Chapter 2

Purification of Tubulin from Porcine Brain

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Abstract

Microtubules, polymers of the heterodimeric protein αβ-tubulin, give shape to cells and are the tracks for vesicle transport and chromosome segregation. In vitro assays to study microtubule functions and their regulation by microtubule-associated proteins require the availability of purified αβ-tubulin. In this chapter, we describe the process of purification of heterodimeric αβ-tubulin from porcine brain.

Key words: Tubulin, Purification, Porcine brain, Microtubules, Phosphocellulose

1. Introduction

The study of microtubules, reconstituted in vitro from purified heterodimeric αβ-tubulin, has provided valuable insights into the properties of microtubules as well as the function of many microtubule-associated proteins (MAPs) (1–5). In particular, the use of fluorescently labelled tubulin in single-molecule in vitro assays has developed our understanding of microtubule polymerisation (6), depolymerisation (7), and the role of MAPs in these processes (3, 5, 8). The lack of an efficient system for the over-expression of αβ-tubulin requires that this protein be obtained by purification from animal brains (usually porcine or bovine). The process of purification (outlined in Fig. 1) consists of homogenisation of the brains, followed by cycles of tubulin polymerisation (in the presence of GTP), centrifugation, and depolymerisation (9–11). Thereby, the particular property of αβ-tubulin to form microtubules is used to isolate soluble tubulin from cell membranes and non-MAPs. The resulting solution of soluble tubulin plus MAPs

Anne Straube (ed.), *Microtubule Dynamics: Methods and Protocols*, Methods in Molecular Biology, vol. 777,
DOI 10.1007/978-1-61779-252-6_2, © Springer Science+Business Media, LLC 2011

