This protocol is based on that originally described by Shalanski et al (1973) and modified by Weingarten et al (1974) and subsequently by Mitchison T. and Kirschner M. (1984b).

All the tubes for a tubulin prep are stored in the cupboard outside the lab. Tony has the key.

Materials and Instrumentation

1. Centrifuges, Rotors and Bottles

    - One JLA8.100 and six 1 litre bottles
    - Four MLA80
    - Three Ultracentrifuges with three Beckman Type 19 rotors and 24 corresponding bottles.
    - Four Beckman Type 45Ti rotors along with 24 (70ml capacity) polycarbonate bottles.

    As can be seen from this list, the rotors required are quite large and heavy, and it is therefore essential to check the vacuum and refrigeration efficiency of all the centrifuges beforehand, using these rotors. With regard to the rotors themselves, these should be inspected beforehand, and any seals and 'O' rings should be checked for signs of perishing, replacing where necessary. It is also important that these 'O' rings are greased lightly with vacuum grease and that the screw-threads for the rotor lids are lightly smeared with Beckman 'Spinkote' to ensure that the samples are sealed and that the vacuum is maintained during the run. Finally the centrifuge rotors should be checked for the condition of the overspeed decals to prevent premature termination of the run. This is particularly true of the Beckman Type 19 rotors which seem especially sensitive on this point.

    The centrifuge bottles too, should be inspected for cracks and warping (particularly true of the polycarbonate Beckman Type 45 bottles), and the lids and caps should be checked for condition and integrity of the 'O' ring.

2. A motor driven Homogenizer such as that made by Waring along with a 4.5L capacity homogenizing beaker.

3. A Continuous flow homogenizer such as that made by Yamato for resuspending tubulin pellets or a very large Dounce hand homogenizer.

4. A 1 Litre Phosphocellulose Column. This is in the Large cold room.

Solutions

The buffer system of choice in the preparation of tubulin is MES for the first step and 1,4-piperazinediethanesulphonic acid (PIPES) for subsequent steps. When making up buffer solutions, it is first necessary to raise the pH of the solution to about pH 6.0 using KOH in order to get the PIPES to go into solution. This initial pH adjustment must be done without the use of a pH meter (ie. using pH indicator paper) since any undisolved PIPES can damage the electrode.

About 4 Litres of Blending buffer are needed

Blending Buffer (BB)

- 0.1M MES pH 6.5
- 2 mM EGTA
- 0.1 mMEDTA
- 0.5 mM MgCl2

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Blending Buffer - 1 Litre

- 21.7 g MES
- 4 ml EDTA from 0.5 M stock
- 0.2 ml EGTA from 0.5 M stock
- 0.5 ml MgCl2 from 1M stock

About Four Litres of Polymerization Buffer (PB) are needed for a large tubulin preparation and if preferred it can be made as a 5X Stock solution and then diluted with water at 4°C before use.

Polymerization Buffer (PB)

- 0.1M PIPES pH 6.8
- 0.5mM MgCl2
- 2.0mM EGTA
- 0.5mM EDTA

Polymerization Buffer - 1 Litre

- 30.2g PIPES pH 6.8
- 0.5ml MgCl2 of 1M Stock
- 0.761g EGTA
- 37mg EDTA

Approximately five Litres of Column Buffer (CB) will be needed for such a preparation, and this is made up as a 10X Solution and diluted with water at 4°C prior to use.

Column Buffer (CB)

- 50mM PIPES pH 6.8
- 1mM EGTA
- 0.2mM MgCl2

Column Buffer - 1 Litre 10X

- 151.2g PIPES pH 6.8
- 3.8g EGTA
- 2ml MgCl2 of 1M Stock

To make 1 Litre of 10X column buffer, weigh out 151.2g of PIPES, 3.8g of EGTA and 2ms of a 1M stock solution of Magnesium Chloride and add 45.6g Potassium hydroxide. This will adjust the pH to about pH 6.7 and further adjustments can be made using a 10M solution of Potassium hydroxide.

BRB80 Conversion Buffer: This is added to the purified tubulin that has been eluted from the Phosphocellulose column to convert the buffer from Column buffer to BRB80 prior to storage of the tubulin. The BRB80 conversion buffer is added in a ratio of one part conversion buffer to 20 parts tubulin in column buffer.

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BRB80 Conversion Buffer (CB) - 150mls

- 47.6g PIPES
- 4.2ml MgCl2 (1M)
- 1.25ml EGTA (0.2M)

This should be brought to pH 6.8 with Potassium hydroxide. Having the correct pH is very important.

