Appl Biochem Biotechnol (2010) 160:122–128
DOI 10.1007/s12010-008-8489-4

Characterization of *Capsicum annuum* Recombinant α- and β-Tubulin

Bon-Sung Koo · Myung-Hyun Jang · Haechul Park · Satish Kalme · Hey-Yeon Park · Jin-Wook Han · Yun-Soo Yeo · Sang-Hong Yoon · Soo-Jin Kim · Chang-Muk Lee · Moon-Young Yoon

Received: 27 September 2008 / Accepted: 11 December 2008 /
Published online: 6 January 2009

Abstract There are several conditions which might modulate polymerization to produce polymers having normal lattice structure. In the absence of 1 mM $MgCl_2$ the assembly was reduced by 36% in *Capsicum annuum* tubulin (CAnm tubulin). There was no significant difference in the final assembly formation in the presence of 5% to 10% glycerol. However, nucleation rate was slow and apparent study state was achieved lately in the presence of 10% glycerol. Taxol at 100 μM concentration increased 23% tubulin assembly. One millimolar $CaCl_2$, ≥1% dimethyl sulfoxide (DMSO) and physiologically low temperature reduced CAnm tubulin assembly. A value of 0.089 mg/ml was obtained as critical concentration for polymerization. Benomyl significantly reduced the number of cysteine residues accessible to 5,5'-dithiobis-(2-nitrobenzoic acid); there were 4.77±0.21 and 3.49±0.35 residues accessible per tubulin dimer in the presence of 50 and 100 μM benomyl respectively.

Keywords Benomyl · *Capsicum annuum* · Polymerization · Sulfhydryl group · Taxol

Introduction

Microtubules are filamentous structure found in virtually all eukaryotic cells. The basic structural unit of microtubules is the αβ-tubulin dimer, each subunit with a molecular mass near 50 kDa. Tubulin dimers arrange head to tail to form protofilaments that associate laterally into a polar microtubule structure. The extent of microtubule polymerization is

B.-S. Koo · Y.-S. Yeo · S.-H. Yoon · S.-J. Kim · C.-M. Lee
Microbial Genetics Division, National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea

M.-H. Jang · H. Park · S. Kalme · H.-Y. Park · J.-W. Han · M.-Y. Yoon (✉)
Department of Chemistry, Hanyang University, Seoul 133-791, Korea
e-mail: myyoon@hanyang.ac.kr

S. Kalme
Research Institute for Natural Sciences, Hanyang University, Seoul 133-791, Korea

dependent upon a critical concentration (Cc) of tubulin dimer, below which tubulin assembly will not spontaneously occur, and the mass of polymer formed above the Cc increases with the tubulin concentration [1]. Although effect of taxol and colchicine on plant tubulin structures is well understood, conditions which might modulate polymerization to produce polymers having normal lattice structure have not been adequately explored. Certain reaction conditions (temperature, pH) and reaction mixture components (Mg^{2+}, glycerol, microtubule stabilizing buffer, DMSO, taxol) are believed to be required for proper microtubule assembly [2, 3]. These factors influencing microtubule polymerization were markedly characterized using tubulin from yeast, fungi, parasites, and mammalian brain.

All the cysteine residues except β239 of type III are highly conserved [4] and many appear to be 'buried' according to the 3.5 Å (1 Å=10^{-10} m) electron diffraction structure [5], but are, nevertheless, accessible to reagents. No definitive function has been ascribed to these cysteine residues; no enzymatic redox function is known; cysteines might be transiently involved in folding [6] or oligomerization [7], and it has been suggested that one or two disulphide bonds are required in vivo to promote optimal polymerization [8]. However cysteine accessibility in protein can be instrumental in understanding the correct folding of recombinant protein at ligand binding site.

Microtubules have been characterized from plants that host pathogenic fungi. Considerable efforts are underway to find an antimitotic agent against plant pathogens like *Phytophthora capsici*. However, screening the effects of antifungal agents on plant tubulin in vivo or using purified native microtubule in vitro is a time-consuming process. A recombinant, correctly folded, microtubule-like structure forming tubulin could accelerate research in this area. The objectives of the present work were to find optimum polymerizing conditions of *Capsicum annuum* tubulin (CAnm tubulin) and to study the effect of benomyl on sulfhydryl group accessibility by DTNB in CAnm tubulin.

