Preparation of Brain Calmodulin

Day 1

Materials (all solutions at 4^oC)

1. 4 mM EDTA, 40 mM Tris-HCl, pH 8.0. Need 4 liters as the 4x base of other buffers.

2. Buffer A: 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.5. Prepare by diluting buffer 1 and adding fresh DTT. Prepare 3 mls per gram of tissue.

3. Buffer B: 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.5. Need 8 liters.

4. PBS Solution A, 2 liters, ice cold.

5. Ultrapure (enzyme grade) ammonium sulfate.

6. 58% saturation ammonium sulfate. Dissolve 6.94 g ammonium sulfate in 20 ml of 2 NH_2SO_4. Save any extra for day 3.

7. 1 M Tris base, a small amount for titration. Save any extra for day 3.

8. Scalpels and dissection scissors.

9. GSA rotor and bottles.

10. Prechilled Waring blender or Polytron with a large generator.

11. DE-52 column, 2.5x20 cm.

Procedure

1. Obtain fresh brain from a local slaughter house, place in a plastic bag and chill in an ice bucket.

2. Remove membrane, meninges and blood, while keeping the tissue on ice or in a cold room. Collect tissue in a chilled plastic pan.

3. Weigh tissue. Two cow brains yield about 400 g.

4. Transfer to a chilled beaker. Rince tissue twice with ice cold PBS.

5. Homogenize brain tissue in 2 volumes of buffer A. Pulse the Waring blender several times then blend for ~60 sec in a cold room at medium speed, or use a Polytron for 20 sec.

6. Centrifuge homogenate in a GSA rotor at 12,000 rpm, $4^{o}C$ for 60 min.

7. Collect pink clear supernatant into a graduate cylinder and read volume. Transfer to a beaker, adjust pH to 7.0 with 1 N NH_4OH.

8. Add, slowly, dry ammonium sulfate to 58% saturation while monitoring the pH. Need 34.7 grams per 100 ml of supernatant. Soak the electrode in high salt after this step.

9. Stir slowly on ice for 15-20 min, then load into GSA centrifuge bottles and let sit on ice for 40 min. Centrifuge in a GSA rotor at 12,000 rpm, $4^{o}C$, for 60 min.

10. Collect supernatant in a beaker, adjust pH to 4.1 with 58% saturation ammonium sulfate in 2 N H2SO4. Soak the electrode in high salt and clean thoroughly.

11. Let proteins precipitate by sitting on ice for 60 min. Centrifuge in a GSA rotor at 12,000 rpm, $4^{o}C$, for 90 min.

12. Resuspend pellets in buffer B and rinse bottles with buffer B. The total volume of buffer used should be ~10% of the starting tissue weight.

13. Adjust pH to 7.5 with 1 M Tris base. Many but not all proteins go back into solution.

14. Dialyze overnight against 2 liters of buffer B. Change buffer twice.

15. Equilibrate the DE-52 column with buffer B.

Day 2

Materials

1. Buffer B, 2 liters.

2. Buffer B, but with 0.5 M NaCl. Need 600 ml.

3. G-100 Sephadex column, 2.5x90 cm.

Procedure

1. Collect solution from the dialysis bag. Centrifuge in a 50.2Ti or 60Ti rotor at 40,000 rpm, $4^{o}C$ for 30 min.

2. Collect supernatant, load to the DE-52 column. Wash the column thoroughly until OD280=0.0 (~500 ml).

3. Elute the column with a 1200 ml gradient of 0.1-0.5 M NaCl in buffer B at a flow rate of ~90 ml/hr. Collect 5-10 ml fractions. Set sensitivity of the UV monitor at OD 2.0. Run the column for 8-10 hr.

4. Equilibrate a G-100 Sephadex column with buffer B, overnight.

Day 3

Materials

1. Buffer B, 2 liters.

2. 1 M Tris base, a small volume for titration, from day 1.

3. 58% saturation ammonium sulfate in H_2SO_4, from day 1.

4. Ultrapure (enzyme grade) ammonium sulfate.

Procedure

1. Run SDS-PAGE of column fractions. Calmodulin should appear at 200-250 mM NaCl.

2. Pool peak calmodulin fractions and measure the volume. Bring ammonium sulfate to 58% saturation (34.7 g / 100 ml), while monitoring and adjusting the pH with NH4OH.

3. Bring the pH to 4.1 with 58% saturation ammonium sulfate in H_2SO_4. Soak the pH electrode in high salt.

4. Let the solution sit on ice for 10-15 min and centrifuge in a SS34 rotor at 17,500 rpm, $4^{o}C$ for 60 min.

5. Resuspend/dissolve the pellets in a small volume (e.g. 4 ml for 400 g brain) of buffer B. Adjust the pH to 7.5 with 1 M Tris base. Soak the electrode in high salt and clean thoroughly.

6. Centrifuge the solution in a 50Ti rotor at 40,000 rpm, 4oC for 30 min.

7. Load the solution into the G-100 column. Elute at ~40 ml/hr, collect 5 ml fractions. Set sensitivity of the UV monitor at 0.5.

Day 4 on

Materials

1. Buffer C: 150 mM NaCl, 1 mM $CaCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 7.5. Need 2 liters.

2. Buffer C with 0.3 M NaCl, 200 ml.

3. DE-52 column, 1.5x12 cm.

4. 58% saturation ammonium sulfate in H_2SO_4, from day 1.

5. Ultrapure (enzyme grade) ammonium sulfate.

6. 10 mM Tris-HCl, pH 7.5, 2 liter.

Procedure

1. Run SDS-PAGE of column fractions. Pool peak fractions.

2. Bring the solution to 2 mM $CaCl_2$ and 150 mM NaCl.

3. Dialyze against buffer C for 6 hr to overnight.

4. Equilibrate the DE-52 column with buffer C.

5. Load the solution into the DE-52 column, wash extensively with buffer C.

6. Elute with a 400 ml gradient of 0.15-0.30 M NaCl in buffer C. Collect 3 ml fractions. Set the sensitivity of UV monitor at <= OD 0.2.

7. Run SDS-PAGE of column fractions. Pool peak fractions. It may be useful to collect also less pure fractions.

8. Concentrate calmodulin with 58% saturation ammonium sulfate and pH 4.1 as before.

9. Resuspend calmodulin in a small volume of 10 mM Tris-HCl, pH 7.5.

10. Dialyze overnight against 10 mM Tris-HCl. Change buffer twice.

11. Clarify in a 50Ti rotor at 40,000 rpm, 4^oC for 30 min.

12. Drop freeze in liquid nitrogen. Stable for months at -80^oC.

Reference

W.H. Burgess, D.K. Jemiolo and R.H. Kretsinger (1980) Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta.* 623:257-270.