BRB80 Buffer

- 80mM K-PIPES (pH6.8)
- 1mM MgCl2
- 1mM EGTA

BRB80 Buffer - 100ml of 5X

- 12.0g K-PIPES (pH6.8)
- 0.5ml MgCl2 1M Stock
- 2.5ml EGTA 0.2M Stock

Adjust the PH to 6.8 with KOH and add water to 100ml

Glycerol PB

- 80mM K-PIPES (pH6.8)
- 5mM MgCl2
- 1mM EGTA
- 1mM GTP
- 33%(V/V) Glycerol

Glycerol PB - 100ml

- 2.4g K-PIPES (pH6.8)
- 0.5ml MgCl2 1M Stock
- 0.5ml EGTA 0.2M Stock
- 52.3mg GTP
- 33mls anhydrous Glycerol

Adjust pH to 6.8 with KOH and add water to 100ml

Nucleotides

These are made as stock solutions as 100mM ATP and 200mM GTP. It is important that the pH of these nucleotides does not become acidic otherwise hydrolysis will result. Consequently they are made up in water and adjusted to pH 7.5 with sodium hydroxide. The ATP solution is made 200mM with respect to MgCl2. This is not the case with the GTP solution since this will cause precipitation. These solutions should be stored at -80°C until needed.

For 20ml ATP dissolve 1.102g ATP with 0.813g MgCl2 and make up to 19 ml with water. Adjust pH to 7.5 with sodium hydroxide and make up the volume to 20ml.

For 20 ml GTP take 2.37g Na3GTP and make up to 18 ml with water and adjust the pH to 7.5 with sodium hydroxide and make up to 20 ml with water.

Protein Concentration Determination Solutions

Protein concentration determinations were made using the BioRad version of the Bradford protein assay , reading the absorption at 595nm using Bovine Serum Albumin as a reference standard.

Preparation of Tubulin from Bovine brain

The week before

Book all the centrifuges and rotors.

The night before

- Move the rotors into the cold room
- Move the waring blender into the cold room
- Move the balance into the cold room.
- Switch the warm room opposite Connie's lab to 37C.
- Move the type 19 rotors into the warm room
- Move the type 19 bottles into the warm room.

It is essential, however, to get the brains as fresh as possible since protein degradation will begin to take place soon after death. The brains should be warm when they are received and should be immediately plunged into a mixture of ice and saline solution for transport back to the laboratory. Do not use cold brains as the prep will fail.

This is the fax that we sent to the boss of the slaughter house before going:

Wir benoetigen 30 bis 40 Schweinehirne fuer die Aufreinigung eines von uns zu Forschungszwecken benoetigten Proteins.

Es ist entscheidend, dass die frischen, warmen Schweinehirne schlagartig auf die Temperatur von Eiswasser (ca. 4 Grad Celsius) gekuehlt werden und danach diese Temperatur behalten bis wir sie erhalten.

Deshalb waere es optimal, wenn die Schweinehirne direkt nach dem Toeten (also noch warm) in eine von uns bereitgestellte eis-kalte Salzloesung gegeben wuerden.

Steps

1. ***Preparation of Brain Tissue for the First Cold Spin***

   Cautionary note: To avoid contact with pathogens associated with nervous tissue, always handle the brains with gloves and dispose of waste tissue by incineration.

   At the laboratory cold-room the brains should be stripped of brain stems, blood clots and meninges (kitchen paper tissue is very good for this purpose). The brains are then weighed, transferred to the Waring blender, and cold blending buffer (BB) containing 1mM ATP but without is added in the ratio of one Litre of buffer per kilogram of brain tissue. The brains are then homogenized twice for approximately 30 seconds each, and then poured directly into the 1L centrifuge bottles. Do not overfill these bottles or they leak. Typically it can be expected to need approximately five Litres of BB for 12 brains giving about ten Litres of total homogenate. These homogenates are then centrifuged at 7000 RPM in the JL8.1000 for 15 minutes. This clears the homogenate of both unhomogenized stuff and bubbles that result from the centrifugation, thus reducing the capacity required for the next step by about one half

   The sups are the transferred to the 250ml bottles and spun at 14,000 for 45 mins.

2. *First Warm Centrifugation*

The supernatants (approximately five Litres for a 12 brain preparation) are pooled and poured into two five-Litre Erlenmeyer flasks. A half volume of anhydrous glycerol that has been prewarmed to 37°C is then added and the GTP, ATP and MgCl2 concentrations are adjusted to 0.5mM, 1.5mM and 3mM respectively. The essential step is to bring the tubulin solution above 30°C as quickly as possible.The tubulin is warmed in a 50C waterbath and the temp monitored. At some point after the tubulin has reached 30°C it will be noticed that the solution becomes noticeably more viscous indicating that polymerization is beginning to take place. The tubulin is incubated for a further 60 minutes at 37°C in a water bath, before recovery by centrifugation at 18,000 rpm for 120 minutes at 35°C in Beckman Type 19 rotors.