Materials and Methods

Materials

Taxol, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and benomyl were purchased from Sigma (Sigma Aldrich, ST. Louis, Missouri). Stock solution of taxol (2.0 mM) and benomyl (20.0 mM) were made in dimethyl sulfoxide (DMSO). The final DMSO concentration was 5% (*v/v*) in polymerization buffer. Stock solutions of DTNB (10.0 mM) were prepared in buffer of pH 8.2 and then adjusted to pH 7.0. The concentration was estimated using ε=17.78 mM^{-1} cm^{-1} at 324 nm [9].

Purification of α- and β-tubulin

The α- and β-tubulin of *C. annuum* were purified as reported previously [10].

Polymerization of Tubulin

The time course of microtubule assembly was monitored at A_{350} [10] using an Optizen 2,120 UV spectrophotometer equipped with a digital temperature control assembly. The reaction mixture (400 μl) contained 0.5 mg/ml of α- and β-tubulin in polymerization buffer (80 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, and 5% *v/v* glycerol). The reaction was initiated by adding guanosine-5'-triphosphate (GTP) at a final concentration of 2 mM. The

contents were mixed and turbidity was measured at 37 °C over a time scale of 95 min, reading at every 5 s. Using this polymerization procedure, the effect of various components and conditions on α- and β-tubulin polymerization were assessed. These included varying glycerol, DMSO, taxol, and tubulin concentrations, and temperature conditions. Effect of $MgCl_2$ and $CaCl_2$ on polymerization was also reported. The degree of polymerization was assessed by subtracting the initial absorbance reading from the final reading obtained after 95 min. The critical protein concentration, Cc, was determined by incubating tubulin samples, at a series of concentrations, at 37 °C for 95 min and measuring the increase in turbidity at 350 nm [11]. Transmission electron microscopy (TEM) analysis of tubulin polymerized at different conditions was carried out as reported previously [10].

Titration of Tubulin Sulfhydryl Groups

DiAminoacid Neural Network Application (DiANNA 1.1) was used to determine the cysteine species (free cysteine, half-cystine, or ligand-bound) in α- and β-tubulin using a support vector machine (SVM) with degree 2 polynomial kernel for the spectrum representation [12]. The SH modifications of tubulin with DTNB was followed in an Optizen 2,120 UV spectrophotometer at 37 °C by following the A_{412} of the product, thionitrobenzoate (TNB; ε=14.15 mM^{-1} cm^{-1}) [13]. Tubulin (1 μM) was incubated in polymerization buffer with and without, 50 and 100 μM benomyl at 4 °C for 15 min. Then 1 mM DTNB was added. The number of sulfhydryl groups modified after 40 min of reaction was determined at 412 nm. As a non-SH-containing control, RNase A was used. DTNB absorbance remained unchanged. All values were given for the SH groups reacted.

Results and Discussion

Effect of $MgCl_2$, Glycerol, and Taxol

Magnesium is believed to be required for microtubule polymerization and stability and to allow correct folding of tubulin [14]. In the absence of 1 mM $MgCl_2$ the assembly was reduced by 36%. Glycerol is believed to lower the ΔG and the critical tubulin concentration required for the polymerization reaction as well as increasing microtubule stability by increasing the strength of interactions between individual tubulin subunits [15, 16]. There was no significant difference in the final assembly formation in the presence of 5% to 10% glycerol. However, nucleation rate was slow and apparent study state was achieved lately in the presence of 10% glycerol. The assembly was reduced by 23% without glycerol (data not shown). Taxol has been used to promote assembly of tubulin into microtubules from a wide variety of organisms [2, 17]. Increasing taxol concentration from 10 to 100 μM resulted in 23% increase in the amount of tubulin assembly. Taxol concentration between 10 and 20 μM was effective in promoting assembly of CAnm tubulin into microtubules; however concentration from 20 to 100 μM did not cause any significant change in the total assembly (Fig. 1a).

