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α- and β-tubulin from *Phytophthora capsici* KACC 40483: molecular cloning, biochemical characterization, and antimicrotubule screening

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Abstract Internal fragments of α- and β-tubulin genes were generated using reverse transcription polymerase chain reaction (RT-PCR), and the termini were isolated using 5′- and 3′-rapid amplification of cDNA ends. *Phytophthora capsici* α- and β-tubulin specific primers were then used to generate full-length cDNA by RT-PCR. The recombinant α- and β-tubulin genes were expressed in *Escherichia coli* BL21 (DE3), purified under denaturing conditions, and average yields were 3.38–4.5 mg of α-tubulin and 2.89–4.0 mg of β-tubulin, each from 1-l culture. Optimum conditions were obtained for formation of microtubule-like structures. A value of 0.12 mg/ml was obtained as the critical concentration of polymerization of *P. capsici* tubulin. Benomyl inhibited polymerization with half-maximal inhibition (IC_{50})=468±20 μM. Approximately 18.66±0.13 cysteine residues per tubulin dimer were accessible to 5,5′-dithiobis-(2-nitrobenzoic acid), a quantification reagent of sulfhydryl and 12.43±0.12 residues were accessible in the presence of 200 μM benomyl. The order of preference for accessibility to cysteines was benomyl > colchicine > GTP > taxol, and cysteine accessibility changes conformed that binding sites of these ligands in tubulin were folding correctly. Fluorescence resonance energy transfer technique was used for high throughput screening of chemical library in search of antimitotic agent. There was significant difference in relative fluorescence by 210-O-2 and 210-O-14 as compared to colchicine.

Keywords *Phytophthora capsici* · Recombinant tubulin · Benomyl · DTNB · FRET

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Introduction

The genus *Phytophthora* includes many notorious plant pathogens, e.g., *Phytophthora capsici*, the causative of *Phytophthora* blight, a devastating disease of pepper (*Capsicum annuum*) in South Korea and worldwide (Hwang and Kim 1995). *Phytophthora* blight has been responsible for major losses of pepper production. Control measures have not been effective since *P. capsici* oospores are resistant to desiccation and can survive in the soil for many years in the absence of the host plant (French-Monar et al. 2007).

Cellular microtubules are known to play an essential role in nuclear division as components of the mitotic spindle and dimeric tubulin is their primary component. A key property of tubulin is its ability to assemble into microtubules via interaction between polymerized α- and β-tubulin monomers (heterodimer), and to undergo disassembly at appropriate times in the cell cycle. Microtubules are the site of action of an important family of agents that include fungicides (Keinath

2007). There are several reports on biological control of root rot caused by *P. capsici* in pepper (Sid et al. 2003; Chung et al. 2008). However, despite several attempts to identify the most suitable biocontrol agent and dose, no single biocontrol agent has proved capable of completely inhibiting infection (Liu et al. 2007). Young and Slawecki (2001) showed that RH-7281 (zoxamide) rapidly arrested nuclear division in *P. capsici* germlings and destroyed the microtubule cytoskeleton. However, there are no reports on the isolation of tubulin from *P. capsici* and attempts by Young and Slawecki (2001), though successful, resulted in insufficient amounts for assembly. The low solubility of this component is a major concern for large-scale in vitro screening of antimicrotubule agents.

Certain conditions are believed to strongly promote microtubule assembly using partially purified tubulin from eukaryotic tissues or cells (Dong et al. 2004; Bellocq et al. 1992). These conditions include temperatures ranging from 30–37°C, a pH of 6.4–6.7, hydrolysis of guanosine triphosphate (GTP), millimolar levels of Mg^{2+}, glycerol, chelating agents such as ethylene glycol tetraacetic acid (EGTA) or ethylenediaminetetraacetic acid, microtubule-stabilizing buffers, and microtubule-associated proteins (MAPs), in addition to a critical concentration of tubulin subunits (Oxberry et al. 2001a). Factors that influence microtubule polymerization have largely been characterized using tubulin purified from mammalian brain and from other eukaryotic organisms such as yeast, fungi, and parasites. The disadvantage of using tubulin purified from these sources is that the effects of other proteins, such as MAPs, protein-bound Mg^{2+}-GTP and Mg^{2+}-GDP, and other cofactors that have copurified with the tubulin or posttranscriptional modifications, cannot be eliminated. This is the first report of the cloning, expression, and purification of *P. capsici* α- and β-tubulin from *Escherichia coli* BL21 (DE3). We show that individually expressed α- and β-tubulins, purified under denaturing conditions, were folding correctly and could assemble into microtubules. These microtubules obtained from *P. capsici* recombinant tubulin can be used for in vitro inhibitor screening using fluorescence resonance energy transfer (FRET) technique.