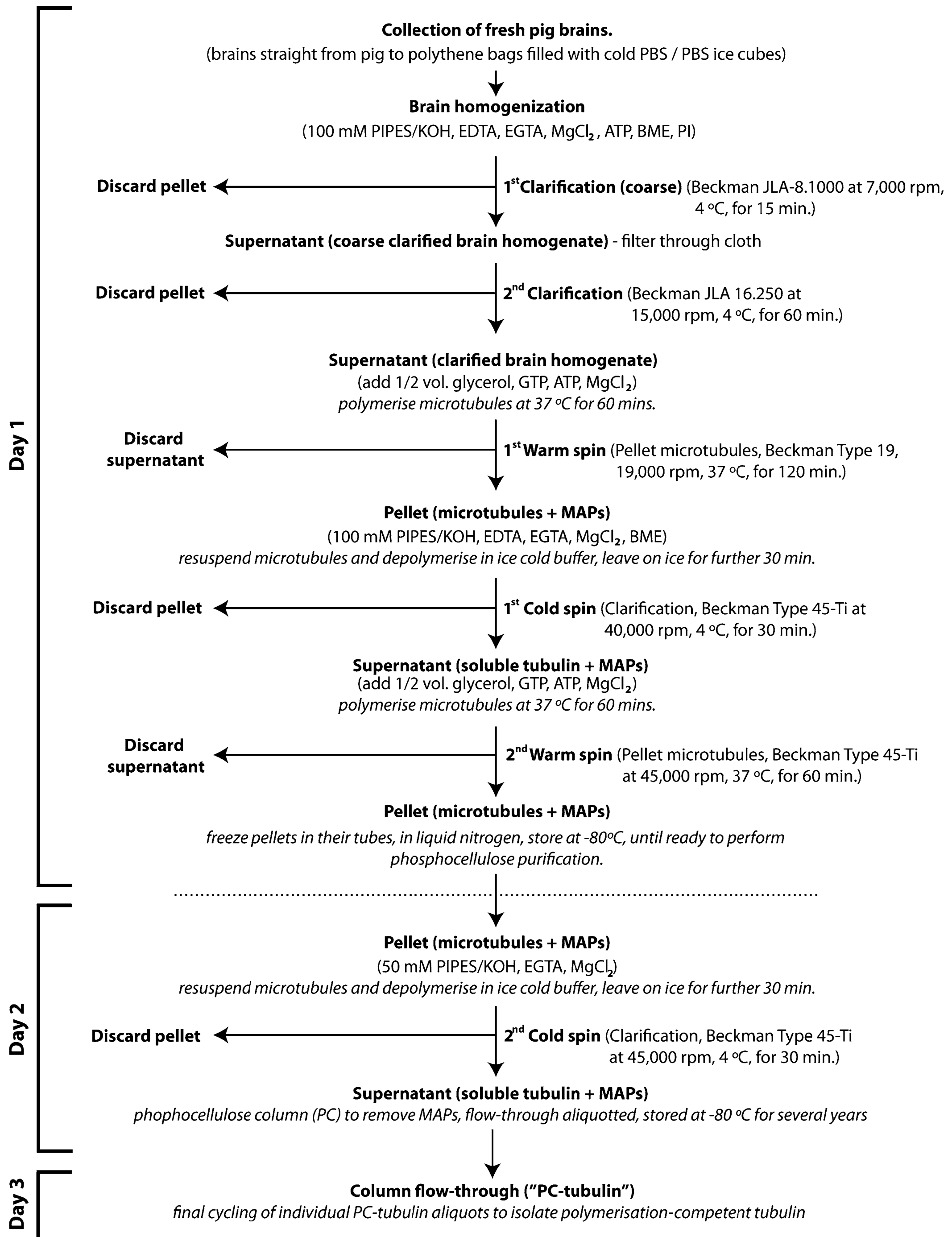

Fig. 1. Outline of the steps in the purification of $\alpha\beta$-tubulin from porcine brains, using alternate steps of polymerisation (in the presence of GTP) and depolymerisation, followed by the removal of MAPs on a phosphocellulose column. Figure style based on ref. 10.

is then passed over a phosphocellulose (PC) column in order to remove the remaining MAPs, which bind to the column while the soluble tubulin flows through. The purified tubulin is generally stored long-term in this state. Prior to use for subsequent in vitro studies, a final cycling (a step of polymerisation–depolymerisation) ensures that only polymerisation-competent tubulin is present. The protocol presented here describes the brain homogenisation, initial cycles of polymerisation/depolymerisation, and phosphocellulose column stages of the purification.

Below is the protocol we use for the purification of porcine brain tubulin on a large scale (typically, starting with ~75 pig brains and resulting in several grams of purified tubulin, see Fig. 1). However, the basic protocol can be scaled down, the most important consideration being the capacity of the available centrifuges; it is essential to consider the reductions and increases in volume expected at each stage and to match this to the available centrifuges to minimise loss. To this end, throughout the protocol, we give an indication of the *typical* volumes expected at each stage (references to Notes). However, significant variability (differing size of the pig brains obtained; amount of supernatant recovered in each step; volume needed for re-suspension of pellets) should be expected. We note that the investment of time necessary for the preparation is somewhat independent of the starting material. Thus, large preparations are an efficient use of time; given the fact that tubulin is stable for years when stored at −80°C.

2. Materials

Unless otherwise noted, the quantities (volumes) given are those that we typically find necessary for a preparation starting with ~75 pig brains (~10 kg). (Items 1–17 and 18–30 detail the requirements for the initial tubulin preparation and phosphocellulose column, respectively).

1. Phosphate-buffered saline (PBS) to make ice cubes (~7.5 L).
2. PBS (~20 L, chilled to 4°C).
3. Large, strong plastic bags, each sufficient to hold 2.5 L PBS ice cubes, 3 L cold PBS, and ~25 pig brains.
4. Sufficient glassware (beakers, measuring cylinders, etc.) pre-cooled or pre-warmed as necessary.
5. Large flasks with magnetic stirrers for tubulin polymerisation; pre-warm flasks to 37°C (e.g. Wheaton Magna Flex Spinner Flasks, 3 L).
6. ~75 pig brains (typically, 10 kg, final yield ~1–3 g of purified tubulin). Brains should be as fresh as possible. It is important

to get the brains into the mixture of chilled/ice PBS as quickly as possible. We achieve a delay of only a few minutes from pig to PBS. The time that the brains spend chilled in this way should also be kept to a minimum. However, in our particular case, transport times dictate a delay of over an hour at this stage and high yields of tubulin are nonetheless obtained.

