NO CALPAIN, NO GAIN:

NEWLY DEVELOPED PROCEDURES FOR THE SEPARATION AND CHARACTERIZATION OF

THE CALPAIN FAMILY OF PROTEINS IN

HUMAN DYSTROPHIC AND NON-DYSTROPHIC MUSCLE

By

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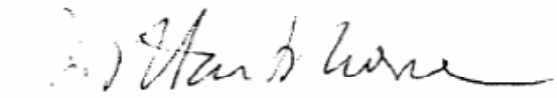

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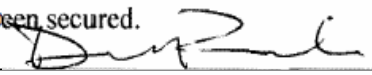
:The University of Arizona Library Release	I hereby grant to the University of Arizona Library the nonexclusive worldwide right to reproduce and distribute my dissertation or thesis and abstract (herein, the "licensed materials"), in whole or in part, in any and all media of distribution and in any format in existence now or developed in the future. I represent and warrant to the University of Arizona that the licensed materials are my original work, that I am the sole owner of all rights in and to the licensed materials, and that none of the licensed materials infringe or violate the rights of others. I further represent that I have obtained all necessary rights to permit the University of Arizona Library to reproduce and distribute any nonpublic third party software necessary to access, display, run or print my dissertation or thesis. I acknowledge that University of Arizona Library may elect not to distribute my dissertation or thesis in digital format if, in its reasonable judgment, it believes all such rights have not been secured. Signed:  Date: 1-21-2010

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Abstract

Muscular dystrophy is a disease which gradually deteriorates skeletal muscle cells, leading to the eventual death of such cells and the surrounding tissue. Calpains are Ca^{2+}-dependent proteases and together with the Ca^{2+}-dependent specific inhibitor of calpains, calpastatin, are widely distributed in eukaryotic cells. It has been suggested that part of the enhanced deterioration in the dystrophic state is due to enhanced calpain activity; therefore analysis of normal and dystrophic muscle was essential. Conventional techniques for the isolation and characterization of calpain and calpastatin utilize relatively large muscle samples (>100g), whereas biopsy or post-mortem samples are considerably less than this. Thus, the initial and main objective of this project was to develop methods suitable for purification and analysis of the calpain family from limited muscle samples. With these restrictions in mind, techniques were developed for samples ranging from 0.2-1g, a realistic biopsy extraction. The hypothesis to be further evaluated is that some dystrophies are characterized by increased calpain activity, caused either by an increased expression of m- or μ- calpain or decreased inhibition by calpastatin, or both. The procedures are now in place to test this hypothesis further and extensive analyses are required using defined dystrophic types and increased sampling numbers.

Introduction

Loss of muscle mass in various muscular dystrophies is caused by a greatly increased rate of muscle protein degradation with little change or even a small increase in rate of muscle protein synthesis (MacLennan and Edwards, 1990 and Wolitsky *et al*., 1984). Muscle atrophy can be initiated by a variety of signaling events, and atrophy ultimately is caused by proteolytic degradation of muscle proteins (Jackman and Kandarian, 2004). There are several major proteolytic systems in muscle. The proteasome degrades myofibrillar proteins only after they have been released from the myofibril (Solomon and Goldberg, 1996). It cannot degrade intact myofibrils that are 10-100nm in diameter because intact myofibrils cannot enter the central cavity of the proteasome which contains the proteolytically active sites of the proteasome and is only 1-2 nm in diameter. The lysosome system can degrade the peptides generated by the proteasome and provide a recycling of amino acids.

A substantial amount of evidence based to a considerable extent on studies using the mdx mouse model of Duchenne dystrophy has indicated that another proteolytic system, the calpains, is responsible for the loss of muscle proteins in different muscular dystrophies (Spencer *et al.*, 1995). The calpains are active at pH 7.0-7.5 and cleave several myofibrillar proteins, including α-actinin and titin, and these two proteins may be critical to the disassembly. Troponin T and the extramyofibrillar protein desmin also are targets for calpain (Huang and Forsberg, 1998). The major myofibrillar proteins, such as actin, are not degraded by calpain, while myosin is but only at high concentrations. Thus there is some controversy concerning the mechanism of myofibrillar disassembly. However, there is a consensus that after disruption of the myofibrillar structure, the

released proteins may then be degraded by the proteasome. Hence, the calpains may have a critical role in initiating degradation of the myofibrillar proteins and may have an important role in muscle wasting in the muscular dystrophies (Goll *et al.*, 2003).

To challenge this hypothesis, it was necessary to develop procedures adapted to small sample sizes and then to compare normal and dystrophic muscle. In previous studies, 100-200g of muscle was required for assaying calpain activity. It is difficult to obtain this quantity of human muscle, thus the development of new procedures was essential. Furthermore, techniques to quantify the proteins have also been explored and will be discussed throughout this analysis. Future analyses will require considerably more samples and discrimination between various forms of dystrophy.

Materials and Methods

The purification procedure is summarized by the flow diagram in Fig.1.

Preparation and Homogenizing

Human muscle samples (dystrophic and non-dystrophic) were stored at -140°C from the time they were obtained from the National Disease Research Interchange. Sample sizes varied but never exceeded 10g. Frozen samples were pulverized into a fine powder, weighed, and placed into individual containers. Each sample has a unique identifying number (e.g. 59303) followed by a dash (e.g. 59303-1) which refers to the container number used when the original muscle was pulverized and separated. Each sample was homogenized twice using a Brinkman Polytron in buffer (125mM KCl, 20mM Tris-HCl, pH 7.5, 1mM EDTA, 0.1% β mercaptoethanol [MCE], 1mM Pefabloc), centrifuged at 48,384G for 20 minutes, and then filtered using an Acrodisc syringe filter [Pall corporation, East Hills, NY].

Hexyl-TSK 650 Column

An aliquot was saved for a net calpastatin, calpain activity, and total protein concentration assays. The remaining sample was loaded onto a hydrophobic hexyl-TSK 650 [Toyopearl, Montgomeryville, PA] column equilibrated with 125mM KCl in 20mM Tris-HCl pH 7.5, 1mM EDTA, 0.1% MCE, 1mM NaN_3 (TEMA) in order to separate calpastatin from μ- and m-calpain. The calpastatin does not bind under these conditions and passed straight through the column. The calpains were eluted using 0.1% Brij-35, 1mM EDTA, 0.1% MCE. Calpastatin activity was assayed using the FITC-casein assay (Wolfe *et al*., 1989) and calpain was assayed using BODIPY-casein assay (Thompson *et al*., 2000).