Materials and methods

Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG), taxol, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), benomyl, and colchicine were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). *E. coli* BL21 (DE3; Novagen, Madison, WI, USA) and plasmid pET28a (Novagen) were used for gene expression. Restriction and modifying enzymes were purchased from Takara Bio (Kyoto, Japan) and used according to supplier recommendations. Other DNA manipulation experiments were performed as described by Sambrook et al. (1989). Stock solutions 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. A chemical library of 395 chemical compounds was obtained from National Institute of Agricultural Biotechnology, Suwon, South Korea. 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 (Riddles et al. 1979).

RNA extraction and cDNA synthesis

Total RNA was isolated from *P. capsici* Korean Agricultural Culture Collection (KACC) 40483 mycelium using an Easy blue™ Kit (iNtRON Biotechnology, Seoul, South Korea). Using liquid nitrogen, 100 mg mycelia were ground to a fine powder, transferred to a microcentrifuge tube, and the extraction procedure suggested by the manufacturer was followed. RNA integrity was determined by resolving approximately 1 μg of the total RNA on a denaturing agarose gel. The first strand of cDNA was synthesized using a Takara RNA PCR kit (AMV) Ver.3.0 (Takara Bio). For the reverse transcription polymerase chain reaction (RT-PCR), an oligo dT (GenoTECH, Seoul, South Korea) primer was used. The PCR conditions were followed as described by manufacturer.

Isolation of partial α- and β-tubulin genes

A partial fragment of *P. capsici* α-tubulin was obtained using primers (forward: GGTTGGCAATGCGTGTTGG GAACT, reverse: TACATCAGGTCGAACTTGTGG TCGAT) based on the *Phytophthora palmivora* α-tubulin gene (GenBank accession no. AY729835). For the β-tubulin gene fragment, primers (forward: GTTTCCAGAT CACCCACTCGCTTGGT and reverse: GTACCAGTG CAAGAAAGCGTTACGAC) were designed based on the *P. capsici* isolate IFO30696 β-tubulin gene (GenBank accession no. DQ071350). Amplification reactions were carried out using *Taq* DNA polymerase in a solution containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 25 mM $MgCl_2$, 0.01% (*w*/*v*) gelatin, 0.25 mM of each deoxyribonucleoside triphosphates, and 5 pmol of each primer. Thirty cycles of amplification were employed, which consisted of denaturing at 93°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s for α-tubulin and that of β-tubulin, other conditions were similar except extension at 72°C for 1 min. These partial gene PCR products were sequenced and used as a template for designing primers for rapid amplification of cDNA ends PCR (RACE-PCR).

3′- and 5′-RACE-PCR

Since the *P. capsici* α- and β-tubulin coding genes are unknown, RACE-PCR was used. 3′ RACE-PCR was initiated using an antisense primer with a specific 5′ oligo (dT) adaptor sequence (5′-GTTTTCCCAGTCACGAC-3′; M13 primer M4, Takara Bio). An internal sense primer (for α-tubulin: 5′-TGTGCATGATCTCGAACACGA-3′, for β-tubulin: 5′-AGAACAGTTTACGGCTATGTT-3′) was then used to generate a short second strand, ending in a sequence complementary to the original adaptor sequence. Thereafter, PCR was initiated using the internal sense primer and an adaptor sequence primer. Amplification reaction mixtures were prepared as described above. Thirty cycles of amplification were employed, which consisted of denaturing at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s for α-tubulin and that of β-tubulin, other conditions were similar except annealing at 53°C for 30 s and extension at 72°C for 1 min.

