

Dimethyl Sulfoxide Is Feasible for Plant Tubulin Assembly *In vitro*: A Comprehensive Analysis

Chun-Hua XU*, Shan-Jin HUANG* and Ming YUAN**

(State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100094, China)

Abstract: It is much more difficult for tubulin from plant sources to polymerize *in vitro* than tubulin from animal sources. Taxol, a most widely used reagent in microtubule studies, enhances plant microtubule assembly, but hinders microtubule dynamics. Dimethyl sulfoxide (DMSO), a widely used reagent in animal microtubule studies, is a good candidate for the investigation of plant microtubule assembly *in vitro*. However, proper investigation is lacking about the effects of DMSO on plant microtubule assembly *in vitro*. In the present study, DMSO was used to establish optimal conditions for the polymerization of plant tubulin. Tubulin, purified from lily pollen, polymerizes into microtubules at a critical concentration of 1.2 mg/mL in the presence of 10% DMSO. The polymers appear to have a normal microtubule structure, as revealed by electron microscopy. In the presence of 10% DMSO, microtubule polymerization decreases when the pH of the medium is increased from 6.5 to 7.4. Both the polymerization rate and the mass of the polymers increase as temperature increases from 25 to 40 °C. Tubulin polymerizes and depolymerizes along with cycling of temperature, from 37 to 4 °C, or following the addition to or the removal of Ca²⁺ from the medium. When incubated with nuclei isolated from tobacco BY-2 suspension cells, tubulin assembles onto the nuclear surface in the presence of 10% DMSO. Labeling lily pollen tubulin with 5- (and 6-) carboxytetramethyl-rhodamine succinimidyl ester (NHS-rhodamine) was performed successfully in the presence of 10% DMSO. Labeled tubulin assembles into a radial structure on the surface of BY-2 nuclei. The polymerization of lily pollen tubulin is also enhanced by microtubule-associated proteins from animal sources in the presence of 10% DMSO. All the experimental results indicate that plant tubulin functions normally in the presence of DMSO. Therefore, DMSO is an appropriate reagent for plant tubulin polymerization and investigation of plant microtubules *in vitro*.

Key words: dimethyl sulfoxide; *Lilium davidii* Duch.; tubulin labeling; tubulin polymerization; tubulin purification.

Microtubules are polymerized by tubulins that are abundant in eukaryotic cells. Many of the cellular functions with which microtubules are associated depend upon the dynamic characteristics of microtubule polymerization/depolymerization. Although tubulin is one of the most conserved proteins among eukaryotes, differences exist between plant and mammalian tubulins.

One of the most remarkable differences is that it is much more difficult for plant tubulin to polymerize *in vitro* than mammalian tubulin. Consequently, it is difficult to obtain large quantities of plant tubulin for analysis. Hence, studies and theories concerning microtubule dynamics come mostly from investigations using mammalian tubulins. However, microtubule

Received 14 Jul. 2004 Accepted 3 Dec. 2004

Supported by the State Key Basic Research and Development Plan of China (G1999011701) and the National Natural Science Foundation of China (30128008 and 30170057).

* Contributed equally to this study.

**Author for correspondence. Tel: +86 (0)10 6273 3436; Fax: +86 (0)10 6273 3491; E-mail: <mingyuan@cau.edu.cn>.

organization, distribution, and dynamics are distinctively different between plant cells and animal cells. For example, the cortical microtubule array, the microtubule preprophase band, and the phragmoplast are unique in plant cells (Staiger and Lloyd 1991; Shibaoka and Nagai 1994). Furthermore, investigations have revealed that plant microtubule turnover may be more rapid in plant cells than in mammalian cells (Hush *et al.* 1994; Yuan *et al.* 1994; Dhonukshe and Gadella 2003). All such questions regarding microtubule organization, distribution, and dynamics in plant cells are related to tubulin assembly and disassembly.

In vitro systems for microtubule formation facilitate more detailed analyses of the molecular mechanism governing tubulin polymerization and further our understanding of the mechanism of microtubule function. Therefore, it is important to establish an *in vitro* system for the polymerization of plant tubulin to investigate the characteristics of plant microtubule dynamics. This will be useful in understanding the functioning mechanism of the microtubule cytoskeleton in plant cells.

Plant tubulin may assemble *in vitro* in the absence of microtubule-stabilizing reagents, but with a very high critical concentration (Huang *et al.* 2000). Therefore, it is difficult to use such a system in plant microtubule studies, considering the difficulties in purifying tubulin from plant cells. Taxol enhances plant tubulin assembly (Bokros *et al.* 1993; Moore *et al.* 1997). However, taxol caused marked changes in the assembly/disassembly equilibrium. Microtubules are usually difficult to disassemble in the presence of taxol. It has been reported that some reagents, other than taxol, can enhance the assembly of mammalian tubulin *in vitro*. Dimethyl sulfoxide (DMSO) is a widely used reagent for investigations into microtubule formation from purified mammalian tubulin (Himes *et al.* 1977; Robinson and Engelborghs 1982). However, there are few reports regarding plant tubulin polymerization in the presence of this reagent. Thus, it is necessary to identify whether DMSO enhances plant tubulin assembly *in vitro* and, then, to characterize the properties of plant

microtubule dynamics in the presence of DMSO.