7. Glycerol (~6 L, pre-warmed to 37°C).
8. Buffer P (polymerisation buffer): 100 mM PIPES/KOH pH 6.85, 0.1 mM EDTA, 2 mM EGTA, 0.5 mM $MgCl_2$ (final concentrations). Prepare 3 L of 5× Buffer P.
9. Protease inhibitors (PI) 1,000× stock solutions: 1 mg/ml aprotinin in water, 1 mg/ml TAME in water, 10 mg/ml trypsin inhibitor in water, 1 mg/ml pepstatin in ethanol, 1 mg/ml TPCK in ethanol, 5 mg/ml leupeptin in ethanol, 100 mM PMSF in ethanol. PI stock solutions can be prepared prior to the purification and stored at −20°C. We typically prepare 10 mL of *each* PI stock solution.
10. 100 mM Mg-ATP (~260 mL, ATP is dissolved in a 100 mM $MgCl_2$ solution and the pH adjusted to 6.8 using KOH). The concentration should be checked by absorption at 260 nm ($\varepsilon = 15{,}400\ M^{-1}\ cm^{-1}$) (12), and solution should be stored at −20°C.
11. 200 mM GTP (~60 mL, GTP is dissolved in distilled water, and the pH adjusted to 6.8 with KOH). The concentration should be checked by absorption at 260 nm ($\varepsilon = 11{,}700\ M^{-1}\ cm^{-1}$) (12], and solution should be stored at −20°C.
12. β-mercaptoethanol (BME).
13. Buffer P+ (prepared on the morning of the preparation): Buffer P, 1× of *each* PI (see Item 9), 1 mM ATP (see Item 10), 0.1% BME.
14. Blender for brain homogenisation (e.g. a 4-L Waring Blender, 38BL30).
15. Dounce homogenisers (Kontes, Vineland, NJ, USA; 885300-0040, 885301-0040, 885302-0040).
16. Appropriate centrifuges, rotors, and centrifuge tubes, pre-warmed or pre-cooled as necessary throughout the preparation.
17. Three large heated water baths for polymerisation. We use large (~50 L) plastic storage boxes, filled with water, each fitted with a heating recirculating water bath head (e.g. Thermo Haake, 003-2859). One bath set to 55°C, and two set to 37°C. Baths must be large enough to accommodate polymerisation flasks (see Item 5).
18. 10× column buffer (CB): 500 mM PIPES/KOH pH 6.85, 10 mM EGTA, 2 mM $MgCl_2$. (Prepare 5 L).

19. CB: 50 mM PIPES/KOH pH 6.85, 1 mM EGTA, 0.2 mM $MgCl_2$ (20 L); prepared from 10× CB.
20. CB + 1 M KCl (12 L); prepared using 10× CB and KCl.
21. Whatman P11 cellulose phosphate (PC, 300 g Whatman 4071050; ~4 mL wet resin/1 g dry weight).
22. 0.5 M NaOH (5 L).
23. 0.5 M HCl (5 L).
24. 0.5 M K-phosphate pH 6.8 (13 L).
25. ddH_2O (5 L).
26. BSA (Fraction V, protease-free; SERVA, 11926).
27. 5 × 2-L beakers, 1 × 4-L beaker, 2× stirring rods.
28. Aspirator pump (Vacuubrand ME16C) with 10 L trap.
29. Peristaltic pump; tubing.
30. Housings for column and pre-column (XK 50/20, XK 50/100 column housings; GE Healthcare 18-8753-01, 18-1000-71).

3. Methods

3.1. Several Weeks Before the Preparation

3.1.1. Equipment and Resource Arrangements

1. Arrange for the collection of pig brains as early as possible on the day of preparation. The use of fresh pig brains is essential.
2. Order sufficient quantities of all reagents.
3. Book all equipment (centrifuges, rotors, warm/cold room time, balances, douncers, blenders, etc. as necessary in your lab; details of the centrifuges, rotors, and speeds we use are stated in the text and notes).
4. Arrange for sufficient manpower for the preparation, ensuring that each person is aware of his or her duties. For a 75 brain prep, we find that the involvement of a minimum of four people is necessary.

