Purification of tubulin from plant material using the TOG column

Takashi Hotta, Hashimoto lab, NAIST
June 2013, revised in April 2016

Recently, the TOG domains of yeast Stu2 protein have been utilized as an efficient tool for tubulin purification (Mol. Biol. Cell, 23: 4393-4401, 2012). I modified the original paper and applied the method to plant material. The column, called TOG column, turned out to be very useful for tubulin purification from plant material especially actively-dividing cultured cells. Plant tubulin purified with TOG-method is highly assembly-competent and thus can be used in various in vitro experiments in which pig-brain tubulin had to be substituted. Our paper of purification and characterization of plant tubulin has been published (Hotta et al., Plant Physiology, 170: 1189-1205, 2016). Here, I summarized a little bit more detailed purification procedure. Should you have any questions, ask me at thotta@bs.naist.jp

Note that the plasmid of GST-TOG1/2 (pGEX-6P-1-Stu2[1-590]) is a kind gift from Dr. Per O. Widlund (Max Planck Institute for Molecular Cell Biology and Genetics). Please request him for permission to obtain the plasmid.

1. Preparation of GST-TOG1/2 proteins

To make 2-ml resin TOG-column (that we call medium-size TOG column), total of ~40 mg GST-TOG1/2 proteins need to be prepared. In my hands, 1L bacterial culture yields ~5 mg of GST-TOG1/2 protein (the original lab seems more successful in terms of the protein yield). So, I usually repeat quite some rounds of induction (1 round = 500 ml x 2) to achieve 7~8 L culture. In reality, preparing 40 mg of protein requires too much work, so I recommend you to prepare 20 mg of protein and make 1-ml resin TOG-column (we call it small TOG column).

1.1 Large scale induction of GST-TOG1/2 proteins (1 round: 500 ml x 2 = 1L culture)
1st day
- Streak the GST-TOG1/2 bacteria (strain Rosetta) on an LB (100 ug/ml carbenicillin + 15 ug/ml chloramphenicol) plate. Incubate at 37ºC, over night.

2nd day
- Start the preculture of the bacteria. 3 ml of LB (100 ug/ml carbenicillin + 15 ug/ml chloramphenicol). 37ºC, over night.
- Prepare 500 ml x 2 of TB medium for the next day.

Solution A	Solution B (for 1 L TB medium)
6 g Bacto tryptone	2.31 g KH_2PO_4
12 g Bacto yeast extract	12.54 g K_2HPO_4
2 ml Glycerol	100 ml DW
450 ml DW in 3L flask (prepare 2 flasks)	

After autoclaving both, add 50 ml of solution B to the solution A (Now you have 500 ml medium x2).

3rd day
- Add 1 ml of the over night culture to each of 500 ml TB medium (containing 100 ug/ml carbenicillin + 15 ug/ml chloramphenicol).
- Culture at 37ºC until OD_{600} reaches 0.6 ~ 0.8.
- Put the flasks in the ice-water for 10 min.
- Culture the bacteria for additional 1 hour at 18ºC.
- Add 500 ul of 0.2 M IPTG (final 0.2 mM) and culture them for over night.

4th day
- Centrifuge the bacterial culture. Resuspend the pellets with 1x PBS, divide them into three 50 ml tubes and centrifuge them (1L culture turns to 3 tubes here). Store rinsed pellets in -80ºC freezer until use.

1.2 Purification of GST-TOG1/2 proteins

To save time, I handle 6 tubes of bacterial stocks (which are from 2 L culture) at one round of purification using total 40 ml resins in 8 columns (each column has 5 ml resins). Because 2 L culture typically yields ~10 mg of GST-TOG1/2 proteins, I repeat this preparation twice to prepare 20 mg of protein (which is required for making 1-ml resin TOG-column). When I make 2-ml resin TOG-column, I repeat the below procedure ~4 times.

