

## **A Simple Protocol for Separation and Assay of $\mu$ -Calpain, m-Calpain, and Calpastatin From Small Tissue Samples**

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### **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  $\mu$ - and m-calpain from each other and from the endogenous inhibitor, calpastatin, and requires only 1 d for a small sample. At this stage,  $\mu$ -calpain, m-calpain, and calpastatin activity may be assayed separately. Both the  $\mu$ -calpain-containing and the calpastatin-containing fractions still contain many other proteins, while the m-calpain is relatively pure. If necessary, further purification of  $\mu$ -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  $\mu$ -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  $-20^{\circ}\text{C}$  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 ( $\text{NaN}_3$ ).
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  $\text{NaN}_3$  (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  $\text{NaN}_3$ .
8. Starting buffer for the phenyl-Sepharose column: 10 mM borate, pH 7.5, 100 mM NaCl, 1 mM EDTA, 3 mM  $\text{NaN}_3$ .
9. Final buffer for the phenyl-Sepharose column: 10 mM sodium borate, pH 8.0, 1 mM EDTA, 3 mM  $\text{NaN}_3$ .
10. Buffer for the Mono Q column: 20 mM sodium borate, pH 8.0, 0.1 mM EDTA, 3 mM  $\text{NaN}_3$ .
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  $4^{\circ}\text{C}$  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 ( $0.8 \times 13$  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  $A_{280\text{ nm}}$  baseline is obtained.
3. Elute m-calpain and  $\mu$ -calpain with a linear gradient of increasing pH and decreasing salt concentration in borate buffer (**I**). 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 mM NaCl, pH 7.5 (**Fig. 1**), as a single broad peak, with very little contaminating protein.  $\mu$ -Calpain is eluted at 10–25 mM 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 mM 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  $\mu$ - or m-calpain sample (3–20 mL) from the phenyl-Sepharose separation step.
3. Wash the column with buffer and elute at 1 mL/min until a stable  $A_{280\text{ nm}}$  baseline is obtained.
4. Elute the calpains with an appropriate linear gradient of NaCl concentration with a total volume of 40 mL. For  $\mu$ -calpain the gradient should be from 0 to ~300 mM NaCl; for m-calpain the gradient should be from 0 to 500 mM NaCl. Under these conditions,  $\mu$ -calpain is eluted at 185–200 mM NaCl and m-calpain at 380 mM NaCl (**Figs. 2 and 3**). Calpastatin (always applied separately) elutes at ~220 mM 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 mM 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- $\mu$ L samples of enzyme solution (tissue extract supernatants or fractions from the phenyl-Sepharose or Mono Q columns), with 25  $\mu$ L of 20 mM Tris-HCl, pH 7.5, 3% (v/v) DMSO, 3 mM  $\text{NaN}_3$  in a white microtiter plate.
2. Preincubate for 10 min at room temperature.
3. Add 100  $\mu$ L of 1 mM Succ-Leu-Tyr-AMC, 1 mM DTT, 3% DMSO and final net

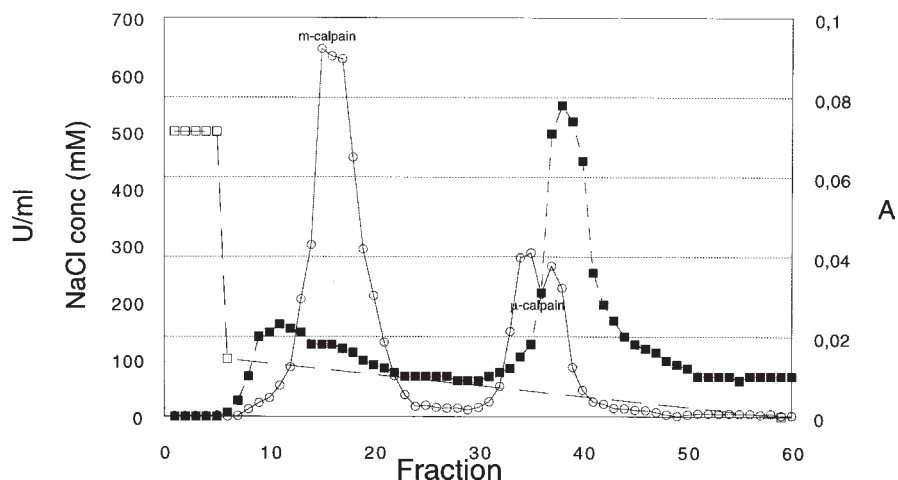


Fig. 1. Separation of calpains on phenyl-Sepharose. The supernatant from 30 g (wet weight) of rabbit lungs was applied to the column and the nonbinding proteins were saved and frozen for assay of calpastatin activity. The gradient of decreasing NaCl concentration was applied at fraction 1 and ended at fraction 60. ■, absorbance at 280 nm; ○, proteolytic activity; □, NaCl concentration.

concentrations of 5 mM EDTA or 1 mM  $\text{CaCl}_2$  (3).