In the present study, we used two-step anion chromatography to purify tubulin from lily pollen and investigated the optimal conditions, such as DMSO concentration, pH, and temperature, to establish an *in vitro* system for lily pollen tubulin assembly in the presence of DMSO. Our experimental results demonstrate that lily tubulin polymerizes into microtubules and that the microtubules function normally in the presence of DMSO. Cycles of lily pollen tubulin polymerization/depolymerization according to temperature or Ca^{2+} , and labeling of tubulin with 5- (and 6-) carboxytetramethylrhodamine succinimidyl ester (NHS-rhodamine) were performed in the presence of 10% DMSO.

1 Materials and Methods

1.1 Materials

Lilium davidii Duch. pollen was collected from the suburban district of Lanzhou City, China, and stored at $-20\text{ }^{\circ}\text{C}$ in a refrigerator after desiccation. Experimental reagents, except those described in detail, were obtained from Sigma (St Louis, MO, USA).

1.2 Purification of lily pollen tubulin

Lily pollen tubulin was purified according to the method of Huang *et al.* (2000). Purified tubulin was aliquoted into 50 μL aliquots and stored in liquid nitrogen after preparation.

Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined using a Bio-Rad (Hercules, CA, USA) protein dye reagent with IgG as the standard.

1.3 Turbidimetric measurement and polymer sedimentation assay of tubulin assembly

The methods used were as described by Huang *et al.* (2000).

1.4 Electron microscopy

The morphology of the plant tubulin polymers was observed with transmission electron microscopy (TEM), as described previously (Kim *et al.* 1979), with some modifications. Microtubules were sedimented as described above. Pellets were fixed overnight at room

temperature with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and then post-fixed for 2 h at 4 °C with 1% osmium tetroxide in the same buffer. Pellets were then embedded in 1% agarose and dehydrated through a graded ethanol series and propylene oxide. Pellets were further embedded in Epon 618 and sectioned with an ultramicrotome (LKB III, Sweden). Sections (100–200 nm) were placed on 200 mesh copper glider grids and stained for 30 min with uranyl acetate and then for 15–20 min with lead citrate. Electron microscopic observations were performed on a Hitachi (Tokyo, Japan) H-7500 electron microscope.

1.5 Determination of the critical concentration for tubulin polymerization

The critical protein concentration, C_c , was determined from the mass of polymerized tubulin in the pellets and the tubulin concentration in the supernatant according to the method of Mitchison and Kirschner (1984).

1.6 Preparation of NHS-rhodamine-labeled lily pollen tubulin

Two-step ion exchange-purified lily pollen tubulin was thawed in wet ice and clarified by centrifugation at 100 000g at 4 °C for 20 min. The supernatant was added to final concentrations of 1 mmol/L GTP and 10% DMSO and incubated at 35 °C for 60 min to allow the tubulin to polymerize. Then, a 1/10 volume of 100 mmol/L NHS-Rhodamine (Molecular Probes, Eugene, OR, USA) in DMSO was added to the mixture and samples were incubated at 35 °C for 10 min, vortexing every couple of minutes. The reaction was terminated by the addition of 100 mmol/L potassium glutamate. Labeled microtubules were sedimented by centrifugation at 25 000g at 35 °C for 30 min. Pellets were resuspended in 5 volumes of cold PEM buffer (0.1 mol/L Pipes, 1 mmol/L GTP, 1 mmol/L MgSO₄, 1 mmol/L EGTA, pH 6.9) and incubated on ice for 10 min. The solution was then sedimented at 25 000g at 4 °C for 20 min to remove the denatured proteins. The supernatant was adjusted to 10% DMSO and 1 mmol/L GTP and incubated at 35 °C for 60 min. Microtubules were pelleted at 25 000g at 35 °C for 30 min. An

additional temperature cycle was repeated, as above. Microtubule pellets were then resuspended in cold PEM and incubated on ice for 10 min before being subjected to centrifugation at 25 000g at 4 °C for 20 min. Labeled tubulin was then aliquoted into 10 µL aliquots, frozen, and stored in liquid nitrogen until future use.

1.7 Assembly of NHS-rhodamine-labeled lily pollen tubulin with nuclei of BY-2 cells

Nuclei were isolated from BY-2 cells according to the method of Mizuno (1993). The NHS-rhodamine-labeled lily pollen tubulin (1 mg/mL) was incubated with isolated nuclei in polymerization buffer (0.1 mol/L Pipes, 1 mmol/L GTP, 1 mmol/L MgSO₄, 1 mmol/L EGTA, 5 µmol/L taxol, pH 6.9) at 30 °C. After incubation, the reaction mixture was examined directly on a confocal microscope (Bio-Rad MRC1024).

Crude microtubule-associated proteins (MAPs) were isolated from pig brain according the method of Vallee (1982). Isolated MAPs were frozen in liquid nitrogen and stored at –80 °C.