Source 15Q Column for Calpain

Next, the calpains were loaded onto an ion-exchange Source 15Q column [GE Healthcare, Uppsala, Sweden] equilibrated with 20mM Tris-MES, pH 6.5, 1mM EDTA, 0.1% MCE, 1mM NaN_3 (TMEMA). μ-calpain was eluted by washing the column with 170mM KCl, TMEMA while m-calpain was eluted with 370mM KCl, TMEMA. The fractions were assayed for activity using CalpainGlo [Promega, Madison, WI] (Moldoveanu and Hosfield, 2002).

Source 15Q Colum for Calpastatin

The calpastatin was loaded onto a separate ion-exchange Source 15Q column equilibrated with 62.5mM KCl, TEMA. Calpastatin was eluted by 250mM KCl, TMEMA The fractions were assayed for activity using FITC-casein and BODIPY-casein.

FITC-Casein Assay

In order to measure the calpastatin activity using FITC-casein, fresh FITC-casein was made using 0.0625g of FITC-casein dissolved in 5ml of H_2O (covered in foil), followed by the addition of 2.5ml of 1M Tris-HCl at pH 7.5, 1.25ml of 2M KCl, and 35μL MCE to a final volume of 10mL. Assays were carried out in 96 well plates. The muscle homogenate, 62.2μg/well, was added in triplicate, followed by a Ca^{2+} blank in quadruplicate, and an EDTA blank in triplicate. 5μL of pure calpain was diluted in fresh TEMA and 20μL of diluted homogenate was added and homogenizing buffer was added to the Ca^{2+} blank. 25μl of FITC-casein solution was added to each tube, followed by 12.5μl of 25mM $CaCl_2$ to Ca^{2+} blanks and samples, and then 12.5μl of 25mM EDTA to EDTA blanks. After incubation in the dark for 30 minutes at room temperature, 62.5μl

of 5% TCA with 0.01% Triton X-100 was added, incubated in ice for 5 minutes and centrifuged at 14,000 rpm for 10 minutes/ 4°C . 50µl of supernatant was added to 2ml of 0.3M K-phosphate at pH 8.5 in each well and read on a Infinite M200 [TECAN, Durham, NC] The fluorescent units of inhibition were calculated in the case of calpastatin.

BODIPY-Casein Assay

As for the BODIPY-casein assay for calpain fraction identification, fresh BODIPY was prepared using 4mL of 5X BODIPY-casein dilution buffer with 20µL of MCE and 100µg of BODIPY-casein and final dilution with H_2O to 20mL. Using a 96-well plate, 100µL of 125mM KCl–TEMA was added in duplicate, as the Ca^{2+} blank. 100µL of each fraction was added to the wells and an EDTA blank was done for every 10^{th} fraction by adding 25µL of 0.1M EDTA. 100µL of BODIPY-casein was added to each well, the plate covered in foil, and incubated at room temperature for 30 minutes. The reaction was stopped by the addition of 25µL of 0.1M EDTA and read using TECAN Infinite M200. Fluorescent units were calculated to identify the calpain fractions.

CalpainGlo Assay

Using a 96-well plate, 50µL of pure m- or µ-calpain was added in duplicate, followed by 50µL of TEMA in duplicate as the Ca^{2+} blank. 50µL of each fraction was added and 50µL of CalpainGlo/Ca^{2+} was added to the Ca^{2+} blank, standards, and samples. Using the Luminometer Shake and Delay Clarity program, the plate was shaken 30 seconds and incubated 12 minutes before reading. The relative luminescence units were calculated and each of the calpain-containing fractions was identified.

Concentration

Following the assays, fractions were concentrated using 5K or 10K cut-off centrifugal concentrators and assayed for protein concentration with the Coomassie Plus [Pierce, Rockford, IL] assay.

Western Blotting for m- and μ-Calpain

Enzyme-Linked ImmunoSorbent Assay (ELISA) procedures were initially attempted to find a more accurate way to quantify the amount of calpain/calpastatin present. However, this method was found to be too variable for calpain, but not calpastatin, as will be discussed below. Thus Western blots were used to determine the concentration of the 80kDa subunit of calpain in the muscle homogenates (the epitopes for the antibodies used are in the large subunit). Western blots were performed, using 8-16% gradient gels, on each sample to determine both m-calpain and μ-calpain. (Pierce, Rockford, IL]. 20μg of sample was loaded in each well. For μ-calpain, a standard curve from the human placental prep 37 (HP 37) was constructed using 100ng, 50ng, 25ng, 12.5ng, and 6.25ng. For m-calpain, the bovine skeletal prep 80 (BSP 80) was used with standards of 100ng, 50ng, 25ng, 12.5ng, and 6.25ng. Tranfers were done using polyvinylidene fluoride (PVDF) membrane with blocking of the membrane by 1% bovine serum albumin (BSA) in 20mM Tris (pH 7.5), 50mM NaCl, 0.05% Tween (1X TTBS). The primary antibodies used included, 9A4 [Affinity BioReagents, Rockford, IL] and B2 [Gift from Ahmed Ouali, French National Institute for Agricultural Research] for μ-calpain and m-calpain, respectively. The secondary antibodies used included anti-mouse [American Qualex, San Clemente, CA] and anti-rabbit [Rockland, Gilbertsville, PA] for μ-calpain and m-calpain, respectively. Washes were done 4 times at 10 minutes per wash

using 1X TTBS between incubation with primary and secondary antibodies. The reaction was completed with FEMTO [Pierce, Rockford, IL] for 5 minutes and the membranes were exposed for varying durations. Using an Epi Chemi II darkroom camera [UVP, Cambridge, UK] and the integrated optical density from the associated LabWorks program, the mass of the calpain could be determined by comparison to the standard curves. Standard curves were adjusted to reflect the percent of the 80kDa (large) subunit by running gels (Coomassie stained) of the purified standard human placental calpain.