In 5′ RACE-PCR, an internal antisense primer (for α-tubulin: 5′-CCTTGCCGATGGTGTAGTG-3′, for β-tubulin: 5′-GACAACGTAGCGTTGTAA-3′) was used for the first round of the cDNA synthesis reaction. Terminal deoxynucleotidyl transferase (Takara Bio) was used to add a poly(dA) chain. For the next PCR reaction, an oligo dT adaptor primer (M13 primer M4) was used to generate ds cDNA. This strand was used as a template for a further synthesis step using the sequence-specific internal primer (for α-tubulin: 5′-TCCTTGCCGGAGATGATCTGCT-3′, for β-tubulin: 5′-CGAATCTTCGAGATAAGAAG-3′) in order to produce a complementary copy of the adaptor sequence. Amplification reaction mixtures were prepared as described above and 30 cycles of amplification were employed, which consisted of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for α-tubulin and that of β-tubulin, other conditions were similar except annealing at 50°C for 30 s.

Cloning of full-length α- and β-tubulin gene

Based on the *P. capsici* specific sequence obtained from the 5′- and 3′-RACE experiments, primers corresponding to the beginning and end of the coding region were generated. For α-tubulin, the 5′-full primer was 5′-CATATGCGTGAGGTCATCTCCATCC-3′ and that of the 3′-full primer was 5′-GCGGCCGCTTAGTACTCCTCGCCCAGTTCCT-3′. These primers contained restriction sites (underlined) for *Nde*I and *Not*I, respectively. For β-tubulin, the 5′-full primer was 5′-AAGTCGACAAATGAGAGAGCTTGTTCACATCCAG-3′ and the 3′-full primer was 5′-AAGCGGCCGCCTACATCATCTCGTCCATCTCCT-3′. These primers contained *Sal*I and *Not*I restriction sites (underlined), respectively. These two primers were then used in the RT-PCR protocol using total RNA isolated from fungal mycelia as described above. Amplification reaction mixtures were prepared as described above except 10 pmol of each primer. Thirty cycles of amplification were employed, which consisted of denaturing at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 90 s for α-tubulin and that of β-tubulin, other conditions were similar except annealing at 55°C for 45 s. Products approximately 1,400 bp in size were identified. The sequencing of both strands was accomplished using BigDye™ terminator chemistry. Reaction products were purified using ethanol precipitation and sequenced using a 3730xl (Macrogen, Seoul, South Korea). The α- and β-tubulin inserts were then cloned into *Nde*I/*Not*I and *Sal*I/*Not*I sites of pET-28a (Novagen), respectively, producing fusion proteins with N-terminal His^6 tags.

Expression and purification of α- and β-tubulin

The expression plasmids were transformed into *E. coli* BL21 (DE3) using the heat shock method. Transformed bacteria were grown at 37°C in Luria–Bertani medium containing 100 μg/ml kanamycin until the D_{600} of the culture reached 0.7. Expression of recombinant α- and β-tubulin was induced by the addition of 1 mM IPTG for 12 h at 18°C. Since α- and β-tubulin are insoluble, purification was done under denaturing conditions with on-column refolding as reported previously (Jang et al. 2008). Briefly, the culture (1 l) was harvested by centrifugation; cells were washed with 20 mM Tris buffer, pH 8.0, and then resuspended in isolation buffer (20 mM Tris [pH 8.0], 300 mM NaCl, 2% Triton X-100). Cells were disrupted briefly by ultrasonication and the pellet obtained after sonication was suspended in 40 ml binding buffer (6 M guanidine hydrochloride, 20 mM Tris [pH 8.0], 300 mM NaCl, 5 mM imidazole, 1 mM β-ME) and incubated at 25°C with gentle stirring for 30 min. The supernatant was collected after centrifugation and passed through a 0.45-μm syringe filter (Millipore, Boston, MA, USA). The clear supernatant, containing α- or β-tubulin, was loaded onto a Ni^{2+}-charged sepharose column pre-equilibrated with binding buffer. After column washing (6 M urea, 20 mM Tris [pH 8.0], 300 mM NaCl, 20 mM imidazole, 1 mM β-ME), on-column refolding of the bound protein was carried out using a decreasing gradient (6–0 M) of urea (refolding buffer; 20 mM Tris [pH 8.0], 300 mM NaCl, 20 mM imidazole, 1 mM β-ME). The refolded protein was eluted using elution buffer (20 mM Tris [pH 8.0], 300 mM NaCl, 0.5 M imidazole, 1 mM β-ME) with a linear gradient from 0.02–0.5 M imidazole. Fractions containing the target protein were dialyzed against 50 mM Tris (pH 8.0) and concentrated by ultrafiltration (YM10: Amicon, Boston, MA, USA). The purity of α- and β-tubulin was analyzed by sodium dodecyl sulfate polyacrylamide gel electropho-