Preparation:

Extraction buffer (200 ml)

10x PBS	20 ml
100 mM $MgCl_2$	2 ml
10% Triron-X100	10 ml
0.1M ATP	2 ml
1M DTT	200 ul
PMSF (final 0.2 mM)	
DW	166 ml

(will be used for column equilibration as well)

Wash buffer I (200 ml)

10x PBS	20 ml
100 mM $MgCl_2$	2 ml
0.1 M ATP	2 ml
1 M DTT	200 ul
PMSF (final 0.2 mM)	
DW	176 ml

Wash buffer II (200 ml)

10x PBS	20 ml
100 mM $MgCl_2$	2 ml
PMSF (final 0.2 mM)	
DW	176 ml

Elution buffer (30 mM glutathione, 120 ml)

Reduced glutathione	368 mg
10x PBS	12 ml
DW	108 ml

Column equilibration (160 ml)

10x PBS	16 ml
DW	144 ml

Dialysis buffer (1 L)

$NaHCO_3$	8.4 g
NaCl	5.8 g
DW	1 L

Column regeneration sol. #1 (100 ml)

1 M Tris pH 8.5	10 ml
NaCl	2.92 g
10% SDS	1 ml
DW	89 ml

Column regeneration sol. #2 (100 ml)

1 M Sodium Acetate pH 4.5	10 ml
NaCl	2.92 g
10% SDS	1 ml
DW	89 ml

- Pour the extraction buffer onto the bacterial pellets (up to total ~30 ml including the bacteria). Apply sonication to break the bacteria (5 minutes or so per tube). If you are worried about insufficient lysis of bacteria, consider adding lysozyme in the extraction buffer.
- Clear the extract (JA20 rotor, 18,000 rpm, 20 min).

- Pack the immobilized glutathione resin (glutathione Sepharose 4B, GE Healthcare) in empty PD-10 columns and equilibrate them with 20 ml 1x PBS followed by each 5 ml extraction buffer (5 ml immobilized glutathione resin per column. I prepare 8 columns for this scale of experiment)
- Load the lysate onto the column at 4ºC (Approximately 20 ml lysate for a 5-ml column). If time permits, recycle the flow-through back onto the column at total of two rounds).
- Wash each column with 25 ml of wash I.
- Wash each column with 25 ml of wash II.
- (Room temperature) Elute with 5 ml of elution buffer per column. Take total 3 fractions (total volume of 15 ml from each column. You should have 120 ml of combined eluate from 8 columns).
- Measure the protein concentration with Bradford assay (for rough estimate).
- Dyalize the eluate against 1L of dialysis buffer at 4ºC for over night.
- Next day, collect the protein sample, concentrate it with ultrafiltration device (Amicon 30K cutoff) and store it in -80ºC. Here I usually try to concentrate the protein slightly more than 4 mg/ml, which is calculated according to the above Bradford assay (Remember that there is always some protein loss during procedures such as dialysis).

- Regenerate the glutathione columns by loading 10 ml of regeneration solution #1, 10 ml of DW, 10 ml of regeneration solution #2 and 10 ml of DW successively. Store them in 4ºC.

2. Preparation of a medium-size TOG column

If you make small TOG-column (1ml-resin), simply half all the volumes described below.

2.1 Solutions to be prepared

- Dialysis buffer (I use the leftover of the latest dialysis).
- 1 mM HCl, 7.5 ml
- Quenching solution (0.5 M ethanolamine, 0.5 M NaCl, pH8.3. Remember that ethanolamine shifts the pH up, so adjust the pH after it is added in the sodium chloride solution.)
- 6x PBS (20 ml) and 1x PBS (10 ml)
- Storage buffer (1x PBS containing 50% glycerol), 10 ml.

2.2 Preparation of the protein (keep the protein solution on ice).
- Repeat the large-scale purification until the total protein amount reaches around 40 mg.
- Combine all the protein samples together and adjust the concentration at 4 mg/ml (total volume should be around 10 ml) using the remaining dialysis buffer.
- Add $MgCl_2$-$6H_2O$ powder at final concentration of 80 mM and gently mix the protein solution.