ELISA for Calpastatin

The detection of the calpastatin within each homogenate sample could be ascertained using a more specific version of the ELISA known as the "sandwich ELISA." A 96-well black strip Costar plate was incubated with 100µl of 0.002µg/µl of mouse anti-human calpastatin antibody [CalBiochem, Gibbstown, NJ] in PBSN overnight at 4°C. After 16 hours, the coat was washed away four times using TTBS and then blocked with 1% BSA for 1 hour at 37°C. Standards were made in blocking solution using human 244-4 starting at 0.0156ng/µl and diluted until 0.00024ng/µl. Homogenate samples were diluted to 0.08µg/µl in 137mM NaCl, 10mM Phosphate, 2.7mM KCl, 1.0mM NaN_3, pH 7.4 (PBSN). After discarding the blocking solution and washing four times with TTBS, the samples and standards were added in triplicate and incubated for three hours followed by another four washes in TTBS. The first antibody was added for three hours at 100µl per well using 244-4 human polyclonal calpastatin [Custom] made in chicken at 10µg/ml in blocking solution. Following this, another four washes were done with TTBS and the secondary antibody was added at 100µl per well using rabbit anti-chicken-HRPO [Rockland, Gilbertsville, PA] at 1:5,000 in blocking solution and incubated for two hours.

Another four washes followed this procedure and then a substrate to peroxide ratio of 9:1 QuantaBlu [Pierce, Rockford, IL] was added at 100µl per well and stopped using QuantaBlu stopping solution at 100µl per well. TECAN Infinite M200 plate reader was used and the calpastatin concentration was determined.

Results

The separation of the two calpains and their endogenous inhibitor, calpastatin, was critical to the proper analysis of the muscle. The column techniques used to separate the three were developed by another member of the lab, Marlon Taylor, and tested by myself with miscellaneous dystrophic and non-dystrophic samples to validate the effectiveness. Figure 2 is a representative profile for the hexyl-TSK 650 column, illustrating the reliability of the separation between calpain and calpastatin. Calpastatin does not bind to the column so it passed through as the column was loaded and flushed with 125mM KCl, TEMA. The calpains eluted together around fraction 35 when using low ionic strength buffer that contained the detergent Brij-35. The fractions were tested for calpastatin activity using the FITC-casein assay. Calpain activity was monitored via the BODIPY-casein assay.

Once the calpastatin and calpain separation was complete after the Hexyl-650 TSK column, each fraction was loaded onto a separate ion-exchange Source 15Q column. The calpain column profile for sample 59263, shown in Figure 3, illustrates the clear separation between μ-calpain and m-calpain as a result in the difference between pI of the two proteins. μ-Calpain came off at a much lower salt concentration (170mM KCl, TMEMA) while m-calpain required a higher salt concentration (370mM KCl, TMEMA) as can be observed by the CalpainGlo activity assay performed for each fraction per the blue line. The relative absorbance was used as a control in order to monitor general protein concentration change.

The general protein concentration within the homogenate and the wet tissue weight was ascertained and summarized in Table 1. Certain information such as age, sex,

and gender was included in an attempt to correlate various phenotypes with the final results. When examining the homogenate solution to ascertain the μ-calpain concentration, a standard curve from HP 37 μ-calpain was run alongside the samples, as can be observed by Figures 4-1 and 4-2. Several samples were examined and each standard curve yielded a highly accurate linear relationship. Using the LabWorks program, the actual amount of the large subunit was quantified as can been seen in Table 1. The values within the table and that of Figure 4-2 represent an adjusted value based on the percent of the 80kDa (large) subunit in the standard. This percentage was determined after running the standards on an SDS-PAGE gel, staining the gel with Coomassie Brilliant Blue R250 and calculating the ratio of IOD of the 80kDa band, per Figure 6. IOD represents the integrated optical density which calculates the amount of fluorescence in a given band as a measure of the protein in that band.

As with μ-calpain, the m-calpain was quantified using Western blots with a respective standard curve but using m-calpain purified from bovine skeletal muscle. Figures 5-1 and 5-2 illustrate the linear relationship of the standard curve and the values gathered can be seen in Table 2. Moreover, this table contains the activity of the calpastatin and calpain gathered from each sample.

Finally, the calpastatin concentration was determined using a "sandwich" ELISA and the results may be seen in Table 3 and the standard curve is shown in Figure 7. No identifiable pattern was noticed with the dystrophic sample and non-dystrophic samples.

Discussion

Making a recipe is often more difficult than following one. Considering that separation and characterization of the calpain family at the 1g range has never been attempted before (Goll *et al.*, 2003), especially with human samples, designing a proper protocol was essential to the project. To establish the appropriate procedures for the columns was laborious, but eventually the right parameters were identified by another lab member, Marlon Taylor. As such, an initial phase of the project was to verify the accuracy and reliability of the protocol by running several non-dystrophic human samples to test separation of the various components. The hydrophobic hexyl column was specifically chosen because several areas on the calpain are largely hydrophobic, specifically in domain III and IV (Imajoh *et al.*, 1988 and Aoki *et al*., 1986) whereas calpastatin is largely unstructured and lacks such a structural feature. Additionally, the hexyl column was chosen because even autolyzed calpain, which is more hydrophobic than unautolyzed calpain, can be eluted quantitatively in an undenatured form, whereas other hydrophobic columns require denaturing conditions to remove autolyzed calpain. This was critical because one aspect of the project was to examine if there was autolysis within the dystrophic samples as a way of determining whether or not the calpain was activated. Unfortunately, this was not observed in the long-run but was a necessary component for understanding the differences between dystrophic and non-dystrophic muscle. Charge separation was the second step. The calpastatin moiety and calpain moiety were loaded on individual Source 15Q ion-exchange columns in order to bind the negatively charged surface residues. μ and m-Calpain separate at distinct salt concentrations due to the unique negative charge composition on each respective

surface—m-calpain requiring a higher salt concentration for elution while μ requires much less. Even though the calpastatin moiety was clearly divided from the calpain fractions with the first column, it was still passed through on a Source 15Q column in order to purify it further at a minimal loss of the protein. The profiles, as illustrated in Figures 2 and 3, clearly support the fact that the separation techniques were effective.