resis (SDS-PAGE; Laemmli 1970), resolved on a 0.75 mm 4% stacking and 12% resolving minigel (Hoefer™: Amersham Biosciences, Boston, MA, USA). Proteins were visualized with Coomassie Blue R-250 stain and sizes estimated from broad range precision standards (Sigma Aldrich, St. Louis, MO, USA).

Spectroscopic and microscopic analysis of microtubule formation

Known concentrations of α- and β-tubulin were mixed with 120 μl polymerization buffer (80 mM pipes [pH 7.4], 1 mM EGTA, 1 mM $MgCl_2$, 5% *v*/*v* glycerol) to give a final concentration of 0.5 mg/ml total tubulin in 300 μl final reaction mixture. Polymerization was initiated by adding 2.5 mM GTP, with or without 100 μM taxol. The effects of GTP (without GTP, 2.5 and 5.0 mM), glycerol (2%, 5%, and 10% *v*/*v*), taxol (without taxol, 20, 50, and 100 μM), temperature (4°C, 25°C, 37°C, and 42°C), DMSO (0.5–5%), colchicine (10–100 μM), and $CaCl_2$ (0.5–2.0 mM) on the polymerization of tubulin were studied. The contents were mixed and the turbidity due to microtubule formation measured at 350 nm (Damien and Allen 2005) using an Optizen 2120 UV spectrophotometer at 37°C over a timescale of 60 min, with readings every 10 s. The critical subunit concentration (Cc) was measured by incubating tubulin samples, at a series of concentrations, at 37°C for 60 min and measuring the increase in turbidity at 350 nm (Banerjee et al. 1992). Completely polymerized (after 60 min) tubulin mixtures were prepared for observation with transmission electron microscopy (TEM) according to the method of Vulevic and Correia (1997).

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) present in α- and β-tubulin using a support vector machine with a degree 2 polynomial kernel for spectrum representation (Ferre and Clote 2006). Sulfhydryl group (SH) modifications of tubulin with DTNB was observed using an Optizen 2120 UV spectrophotometer at 37°C by following the D_{412} of the product, thionitrobenzoate (ε=14.15 mM^{-1} cm^{-1}; Eyer et al. 2003). Tubulin (1 μM) was incubated in pipes buffer (80 mM pipes [pH 7.4], 1 mM EGTA, 1 mM $MgCl_2$) with and without 50–200 μM benomyl, or with 100 μM colchicine, or with 50 μM and 2.5 mM GTP at 4°C for 15 min. One millimolar DTNB was then added. The number of sulfhydryl groups modified after 35 min of reaction was determined at 412 nm. Tubulin was incubated with 100 μM taxol in pipes buffer at 37°C for 30 min. Taxol-polymerized microtubules were collected by centrifugation at 14,000×*g* for 30 min at 37°C, resuspended in pipes buffer, and accessibility to DTNB was measured. As a non-SH-containing control, RNase A was used. DTNB absorbance remained unchanged. All values are given for the reacted SH groups.

