Preface

The purpose of *Calpain Methods and Protocols* is quite straightforward: it is to present the actual experimental methods used in many different laboratories for the study of calpain. It will provide the vital experimental detail, and the discussion of possible pitfalls, for which the standard journals no longer provide space. This will make it as easy as possible for investigators interested in calpain to adopt established methods without repeating old mistakes, and to adapt and apply these methods in novel approaches to the many outstanding calpain questions.

These questions range from purely biochemical problems of protein structure and enzyme regulation at the molecular level, through large areas of cell biology, to applied and clinical aspects of calpain function in human disease. Within this panoply of topics, a wide range of investigators will find many fascinating and as yet unanswered questions about calpain. *Calpain Methods and Protocols* will provide instant access to many essential techniques, while saving them the time and effort involved in developing a new method.

In addition to questions relating to the normal physiological roles of the calpains, there is considerable evidence that inappropriate calpain activity may have pathological effects in many tissues, for example, following ischemia. This provides a major stimulus for the development of specific calpain inhibitors for therapeutic purposes, and for the development of methods to evaluate such inhibitors.

The two most studied calpains, μ- and m-calpain, and the inhibitor calpastatin, are present in essentially every cell of a mammalian organism, although in varying relative amounts. This variation is itself a very interesting problem. Related enzymes have been described in birds, crustaceans, and insects, but not in plants or bacteria. In addition to the well characterized calpains, there is a steadily increasing family of calpain-related genes, many of them apparently tissue-specific, that have been characterized as cDNA sequences, but not yet in most cases as proteins.

Perhaps the most challenging aspect of the calpains, is that we still do not know what they do. There are fundamental difficulties in principle in proving that cleavage of a given target protein inside a cell arises entirely and

exclusively from the action of calpain; and it has proved equally difficult to find out how a particular calpain molecule is activated and directed to choose a given substrate at a given time, and how it escapes inhibition by calpastatin. The existence of many other intracellular proteases, such as caspases and the proteasome, as well as the absence of absolutely specific calpain inhibitors, add to the difficulty of designing unambiguous experiments. These obstacles have been widely recognized, and methods still need to be found to surmount them. We feel that this collection of established calpain methods will provide a solid foundation for future work.

Calpain Methods and Protocols is organized into four sections, though not every chapter can be so rigorously classified. In Section I are gathered methods of purification of the calpains, calpastatin, and the calpain activator protein, from sources ranging from beef heart and lobsters to fruit flies and *E. coli*. Section II includes analytical techniques, such as casein zymography, immunofluorescence, and calpain activity assays, for both in vivo and in vitro use, although further calpain assays are found in several other chapters. Section III presents methods of calpain research applied to specific systems, often in connection with hypoxia or other injury. These systems include neural tissue, kidney, liver, the eye, and membrane fusion in muscle and erythrocytes. Finally, in Section IV are considered some specific substrates that have been proposed for the calpains.

Several recent reviews and monographs provide excellent summaries of the theoretical and historical calpain background, and of the major outstanding problems in the calpain field. The authors in this book were therefore asked to omit all but the briefest rationale of their work, and to confine themselves simply to: "this is how we do it." Given the difficulties of the topic, controversies are bound to arise, and no attempt has been made to resolve them here. At least two examples might be mentioned: there is an extensive and contradictory literature on the physiological relevance of the interplay of calpain and protein kinase C, and another contradictory set relating to the role of calpain in apoptosis. The chapter by Dr. Shea provides methods of looking at PKC, and the chapter by Drs. Squier and Cohen provides methods of assessing apoptosis, while remaining carefully neutral on the issue of 'relevance.' We hope that chapters such as these will help future investigators to resolve these important questions.

For the purposes of *Calpain Methods and Protocols*, some methods are either too specialized or too vast for inclusion. Cloning is barely mentioned, except for the chapter by Dr. Sorimachi on calpain 3, or p94, the best known of the newer calpains, even though these methods have made major contributions to the study of calpain. X-ray crystallography and methods

relating to transgenic mice are not described, even though these areas are also likely to make major contributions to the understanding of calpain in the near future. In contrast, some methods are so widespread, for example gel electrophoresis and Western blotting, that they have been taken as known, and the details omitted to save space.