2 Results

2.1 Purification of lily pollen tubulin

A two-step anion exchange method was used to isolate plant tubulin. The suspension of lily pollen acetone powder was centrifuged and the supernatant was collected and loaded onto DEAE-Sephadex A-50. After chromatography at this step, partially purified tubulin was obtained with a purity of approximately 80% (Fig. 1). This partially purified tubulin was then rechromatographed (after desalting with Sephadex G-25) on a second, high-resolution anionic exchange column. Tubulin with a high purity was obtained after the second chromatography (Fig. 1). The yield of tubulin with 98.5% purity from lily pollen was approximately 5 mg from 10 g lily pollen acetone powder. However, the purity of tubulin had already reached approximately 80% with a yield of 12 mg tubulin from 10 g lily pollen acetone powder after the first DEAE-Sephadex A-50 chromatography. Furthermore, up to 60% of tubulin could be lost during the second step of chromatography. Therefore, in many cases, the

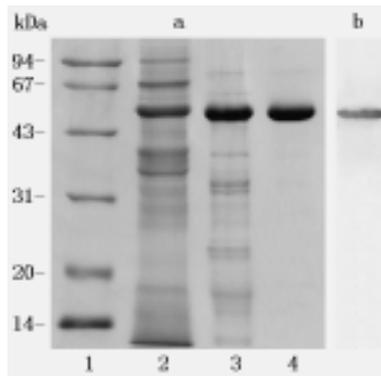


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purification of tubulin from lily pollen. **a.** The SDS-PAGE analysis. Lane 1, molecular weight standards; lane 2, total proteins of acetone powder; lane 3, tubulin fractions after DEAE-Sephadex A-50; lane 4, tubulin fractions after DEAE-toyopearl. **b.** Immunoblot of tubulin from lily pollen probed with a monoclonal antibody raised against rat β -tubulin.

second purification step can be avoided if purity is not crucial for the experiments.

2.2 Dimethyl sulfoxide enhances the polymerization of lily pollen tubulin

The effect of the concentration of DMSO on lily pollen tubulin assembly was investigated using the sedimentation assay. Tubulin was assembled in PEM buffer with various DMSO concentrations, ranging from 2% to 14%. The mass of the polymerization was measured using microtubule pellets. The data obtained showed that the amount of microtubules increased with increasing DMSO concentration (Fig. 2a). Considering that the amount of denatured tubulin also increased when the concentration of DMSO was higher than 10% (data not shown), 10% DMSO was used in the present series of experiments.

To further determine the effect of DMSO on lily pollen tubulin assembly, turbidity analysis experiments were undertaken. The results demonstrated that, as shown in Fig. 2b, 10% DMSO significantly promoted lily pollen tubulin assembly, as well as increasing the rate of lily pollen tubulin assembly and into larger amounts of microtubules. Results of TEM showed that the microtubules that assembled in the presence of 10%

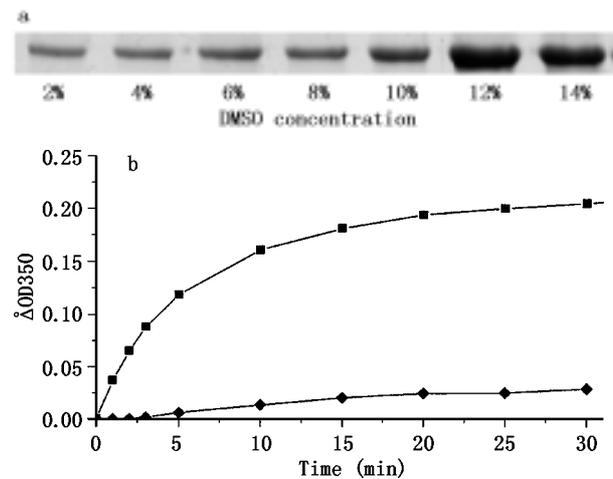


Fig. 2. Lily pollen tubulin assembly is enhanced in the presence of dimethyl sulfoxide (DMSO). **a.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of microtubules in the pellets of sedimentation assay with various concentrations of DMSO. Polymerized lily pollen tubulin increased with higher DMSO concentrations. Although the tubulin polymerized into most abundant microtubules in the presence of 12% DMSO, the amount of denatured protein also increased when the DMSO concentration was higher than 10% (data not shown). **b.** Polymerization of lily pollen tubulin was monitored by turbidimetric measurement. \blacklozenge , lily pollen tubulin assembled in the absence of DMSO; \blacksquare , lily pollen tubulin assembled in the presence of 10% DMSO. Tubulin assembly with 10% DMSO resulted not only in many more microtubules, but also in a higher speed of polymerization. The solution for tubulin polymerization contained 3.2 mg/mL of lily pollen tubulin.

DMSO appeared to have normal microtubule structure (Fig. 3). In all cases, when the apparent absorbance increased to a plateau value, there was no continual increase because of non-specific aggregation of the protein.