Screening of microtubule inhibitors

P. capsici tubulin (0.3 mg/ml) was mixed with different concentrations of benomyl (0–1 mM) at 0°C in an assembly buffer (80 mM pipes [pH 7.4], 1 mM EGTA, 1 mM $MgCl_2$, 2.5 mM GTP, 5% *v*/*v* glycerol). Polymerization was initiated at 37°C in a thermostatically controlled cuvette. The rate and extent of the polymerization reaction was monitored by light scattering at 350 nm (Damien and Allen 2005).

To establish a high throughput screening assay for antimicrotubule inhibitors using FRET technique, we conjugated the fluorescence probe Alexa 488 to purified α-tubulin and Alexa 514 to β-tubulin in 0.1 M sodium bicarbonate buffer, pH 8.5, by incubating at 25°C for 2 h. Other conditions during protein labeling were followed as given by manufacturer (Molecular Probes, Invitrogen). Fluorescence measurements were performed in a Fluoroskan Ascent (Thermo) fluorescence microplate reader using microfluor white 96-well plates (Thermo). The well volume was 100 μl. For the assay, the final concentrations were as follows: 0.15 mg/ml each tubulin, 10 μl library compound in polymerization buffer (pH 7.4), and 1 mM GTP incubated for 1 h at 37°C. Control without library compound contained 2% DMSO (*v*/*v*) and a known inhibitor control contained 4 mM colchicine. Alexa 488 was excited at 495 nm (bandwidth 0.5 nm), and emission of Alexa 514 was recorded at 542 nm (bandwidths 0.5 nm). Spectra were recorded with 2 nm resolution from 520 to 600 nm.

Nucleotide sequence accession number

The cDNA and deduced amino acid sequences for α- and β-tubulins described in this report have been submitted to GenBank and assigned the accession numbers EF495260 and EF495258, respectively.

Results

Cloning and sequencing of α- and β-tubulin

Once the 5′- and the 3′-ends of the coding region were cloned, primers were designed corresponding to both ends of the coding region of *P. capsici* α- and β-tubulin. Use of these primers in RT-PCR yielded a product of the predicted size of about 1,400 bp (data not shown). The tubulin insert, cloned into the pET28a bacterial expression vector, provided

an N-terminal His-tagged protein that was purified in one step. The 1,362 bp α-tubulin gene had a G+C content of 59% and predicted to encode a 453-amino acid protein and 1,341-bp β-tubulin gene, with a G+C content of 56%, encoded a putative protein of 446 amino acids.

Analysis of conserved residues

Basic Local Alignment Search Tool of *P. capsici* α- and β-tubulin nucleotide sequences resulted in similarity with many other tubulins reported. The *P. capsici* α- and β-tubulin genes share 89% and 90% nucleotide sequence identity with corresponding genes from *P. palmivora* (accession no. AY729835 and AY729822, respectively). *P. tropicalis* β-tubulin (accession no. AY564046) had 97% nucleotide sequence identity. *Pythium* Sp. (accession no. EF434977), *Leishmania infantum* (accession no. XM001464016), and wheat (*Triticum aestivum*; accession no. DQ435665) α-tubulin showed 84%, 80%, and 81% nucleotide sequence identity, respectively, and pig (*Sus scrofa*) β-tubulin (accession no. NM001044612) was 76% identical. *P. capsici* α-tubulin showed 98% amino acid sequence identity with *P. palmivora*, while the identity of the *P. capsici* β-tubulin to those from *P. palmivora*, *Phytophthora cinnamomi*, *Phytophthora cactorum*, *Phytophthora infestans*, and *Phytophthora mirabilis* was ≥98%. The GTP binding motif in *P. capsici* α-tubulin was between 140–146 amino acids (data not shown).