4.  $\text{Ca}^{2+}$ -dependent proteolysis is determined with 1 mM  $\text{Ca}^{2+}$  in excess of the EDTA concentration.  $\text{Ca}^{2+}$ -independent proteolysis is measured as a control with 5 mM EDTA in the incubation mixture instead of  $\text{Ca}^{2+}$ .
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  $\text{NaN}_3$ , 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  $\text{CaCl}_2$  to 2 mM to an aliquot of casein substrate solution.
3. Add 80  $\mu\text{L}$  of enzyme sample to a 96-well microtiter plate.
4. Add 200  $\mu\text{L}$  of casein substrate solution, 1 mM DTT, 2 mM  $\text{Ca}^{2+}$ , or 5 mM EDTA, and incubate for 1–2 hours or overnight at room temperature.

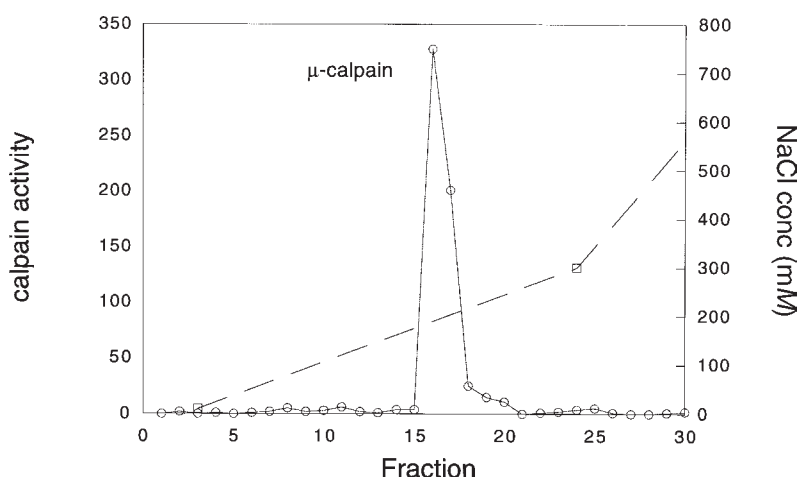


Fig. 2. Purification of  $\mu$ -calpain on Mono Q. The peak of  $\mu$ -calpain activity from a phenyl-Sepharose column was applied to the Mono Q column. A gradient from 10 to 300 mM NaCl was applied to elute  $\mu$ -calpain activity at  $\sim$ 200 mM NaCl. ○, proteolytic activity; □, NaCl concentration.

5. Add 40  $\mu$ L of 40% TCA to stop the proteolysis. Centrifuge the microtiter plates at 750g for 20 min.
6. Transfer 30  $\mu$ L of the TCA supernatants (*see Note 10*) to a white microtiter plate, and add 225  $\mu$ L of 0.5 M sodium borate, pH 10.
7. Add 30  $\mu$ L 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  $-20^{\circ}\text{C}$ .
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  $\mu$ L) with a fixed amount of  $\mu$ -calpain (*see Note 12*) and incubate with substrate (synthetic peptide or casein) and 1 mM  $\text{Ca}^{2+}$  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  $\mu$ -calpain under the described conditions (*see Note 13*).

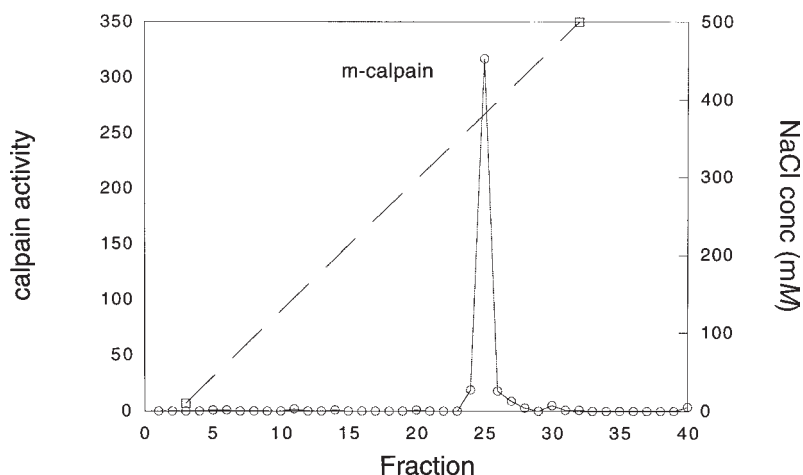


Fig. 3. Purification of m-calpain on Mono Q. The peak of m-calpain activity from a phenyl-Sepharose column was applied to the Mono Q column, followed by a gradient from 10 to 500 mM NaCl, eluting m-calpain activity at ~400 mM NaCl. ○, proteolytic activity; □, NaCl concentration.

#### 4. Notes

1. No additional salt is included in the homogenization buffer, in order to decrease possible binding of calpains to membranes. 1 mM DTT and 0.1 mM *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  $\text{Ca}^{2+}$  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  $-80^{\circ}\text{C}$ . 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  $25^{\circ}\text{C}$ . 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  $0.8 \times 30$  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  $\mu$ -calpain or m-calpain may be stored for long periods (several months) at  $4^{\circ}\text{C}$  in tightly

capped tubes with no loss of activity. Fractions containing calpastatin should be frozen immediately. Pure fractions of  $\mu$ -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 mM sodium borate buffer, pH 8.0, 1 mM NaN<sub>3</sub>, 0.1 mM EDTA, 1 mM DTT.
12. Make a preliminary titration first to determine a suitable amount of  $\mu$ -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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