$CaCl_2$ and DMSO Decreases the Polymerization

It has been proposed that Ca^{2+} acts to promote disassembly by binding to free GTP which prevents it from contributing to polymerization and increases the hydrolysis of stabilizing GTP in the microtubule cap [18]. $CaCl_2$ at 1 mM concentration reduced assembly by 69% for initial 20 min incubation period which was further reduced to promote complete disassembly.

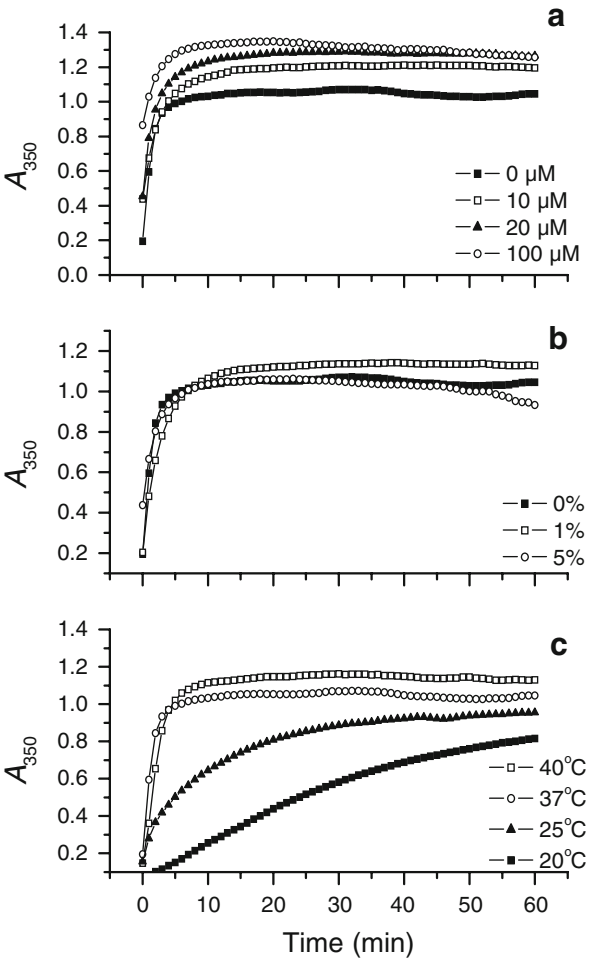
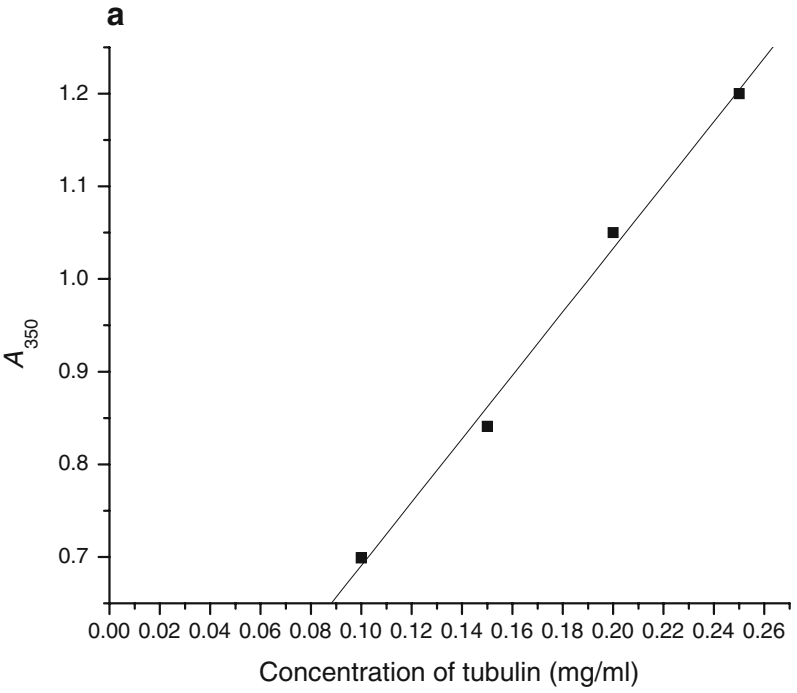
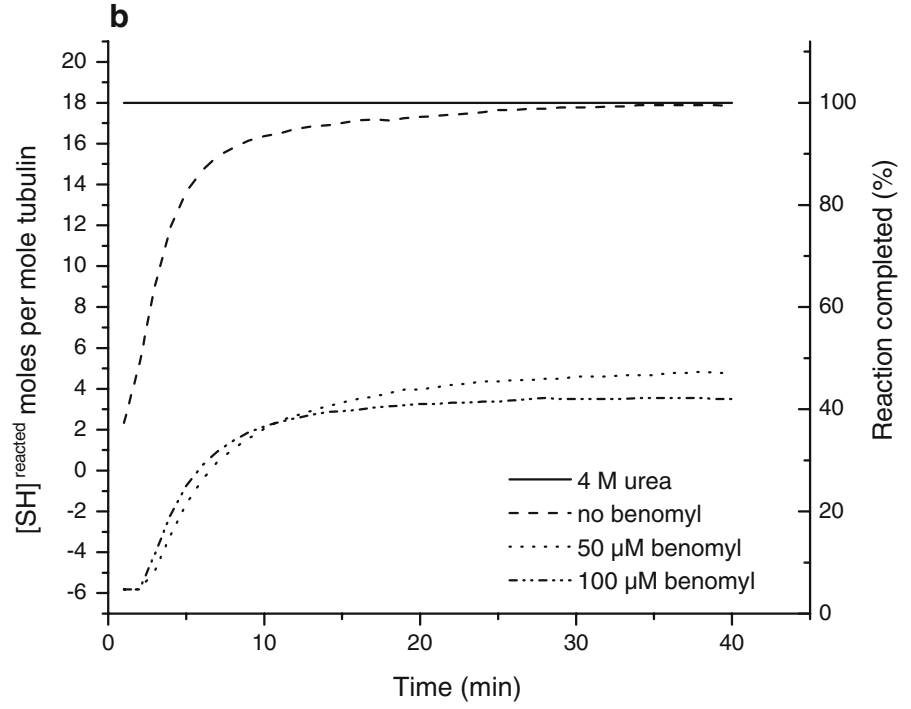