P. capsici α- and β-tubulin amino acid sequences were compared with known regions of *S. scrofa* tubulins to study residues directly involved in nucleotide binding. *P. capsici* α- and β-tubulin showed 83% and 89% sequence identity with *S. scrofa* tubulins, 1TUBA and 1TUBB, respectively (data not shown). Conserved domain analysis (Marchler-Bauer et al. 2007) and sequence alignment of *P. capsici* α- and β-tubulin revealed 40% identity among them; 30 residues were involved in nucleotide binding in each tubulin, of which 22 residues were identical. The residues involved in nucleotide binding and hydrolysis are highly conserved between α- and β-subunits, with some interesting exceptions. Two nonconserved residues were observed: αGly12 was βCys12 and αThr16 was βIle16. Four conserved substitutions in *P. capsici* α- and β-tubulin were observed: αAla140 was βSer138, αVal141 was βLeu139, αAsp206 was βAsn204, and αIle231 was βVal229. Two residues were semiconserved: αVal74, which is involved in phosphate binding, was substituted by βThr72, and αAla100, a hydrophobic residue likely contributing to the instability of tubulin monomers, was substituted by β98Gly (Fig. 1). Clustal W (Thompson et al. 1994) alignment revealed that the codons encoding conserved amino acids from wild-type strains of different fungi [*Neurospora*

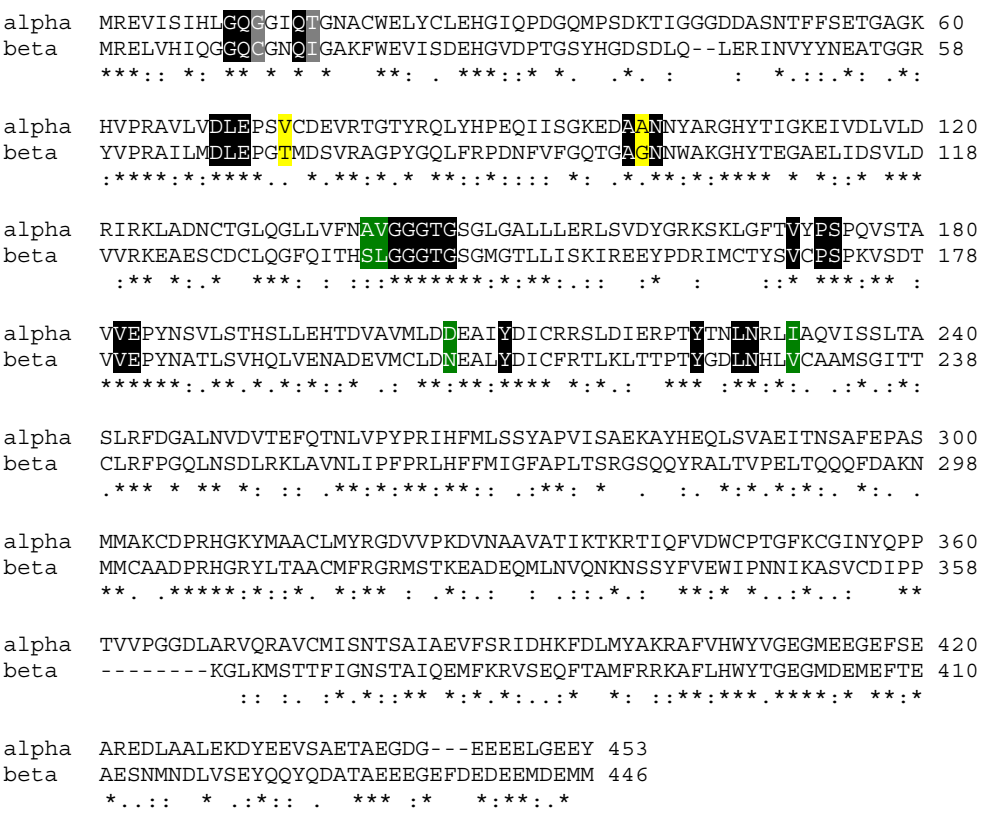

Fig. 1 The amino acid sequence alignment of the *P. capsici* α- and β-tubulin. Identical and conserved amino acids are denoted as * and :/., respectively, and alignments were performed with the aid of the program, Clustal W. Highlighted amino acids are involved in nucleotide binding. Highlighted as *black*, identical residues; *gray*, nonconserved residues; *green*, conserved-substitutions; and *yellow*, semiconserved residues

crassa (AAA33617); *Colletotrichum gloeosporioides* (AAA62875); *Aspergillus nidulans* (1312295B); *Saccharomyces cerevisiae* (CAA24603)], which upon mutation express benomyl resistance (Yan and Dickman 1996; review within), were located at the similar positions in *P. capsici* β-tubulin. Such benomyl sensitive amino acids in *P. capsici* β-tubulin were His6, Glu198, and Arg241 (data not shown).