3.1.2. Phosphocellulose Column Preparation

Column preparation (Items 18–30) largely follows that described by the Mitchison Lab, with modifications (13). The dry resin is acid/base-treated, defined, and then packed into the column housing. After packing, the resin beds are washed with high-salt, then low-salt buffer, treated with BSA to block non-specific binding to the phosphocellulose, and finally equilibrated with buffer.

Due to the high viscosity of the crude microtubule protein loaded on the PC column, a smaller guard column containing 100 mL of the same cycled resin is connected upstream of the main 1 L column.

1. Base treatment of PC: Add 50 g of dry PC to each of 5 × 2-L beakers containing 1 L of 0.5 M NaOH. Stir gently until resin is wetted and re-suspended into a uniform slurry. Let the resin settle for 5 min and then aspirate the supernatant, including any fines. Quickly add 1 L 0.5 M K-phosphate to the settled resin, stir, check that the pH is near neutral, and then let stand for 5 min. Again aspirate the supernatant, add 1 L of H_2O, re-suspend the resin by stirring, and then allow to settle for 5 min prior to aspirating the supernatant.
2. Acid treatment of PC: Add 1 L 0.5 M HCl to the settled resin, re-suspend, and allow to settle for 5 min. Aspirate the supernatant, add 1 L 0.5 M K-phosphate, stir, check the pH is near neutral, and let settle again for 5 min prior to aspirating the supernatant. Then, combine the resin in all the beakers into a single 4-L beaker and add 3 L 0.5 M K-phosphate. Allow to settle, and aspirate the supernatant. Add 1 L CB + 1 M KCl (see Item 20), stir the resin, allow to settle, and then aspirate. Repeat this wash step with 1 L CB + 1 M KCl twice more.
3. Packing the columns: Pour the resin slurry into the column housings in the cold room and then pack the bed at ~15 mL/min using a peristaltic pump attached to the column outlet to control the flow rate. Attach a flow adapter to the top of the column housing and run 7 L of CB + 1 M KCl through at a flow rate of 5–10 mL/min.
4. Washing, blocking, and equilibrating the column: Continue washing with 10 L of CB monitoring the conductivity to ensure that KCl is fully eluted. Adjust the level of the flow adapter to allow an adequate reserve of buffer at the top of the bed, as the resin expands when going from high to low salt. Load 300 mL of 30 mg/mL BSA in CB, chase with 700 mL of CB, and then stop the flow for 2 h to allow adsorption of the BSA. This step blocks tubulin from binding irreversibly to the fresh resin and needs to be performed only once prior to fractionating crude microtubule protein on the PC column. Finally, wash out the BSA with 2 L CB + 1 M KCl and then equilibrate the column with 10 L of CB. *This last CB wash should be done in the evening before the preparation.*

3.2. Several Days Before the Preparation

1. Freeze 7.5 L PBS in plastic bags, ~1 L per bag, break into rough ice cubes.
2. Chill a further 20 L of PBS.
3. Prepare all stock solutions: buffers, nucleotides, and protease inhibitors; ensure that an excess of these solutions is available, see Materials for details.
4. Pack the PC column, block with BSA, and wash with high salt as described in Subheading 3.1.2, steps 3 and 4.

3.3. Evening Before the Preparation

1. Collect all centrifuge rotors to be used in the preparation and pre-warm/cool as necessary.
2. Check the operation of all centrifuges and other essential equipment (e.g. blenders).
3. Place glycerol at 37°C.
4. Equilibrate PC column with 1× CB as described in Subheading 3.1.2, step 4.

3.4. Morning of the Preparation

Early on the morning of the preparation, one team can collect the brains (see Item 6) while a second makes the following preparations:

1. Pre-cool centrifuges necessary for the first two coarse clarification spins to 4°C.
2. If additional centrifuges are available, pre-warm to 37°C ready for subsequent warm spin (microtubule pelleting).
3. Thaw any frozen stock solutions.
4. Prepare cold and warm rooms with all necessary equipment.
5. Prepare Buffer P+ (see Item 13).