2.3 Making the Column (Work at room temperature unless otherwise noted).
- Take 2 ml of resin (NHS-activated sepharose 4 Fast Flow, GE healthcare: the original slurry seems 62.5%, so take 3.2 ml of slurry. Note that the slurry percentage may change after long-term storage) and put them in a column (I use empty PD-10 column). At this moment, do not install the upper filter yet.

- Rinse the column with 7.5 ml of pre-chilled 1 mM HCl.
- Coupling: apply the GST-TOG solution onto the resin. Take the flow-through and recycle it back onto the resin to couple as much protein as possible with the resin. Repeat this manually for 30-40 minutes (Do not think that it is silly!).
- Take the final flow-through, measure its protein concentration with Bradford assay and compare it with the initial concentration to evaluate the coupling reaction. In my hands, typically 76-78 % of the proteins are coupled with the resin. Optional: Keep the unbound proteins for the next use.
- Quenching: Set the upper filter (which came with the original PD-10) and load 6 ml of the quenching solution and close the bottom cap. Incubate for 15 minutes. Remove the bottom cap to let the solution flow through. Repeat two more times (total 3 times, I know this is more than enough, but just in case).
- Wash: Wash with 20 ml of 6x PBS and 10 ml of 1x PBS. Now the column is ready to use.
- If you do not use the column immediately, load 10 ml of the storage buffer. Close the bottom cap when 1-2 ml of the storage buffer remains on the column.

3. Tubulin purification using a TOG column

Here I show the method of tubulin purification from Arabidopsis seedlings or suspension cultured cells. The use of cultured cells such as Arabidopsis MM2d or T87 or tobacco BY-2, is strongly recommended as they yield much more tubulin than seedlings do. Binding capacity of the small TOG-column (1 ml resin) is 600 ug of tubulin. My rules of thumb is that ~15 g (fresh weight) of MM2d or T87 cells or >500 g of Arabidopsis seedlings can satisfy this capacity (although I have never tried such a big scale of seedling experiment). For some reason, the tubulin-binding capacity of my medium TOG-column (2 ml resin) is less than what is expected from small column, but still it can capture 900~1,000 ug of tubulin.

There are two slightly different methods (normal method and phospho-preservation method). In the phospho-preservation method, phosphatase inhibitors are added to keep the phosphorylation state of the tubulin. If no special care for the tubulin dephosphorylation is needed, the normal method seems good enough.

3.1 Things to be prepared beforehand

A medium-size TOG column
5x Mg^{2+} free BRB80
5x BRB80
PD-10 desalting columns
Amicon ultrafiltration device 10k cutoff
Vivaspin 500 10k cutoff (optional)
Arabidopsis seedlings: 7-day old, grown under a light condition. Frozen with liquid nitrogen. Could be stored in -80ºC freezer until use.
Arabidopsis MM2d cells: Generally young culture is preferred. I usually use 3-5 day old culture. In case of BY-2 cells, 3 or 4 day old cells is better than 5-day old cells. Cultured cells also can be stored in -80ºC freezer after removal of the culture medium.

3.2 Solutions to be prepared

Extraction buffer: Prepare the same volume as the weight of the ground powder

Normal (none-phospho-preservation method)

	Total 30 ml
5x BRB80	12 ml
Complete Mini EDTA-free	2 tablets
PMSF (final 0.2 mM)	
1 M DTT	60 ul
DW	17.9 ml

Phospho-preservation method

	Total 30 ml
5x Mg^{2+}-free BRB80	12 ml
Complete Mini EDTA-free	2 tablets
PMSF (final 0.2 mM)	
1 M DTT	60 ul
100 mM glycerophosphate	660 mg
100 mM Na_3VO_4	60 ul
500 mM NaF	600 ul
DW	17.3 ml

Solutions for TOG column: All must be ice-cold (Volumes given are for a medium column. If you use small TOG column, half all the solution colume.) GTP and ATP should be added right before use.

Phospho-preservation method

- Equilibration, 20 ml 1x Mg^{2+} free BRB80 [5x Mg^{2+} free BRB80, 4 ml; DW, 16 ml]
- Wash #0, 10 ml 1x Mg^{2+} free BRB80, 100 uM GTP
  [5x Mg^{2+} free BRB80, 2 ml; 100 mM GTP, 10 ul; DW, 8 ml]

After wash #1: Same as below.