Fig. 1 Spectrophotometric analysis of polymerization of *C. annuum* tubulin at different taxol concentration (**a**), DMSO concentration (**b**), and temperature (**c**)

DMSO is believed to behave like D_2O in that it forms stronger hydrogen bonds with protein functional groups than does H_2O [19]. DMSO is demonstrated as a proper reagent to enhance lily pollen tubulin assembly [20]. The effect of the concentration of DMSO on CAnm tubulin assembly was investigated using the polymerization assay. Tubulin was assembled in pipes buffer with 1% and 5% DMSO concentration. The amount of polymerization decreased with ≥1% DMSO (Fig. 1b). 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. TEM analysis of sample prepared with >1% DMSO showed distorted structure of tubulin (data not shown).

Fig. 2 **a** Critical concentration of polymerization of *C. annuum* tubulin. Tubulin (0.1–0.25 mg/ml) was polymerized at 37 °C as described in the "Materials and Methods". A_{350} at saturation of polymerization (60 min) was plotted as a function of tubulin concentration. **b** Reaction kinetics of DTNB with tubulin-SH in the presence of benomyl. Tubulin (1 μM) was incubated with 4 M urea, without benomyl, 50 μM benomyl, and 100 μM benomyl at 4 °C for 15 min, and then treated with 1 mM DTNB. The rate and extent of sulfhydryl group modification were monitored by measuring the absorbance at 412 nm. Data is generated from three independent measurements


a
1.2
1.1
1.0
0.9
0.8
0.7
A_{350}
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20 0.22 0.24 0.26
Concentration of tubulin (mg/ml)


b
20
18
16
14
12
10
8
6
4
2
0
-2
-4
-6
$[SH]^{reacted}$ moles per mole tubulin
100
80
60
40
20
0
Reaction completed (%)
0
10
20
30
40
Time (min)
4 M urea
no benomyl
50 μM benomyl
100 μM benomyl

Effect of Temperature

Tubulin assembly in vitro does not occur when temperature is decreased to a physiologically low temperature (Fig. 1c). At the temperature ≤25 °C, the net polymerizing system showed a slow nucleation rate. TEM analysis showed that long microtubule-like structures were increased markedly when the temperature increases from 20 to 40 °C (data not shown).