3.5. Purification of Tubulin and MAPs from Brains

3.5.1. Brain Homogenisation

1. Store pig brains in PBS ice/chilled PBS-filled bags until ready for use.
2. Add a mixture of brains (see Note 1) and Buffer P+ to the blender using a ratio of 1 L of Buffer P+ to 1 kg of brains; this typically results in ~18 L final volume for ~75 brains.
3. Homogenise the brain/buffer mixture using short pulses of the blender, separated by pauses in order to avoid heating the homogenate.
4. The final homogenate should have the colour and consistency of a good strawberry milkshake.

3.5.2. Coarse Clarification of Homogenate

1. Clarify the brain homogenate (~18 L total volume) by centrifugation at ~7,000 rpm (~12,000 × *g*, Beckman JLA-8.1000 rotor, see Note 2) for 15 min at 4°C. (Also see Note 3 for important tips related to this and all subsequent centrifugation steps).
2. Recover the supernatant from the centrifuge bottles by pouring carefully into chilled glass beakers. During this step, we filter the supernatant through coarse cotton as it is poured (we use cloth diapers) to prevent any of the loose pellet being collected (see Note 4).
3. Perform a second clarification step of the recovered supernatant (clarified brain homogenate, total volume now ~7 L) by centrifugation at ~15,000 rpm (~33,000 × *g*, Beckman JLA 16.250 rotor) for 60 min at 4°C (see Note 5).

4. Recover the supernatant which contains the soluble protein (typically, ~6 L of supernatant is recovered). This is used for the first tubulin polymerisation step.
5. Check the temperature of the centrifuges required for the next warm spin and ensure that they are at 37°C. Clean and pre-warm centrifuge tubes/rotors as necessary.

3.5.3. Microtubule Polymerisation/ Depolymerisation Cycles

1. To the chilled supernatant, add (final concentrations) 1 mM GTP, 1.5 mM ATP, and 4 mM $MgCl_2$.
2. Add a half volume of pre-warmed glycerol (33% v/v final).
3. Transfer the mixture to a suitable stirring flask (see Note 6).
4. Warm the mixture as quickly as possible to 30°C; we do this by placing the flask in a water bath at 55°C. The polymerisation mixture should be continuously stirred and the temperature monitored closely (see Note 7).
5. Once the solution has reached 30°C (and no higher), transfer the flask to a water bath at 37°C. Incubate for 1 h at 37°C, stirring occasionally (see Notes 7 and 8).
6. After incubation, transfer the viscous solution (now a total volume of ~9 L) to suitable centrifuge tubes, and pellet the microtubules (and MAPs) by centrifugation at ~19,000 rpm (~53,000 × *g*, Beckman Type 19 rotor) for 120 min at 37°C (see Note 9).
7. After centrifugation, ensure that centrifuges and rotors reused in the subsequent step are pre-cooled to 4°C.
8. Discard the supernatant (see Note 10).
9. Store the pellets on ice and re-suspend in ice-cold Buffer P with additionally 0.1% (v/v) BME; typically, ~10 mL per pellet is sufficient for re-suspension (see Note 11).
10. Dounce the re-suspended microtubule/MAPs solution (working in a cold room) until a homogeneous solution is obtained; thorough re-suspension of the pellets is essential (see Note 11).
11. If the pellets are re-suspended separately or in batches (see Note 11), pool the entire solution (keeping on ice), mix well, and determine the approximate concentration using a Bradford assay (see Note 12). If necessary, lower the concentration to ~25 mg/ml (see Note 13) with the addition of cold Buffer P.
12. Incubate the solution for a further 30 min on ice to ensure complete depolymerisation of the microtubules. The total volume is now typically ~840 mL.
13. Clarify the soluble tubulin/MAPs solution by centrifugation at 40,000 rpm (~186,000 × *g*, Beckman Type 45-Ti rotor) for 30 min at 4°C (see Note 14 and 15). After this spin, set the centrifuges to 37°C as required for the next warm spin.