2.3 Dimethyl sulfoxide decreases the critical concentration of lily pollen tubulin polymerization

The critical concentration for lily pollen tubulin assembly in the presence of 10% DMSO was determined by measuring the mass of microtubules in pellets and tubulin in the supernatant and then plotted as the microtubule concentration versus tubulin concentration. The linear line that best fit the data was used to determine the intercept with the x -axis. The C_c for lily

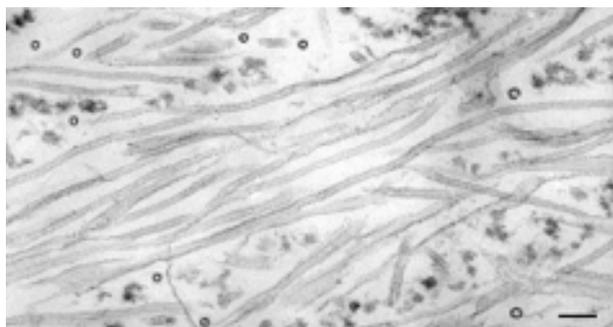


Fig. 3. Observations of microtubules polymerized in the presence of 10% dimethyl sulfoxide (DMSO). The structure of microtubules assembled in the presence of 10% DMSO was examined by electron microscopy. Microtubules appear normal when tubulin polymerized with DMSO. Bar = 100 nm.

pollen tubulin assembly in the presence of 10% DMSO was estimated to be 1.2 mg/mL (Fig. 4), which is considerably lower than that determined in the absence of a microtubule-stabilizing reagent (Huang *et al.* 2000).

2.4 Temperature Affects the polymerization of lily pollen tubulin in the presence of DMSO

It is well known that mammalian tubulin assembly is sensitive to temperature; the assembly of tubulin does not occur at physiologically low temperatures. However, little is known about the effects of temperature on the polymerization of plant tubulin. The results of the present study showed that, in the presence of 10% DMSO, pollen tubulin polymerization was very low when the temperature was below 25 °C, but that when the temperature was increased to 30, 35, and 40 °C, pollen tubulin assembled faster and into a larger amount of microtubules (Fig. 5). At low temperatures, such as 2 °C, microtubules virtually disassembled (Fig. 6A).

2.5 The polymerization of lily pollen tubulin is affected by the pH of the medium in the presence of DMSO

Considering that, generally, media with a pH 6.9 are used in tubulin assembly, media with a series of pH, ranging from 6.5 to 7.4, were used in the present study to investigate the effect of pH on lily pollen tubulin

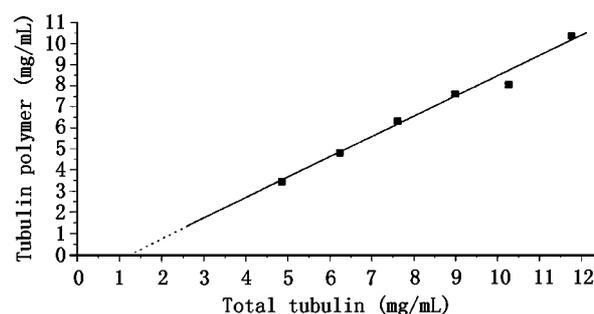


Fig. 4. Determination of the critical concentration (C_c) for lily pollen assembly in the presence of 10% dimethyl sulfoxide (DMSO). The C_c for lily pollen tubulin assembly in the presence of 10% DMSO was determined by measuring the mass of microtubules in pellets and tubulin in the supernatants, and then plotted as the microtubule concentration versus tubulin concentration. The linear line best fit to the data was used to determine the intercept with the x-axis. The C_c for lily pollen tubulin assembly in the presence of 10% DMSO was estimated as 1.2 mg/mL.

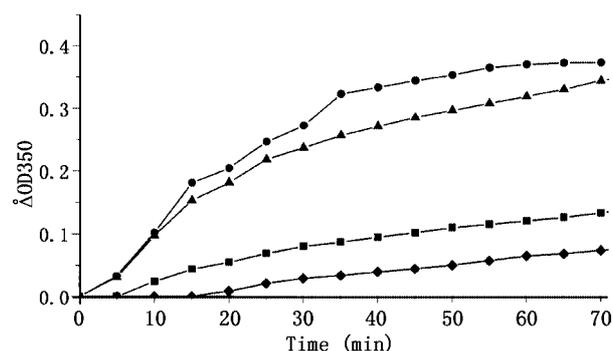


Fig. 5. Effect of temperature on lily pollen tubulin polymerization in the presence of dimethyl sulfoxide (DMSO). The polymerization of lily pollen tubulin was monitored by turbidimetric measurement. All assembly solutions contained 3.9 mg/mL lily pollen tubulin and 10% DMSO. ◆, 25 °C; ■, 30 °C; ▲, 35 °C; ●, 40 °C.

assembly in the presence of 10% DMSO. The tubulin was polymerized at 25 °C and the polymerization was measured by the quantity of pellet microtubules obtained by centrifugation after the tubulin polymerization had reached steady state. The results showed that tubulin polymerization declined considerably as pH increased from 6.5 to 7.4 (Fig. 7). Tubulin polymerization occurred much more favorably in an acidic medium.