Normal method

- Equilibration, 20 ml 1x BRB80 [4 ml 5xBRB80, 16 ml DW]
- Wash #1, 10 ml 1x BRB80, 100 uM GTP [5xBRB80, 2 ml; 100 mM GTP, 10 ul; DW, 8 ml]
- Wash #2, 24 ml 1x BRB80, 10 uM GTP [5xBRB80, 4.8 ml; 100 mM GTP, 2.4 ul; DW, 19.2 ml]
- Wash #3, 8 ml 1x BRB80, 100 uM GTP, 10 mM $MgCl_2$, 5 mM ATP
  [5xBRB80, 1.6 ml; 100 mM GTP, 8 ul; 1 M $MgCl_2$, 80 ul; 0.1 M ATP, 400 ul; DW, 5.2 ml]
- Wash #4, 12 ml 1x BRB80, 10 uM GTP [5xBRB80, 2.4 ml; 100 mM GTP, 1.2 ul; DW, 9.6 ml]
- Elution, 5 ml 1x BRB80, 10 uM GTP, 500 mM $(NH_4)_2SO_4$
  [5xBRB80, 1 ml; 100 mM GTP, 0.5 ul; $(NH_4)_2SO_4$, 330 mg; DW, 4 ml]

- Regeneration I, 25 ml 1x PBS
- Regeneration II, 30 ml 10x PBS
- Regeneration III, 25 ml 1x PBS
- Storage buffer, 20 ml 1x PBS, 50% glycerol

Solution for desalting and ultrafiltration: on ice

- Equilibration for two PD-10 columns, 25 ml 1x BRB80 for each
- Elution for two PD-10 columns, 3.5 ml 1x BRB80 for each

(So, make total 30 ml of 1x BRB80 for a PD-10 column)

3.3 Procedures
- Grind the seedlings or cultured cells with liquid nitrogen using mortar and pestle.
- Transfer the ground powder to 50 ml tubes (I usually do not put too much powder in a 50 ml tube. I limit about 9 g of powder with 9 ml of extraction buffer in a tube).
- Set the tubes on a rotator and incubate them at 4ºC for 20-30 minutes. Meanwhile, turn the centrifuge on and keep it at 4ºC.
- Centrifuge at 18,000 rpm for 10 min at 4ºC. During this centrifuge, equilibrate the column.
- Filter the supernatant with 0.45 um syringe filter.
- Roughly measure the lysate volume. Take 1 ul for Bradford assay. Also I take 3.5~5 ul for SDS-PAGE (good for 1 lane).

- Equilibration: (During the above centrifugation) Take the TOG column out of the freezer and remove the storage buffer. Load the equilibration buffer. Also remember to equilibrate the desalting columns PD-10 with 25 ml of 1x BRB80.
- Loading: load the lysate on the TOG column. If time permits, recycle the flow-through back on the column. Keep the flow-through fraction and take 1 ul for Bradford assay (optional) and 3.5~5 ul for SDS-PAGE.
- Successive washes #1-4. Note that if you are trying phospho-preserving method, you need wash#0 before washes #1.
- Elution. Take the eluate in a 5 ml tube, mix it well and take 10 ul for SDS-PAGE. Rush!!
- Desalting: Immediately after the elution, apply each 2.5 ml of eluate onto a PD-10 (use two PD-10s).
- Load 3.5 ml of 1x BRB80 on each PD-10 column and take the eluted fraction.
- Ultrafiltration (concentration) with Amicon 10K cutoff. 3,500 rpm ~90 minutes. The concentrated tubulin solution should be around 50-100 ul. If you still need to concentrate it, use vivaspin 500.
- Take 1 ul for Bradford assay and estimate the tubulin concentration. Based on this estimate, prepare 1 ug tubulin for SDS-PAGE (for 1 lane). Prepare 0.375, 0.75 and 1.5 ug of BSA for quantification standard. After running the SDS-PAGE, I quantify more accurate concentration of tubulin by densitometric analysis.