A simple search for the word 'calpain' in the Medline data base in October 1999 yielded over 2200 references, all of which I cannot claim to have read, and the list of substrates proposed for the calpains is almost as long. I have attempted to gather a wide and representative set of methods, while avoiding direct duplication of a given topic, and apologize for any real or perceived omissions. It is important to acknowledge the prompt and willing response of all those who were approached. If their wide experience distilled here is able to help other investigators to solve the various calpain problems, *Calpain Methods and Protocols* will have proved its worth.

John S. Elce

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A Simple Protocol for Separation and Assay of μ-Calpain, m-Calpain, and Calpastatin From Small Tissue Samples

Jan-Olof Karlsson

1. Introduction

In many cases separation of calpains and calpastatin has to be performed with a relatively small amount of tissue, so that large-scale and complicated multiple-column procedures are not appropriate. In designing a method suitable for small amounts of tissue, it is important that the extraction and separation methods should be rapid in order to avoid postmortem changes in the subcellular distribution of the enzymes, and also to avoid autolysis or degradation of the enzymes themselves. The method presented here uses relatively simple and inexpensive equipment and reagents, although the assay of calpain activity is greatly simplified by access to a fluorometer with the ability to read microtiter plates.

The procedure includes rapid perfusion of the tissue with EDTA before removal of the sample, homogenization, and centrifugation, followed by a single step of hydrophobic interaction chromatography on a column of phenyl-Sepharose *(1)*. The method separates μ-and m-calpain from each other and from the endogenous inhibitor, calpastatin, and requires only 1 d for a small sample. At this stage, μ-calpain, m-calpain, and calpastatin activity may be assayed separately. Both the μ-calpain-containing and the calpastatin-containing fractions still contain many other proteins, while the m-calpain is relatively pure. If necessary, further purification of μ-calpain and m-calpain is performed by means of ion-exchange chromatography on a Mono Q column *(2)*. This procedure results in electrophoretically pure preparations of μ-calpain and m-calpain.

2. Materials

1. Casein (Hammersten preparation) (Merck, Darmstadt, Germany).
2. DTT, (Boehringer-Mannheim, Mannheim, Germany): 100 mM in water, stored at –20°C in small tightly capped aliquots; after thawing, the DTT solution is used only for 1 d.
3. Phenyl-Sepharose CL-4B, Sephadex G-25, and the FPLC anion column (Mono Q; HR 5/5) were obtained from Pharmacia (Uppsala, Sweden). Other chemicals were of analytical grade.
4. The synthetic calpain substrate succinyl-Leu-Tyr-7-amino-4-methylcoumarin (Succ-Leu-Tyr- AMC) was obtained from Bachem (Bubendorf, Switzerland) AG *(3)*. A 1 mM solution is prepared in 20 mM Tris-HCl, pH 7.5, 3% (v/v) DMSO, 1 mM DTT, 3 mM sodium azide (NaN$_3$).
5. White microtiter plates (Microfluor) were obtained from Dynatech (Chantilly, VA).
6. Homogenization buffer: 10 mM sodium borate, pH 8.0, mM EDTA, 3 mM NaN$_3$ (*see* **Note 1**).
7. Equilibration buffer for the phenyl-Sepharose column: 10 mM sodium borate, pH 7.5, 500 mM NaCl, 1 mM EDTA, 3 mM NaN$_3$.
8. Starting buffer for the phenyl-Sepharose column: 10 mM borate, pH 7.5, 100 mM NaCl, 1 mM EDTA, 3 mM NaN$_3$.
9. Final buffer for the phenyl-Sepharose column: 10 mM sodium borate, pH 8.0, 1 mM EDTA, 3 mM NaN$_3$.
10. Buffer for the Mono Q column: 20 mM sodium borate, pH 8.0, 0.1 mM EDTA, 3 mM NaN$_3$.
11. Perkin-Elmer (Norwalk, CT) LS 50B luminescence spectrometer with a microtiter plate attachment.