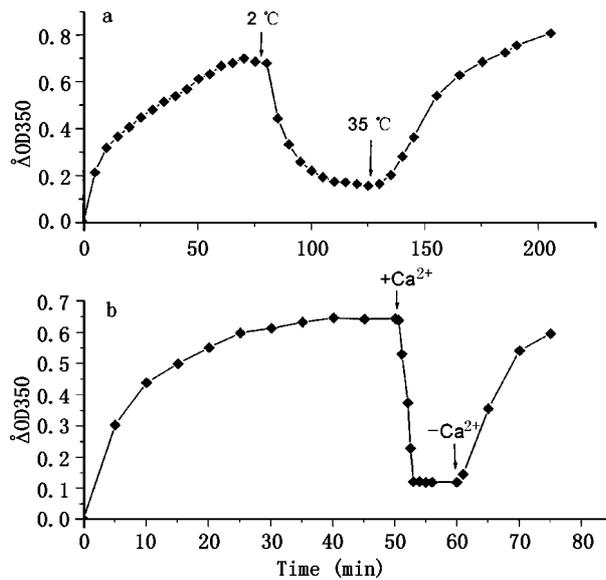


Fig. 6. Polymerization/depolymerization cycle of lily pollen tubulin according to temperature or the presence of Ca²⁺ in the presence of 10% dimethyl sulfoxide (DMSO). The process of tubulin polymerization and depolymerization was monitored turbidimetrically. **a.** Lily pollen tubulin (5.3 mg/mL) assembled first at 35 °C. When the incubating temperature declined to 2 °C (arrow), microtubule disassembly occurred. Raising the temperature to 35 °C (arrow) resulted in tubulin polymerization into microtubules again. **b.** Lily pollen microtubules preformed in the presence of 10% DMSO are sensitive to Ca²⁺. Lily pollen tubulin (5.3 mg/mL) assembled first at 35 °C. After the addition of 3 mmol/L Ca²⁺ to the medium (arrow), microtubule disassembly occurred. The tubulin polymerized into microtubules again when 5 mmol/L EGTA was added (arrow) to chelate the Ca²⁺.

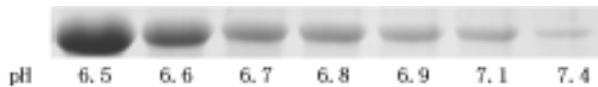


Fig. 7. Effect of pH on dimethyl sulfoxide (DMSO)-induced lily pollen tubulin assembly. Lily pollen tubulin (3.4 mg/mL) was assembled in the presence of 10% DMSO in media with different pH. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to determine the mass of microtubules in the pellets of the sedimentation assay.

2.6 Lily pollen tubulin functions normally in the presence of 10% DMSO

Cycling of tubulin polymerization and

depolymerization is essential in microtubule studies, such as tubulin purification, tubulin labeling, and isolation of MAPs. A previous report has indicated that plant microtubules polymerized with taxol can be disassembled, but that both Ca²⁺ and low temperature are necessary for this to occur (Moore *et al.* 1997).

In the present study, we tested the cycling of the polymerization and depolymerization of lily pollen tubulin with temperature and Ca²⁺ in the presence of 10% DMSO. The results show that, in the presence of 10% DMSO, lily pollen tubulin assembles at higher temperature and disassembles when the temperature drops to 2 °C. The tubulin assembled into microtubules again when the temperature was increased to 35 °C (Fig. 6a). However, cooling does not reduce the turbidity to the starting level, suggesting an irreversible aggregation of tubulin because of the denaturing of the protein at high temperatures. It is normal that a small amount of tubulin denatures at high temperatures. Nevertheless, tubulin runs the cycle of polymerization and depolymerization in the presence of 10% DMSO.

The DMSO-induced plant tubulin assembly was also sensitive to Ca²⁺ (Fig. 6b). The addition of 3 mmol/L Ca²⁺ led to a quick depolymerization of assembled lily pollen microtubules. However, the depolymerized tubulin assembled again after the addition of 5 mmol/L EGTA to chelate the Ca²⁺. Hence, cycling of polymerization/depolymerization of lily pollen tubulin can be achieved by manipulating either the temperature or the Ca²⁺ concentration in the medium in the presence of 10% DMSO, but both maneuvers are not necessary.

When incubated with nuclei isolated from tobacco BY-2 suspension cells in the presence of 10% DMSO, lily pollen tubulin assembled onto the surface of the nuclei and exhibited a radial structure (Fig. 8a).

2.7 NHS-rhodamine-labeled plant tubulin prepared with DMSO

Labeled mammalian brain tubulin has been proved a good probe and has been used in studies of plant microtubule dynamics. However, it would be much more reliable if plant tubulin could be labeled for plant microtubule studies, considering the differences between plant

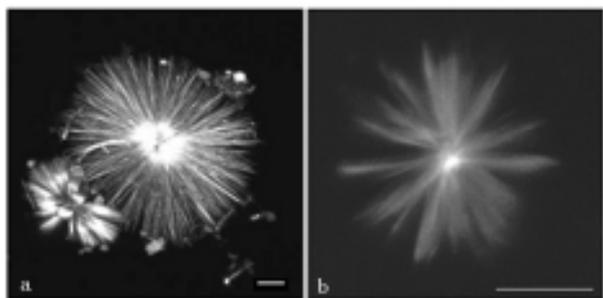


Fig. 8. Lily pollen tubulin assembles onto the surface of isolated nuclei of BY-2 cells. **a.** Lily pollen tubulin reassembles into a radial microtubule structure surrounding the BY-2 nucleus, as visualized using monoclonal anti- α -tubulin Cy3-conjugated mouse antibody (Sigma, St Louis, MO, USA). **b.** A similar structure of microtubules is observed when NHS-rhodamine-tagged lily pollen tubulin is incubated with pieces of BY-2 nucleus, indicating that NHS-rhodamine-tagged lily pollen tubulin is biologically active. Bar=10 μ m.

tubulin and animal tubulin.