3. *Second Cold Centrifugation*

The pellets are resuspended in approximately in about 500 mls PB at 4°C using either a Dounce or a continuous flow homogenizer (such as the Yamato). At this point the protein concentration of the resuspended pellet solution should be determined and the concentration lowered to 25mg/ml by addition of cold PB. The solution is then left on ice for 40 minutes to allow for complete depolymerization before centrifuging at 40,000 rpm for 30 minutes in Beckman Type 45Ti rotors at 4°C.

4. *Second Warm Centrifugation*

Supernatants from this centrifugation are then pooled and the concentrations of ATP, GTP and MgCl2 adjusted to 1mM, 0.5mM and 4mM respectively. As before, a half volume of anhydrous glycerol prewarmed to 37°C is added and the entire mixture is then warmed to 37°C and the tubulin allowed to polymerize at this temperature for a further 40 minutes prior to centrifugation at 40,000 rpm at 35°C for one hour in Beckman Type 45Ti rotors. If time is short, the polymerized tubulin pellets can be frozen in liquid nitrogen and stored at -80°C.

5. *Third Cold Centrifugation*

The polymerized tubulin pellets are collected and resuspended in a total volume of 200mls of PB using either the continuous flow or Dounce homogenizer. The protein concentration should then be determined and the solution diluted with PB as necessary to 35mg/ml. The tubulin is allowed to depolymerize on ice for a further 40 minutes and then centrifuged at 40,000 rpm for 30 minutes in the Beckman Type 45Ti rotor. The supernatant is then ready for loading onto the Phosphocellulose column to separate the tubulin from the microtubule associated proteins (MAPs).

6. *Purification of Tubulin over a Phosphocellulose Column*

The depolymerised tubulin should then be loaded onto a one Litre sized column (at 4°C) that has been previously equilibrated in column buffer (CB). the column is strored in CB + azide. The best thing is to start the column dripping at a low flow rate in the morning. Tubulin should be loaded onto the column at a flow rate of approximately 1.5mls/minute. Once loaded however, the flow rate can be increased to 6mls/minute or even faster if the Phosphocellulose shows little sign of compressing, although some compression is inevitable. Purified tubulin is eluted in the flow through and the protein peak is pooled and the protein concentration determined. The purified tubulin in CB can then be snap frozen in liquid nitrogen or converted to tubulin in BRB80 using BRB80 conversion buffer prior to freezing and subsequent storage at -80°C.

Cycling Tubulin

It is inevitable, however that some tubulin will become inactivated or denatured during passage through the Phosphocellulose column, since tubulin is an unstable entity. It is therefore recommended to 'cycle' the tubulin after it passes through the column.

Protocol

Prewarm the MLA80 in a water bath at 37°C. Fill the rotor tubes to half their volume with the glycerol cushion consisting of 60% glycerol in BRB80 (no GTP), and place these in the rotor and allow them to equilibrate to 37°C. While this is happening proceed with the polymerization of the tubulin.

1. Thaw the tubulin rapidly using a 37°C water bath until the tube is half full of ice and then continue to thaw the remainder on ice. Adjust the solutes to Glycerol PB using anhydrous (100%) glycerol. Allow polymerization to occur for 40 minutes at 37°C

2. Layer the polymerized tubulin onto the prewarmed cushions using tips with large openings to avoid depolymerizing the microtubules. Centrifuge at 226,240g (50,000 RPM in the Beckman Ti50 rotor) for 60 minutes, or alternatively at 70,000 RPM for 30 minutes in the TLA100 rotor, at37°C.

3. Aspirate away the supernatant above the cushion. Rinse the cushion interface twice with water. Aspirate away the cushion. Resuspend the pellet in 0.25X BRB80+0.1% β-mercaptoethanol on ice, using an homogenizer to depolymerize the microtubules. The volume of buffer used for the resuspension is chosen so that the final tubulin concentration is between 10 and 20 mg/ml (based on the assumption that approximately half the tubulin from the Phosphocellulose column will polymerize). Incubate on ice for 15 minutes and then add 5X BRB80 to adjust the buffer to1X BRB80 (note: The volume of 5X BRB80 to add is 3/16ths of the volume of 0.25X BRB80-tubulin).

4. Sediment the undepolymerized microtubules by centrifuging the sample at 213,483g (70,000 RPM in the Beckman TLA100.2 rotor) for 15 minutes at 4°C.

5. Aliquots (10-200µl) of the concentrated tubulin can be made, which should then be snap frozen in liquid nitrogen. The tubulin can then be stored either in liquid nitrogen (indefinitely) or at -80°C (for at least 12 months).