3. Method

3.1. Homogenization

1. If possible, it is an advantage to perfuse the experimental animal with ice-cold 150 mM NaCl, 5 mM EDTA (pH adjusted to 8.0 with NaOH) via the heart ventricle before dissection of tissue (*see* **Note 2**).
2. Take tissue samples of approximately 0.5 g (wet weight) (*see* **Note 3**).
3. Homogenize the tissue in a loose-fitting Teflon-to-glass homogenizer (1500 rpm, ~6 strokes) with 5 mL ice-cold homogenization buffer. For larger samples, use 10 vol of homogenization buffer.
4. Centrifuge the homogenate at 100,000g at 4°C for 1 h.
5. Decant the supernatant and add solid NaCl to a final concentration of 500 mM.
6. Permit the sample to warm to room temperature, and adjust to pH 7.0 with NaOH or HCl. All subsequent work is carried out room temperature with minimum delay.

3.2. Separation on Phenyl-Sepharose *(See* **Note 4***.)*

1. Apply the sample at a rate of 50 mL/h to a column (0.8 × 13 cm, **Note 5**) of phenyl-Sepharose (~26 mL) that has been equilibrated at room temperature in the equilibration buffer. If calpastatin is to be purified or assayed, the flow-through

fractions containing protein not bound to the phenyl-Sepharose column should be frozen immediately.

2. Wash the column thoroughly with equilibrating buffer until a stable $A_{280\ nm}$ baseline is obtained.
3. Elute m-calpain and μ-calpain with a linear gradient of increasing pH and decreasing salt concentration in borate buffer *(1)*. Use 150 mL of starting buffer and 150 mL of final buffer.
4. Collect fractions of 3–6 mL and analyze aliquots for proteolytic activity.

The enzymes are isolated by virtue of their different hydrophobic characteristics. Using a phenyl-Sepharose column with the conditions described, m-calpain is eluted first, at 50–80 m*M* NaCl, pH 7.5 (**Fig. 1**), as a single broad peak, with very little contaminating protein. μ-Calpain is eluted at 10–25 m*M* NaCl, pH 7.9, as one or two peaks, just before and partly overlapping a major peak of hydrophobic protein at the end of the gradient. A further major peak of more firmly bound protein may be eluted with distilled water or 1 m*M* NaOH, but no additional calcium-activated proteolytic activity is detected.

3.3. Further Purification on Mono Q

1. Equilibrate the Mono Q column (HR 5/5) in Mono Q buffer at a rate of 1 mL/min.
2. Apply the μ- or m-calpain sample (3–20mL) from the phenyl-Sepharose separation step.
3. Wash the column with buffer and elute at 1 mL/min until a stable $A_{280\ nm}$ baseline is obtained.
4. Elute the calpains with an appropriate linear gradient of NaCl concentration with a total volume of 40 mL. For μ-calpain the gradient should be from 0 to ~300 m*M* NaCl; for m-calpain the gradient should be from 0 to 500 m*M* NaCl. Under these conditions, μ-calpain is eluted at 185–200 m*M* NaCl and m-calpain at 380 m*M* NaCl (**Figs. 2** and **3**). Calpastatin (always applied separately) elutes at ~220 m*M* NaCl *(2)*.
5. Collect fractions of 1 mL and assay aliquots for proteolytic activity (*see* **Note 6**).
6. For Western blotting, the enzyme-containing fractions can be desalted on a small Sephadex G-25 column (PD-10, Pharmacia) according to the manufacturer's instructions. The columns are equilibrated in 0.1% (w/v) SDS containing 0.01 m*M* EDTA (allowing for the subsequent concentration by lyophilization). The desalted proteins are immediately lyophilized and subjected to SDS-PAGE.