Based on the experimental results above, it is indicated that lily tubulin can run through polymerization and depolymerization along with the temperature cycle with a relatively low critical concentration in the presence of 10% DMSO, we labeled microtubules assembled from lily tubulin with NHS-rhodamine and ran the labeled lily tubulin with temperature cycles to remove denatured protein and free dye. When incubated with isolated BY-2 nuclei, the labeled lily tubulin assembled into radial structures on the nuclear surface (Fig. 8b), demonstrating that the labeled plant tubulin was biologically active.

2.8 Animal MAPs enhance plant tubulin assembly in the presence of DMSO

To investigate further whether lily tubulin functions normally in the presence of 10% DMSO, we applied crude MAPs from pig brain to lily tubulin with 10% DMSO and examined the effect on tubulin polymerization. Turbidity analysis showed that the MAPs markedly enhanced tubulin assembly. The tubulin assembled into a much larger mass and at much faster rate when MAPs were added (Fig. 9).

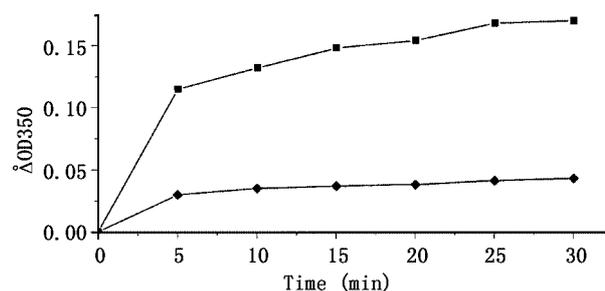


Fig. 9. Animal microtubule-associated proteins (MAPs) promote lily pollen tubulin assembly in the presence of 10% dimethyl sulfoxide (DMSO). The polymerization of lily pollen tubulin in the presence of 10% DMSO was monitored turbidimetrically. The tubulin assembled into a much larger mass and at a much faster rate when the MAPs from an animal source were added (■) compared with control (◆). The solutions contained 1.4 mg/mL lily pollen tubulin.

3 Discussion

3.1 Purification of plant tubulin compared with mammalian tubulin

Certain animal organs, such as the brain, are disproportionately rich in tubulins compared with plant tissues. Consequently, animal tubulins are readily isolated by direct polymerization of endogenous tubulin in crude cellular supernatants. Application of this method to plant crude supernatants has met with limited success because plant tissues are not particularly rich in tubulin and many possess factors that inhibit tubulin polymerization (Morejohn and Fosket 1991). Nevertheless, tubulin can be isolated from cultured higher plant cells by ion-exchange chromatography and, subsequently, polymerized into microtubules (Morejohn and Fosket 1982). However, the central vacuoles and cell wall occupy most of the plant cell volume and the cytoplasm of plant cells usually comprises only a small portion. In addition, there are a lot of proteinases in the vacuole, which may have a serious effect on tubulin proteins. Therefore, a large quantity of plant materials is needed, along with the addition of large amounts of proteinase inhibitors to separate and purify tubulins from plant cells (Bokros *et al.* 1993).

Pollen cells have been proven to be good experiment material for tubulin purification (Huang *et al.* 2000). The two-step ion-exchange chromatography modified by Moore *et al.* (1993) may shorten the time of tubulin purification, which benefits tubulin activity. In the present study, partially purified lily pollen tubulin was obtained by DEAE-Sephadex A-50 chromatography with a purity of 80%. The yield of 12 mg tubulin from 10 g lily pollen acetone powder is reasonably high for many experiments in which the tubulin purity is not crucial. A high purity of lily pollen tubulin can be achieved by the step of second chromatography, but up to 60% of the tubulin may be lost during the second chromatography.

3.2 Plant tubulin functions normally with DMSO

Although reversible taxol-induced polymerization of plant tubulin has been achieved, the conditions for depolymerization are critical (Moore *et al.* 1997), needing the combination of calcium ions and low temperature. In addition, microtubules formed in the presence of taxol consist of a large number of polymorphic structures, such as sheets, ribbons, or free protofilaments. Direct support for this notion also comes from studies demonstrating that different lattice structures of mammalian tubulin polymer markedly affect kinesin-dependent motility (Kamimura and Mandelkow 1992). Such abnormal structures may also affect the binding of MAPs to microtubules. Therefore, it is difficult to investigate whether and how the putative MAPs influence the kinetics of plant tubulin assembly because of the interference of taxol. A previous study indicated that tubulin purified from lily pollen could assemble in the absence of MAPs or microtubule-stabilizing reagents, but with a very high Cc (2.8 mg/mL; Huang *et al.* 2000). This increases the difficulties in studying plant microtubule dynamics *in vitro*.