In an attempt to quantify the calpain amount in the homogenate, several ELISA procedures were developed and tested but proven to be unsuccessful. One main reason for this was that flow through from the hexyl column was used as a control in order to test for cross reaction. Every time an ELISA was attempted there seemed to be a high cross reaction with another protein in the mixture that could not be eliminated by various washing procedures including higher salt washes, changing antibodies, or using other fluorescent agents. The inconsistency within each sample was another factor which contributed to the elimination of the ELISA as a means to characterize m and μ-calpain in the mixture; therefore, Western blotting was the method of choice. Often western blots are used in a qualitative way to establish the presence of a given antigen, however, quantitative procedures are also used and this appeared to be best option. A human placental standard was used for μ-calpain while a bovine skeletal standard was used for m-calpain. The rationale behind using a bovine standard was that the m-calpain human placental standard degraded over time and purifying a new batch would have taken several weeks. Nevertheless, the bovine calpain had the same sequence recognized by the antibody (Ouali) so the calculations were not affected. While the ELISA was unsuccessful for calpain, it served well to identify calpastatin due to the unique properties of the "sandwich" ELISA which was employed. The idea of a coating the wells with an

antibody acts to bind the antigen initially served well to eliminate cross reaction and allowed for a more sensitive analysis.

The calpain results, along with the activity assays, did not indicate a correlation between dystrophic and non-dystrophic samples. However, this does not prove or disprove the hypothesis of increased calpain activity/amount within dystrophic muscle, but it is rather an indicator that larger sampling is needed. There are several factors to be considered in the future. While larger amounts of normal and dystrophic muscle would be preferred (Kent *et al*., 2005) it is unrealistic to expect that this can be achieved from human donors. Another variable that cannot be controlled with the current protocols is that we have no control over which samples are received. Even for normal muscle there are variations in age and condition of the donor (other pathologies etc). For the dystrophic muscle samples we have no control over which dystrophy affects(ed) the donors and how severe (i.e. at which stage) the disease had progressed. Many samples are post mortem but the cause of death is not defined. It may be difficult to satisfy all of these problems and new collection protocols need to be established. Given that such is possible, the next step in the project will be to repeat these analyses several times over for the same muscle extract in order to have enough data points to be able to see a pattern emerge. As alluded to above it is important to collect several muscle samples from the same donor, either normal or dystrophic, optimally from the same muscle and obtain sufficient data for significant statistical analyses and determine if there is a difference in a given dystrophy in calpain activity compared to control muscle. Currently this has proven to be difficult because we have had to rely upon the donor bank for limited quantities.

Figure Legend

Figure 1: Purification Flow Chart

The following flow chart serves as a general summary for the procedures necessary from beginning to end for the purification and characterization of the calpains and calpastatin.

Figure 2: Hexyl-TSK 650 Column Profile for Sample 59263

Elution profile of a muscle extract from patient 59263 using a 1.0x8.7cm hexyl-TSK 650 hydrophobic column at a flow rage of 10mL/hour . The column was equilibrated in 125 mM KCl, TEMA. The KCl concentration of the sample was adjusted to 125 mM before loading. Flushing was done with 125mM KCl, TEMA to remove unbound protein including calpastatin which was identified using an activity assay to find the percent inhibition (red line) and collect fractions 8 to 14. Flushing for μ- and m-calpain was done with 0.1% Brij-35, 1mM EDTA, 0.1% MCE and then identified using an activity assay to find the fluorescence (green line) in order to collect fractions 35 to 42. The absorbance of proteins at 280nm, as represented by the black line, was performed in order to monitor the general flux of protein loss or gain. The blue line represents the KCl concentration used throughout the procedure.

Figure 3: Source 15Q Calpain Column Profile for Sample 59263

The calpain fractions collected from the hydrophobic Hexyl-TSK 650 column were loaded onto a 0.5x10.7cm ion-exchange Source 15Q column at a flow rate of 10mL/hour in order to separate out μ and m calpain. After an equilibration using

TMEMA, the KCl concentration was gradually increased in order to elute the two, as can be seen by the black dotted line. μ-calpain was eluted off using 170mM KCl, TMEMA while m-calpain was eluted off using with 370mM KCl, TMEMA as can be seen by the two respective calpain activity assays performed per the blue line. The absorbance of proteins at 280nm, as represented by the doted red line, was performed in order to monitor the general flux of protein loss or gain.

Figure 4-1: μ-Calpain Homogenate Western Blot

Samples were loaded onto an 8-16% gradient SDS-PAGE gel and transferred onto PVDF membrane. The membranes were blocked using 1% BSA blocking solution. μ-Calpain was detected by incubation with 9A4 at 1:20,000 dilution in 1% BSA and APP labeled secondary antibody at 1:50,000 dilution of 1% BSA. Lanes 1-5 represent the standards from HP 37. The lanes contain (1) 100ng, (2) 50ng, (3) 25ng, (4) 12.5ng, and (5) 6.25ng of HP 37 standard. Samples (6) 59149-5, (7) 59263-5, (8) 59149-1, (9) 59149-8, and (10) 52499-1 were loaded at 20μg protein per lane.

Figure 4-2: μ-Calpain Standard Curve from Western Blot

Using a western blot and LabWorks program, the integrated optical density was found for each standard and plotted against the mass of the 80kDa (large) subunit. The percent 80kDa (large) subunit of the standards had to be accounted for by using a previously determined SDS-PAGE gel of the purified fraction (see Figure 5).

Figure 5-1: m-Calpain Homogenate Western Blot

Samples were loaded onto an 8-16% gradient SDS-PAGE gel and transferred onto PVDF membrane. The membranes were blocked using 1% BSA blocking solution. m-Calpain was detected by incubation with B2 at 1:100,000 dilution in 1% BSA and APP labeled secondary antibody at 1:50,000 dilution of 1% BSA. Lanes 1-5 represent the standards from BSP 80. The lanes contain (1) 100ng, (2) 50ng, (3) 25ng, (4) 12.5ng, and (5) 6.25ng of BSP 80 standard.

Figure 5-2: m-Calpain Standard Curve from Western Blot

Using a western blot and LabWorks program, the integrated optical density was found for each standard and plotted against the mass of the 80kDa (large) subunit. The percent 80kDa (large) subunit of the standards had to be accounted for by using a previously determined SDS-PAGE gel of the purified fraction.

Figure 6: Percentage of 80kDa Subunit from Gel for Human Placental Prep 37 and Bovine Skeletal Prep 80

Binding of the primary antibody occurs at the 80kDa subunit for both human and bovine calpain. As such, the percentage of each had to be determined using IOD by summing the 80kDa IOD band from each purified prep and dividing it by the total IOD throughout the lane. HP 37 and BSP 80 final fractions represent each gel lane, respectively. Both HP 37 and BSP 80 were loaded at 2µg of protein per lane in an 8-16% gradient SDS-PAGE gel and stained with Coomassie Brilliant Blue 250.