3.4. Assay of Proteolytic Activity with Synthetic Peptide Substrate
(See **Note 7.***)*

1. Mix 25-μL samples of enzyme solution (tissue extract supernatants or fractions from the phenyl-Sepharose or Mono Q columns), with 25 μL of 20 m*M* Tris-HCl, pH 7.5, 3% (v/v) DMSO, 3 m*M* NaN_3 in a white microtiter plate.
2. Preincubate for 10 min at room temperature.
3. Add 100 μL of 1 m*M* Succ-Leu-Tyr-AMC, 1 m*M* DTT, 3%DMSO and final net
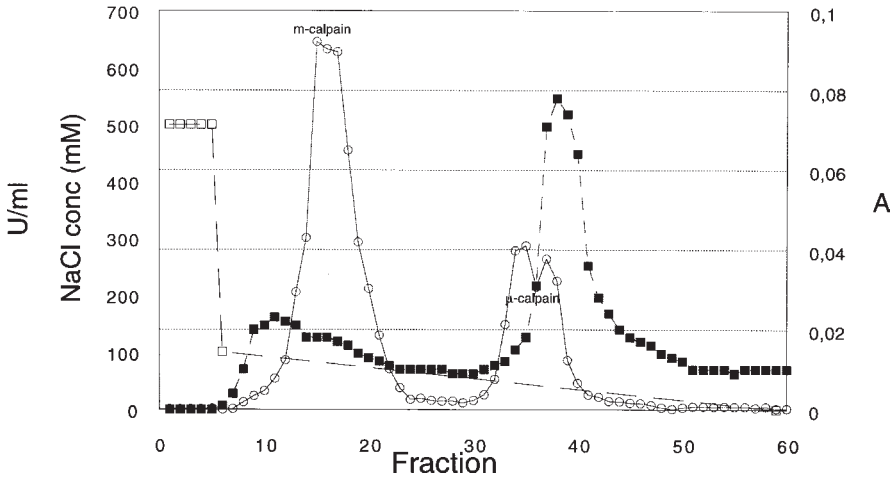
Fig. 1. Separation of calpains on phenyl-Sepharose. The supernatant from 30 g (wet weight) of rabbit lungs was applied to the column and the nonbinding proteins were saved and frozen for assay of calpastatin activity. The gradient of decreasing NaCl concentration was applied at fraction 1 and ended at fraction 60. ■, absorbance at 280 nm; ○, proteolytic activity; □, NaCl concentration.

concentrations of 5 mM EDTA or 1 mM CaCl$_2$ *(3)*.

4. Ca^{2+}-dependent proteolysis is determined with 1 mM Ca^{2+} in excess of the EDTA concentration. Ca^{2+}-independent proteolysis is measured as a control with 5 mM EDTA in the incubation mixture instead of Ca^{2+}.
5. Set the spectrometer with an excitation wavelength of 380 nm (slit 5 nm) and an emission wavelength of 460 nm (slit 10 nm).
6. Observe hydrolysis of the substrate at intervals of 2–3 min at a rate that is linear for at least 2 h (*see* **Note 8**).