Preparation of a Phosphocellulose column

For large scale preparations of tubulin (finally giving 1g or more of purified tubulin) a Phosphocellulose (PC) column of approximately one litre volume will be necessary. For successful preparation of PC, it has to be equilibrated for short periods first in base and then in acid, interspersed with water washes. This is normally achieved by suspension of the PC in either the acid or the base solution and then rapid filtration over a sintered glass funnel where the PC can be washed with large volumes of water. Large volumes of PC are, however, quite cumbersome to handle and the importance of setting up an efficient filtration system before commencing the PC preparation cannot be overemphasised. With volumes as large as one litre it may be unwise to rely on running water aspirators since the vacuum produced may be insufficient (but for small volumes of PC these may be suitable). Better is to use a membrane type pump and if, possible, connect this to a wide, sintered glass funnel over which Whatman 3MM filter paper has been placed. Alternatively, and we have used this quite successfully, is to use some nylon or polypropylene meshing (available from SpectraMesh) since this allows rapid filtration rates with little risk of tear as there is when using the Whatman filter paper.

Useful figures when considering the amount of Phosphocellulose to prepare

How much Phosphocellulose is needed?

When considering what volume of Phosphocellulose is needed, our lab uses 200mls Phosphocellulose to obtain 300-600mg of purified tubulin.

Characteristics of Phosphocellulose

One gram of PC powder when equilibrated in buffer will give between five and six millilitres of PC column matrix (depending on the salt concentration) but allow for some loss due to the removal of fines when calculating the amount of PC to prepare.

Materials

1. Phosphocellulose was obtained from Whatman Scientific Ltd, Whatman House, St. Leonard's Road, 20/20 Maidstone, Kent ME16 OLS, England.

2. Column and Adaptors were obtained from Kontes but any column with similar dimensions (10 x 30 x 4.8cm) should be suitable.

Protocol for Preparation of Phosphocellulose

Note: All stages at 4C.

Having determined the amount of Phosphocellulose that is needed using the information above, the dry powder should slowly be hydrated by washing twice in 95% ethanol, once in 50% ethanol, and one final wash in water.

Resuspend the hydrated Phosphocellulose in 25 volumes (liquid volume per original dry weight of Phosphocellulose) of 0.5M NaOH and stir gently (stirring too fast produces fines which will result in slow column

flow rates if not removed later) for 5 minutes. Filter the Phosphocellulose rapidly to remove the NaOH solution, and continue to rinse with water until the pH of the washings is lower than 10 (usually at least three times the volume of the NaOH solution used).

As soon as the pH is sufficiently low, resuspend the Phosphocellulose in 25 volumes of 0.5M HCl (i.e. the same volume as the NaOH solution as used above) and again stir gently for 5 minutes and quickly filter. Continue to wash with water until no longer acid-usually around ten times the volume of the HCl solution used.

If the column is not to be poured at this stage the Phosphocellulose should be resuspended and stirred for five minutes in a 2M solution of Potassium phosphate pH7.0. The Phosphocellulose can then be resuspended and stored in a solution of 0.5M Potassium phosphate pH 7.0 containing 20mM Sodium azide as preservative. When the Phosphocellulose is removed from storage it should be resuspended and washed in at least three volumes of water.

At this stage whether the column was stored or not, the Phosphocellulose should be resuspended in at least three volumes of water and transferred to a large measuring cylinder. The Phosphocellulose should be stirred and then allowed to settle naturally. After the bulk of the Phoshocellulose has settled, it will be noticed that there is a cloudy layer just above it. These are the fines and should be removed by aspiration to ensure fast column flow rates. This cycle of resuspension,stirring and aspiration of the fines should be repeated until no more fines are visible.

The Phosphocellulose can now be resuspended in Column Buffer (see below) solution and then degassed for 30 minutes prior to pouring the column.

After use, the Phosphocellulose column should be extensively washed and the buffer replaced with 50mM Potassium phosphate buffer containing 20mM Sodium azide.

Chemical Suppliers

All chemicals should be of analytical grade and could be obtained from a variety of suppliers. Chemicals for the solution given below were obtained from the following suppliers:

From Sigma-Aldrich

- PIPES P-6757
- EGTA E-4378
- MgCl2 104-20
- GTP G-8877
- ATP A-1388
- β-mercaptoethanol M-6250

From Merck

- EDTA 1.08418.1000
- Anhydrous Glycerol 1.04093.2500
- NaCl 1.06404.1000
- NaOH 1.06498.1000
- HCl 1.00319.2500
- KH2PO4 4873.1000
- *K2HPO4 5099.1000*

From BioRad

Protein Assay Kit, product number 500-0006)

Phosphocellulose

was obtained from Whatman Scientific Ltd, Whatman House, St. Leonard's Road, 20/20 Maidstone, Kent ME16 OLS, England.

Tel. 01622 676670; Fax. 01622 677011

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