *et al.,* 1995), multiple modifications of the parent protocol have emerged in literature, yet none of them have reported to be effective for skeletal muscle specific calpain isoform; Calpain-3. Calpain-3 is extremely exhaustive in self-cleavage and have a half-life of few milliseconds only (Ono *et al.,* 2016). Calpain-3 holds a distinctive role in skeletal muscle physiology. Opposite to the cleavage action of µ & m-calpain, p94 maintains the integrity of key z-disk protein; Titin (Duguez *et al.,* 2006). Moreover, Calpain-3 null mutations have been reported to generate Limb-Girdle muscle atrophy Type -2A (Kramerova *et al.,* 2016). Therefore, it is crucial to obtain a comprehensive understanding of the activity of this enzyme. Until now, no published protocol has been applicable for the detection of calpain-3 band in zymograms. The lack of available information necessitated the development and modification of the zymography assay to assess calpain activity in skeletal muscle samples.

In current study, we produced an improvised protocol applicable for detection of all three calpain isoforms reported in skeletal muscles. This article represents a significant contribution in the field of casein zymography

as it is the first of its kind to report the detection of all three skeletal muscle isoforms of calpains in a single gel using an in-gel assay. This refined casein zymography protocol allows for the visualization and quantification of all three skeletal muscle calpain isoforms. The modified protocol presented in this study is straightforward and user-friendly, providing a well-defined and effective method to assess calpain activity even at very low total protein concentrations (10µg) in skeletal muscle lysates. By comparing the band intensities, researchers can assess the relative expression and activity of different calpain isoforms under various experimental conditions. Sensitive protocols of this nature are in high demand for diverse studies involving skeletal muscle atrophy models.

The cost-effective nature of this protocol is a notable advantage. The use of casein, a readily available and inexpensive substrate, reduces experimental costs compared to other methods requiring specialized substrates. This affordability facilitates broader adoption of the protocol by research groups with limited resources. Moreover, the refined protocol offers improved sensitivity and resolution compared to previous methodologies. It provides a clearer visualization of calpain activity, enabling the identification of specific isoforms and their potential involvement in muscle atrophy. This protocol's affordability and improved sensitivity make it a valuable tool for researchers working on skeletal muscle atrophy and related fields, contributing to a better understanding of this complex process and potentially aiding the development of therapeutic interventions.

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