14. Recover the supernatant for the second round of tubulin polymerisation (the reduced volume of the clarified solution is typically now ~560 mL from 840 mL); repeat Subheading 3.5.3, Steps 1–5.
15. After incubation, transfer the viscous solution (typically, ~840 mL) to suitable centrifuge tubes and pellet the microtubules (and MAPs) by centrifugation at ~45,000 rpm (~235,000 × *g*, Beckman Type 45-Ti rotor) for 60 min at 37°C (see Notes 15 and 16).
16. Discard the supernatant: At this point, the purification can be paused. We do this by freezing the pellets still in their centrifuge tubes. First, cool the pellets and tubes by placing them on ice for 5 min. Then, snap-freeze in liquid nitrogen and store at −80°C. We have successfully paused the protocol at this point for several months with no apparent loss in final tubulin yield.
17. Re-suspend half (six) of the pellets in buffer 1× CB and using ~20 mL/pellet. (This typically results in ~200 mL total volume, see Note 17.) The remaining frozen pellets can be re-suspended and purified at a later date in a second run over the PC column after cleaning and re-equilibration of the resin.
18. Dounce the re-suspended microtubule/MAPs solution (working in a cold room) until a homogeneous solution is obtained.
19. Pool the solution (keeping on ice), mix well, and allow the microtubules in the solution to depolymerise for a further 30 min on ice (see Note 17).
20. Clarify the soluble tubulin/MAPs solution one final time at 45,000 rpm (~235,000 × *g*, Beckman Type 45-Ti rotor) for 30 min at 4°C (see Note 18).
21. Recover the supernatant, taking care not to disturb the unstable pellet, into a pre-cooled glass bottle (recovered volume ~150 mL) kept on ice discarding the pellets. Check the protein concentration by Bradford assay (see Note 12) and if necessary, adjust to 20 mg/mL with 1× CB. Solution (soluble tubulin/MAPs) is now ready for phosphocellulose column chromatography.

3.5.4. Purification of Tubulin on a Phosphocellulose Column

1. Introductory note: Due to the high viscosity of the tubulin sample and the compressibility of the PC column bed, a pre-column, one tenth the volume of the main column, is used. The pre-column binds much of the pink, smelly, sticky stuff that normally clogs the column and leads to low flow rates and resin compression. The pre-column can either be run alone or in-line with the main column. If run alone, the flow through is pooled and loaded immediately onto the main PC

column. For a 100 mL pre-column, tubulin begins to elute ~70 mL after loading.

2. The sample is then applied at 5 mL/min to the large 1 L column and then, after washing with 150 mL of 1× CB, fraction collection can be started (8 mL/fraction). The flow rate may need to be lowered while loading or running the column in order to keep the back pressure below the limit for the column housing. Generally, a flow rate of at least 3 mL/min can be maintained.

3. The tubulin elutes as a clear, slightly yellow solution. Check the protein concentration by Bradford Assay using BSA as the standard (see Note 12) and pool the most concentrated fractions (typically, ~160 mL total) as well as side fractions (~50 mL) with less tubulin. Aliquots are fast-frozen in cryotubes (4 mL) by immersion in liquid N_2 and stored at −80°C.

4. Analysis of the fractions eluted from the PC column by SDS-PAGE shows that the bulk of the MAPs and other contaminants are bound by the PC column, leaving a highly purified tubulin preparation (Fig. 2).

