PREPARATION OF TUBULIN

Materials

1. Cow (calf) brains, 4-6. Calf brains are preferred as they are less fatty. Should be fresh and chilled in ice (in a clean plastic bag) as soon as possible.

2. Dissection trays, scissors, fine tweezzers, scalpels. One set per person. Set up in the cold room.

3. Clean plastic pans for collecting dissected brains, 2 ea. Record the weight of the empty pan.

3. Two chilled GSA rotors and chilled bottles.

4. A Type 45Ti (564 ml capacity) and a Type 60Ti (462 ml capacity) rotor, and prechilled tubes. Several sets of tubes for Type 60Ti will be needed.

5. Brinkman Polytron and large generator. The generator should be pre-chilled in the cold room.

6. Water bath, set for 37°C.

7. Several dish pans for holding ice and chilling/warming rotors.

8. Large 4-5 liter plastic beakers, 2-3 ea. Tall 3-liter plastic beaker, one ea.

9. Glass rods and rubber policemen for the glass rods.

10. Disposable plastic transfer pipets, 20-30.

11. Dounce homogenizer, 40 ml capacity.

12. Graduated cylinders, 1000 ml, 100 ml.

13. Liquid nitrogen.

14. Powder-free gloves.

15. PBS, 4°C, need 5-10 liters. Prepare 10x PBS and store at room temperature. Dilute with cold distilled water before use.

16. GTP, 50 mM, need ~20 ml or 500 mg, dissolved fresh in cold distilled water and store on ice.

17. Glycerol, 1000 ml.

18. 100 mM PIPES-NaOH, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM DTT, pH 6.9 at room temperature (PEM buffer), 250 ml at 4^oC.

19. 100 mM PIPES-NaOH, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM DTT, 4 M glycerol, pH 6.9 at room temperature (PEM-4G buffer), 500 ml at 4^oC. The amount of glycerol should be determined by weight rather than volume.

20. 100 mM PIPES-NaOH, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM DTT, 8 M glycerol, and 1 mM GTP, pH 6.9 at room temperature (PEM-8G buffer). Keep 250 ml at 4^oC.

Procedure

1. In a cold room, collect brain in two large plastic beakers and thoroughly rinse with 3-5 changes of chilled PBS to remove most of the blood. Keep brains in cold PBS until dissection.

2. Place brains one at a time on the dissection tray. Remove surface membranes, blood vessels, and blood clots with tweezers. Rinse the tissue during dissection to remove blood. Discard parts with high fat content near the spinal cord.

3. Mince the tissue coarsely using dissection scissors. Determine the weight. The total weight should be around 900-1000 g from 6 calf brains.

4. Transfer about 50% of the tissue into a tall 3-liter plastic beaker set in an ice bucket. Add 75 ml of chilled PEM-4G buffer per 100 g tissue. Homogenize with a Polytron with several bursts at a high speed. The head of Polytron should be partially submerged in the liquid and there should be a vortex generated in the homogenate. Repeat this for the rest of the tissue and combine the homogenate in a beaker in the ice bucket.

5. Load the homogenate into pre-chilled GSA bottles, balance. Centrifuge at 8,000 rpm for 15 min, 4^oC.

6. Collect supernatants into ultracentrifuge tubes on ice. The pellets are very loose, so avoid decanting. Use 10 or 25 ml pipets instead. Load a set of tubes for the Type 45Ti rotor and a set for the Type 60Ti rotor, for a total volume of ~1,000 ml.

7. Centrifuge at 35,000 rpm for 75 min, 4^oC. Leave the centrifuge vacuum on and the refrigeration off after the spin.

8. Collect supernatant into a chilled graduated cylinder, measure the volume (typically 600-700 ml).

9. While stirring gently, add 50 mM GTP stock solution to a final concentration of 1 mM, i.e. 1/50 of the volume measured in the previous step.

10. Load the supernatant into ultracentrifuge tubes. Balance the tubes.

11. Incubate for 30-45 min in a 37°C water bath. During the incubation, warm both ultracentrifuge rotors to 30°C, in a dish pan filled with warm water.

12. Centrifuge at 35,000 rpm for 60 min, 27°C. Leave the vacuum on and set the temperature to 4°C after the spin.

13. Discard the supernatant. Dislodge pellets with glass rods fitted with rubber policemen. Collect as much material as possible into the bottom of the Dounce homogenizer in an ice bucket.

14. Rince the centrifuge tubes with a small volume of cold PEM buffer. Collect the buffer into the homogenizer.

15. Homogenize with ~5 strokes with the A pestle. Collect the solution in a beaker on ice.

16. Rince the tubes several more times with PEM buffer. Repeat the homogenization. The total volume of PEM buffer used should not exceed 1/4 volume of the volume measured in step 8.

17. Incubate the solution on ice for 30 min. Also chill down the rotor on ice.

18. Load into Type 60Ti tubes. Centrifuge for at 35,000 rpm for 60 min, 4°C. Leave the centrifuge vacuum on and refrigeration off.

19. Carefully pool supernatants into a graduated cylinder, measure volume (~100 ml).

20. While stirring slowly, add an equal volume of PEM-8G buffer. Stir until the solution is uniform. The solution may be stored at -20°C overnight.

21. Transfer the solution into Type 60Ti tubes. Balance the tubes.

22. Incubate for 30-45 min at in a 37°C water bath. During the incubation, warm the Type 60Ti rotor to 30°C, in a dish pan filled with warm water.

23. Centrifuge at 35,000 rpm for 90 min, 27°C. Leave the centrifuge vacuum on and set the temperature to 4°C.

24. Remove supernatants carefully with a pipet (the pellets may be loose). Dislodge pellets with glass rods fitted with rubber policemen. Collect as much material as possible into the bottom of the Dounce homogenizer in an ice bucket.

25. Rince the centrifuge tubes with a small volume of cold PEM buffer. Collect the buffer into the homogenizer.

26. Homogenize with ~5 strokes with the A pestle. Collect the solution in a beaker on ice.

27. Rince the tubes with PEM buffer. Repeat the homogenization. The total volume of PEM buffer used should not exceed 1/4 volume of the volume measured in step 19.

28. Incubate on ice for 30 min.

29. Transfer to clean Type 60Ti tubes. Balance.

30. Centrifuge at 35,000 rpm for 60 min, $4^{o}C$.

31. Collect supernatants in a chilled 100 ml graduated cylinder. Measure volume. Save ~100 ul for Lowry assay.

32. Drop-freeze the rest. Keep the collecting bottle (a GSA bottle) on dry ice while collecting the frozen pellets. Store in $-80^{o}C$ freezer in a GSA bottle.

33. Tubulin can be stored for months in this form, referred to as "twice cycled", which still contains many MAPS.

34. Perform Lowry assay and estimate the total yield (~400 mg total).