Critical Concentration for Polymerization of CAnm Tubulin

We measured the critical subunit concentration for tubulin polymerization by measuring the A_{350} as a function of the initial tubulin concentration (Fig. 2a). At tubulin concentrations <0.01 mg/ml, no polymeric structures were observed. From 0.01 to 0.2 mg/ml each tubulin concentration assembly increased linearly with typical long microtubule-like structures. A value of 0.089 mg/ml was obtained as critical concentration for polymerization. In the absence of taxol Cc for purified maize and tobacco tubulins was in the range of 0.8 to 0.9 mg/ml [2], and that of lily pollen tubulin was 1.2 mg/ml [20].

Binding of Benomyl to CAnm Tubulin Sulfhydryl Groups

CAnm α- and β-tubulin contains 12 and 11 cysteines, respectively, where 18 cysteines are free in sulfhydryl state (data not shown). We used the sulfhydryl-specific reagent DTNB to determine the accessibility of the cysteine residues of tubulin toward modification in association with benomyl binding. Figure 2b depicts a comparison of the reaction of 1.0 μM purified CAnm tubulin with DTNB (1 mM), at a molar ratio (reagent/SH) of 50:1 in the presence or absence of benomyl. The reaction was >99% complete for DTNB without benomyl by 40 min reacting 17.82±0.04 cysteine residues per tubulin dimer. The remaining approximately one cysteine equivalent of tubulin become instantly accessible in 4 M urea. Benomyl significantly reduced the number of cysteine residues accessible to DTNB; there were 4.77±0.21 and 3.49±0.35 residues accessible per tubulin dimer in the presence of 50 and 100 μM benomyl, respectively. The difference in the number of modified cysteine residues in the absence and presence of 50 μM benomyl was 13.05, and that of 100 μM benomyl was 14.33 ($P<0.001$, Tukey–Kramer Multiple Comparisons Test, one-way ANOVA).

There are some reports on herbicide binding to plant tubulin but there is no insight on antifungal binding to plant tubulin. It is believed that the benzimidazole drugs bind to the colchicine binding site on the tubulin molecule [21]. It is known that colchicine protects one sulfhydryl group from reaction with other sulfhydryl reagents [22]. Our report indicates that benomyl binding site is different as compared to known benzimidazole binding site as the number of sulfhydryl group protected in the presence of benomyl are significantly high. There has been much speculation regarding the position of the benzimidazole binding site on the tubulin molecule. The majority of studies have proposed that benzimidazoles bind to β-tubulin. However, others have suggested that the binding site is situated; on α-tubulin [23], on α-tubulin and regulated by the β-subunit [24], on both α- and β-tubulin [25], or at the α/β-tubulin interface [26]. In this study, the binding of benomyl to CAnm tubulin affected the accessibility of the sulfhydryl groups of tubulin to DTNB, indicating that benomyl binding to the tubulin induced a conformational change in the tubulin. Accessibility of cysteine residues of tubulin revealed that important ligand binding sites were folded correctly. This recombinant tubulin could serve as a control of phytotoxicity of selected antimitotic fungicide compounds during in vitro screening experiments.

Acknowledgements This study was carried out with the support of the On-Site Cooperative Agriculture Research Project (20070201080024) and the National Institute of Agricultural Biotechnology, RDA, of the Republic of Korea.