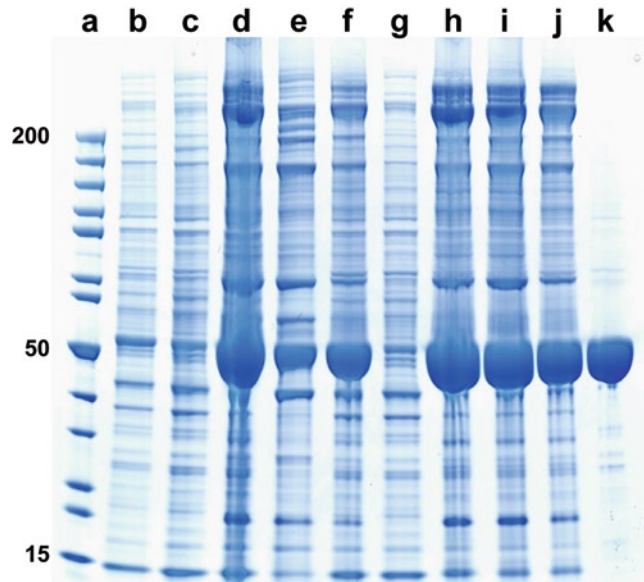

Fig. 2. SDS-PAGE gel showing protein content at each stage in a typical tubulin purification. Lanes: (**a**) Fermentas protein ladder 10–200 kDa; (**b**) clarified brain homogenate; (**c**) first warm spin supernatant; (**d**) first warm spin pellet; (**e**) first cold spin pellet; (**f**) first cold spin supernatant; (**g**) second warm spin supernatant; (**h**) second warm spin pellet; (**i**) second cold spin pellet; (**j**) second cold spin supernatant (PC column load); (**k**) PC column flow through ("PC tubulin"). The tubulin band at ~50 kD is overloaded in order to monitor the level of contaminating MAPs that are largely removed after chromatography on PC.

5. To regenerate the column, wash with 3 CV of 1 M KCl in 1× CB at 3 mL/min; this high salt wash contains the MAPs that can be collected if desired. Re-equilibrate the resin by washing with 3–5 CV of 1× CB and monitor the conductivity to ensure that the KCl is removed. Finally, wash with 5 CV of 1× CB containing 0.1% NaN_3 for long-term storage at 4°C.

4. Notes

1. Some protocols suggest dissection of the brains and removal of the meninges and blood clots. However, using pig brains and our particular blender, we have found this to be an unnecessary delay. Such steps may be necessary when using bovine brain or different equipment.
2. We have three Beckman Avanti J-20 centrifuges available with JLA-8.1000 rotors, giving a capacity of 18 × 1 L, usually sufficient for the coarse clarification of the homogenate from ~75 brains. Note that this first clarification is not strictly necessary; large centrifuges, like the J-20s, may not be available. However, this step does result in a significant initial reduction of volume (~18 to 7 L), resulting in an increased final tubulin yield using a smaller number of centrifuges in the subsequent steps.
3. In this and all subsequent centrifugation steps, we state the rpm, the relative centrifugal force, the rotor used, and the total spin time in our preparation; of course, any suitable rotor/rpm/time combination can be substituted. A number of checks should be carried out prior to each spin: all centrifuge tubes should be balanced to at least 1 mg; rotors should be checked for damage, particularly to their over-speed discs; o-rings should be checked for wear, re-greased, and replaced as necessary; all threads should be treated with spincote; each centrifuge tube should be checked for wear (including o-rings, if present); and typically, centrifuge tubes should always be filled completely to reduce the chance of collapse and if insufficient solution is present, then the tubes should be topped up with the appropriate buffer.
4. This first coarse clarification results in a small dense pellet with a much larger loose foam-like pellet. Discard both the pellet and the foam.
5. We use five Beckman Avanti J-25 centrifuges with JLA 16.250 rotors, giving a maximum capacity of 30 × 250 mL. After this second clarification, small pellets are obtained and the volume of recovered supernatant is typically ~6 L.