In the present study, we demonstrated that DMSO is a proper reagent to enhance plant tubulin assembly. We used a series of DMSO concentrations, ranging from 2% to 14%, to identify an optimal DMSO concentration for plant tubulin assembly. Although 12% DMSO had the greatest enhancing effect on plant

tubulin assembly (Fig. 2), the amount of denatured protein was also increased when the DMSO concentration was higher than 10%. Therefore, 10% DMSO is a suitable concentration in investigations of plant tubulin assembly and this concentration was used in the present study. The data from the Cc determination experiments show that lily pollen tubulin assembly in the presence of 10% DMSO has a markedly lower Cc (1.2 mg/mL) than that in the absence of microtubule-stabilizing reagents. Direct observation of the microtubules formed with DMSO by TEM showed that the polymers exhibit a normal microtubule structure, suggesting that the microtubules are functionally normal. The situation is quite different when taxol is used (Moore *et al.* 1997; Huang *et al.* 2000).

The pH and temperature are important conditions for tubulin assembly. The effects of pH and temperature on the polymerization of lily pollen with DMSO were also investigated in the present study. At pH ranging from 6.5 to 7.4, the polymerization of lily pollen tubulin decreases when the pH is increased. A similar observation was reported for animal tubulin assembly (Regula *et al.* 1981), although there is a report indicating that the pH necessary for an optimum yield of microtubules in sea urchin egg extracts is 7.1–7.3 (Suprenant and Marsh 1987). It is well known that temperature affects mammalian tubulin assembly. Tubulin assembly *in vitro* does not occur when temperature is decreased to a physiologically low temperature. Similarly, lily pollen tubulin also assembles at high temperature and disassembles at low temperature. Polymerization increases markedly when the temperature increases from 25 to 40 °C. Because plants have to survive at low temperatures, we infer that the ability of the plant microtubule system to resist coldness does not rely on the properties of plant tubulin, it may be otherwise due to other stabilizing factors, such as plant MAPs (Fosket and Morejohn 1992; Rutten *et al.* 1997).

As opposed to taxol, the temperature-dependent cycle of plant tubulin assembly can occur in the presence of 10% DMSO without the need for calcium, although DMSO-induced plant tubulin assembly is also

sensitive to calcium. This suggests that DMSO does not hinder microtubule dynamics seriously. The present experiments also demonstrate that MAPs from animal sources have a significant enhancing effect on the polymerization of lily pollen tubulin. Therefore, the function of tubulin or microtubules to interact with MAPs remains in the presence of DMSO.

In conclusion, the present study showed that microtubules assembled *in vitro* from purified plant tubulin with 10% DMSO have properties similar to those of plant microtubules and mammalian microtubules assembled in the absence of microtubule-stabilizing reagents (Herzog and Weber 1977; Huang *et al.* 2000), indicating that these properties are due to the nature of tubulin itself. Therefore, the system can be used to analyze the properties of plant microtubule dynamics *in vitro*.

3.3 Functional studies with plant tubulin assembly with DMSO

Microinjection of fluorescently labeled exogenous tubulin has deepened our understanding of the dynamic properties of microtubules in the plant cell. However, it is still easy to imagine that there could be subtle differences between exogenous tubulin and native plant tubulin. Therefore, producing fluorescently labeled plant tubulin should provide a more appropriate probe for plant studies than a probe derived from mammalian brain (Hepler and Hush 1996). To date, there have been no reports regarding the preparation of fluorescently tagged plant tubulin because of the difficulties of purification and assembly of plant tubulin. In the present study, we labeled plant tubulin with NHS-rhodamine in the presence of DMSO. The tubulin assembled into a radial structure on the surface of isolated tobacco BY-2 cells, suggesting that the labeled tubulin has biological activity. So, this provides a more faithful probe for us in investigations of plant microtubule dynamics *in vivo*. In addition, it is a good probe for the investigation of plant microtubule dynamics *in vitro*, with which whether and how some factors, such as MAPs, modify the dynamics of plant microtubules can be examined.

A comprehensive understanding of microtubule

function during plant cell division and differentiation will come from further identification of plant MAPs (Lloyd and Hussey 2001). Many MAPs were identified by cyclic copolymerization with microtubules in cellular extracts. However, the relatively low tubulin concentrations in whole plant cell extracts present a major limitation to plant MAP isolation. To date, the isolation and analysis of putative higher plant MAPs have relied heavily on animal microtubule affinity (Cyr and Palevitz 1989; Chan *et al.* 1996, 1999; Marc *et al.* 1996). Hugdahl *et al.* (1993) reported that the MAP-binding region in the regulatory domain of maize tubulin is distinct from that of brain tubulin. This result shows that plant MAPs have tubulin-binding domains that are structurally and functionally distinct from those of mammalian MAPs. Therefore, it is reasonable to presume that plant and animal microtubules will bind different MAPs. The method of plant MAPs isolation and analysis relying on animal microtubule affinity preferentially enriches only those MAPs having the most conserved microtubule-binding domains. For this reason, it is important to use a homologous source of tubulin for plant MAP isolation studies, to ensure a high-fidelity interaction of plant MAPs with plant microtubules. Genomic data provide considerable information regarding the sequences that contain some putative plant MAP genes. However, biochemical work to characterize the gene products is necessary before any conclusions are drawn. The DMSO-induced plant tubulin assembly system provides a good experimental system for the isolation and characterization of putative plant MAPs.