References

1. Davis, A., Sage, C. R., Wilson, L., et al. (1993). *Biochemistry*, *32*, 8823–8835. doi:10.1021/bi00085a013.
2. Bokros, C. L., Hugdahl, J. D., Hanesworth, V. R., et al. (1993). *Biochemistry*, *32*, 3437–3447. doi:10.1021/bi00064a030.
3. Oxberry, M. E., Geary, T. G., Winterrowd, C. A., et al. (2001). *Protein Expression and Purification*, *21*, 30–39. doi:10.1006/prep.2000.1347.
4. Cowan, N. (1984). *Oxford surveys on eukaryotic genes*. In N. Maclean (Ed.), Tubulin genes and the diversity of microtubule function (pp. 26–60). Oxford: University of Oxford Press.
5. Lowe, J., Li, H., Downing, K. H., et al. (2001). *Journal of Molecular Biology*, *313*, 1045–1057. doi:10.1006/jmbi.2001.5077.
6. Robinson, A. S., & King, J. (1997). *Nature Structural Biology*, *4*, 450–455. doi:10.1038/nsb0697-450.
7. Li, P. P., Nakanishi, A., Clark, S. W., et al. (2002). *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 1353–1358. doi:10.1073/pnas.032668699.
8. Chaudhuri, A. R., Khan, I. A., & Ludue˜na, R. F. (2001). *Biochemistry*, *40*, 8834–8841. doi:10.1021/bi0101603.
9. Riddles, P. W., Blakeley, R. L., & Zerner, B. (1979). *Analytical Biochemistry*, *94*, 75–81. doi:10.1016/0003-2697(79)90792-9.
10. Jang, M. H., Kim, J., Kalme, S., et al. (2008). *Bioscience, Biotechnology, and Biochemistry*, *72*, 1048–1055. doi:10.1271/bbb.70794.
11. Banerjee, A., Roach, M. C., Trcka, P., et al. (1992). *The Journal of Biological Chemistry*, *267*, 5625–5630.
12. Ferre, F., & Clote, P. (2006). *Nucleic Acids Research*, *34*, W182–W185. doi:10.1093/nar/gkl189.
13. Eyer, P., Worek, F., Kiderlen, D., et al. (2003). *Analytical Biochemistry*, *312*, 224–227. doi:10.1016/S0003-2697(02)00506-7.
14. Correia, J. J., Baty, L. T., & Williams Jr., R. C. (1987). *The Journal of Biological Chemistry*, *262*, 17278–17284.
15. Himes, R. H., Burton, P. R., & Gaito, J. M. (1977). *The Journal of Biological Chemistry*, *252*, 6222–6228.
16. Li, J., Katiyar, S. K., & Edlind, T. D. (1996). *FEBS Letters*, *385*, 7–10. doi:10.1016/0014-5793(96)00334-1.
17. Dawson, P. J., & Lloyd, C. W. (1985). *The EMBO Journal*, *4*, 2451–2455.
18. O'Brien, E. T., Salmon, E. D., & Erickson, H. P. (1997). *Cell Motility and the Cytoskeleton*, *36*, 125–135. doi:10.1002/(SICI)1097-0169(1997)36:2<125::AID-CM3>3.0.CO;2-8.
19. Rammler, D. H., & Zaffaroni, A. (1967). *Annals of the New York Academy of Sciences*, *141*, 13–23. doi:10.1111/j.1749-6632.1967.tb34861.x.
20. Xu, C.-H., Huang, S.-J., & Yuan, M. (2005). *Journal of Integrative Plant Biology*, *47*, 457–466. doi:10.1111/j.1744-7909.2005.00086.x.
21. Lacey, E. (1988). *International Journal for Parasitology*, *18*, 885–936. doi:10.1016/0020-7519(88)90175-0.
22. Basusarkar, P., Chandra, S., & Bhattacharyya, B. (1997). *European Journal of Biochemistry*, *244*, 378–383. doi:10.1111/j.1432-1033.1997.00378.x.
23. Serrano, L., De La Torre, J., Maccioni, R. B., et al. (1984). *Proceedings of the National Academy of Sciences of the United States of America*, *81*, 5989–5993. doi:10.1073/pnas.81.19.5989.
24. Banerjee, A., & Luduena, R. F. (1992). *The Journal of Biological Chemistry*, *267*, 13335–13339.
25. Floyd, L. J., Barnes, L. D., & Williams, R. F. (1989). *Biochemistry*, *28*, 8515–8525. doi:10.1021/bi00447a037.
26. Sackett, D. L., Bhattachararyya, B., & Wolff, J. (1994). *Biochemistry*, *33*, 12868–12878. doi:10.1021/bi00209a019.