Figure 7: Calpastatin Standard Curve from ELISA

Pure human calpastatin was used as the standard for the ELISA by serially diluting it from 0.0156ng/µl to 0.00024ng/µl in 1% BSA blocking solution. This was only added after first coating the initial wells with 0.002µg/µl of mouse anti-human calpastatin antibody per well. The first and second antibodies used were 10µg/ml of human polyclonal made in chicken and Rockland rabbit anti-chicken-HRPO at 1:5,000, respectively. The samples were reacted with QuantaBlu fluorescent reagent and read on a TECAN plate reader using $\lambda_{Excitation}$ of 325nm and $\lambda_{Emission}$ of 420nm.

Figures

Figure 1

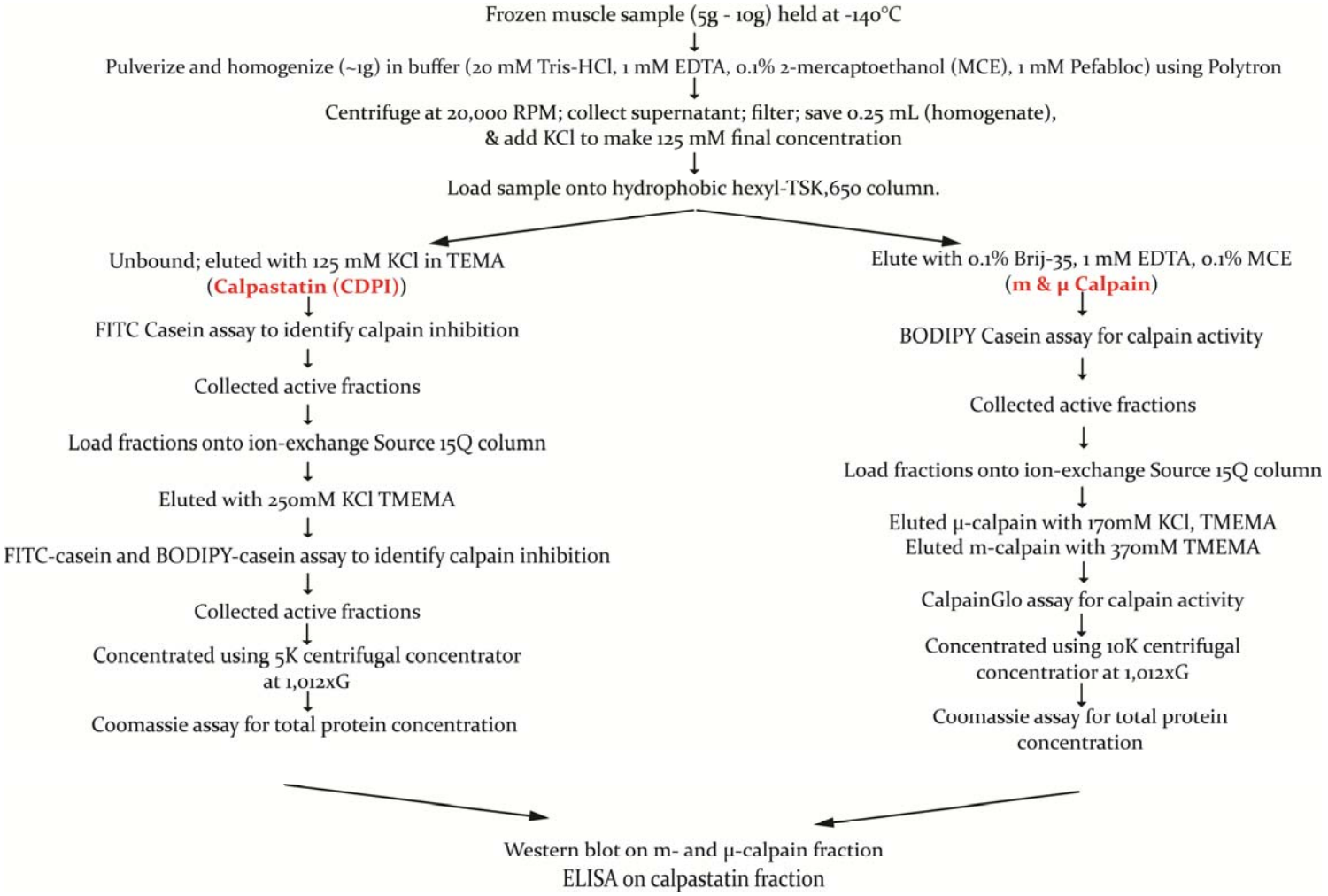


Figure 2

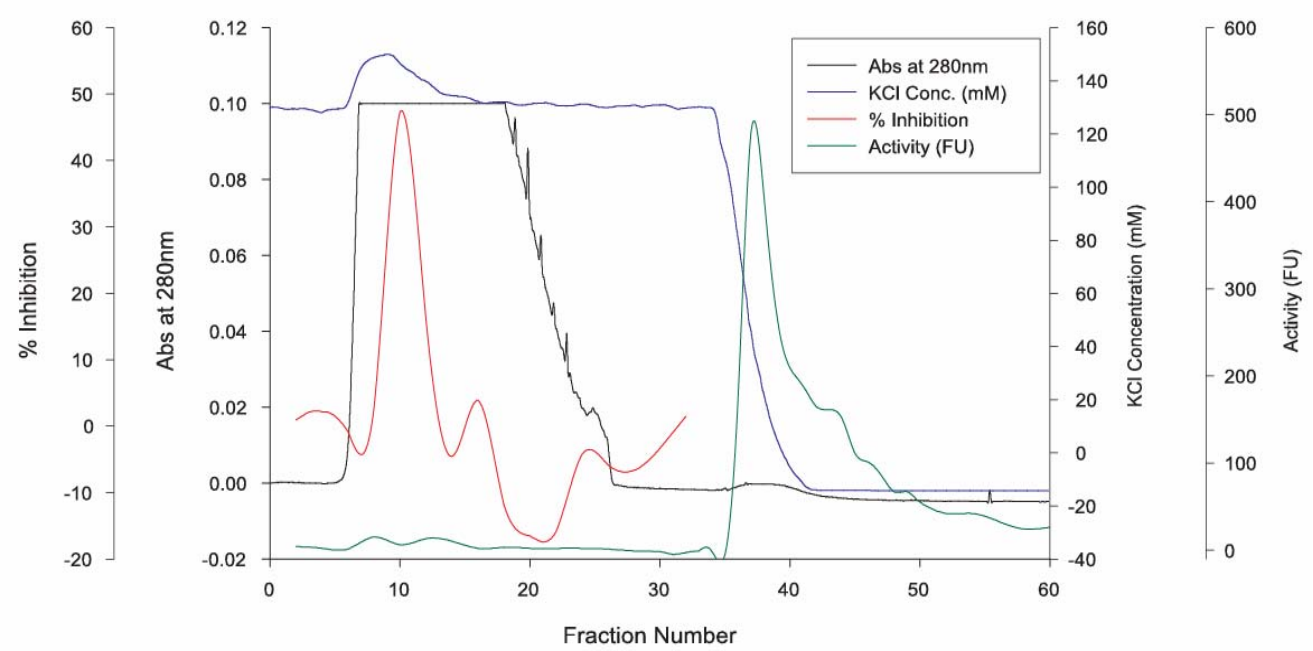


Figure 3

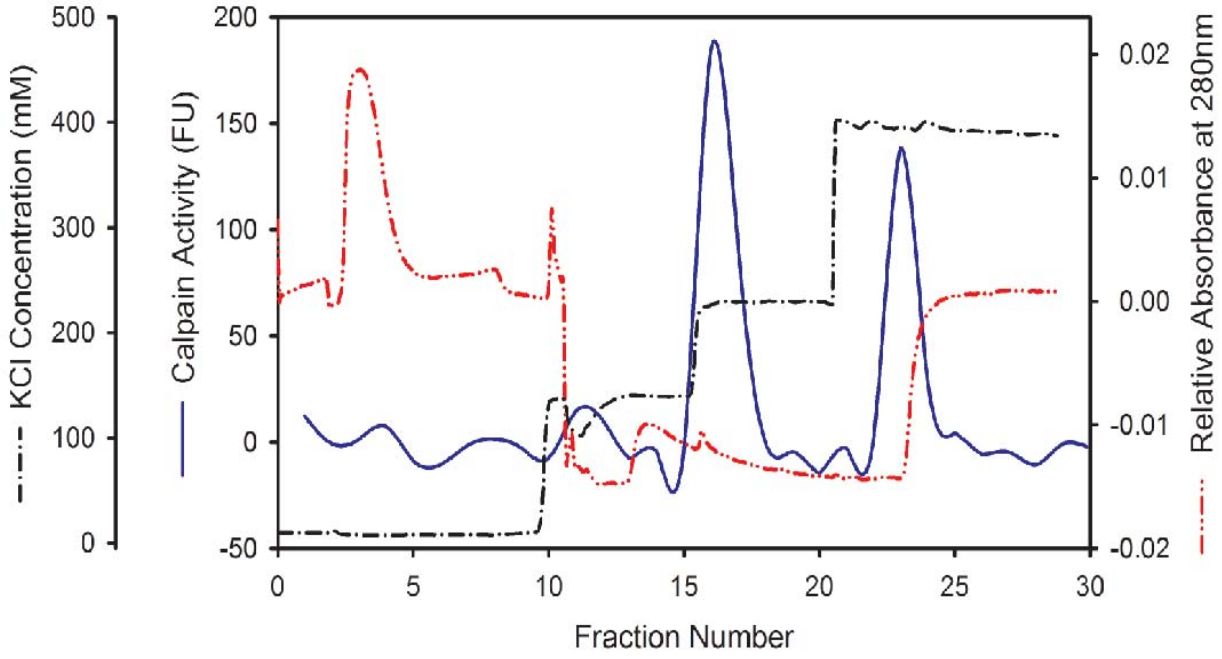
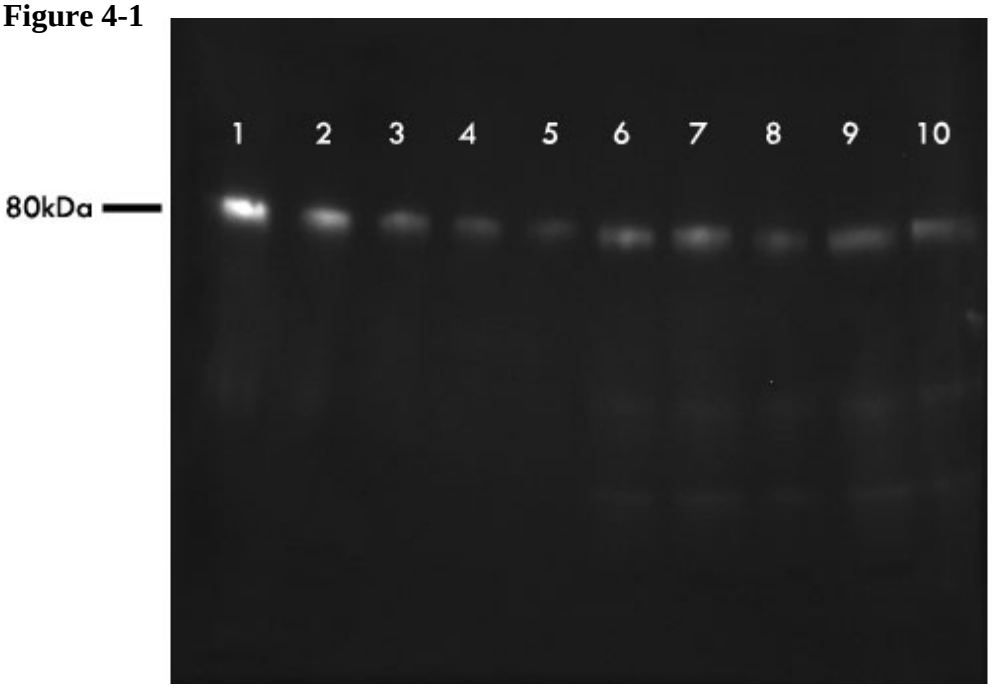


