FILE:CALPAIN.PRT
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Calpain/Calpastatin Extraction and Assay

1. In a metal Waring Blendor jar,extract 10 grams muscle in 3 times the volume of extraction buffer plus the following inhibitors: 2mM PMSF, 6 mg/liter leupeptin and 100 mg/liter ovomucoid. Homogenize 4 times on high for 30 secs., with 30 secs. of cooling in between bursts.

2. Pour homogenate into centrifuge tubes. Using a rubber policeman and extraction buffer (~10 ml) to remove all of the homogenate. May need to use 2 centrifuge tubes.

3. Centrifuge in Beckman JA-17 rotor for 2 hrs at 17,000 rpm

4. Filter through cheesecloth/glasswool sandwich directly into dialysis tubing.

5. Dialyze against dialysis buffer for 18-24 hours.

6. Remove (we pipet it) and spin as above.

7. Load on a small column (1.5x20 cm) packed with DEAE- sephacel and equilibrated with 1X elution buffer. Remove unbound proteins by washing column with 1X elution buffer until absorbance of the outflow at 278 nm is <.2. NOTE: If you want to express the data in terms of activity per mg protein extracted, prior to loading determine volume and protein concentration.

8. Elute bound proteins with an increasing gradient of NaCl consisting of 1X+25mM NaCl to 1X+350mM NaCl(250 ml of each). Collect 140, 3 ml, fractions.

9. Assay for calpastatin in fractions 15-55. Sequentially add the followings:

   *1.0 ml of each fraction + sufficient volume of m-calpain having net activity of 0.35 (for positive control use 1.0 ml of 1x+25 mM NaCl instead of the fraction), add 1.0 ml of assay media and then 100 ul of 100 mM CaCl2, vortex and then incubate at 25 C for 60 min. Stop the reaction with 2 ml of 5% TCA (Trichloroacetic acid). Centrifuge for 30 min. at 2000 rpm. Read A278. Fraction having calpastatin activity will be clear, while those without calpastatin activity will be turbid due to casein hydrolysis. Pool all fraction having calpastatin activity and assay again as above, but this time use various volume and use the reading which represent 50% or greater inhibition to calculate total calpastatin activity. The relation

between amount of calpastatin (i.e. µg calpastatin) and m-calpain inhibition activity is not linear.

Let us assume you pool 15 fractions, positive m-calpain activity was 0.33 and 100, 200, 300 and 400 µl of the pooled fractions were assayed and had the following reading 0.22, 0.15, 0.05, and 0.05, respectively.

Total Calpastatin activity = (0.33 - 0.15) x 5 (per ml) x 15 (fracxtions) x fraction volume/ 10 (per gram muscle) = 1.35

10. Assay for m-calpain in fractions 101-130. Use 1.0 ml of fractions plus 1.0 ml of assay media, add 100 ul of 100 mM CaCl2 and incubate at 25˚C for 60 min. Stop the reaction with 2.0 ml of 5% TCA , centrifuge and read as for calpastatin.

11. Assay for µ-calpain from the end of calpastatin + 30 fractions. Assay as for m-calpain.

* calpain activity is only linear up to 0.45. Therefore, if fraction contain >0.45 reading they will need to be assayed again

For further details see Koohmaraie, 1990. J. Anim. Sci. 68:659-665