3.5. Microtitre Plate Calpain Assay with Casein and Fluorescamine
(See **Note 9.**)

1. Preparation of substrate *(1)*. Suspend 1.25 g of casein in ~500 mL of water and heat to 80°C while stirring. Adjust pH to 9.5 with 1 *M* KOH, and allow to cool. Add 1 mL of 100 mM EDTA, 10 mL of 300 mM NaN$_3$, 7.5 g of KCl and adjust pH to 7.5 with 0.5 *M* boric acid. Dilute to 1000 mL, filter and freeze at –20°C in 10-mL portions.
2. Add DTT to 1 mM and CaCl$_2$ to 2 mM to an aliquot of casein substrate solution.
3. Add 80 μL of enzyme sample to a 96-well microtiter plate.
4. Add 200 μL of casein substrate solution, 1 mM DTT, 2 mM Ca^{2+}, or 5 mM EDTA, and incubate for 1–2 hours or overnight at room temperature.
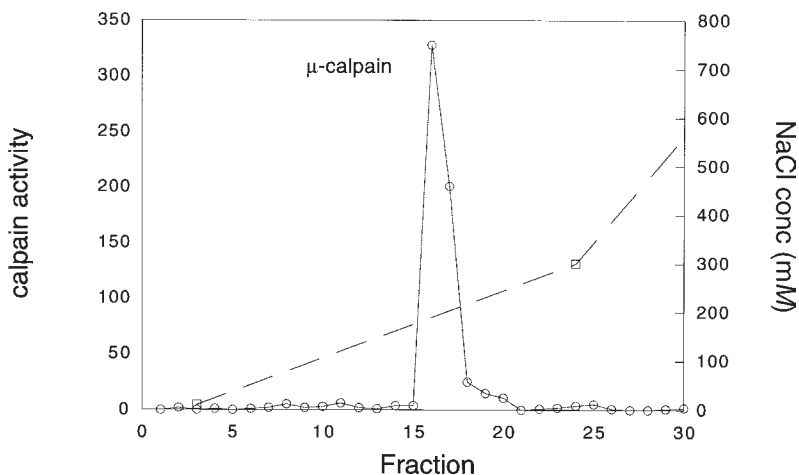
Fig. 2. Purification of μ-calpain on Mono Q. The peak of μ-calpain activity from a phenyl-Sepharose column was applied to the Mono Q column. A gradient from 10 to 300 mM NaCl was applied to elute μ-calpain activity at ~200 mM NaCl. ○, proteolytic activity; □, NaCl concentration.

5. Add 40 μL of 40% TCA to stop the proteolysis. Centrifuge the microtiter plates at 750g for 20 min.
6. Transfer 30 μL of the TCA supernatants (*see* **Note 10**) to a white microtiter plate, and add 225 μL of 0.5 M sodium borate, pH 10.
7. Add 30 μL fluorescamine (1 mg/mL in acetone) and mix rapidly *(4)* with the pipette.
8. Measure the fluorescence within 40 min at an excitation wavelength at 390 nm (slit 15 nm) and at an emission wavelength of 480 nm (slit 20 nm). Subtract appropriate zero time values (TCA added before the sample; controls with 5 mM EDTA). Use a standard of 0–2 mM glutamate.

3.6. Assay of Calpastatin

1. Heat samples (0.2–0.5 mL) of homogenates, supernatants, or fractions containing protein not bound to the phenyl-Sepharose column at 96°C for 10 min to destroy endogenous proteolytic activity *(5)*.
2. Centrifuge at 10,000g for 5 min and freeze at –20°C.
3. Thaw the heated samples and centrifuge again (*see* **Note 11**).
4. Mix aliquots of the heated samples (0.5, 1, 2, 5, 10, 20, and 50 μL) with a fixed amount of μ- calpain (*see* **Note 12**) and incubate with substrate (synthetic peptide or casein) and 1 mM Ca^{2+} as described above.
5. Plot the observed residual proteolytic activity against the amount of added calpastatin sample. Calculate the inhibitory capacity from the straight portion of the titration curve *(5)*. 1 unit of calpastatin is defined as that which inhibits 1 unit of μ-calpain under the described conditions (*see* **Note 13**).
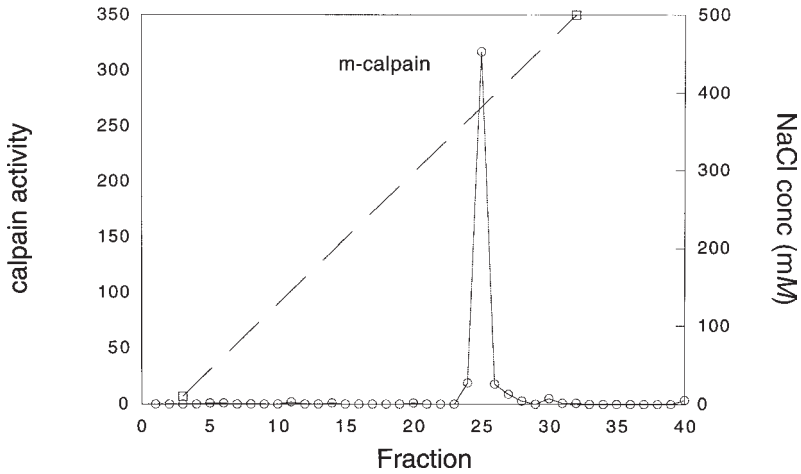
Fig. 3. Purification of m-calpain on Mono Q. The peak of m-calpain activity from a phenyl-Sepharose column was applied to the Mono Q column, followed by a gradient from 10 to 500 m*M* NaCl, eluting m-calpain activity at ~400 m*M* NaCl. ○, proteolytic activity; □, NaCl concentration.