Figure 4-1



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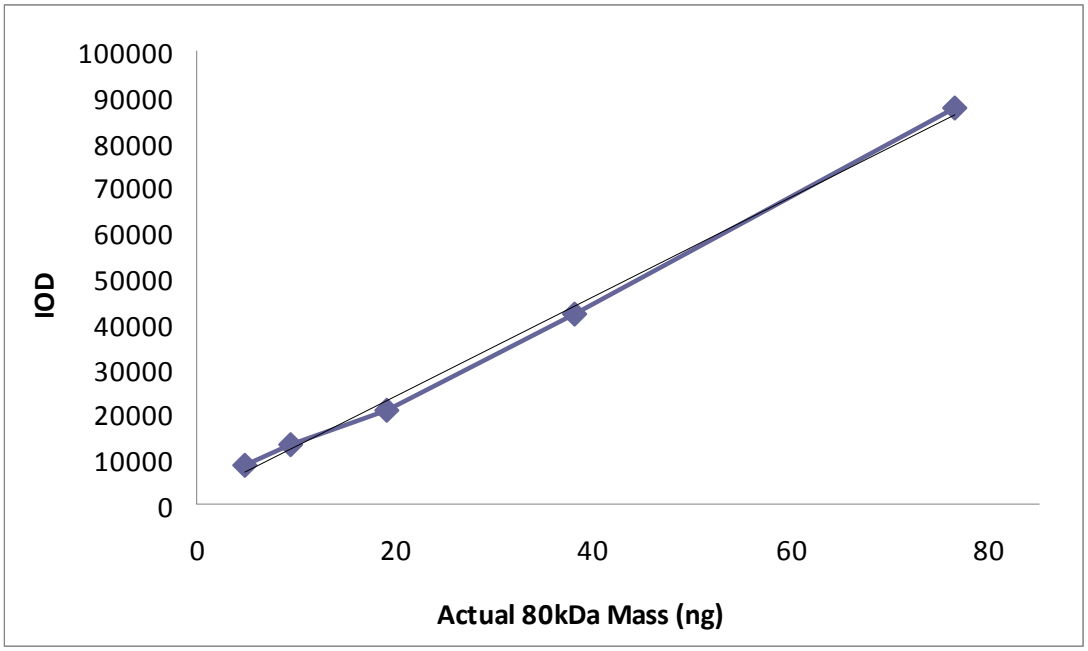

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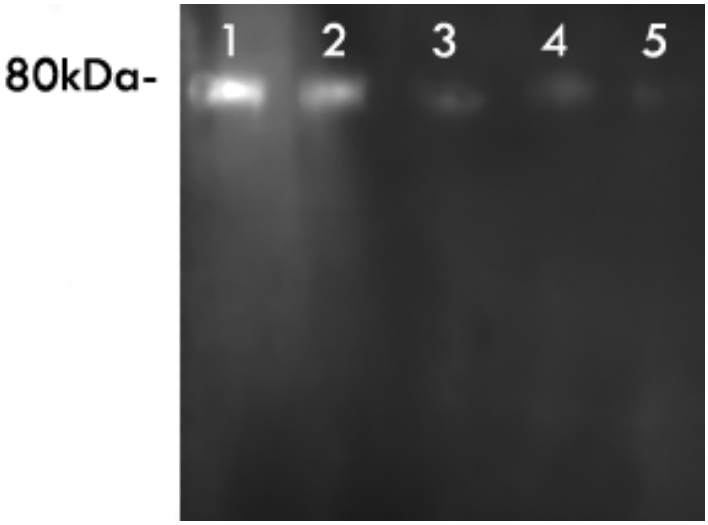