6. See Item 5. However, any container is suitable as long as it is possible to continuously stir the solution and monitor the temperature (see Note 7).
7. It is essential to bring the tubulin solution above 30°C quickly; slow polymerisation affects the final tubulin yield. We have had good results by ensuring that the glycerol, added to the chilled supernatant, is at 37°C and the polymerisation solution is added to a pre-warmed (37°C) flask. The flask is then transferred to a large water bath set at 55°C. This raises the temperature of the solution quickly (~5 min). It is essential to ensure that the solution is stirred continuously during this time and that the temperature inside the flask is closely monitored. The flask must be removed from the 55°C bath when the temperature reaches 30°C, or there is a risk that the high temperature will denature the tubulin close to the glass; the flask is then transferred to a separate water bath set at 37°C.
8. As polymerisation proceeds, the solution becomes more viscous and undergoes a slight colour/clarity change.
9. The total volume of the polymerisation mixture is ~9 L, requiring 36 × 250-mL centrifuge bottles. We use four Beckman Optima LE-80K centrifuges with Type 19 rotors and two Beckman Avanti J-25s with JLA-16.250 rotors (resulting in a reduced *g*-force of 33,000 × *g* for the JLA-16.250). Note that Type 19 centrifuge tubes will likely collapse slightly at 37°C in this spin (even when filled) and must not be reused. Also note that Beckman Avanti J-25 centrifuges have no facility for heating. However, using a pre-warmed rotor and pre-warmed tubes (37°C) and the centrifuge at room temperature, there is minimal loss of tubulin.
10. It is sensible to retain a small volume of this supernatant and all subsequent supernatants (and re-suspended pellet solutions) to perform gel electrophoresis to monitor the progress of the purification (Fig. 2).
11. It is essential to re-suspend the pellets completely. Similarly, it is desirable to re-suspend in as low a volume that allows complete re-suspension. Typically, we begin by attempting re-suspension using 5 mL of Buffer P for each pellet and use a soft spatula to scrape and break the pellet from the walls of the centrifuge tube. Then, homogenise the solution using a dounce homogeniser; use the dounce slowly to avoid pulling a vacuum. Additional dilution may then be necessary, particularly if the re-suspension is “holding” bubbles. This dilution, along with additional “washing” of the tubes and dounces, typically results in using the equivalent of ~10 mL per pellet. Conveniently (for the next centrifugation step), the total volume (including the additional volume of the pellets) is typically 800–900 mL. Several steps of douncing/incubation

on ice may be necessary to maximise re-suspension and depolymerisation of the microtubule pellet.

12. The total protein concentration (consisting of tubulin and any MAPs) can be determined using a standard Bradford assay (14).
13. Lowering the concentration assists depolymerisation, as well as diluting out glycerol and other components prior to clarification; nevertheless, the concentration should not be lowered below 25 mg/mL; otherwise, the efficiency of polymerisation will be affected in the next steps.
14. We now typically use two Beckman LE-80K centrifuges with Type 45-Ti rotors (giving a total capacity of 12×70 mL= 840 mL). The recovered volume of the supernatant is typically ~560 mL.
15. In the Type 45-Ti rotor, at the required centrifugation speeds, it is *essential* to fill the centrifuge tubes completely; otherwise, collapse *will* occur. If necessary, tubes can be topped up with *cold* or *warm* buffer P, as appropriate.
16. The volume of the polymerisation solution is now ~840 mL due to the added glycerol. We use two Beckman LE-80K centrifuges with Type 45-Ti rotors (giving a total capacity of 12×70 mL = 840 mL).
17. The same advice applies to the re-suspension as before (see Note 11); note, however, the different buffer and final volumes. The increased re-suspension volume (20 mL/pellet compared to 10 mL/pellet) is to ensure depolymerisation, but also to ensure that the solution is not too viscous, improving the effectiveness of the final clarification spin. It is particularly important to re-suspend the pellets well at this stage in order to minimise the viscosity of the solution loaded onto the PC column.
18. Re-suspension of half of the pellets (six) with around 20 mL of buffer results in a total volume of ~200 mL. Thus, the clarification can easily be carried out in a single Beckman LE-80K centrifuge with a Type 45-Ti rotor. If necessary, to ensure that three tubes are filled completely (3×70 mL = 210 mL), the volume of the re-suspension should be adjusted to 210 mL. Significant pellets are obtained after this clarification, and the volume of supernatant recovered is typically ~150 mL.

Acknowledgements

The authors acknowledge the input of many people in the development of these protocols. Protocols published on the Web, especially by the Mitchison lab, have proved invaluable in the development of this protocol. In more recent years, many

members, past and present, of the Howard, Diez, and Hyman labs at the MPI-CBG have been involved in performing this purification. In particular, we thank Regine Hartmann, Corina Braeuer, Marija Zanic, Gero Fink, Tony Ashford, and Andy Hunter. Finally, C.G. and C.T.F. thank Gary Brouhard, both for sharing the misery of getting this protocol up and running (again) and for promising (after "the incident") never to run another PC column.

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