4. Notes

1. No additional salt is included in the homogenization buffer, in order to decrease possible binding of calpains to membranes. 1 m*M* DTT and 0.1 m*M PMSF* may be added to the homogenization solution, although in our hands no improvement of the separation has been seen with these additions. By contrast, it may be an advantage to delay addition of DTT until assay of the partially purified enzyme, since this may reduce the activity of other thiol proteases in the crude extracts.

2. Fresh tissue from nonperfused animals may be used, but perfusion of the animal removes most of the contaminating blood and Ca^{2+} from the tissue and also chills the samples.

3. The protocol works with relatively small amounts of tissue, normally from 0.5 to 30 g wet weight. Fresh tissue is preferable but samples may be stored in sealed vials at –80°C. We have successfully analyzed brain samples stored frozen for 2 yr or more.

4. It is essential that the hydrophobic interaction chromatography is carried out between 20 and 25°C. At lower temperatures the hydrophobic interaction with the phenyl-Sepharose is significantly decreased, which will affect the elution pattern.

5. For larger amounts of sample (up to 30 g wet weight) the column size should be increased to 0.8 × 30 cm. In this case the column may be washed overnight with a flow rate of 10–30 mL/h to obtain a stable baseline before starting the gradient.

6. Fractions obtained from the phenyl-Sepharose column containing μ-calpain or m-calpain may be stored for long periods (several months) at 4°C in tightly

capped tubes with no loss of activity. Fractions containing calpastatin should be frozen immediately. Pure fractions of μ-calpain or m-calpain obtained after the separation on the Mono Q column are less stable and they should be used within a week when stored at 4°C. They can be stored indefinitely in the presence of 50% glycerol at –70°C. Freezing of enriched or pure calpain fractions in the buffer results in the loss of most (>75%) of the enzymatic activity.

7. Both this assay, and the following assay with casein substrate, can be scaled up for use with a conventional fluorometer, without the use of a microtiter plate attachment.

8. Zero time values are subtracted from each individual well. Standard curves with AMC in the appropriate buffer are used to express the data in picomoles of AMC formed per minute and per milliliter of enzyme solution (unit per milliliter). Addition of a known amount of AMC to the complete incubation mixture gives a fluorescence recovery better than 95%.

9. Do not use Tris or other buffers containing amino groups in this assay, as these groups react with fluorescamine.

10. For higher signals, more of the TCA supernatant may be used, but the pH of the final mixture should be checked (pH 10).

11. For samples containing a substantial amount of low molecular weight material, which may disturb the assay, it is an advantage to perform a buffer exchange on a small gel filtration column. A 2.5-mL portion of the supernatant is applied to a small Sephadex G-25 column (PD-10, Pharmacia) equilibrated in 3 m*M* sodium borate buffer, pH 8.0, 1 m*M* NaN$_3$, 0.1 m*M* EDTA, 1 m*M* DTT.

12. Make a preliminary titration first to determine a suitable amount of μ-calpain for the particular assay.

13. It is usual to determine the volume of calpastatin which gives 50% inhibition of the enzyme and then to make the calculations.

References

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