Figure 5-2

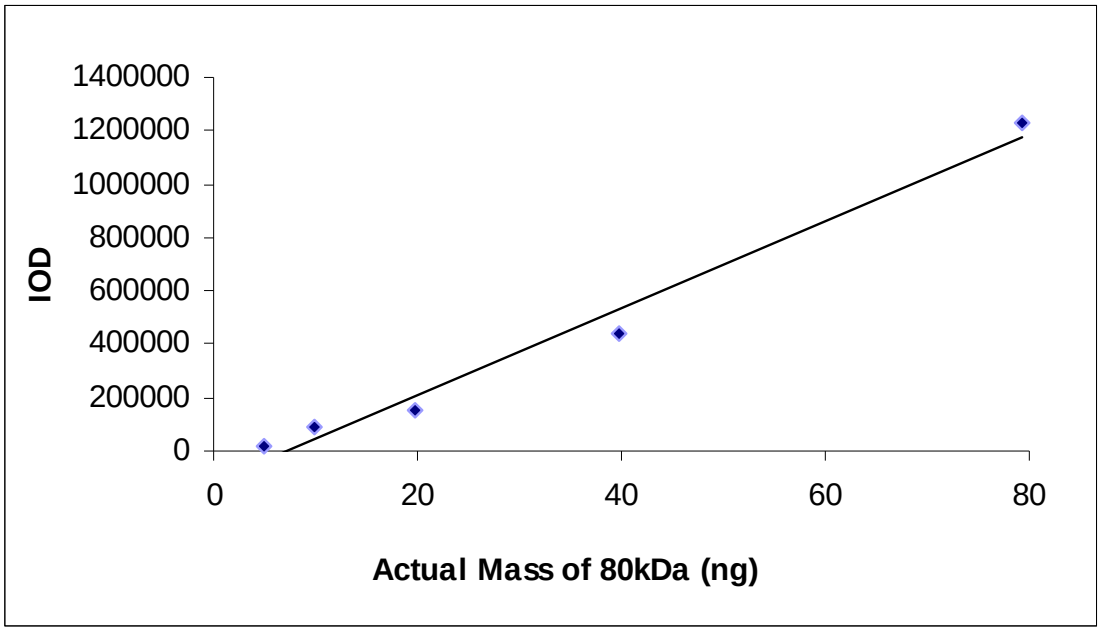

Figure 6

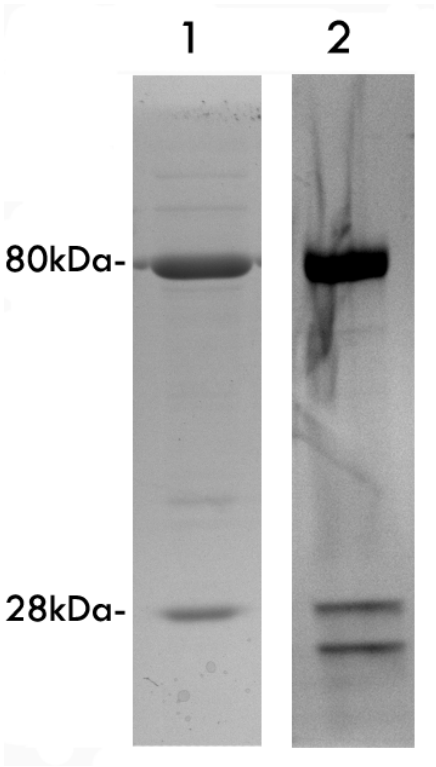

Figure 7

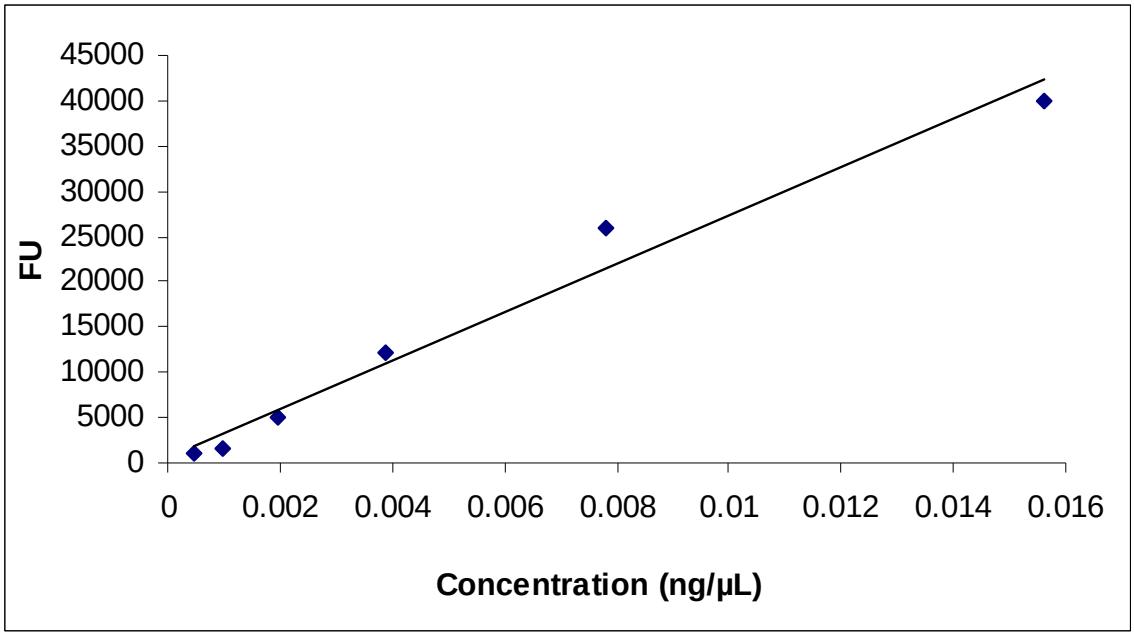

Table Legend

Table 1

Each sample gathered was given a unique identification number and then pulverized into several smaller pieces that can be represented by the notation following the dash. Limited information was given for each patient such as age, gender, and the tissue type. Every aliquot was weighed before homogenizing and then the total protein extracted was assayed. The soluble protein per wet tissue was determined by dividing the latter by the former.

Table 2

From each Western blot performed, the IOD of the 80kDa band was quantified from the standard curve and then divided by the total protein extracted in the initial homogenate. The activity assays were performed and serve to illustrate the relative amount of activity for calpastatin and calpain with regards to the total protein extracted and the total wet tissue.

Table 3

While the attempts at quantifying the calpains using ELISA procedures was unsuccessful due to excessive cross reaction, the special case of a "sandwich" ELISA seemed to yield positive results.

Tables

Table 1: General Information for Each Sample

Sample	Gender	Age	Tissue Type	Wet Tissue Weight (mg)	Total Protein Extracted (mg)	Soluble Protein per Wet Tissue (µg/mg)
61658-3	M	48	Myotonic Dystrophy	1007.4	56.5	56.1
61658-5	M	48	Myotonic Dystrophy	1073.1	58.4	54.4
59149-7	F	61	Dystrophic	1253	41.2	32.9
59149-5	F	61	Dystrophic	1509	58.4	38.7
59149-1	F	61	Dystrophic	1130	47.5	42.0
59149-8	F	61	Dystrophic	1450	38.6	26.6
62499-2	M	52	Limb-Girdle Dystrophy	1518	6.9	4.5
62499-1	M	52	Limb-Girdle Dystrophy	1122	4.2	3.7
59263-1	M	60	Non-Dystrophic	1085.5	46.2	42.6
59263-5	M	60	Non-Dystrophic	811	43.5	53.6
54734-1	F	44	Non-Dystrophic	1194	57.2	47.9
59303-10	M	57	Non-Dystrophic	1122	47	41.9
59303-12	M	57	Non-Dystrophic	1054.8	54	51.2

Table 2: Western Blot and Activity Assay Results

Sample	µ-CDP 80kDa/Total Protein Extracted (ng/mg)	m-CDP 80kDa/Total Protein Extracted (ng/mg)	FU Inhibit/µg Protein	FU Inhibit/mg Wet Tissue	FU Inhibit/µg Protein	FU Inhibit/mg Wet Tissue
59149-7	0.04570	0.06913	21	706	17.11	563
59149-5	0.03224	0.04877	101	3977	8.71	337
59149-1	0.03964	0.05996	15.8	661	15.3	641
59149-8	0.04878	0.07379	156	4133	20.5	545
62499-2	0.27290	0.41279	24	108	44	203
62499-1	0.44834	0.67815	28	360	50.14	652
59263-1	0.04076	0.06165	16.6	702	31.5	1340
59263-5	0.04329	0.06548	110	5845	20	1062
54734-1	0.03292	0.04979	8	387	17.78	851

Table 3: ELISA Calpastatin Results

Sample	Homogenate [CDPI] (ng/µL)
61658-3 Pt.1	0.0012815
61658-5 Pt.1	0.0012863
59303-10 Pt.2	0.0011062
59303-12 Pt.2	0.0010733

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