References

- Bokros CL, Hugdahl JD, Hanesworth VR, Murthy JV, Morejohn LC (1993). Characterization of the reversible taxol-induced polymerization of plant tubulin into microtubules. *Biochemistry* **32**, 3437–3447.
- Chan J, Rutten T, Lloyd CW (1996). Isolation of microtubule-associated proteins from carrot cytoskeleton: A 120 kDa map decorates all four microtubule arrays and the nucleus. *Plant J* **10**, 251–259.
- Chan J, Jensen CG, Jensen LCW, Bush M, Lloyd CW (1999).

- The 65-kDa carrot microtubule-associated protein forms regularly arranged filamentous cross-bridges between microtubules. *Proc Natl Acad Sci USA* **96**, 14931–14936.
- Cyr RJ, Palevitz BA (1989). Microtubule-binding proteins from carrot. I. Initial characterization and microtubule bundling. *Planta* **177**, 245–260.
- Dhonukshe P, Gadella TW Jr (2003). Alteration of microtubule dynamic instability during preprophase band formation revealed by yellow fluorescent protein — CLIP170 microtubule plus-end labeling. *Plant Cell* **15**, 597–611.
- Fosket DE, Morejohn LC (1992). Structural and functional organization of tubulin. *Annu Rev Plant Physiol Plant Mol Biol* **43**, 201–240.
- Hepler PK, Hush JM (1996). Behavior of microtubules in living plant cells. *Plant Physiol* **112**, 455–461.
- Herzog W, Weber K (1977). *In vitro* assembly of pure tubulin into microtubules in the absence of microtubule-associated proteins and glycerol. *Proc Natl Acad Sci USA* **74**, 1860–1864.
- Himes RH, Burton PR, Gaito JM (1977). Dimethyl sulfoxide-induced self-assembly of tubulin lacking associated proteins. *J Biol Chem* **252**, 6222–6228.
- Huang S, Ren H, Yuan M (2000). *In vitro* assembly of plant tubulin in the absence of microtubule-stabilizing reagents. *Chin Sci Bull* **45**, 2258–2263.
- Hugdahl JD, Bokros CL, Hanesworth VR, Aalund GR, Morejohn LC (1993). Unique functional characteristics of the polymerization and MAP binding regulatory domains of the plant tubulin. *Plant Cell* **5**, 1063–1080.
- Hush JM, Wadsworth P, Callahan DA, Hepler PK (1994). Quantification of microtubule dynamics in living plant cells using fluorescence redistribution after photobleaching. *J Cell Sci* **107**, 775–784.
- Kamimura S, Mandelkow E (1992). Tubulin protofilaments and kinesin-dependent motility. *J Cell Biol* **118**, 865–875.
- Kim H, Binder LI, Rosenbaum JL (1979). The periodic association of MAP2 with brain microtubules *in vitro*. *J Cell Biol* **80**, 266–276.
- Lloyd CW, Hussey PJ (2001). Microtubule-associated proteins in plants: Why we need a MAP. *Nat Rev Mol Cell Biol* **2**, 40–47.
- Marc J, Sharkey DE, Durso NA, Zhang M, Cyr RJ (1996). Isolation of a 90-kDa microtubule-associated protein from tobacco membranes. *Plant Cell* **8**, 2127–2138.
- Mitchison TJ, Kirschner MW (1984). Microtubule assembly nucleated by isolated centrosomes. *Nature* **312**, 232–237.
- Mizuno K (1993). Microtubule-nucleation sites on nuclei of higher plant cells. *Protoplasma* **173**, 77–85.
- Moore RC, Zhang M, Cassimeris L, Cyr RJ (1997). *In vitro* assembled plant microtubules exhibit a high state of dynamic instability. *Cell Motil Cytoskel* **38**, 278–286.
- Morejohn LC, Fosket DE (1982). High plant tubulin identified by self-assembly into microtubules *in vitro*. *Nature* **297**, 426–428.
- Morejohn LC, Fosket DE (1991). The biochemistry of compounds with anti-microtubule activity in plant cells. *Pharmacol Theor* **51**, 217–230.
- Regula CS, Pfeiffer JR, Berlin RD (1981). Microtubule assembly and disassembly in alkaline pH. *J Cell Biol* **89**, 45–53.
- Robinson J, Engelborghs Y (1982). Tubulin polymerization in dimethyl sulfoxide. *J Biol Chem* **257**, 5367–5371.
- Rutten T, Chan J, Lloyd CW (1997). A 60-kDa plant microtubule-associated protein promotes the growth and stabilization of neurotubules *in vitro*. *Proc Natl Acad Sci USA* **94**, 4469–4474.
- Shibaoka H, Nagai R (1994). The plant cytoskeleton. *Curr Opin Cell Biol* **6**, 10–15.
- Staiger CJ, Lloyd CW (1991). The plant cytoskeleton. *Curr Opin Cell Biol* **3**, 33–42.
- Suprenant KA, Marsh JC (1987). Temperature and pH govern the self-assembly of microtubules from unfertilized sea-urchin egg extracts. *J Cell Sci* **87**, 71–84.
- Vallee RB (1982). A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J Cell Biol* **92**, 435–442.
- Yuan M, Shaw PJ, Warn RM, Lloyd CW (1994). Dynamic reorientation of cortical microtubules, from transverse to longitudinal, in living plant cells. *Proc Natl Acad Sci USA* **91**, 6050–6053.

(Managing editor: Li-Hui ZHAO)