-Regeneration of TOG-column: regeneration I, II, III and fill the column with the storage buffer. Store the column at -20ºC until next use. Column can be used many time (~50 times or more) over at least 3 years.

4. Selection of internally His-tagged tubulin from purified tubulin fraction

To isolate tubulin dimers containing particular isoform of a-tubulin from TOG-purified total tubulin fraction, the 6xHis-tag inserted between Val-42 and Gly-43 of Arabidopsis a-tubulin worked well. We constructed pGWB2-AtTUA6^{6xHis} and transformed it in Arabidopsis T87 cell line. After purifying total tubulin pool using TOG method, I further applied it to Nickel column to select His-tagged tubulin. Here, I show the 2-step purification method.

4.1 Things to be prepared beforehand
- Two TOG columns
- Three PD-10 desalting columns
- Ni Sepharose 6 Fast Flow resin (GE Healthcare)

4.2 Solutions to be prepared

- Wash #5, 18 ml 80 mM PIPES, 1 mM $MgCl_2$, 10 uM GTP, pH 6.8
- EGTA free elution buffer, 10 ml 80 mM PIPES, 1 mM $MgCl_2$, 10 uM GTP, 500 mM $(NH_4)_2SO_4$, pH 6.8
- 2 M Imidazole (in DW)
- Ni-binding buffer, 6 ml 80 mM PIPES, 1 mM $MgCl_2$, 500 mM $(NH_4)_2SO_4$, 30 mM imidazole pH 6.8
- Ni-wash buffer #1, 35 ml 80 mM PIPES, 1 mM $MgCl_2$, 500 mM $(NH_4)_2SO_4$, 30 mM imidazole, 10 uM GTP pH 6.8
- Ni-wash buffer #2, 12 ml 80 mM PIPES, 1 mM $MgCl_2$, 30 mM imidazole, 10 uM GTP pH 6.8
- Ni-elution buffer, 7.5 ml 80 mM PIPES, 1 mM MgCl2, 250 mM imidazole, 10 uM GTP, pH 6.8
- 100 mM EGTA
- 1x BRB80, 90 ml (to equilibrate three PD-10 columns)

4.2 Procedures

Purification of total tubulin is essentially the same as described above. To increase total tubulin amount, I usually use two TOG columns (one each small and medium column) in parallel. Initial cell weight of 45 ~ 60 g would be good. Typically 18 ~ 33 ug of tagged-tubulin can be recovered.

- Wash the columns with wash buffers #1-#4 successively.
- Load 6 ml (for a small column) or 12 ml (for a medium column) of wash buffer #5.
- Elute tubulin with 4 ml (for a small column) and 6 ml (for a medium column) of EGTA-free elution buffer and combine the eluate together.
- Add 150 ul of 2 M imidazole (final 30 mM) and mix gently.

- Equilibrate 1.2 ml resin volume of Ni Sepharose (1.5 ml suspension) by rinsing resins with 2 ml of Ni-binding buffer in a 5-ml sample tube and centrifuging it at 1,000 xg for 1 min. Repeat total three times.
- Gently mix the resins with the above tubulin solution in a 15-ml tube and slowly rotate it at 4ºC for 30 min.
- Centrifuge at 1000 xg for 2 min at 4ºC and discard the supernatant (= flow-through fraction).
- Resuspend the resin in small volume of Ni-wash buffer #1 and transfer it to an empty PD-10 column.
- Wash with the remaining wash buffer #1 (total 35 ml).
- Wash the resin with wash buffer #2 (12 ml)
- Elute tagged-tubulin with 7.5 ml of elution buffer and add ~75 ul of 100 mM EGTA.
- Apply this to three PD-10 desalting columns (pre-equilibrated with 25 ml of 1x BRB80).
- Elute with 3.5 ml of BRB80 per column (total 10.5 ml should be recovered).
- Concentrate the tagged-tubulin fraction with Amicon (10K cutoff) to approximately 50 ul. If necessary, switch to smaller device such as vivaspin 500.