Expression and purification of α- and β-tubulin

In the present study, a temperature range of 10–30°C did not increase the amount of soluble protein, whereas the amount of induced tubulin in the pellet decreased at 10°C (data not shown). A range of IPTG concentrations were tested to find the optimal concentration for induction, which was determined to be 1 mM. As attempts to produce recombinant protein in soluble form were unsuccessful, conditions were adjusted to form an excessive inclusion body. Fortunately, the inclusion body was solubilized upon treatment with 6 M urea. The His-tagged tubulin was purified to apparent homogeneity in one step using Ni-NTA agarose, a form of immobilized metal affinity chromatography. The Ni-NTA agarose-bound protein was eluted with increasing imidazole concentrations. There was no loss of protein carrying the His^6-tag in the flow through (Fig. 2a, lanes 4–5). Analysis of fractions by SDS-PAGE under reducing conditions showed that elution with 180 mM imidazole and higher resulted in a single band around 55 kDa for α-tubulin (Fig. 2a, lanes 6–9) and 57 kDa for β-tubulin (Fig. 2b, lanes 4–9). This process typically yielded 3.38–4.5 mg α-tubulin and 2.89–4.0 mg β-tubulin per liter culture, which corresponds to yields of 2% and 1.72%, respectively, of total soluble protein.

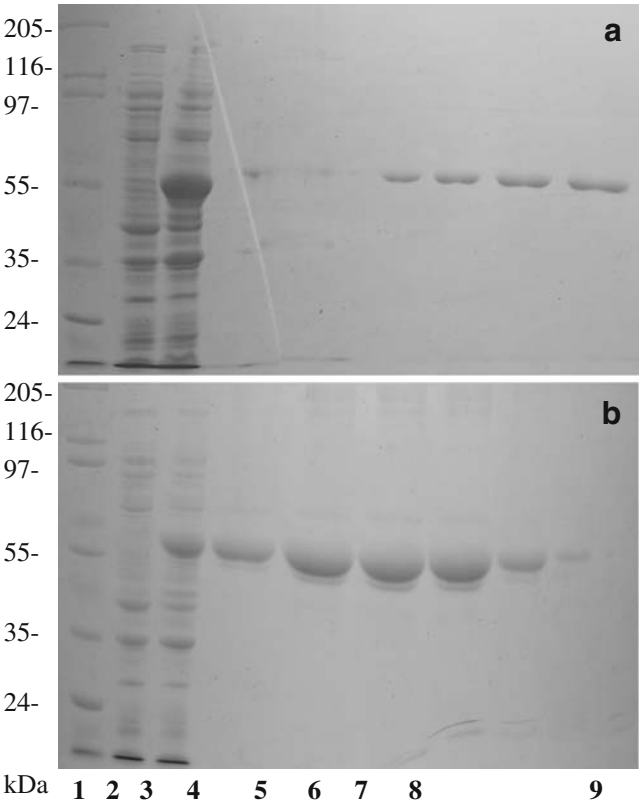

Fig. 2 Assessment of a α- and b β-tubulin purity by SDS-PAGE. **a** *Lanes 1* molecular size markers, *2* total cellular protein without IPTG induction, *3* total cellular protein induced by 1 mM IPTG, *4* and *5* flow through of Ni^{+2} charged sepharose column, and *6–9* elute with liner gradient of imidazole containing α-tubulin. b *Lanes 1–3* same as above and *4–9* elute with liner gradient of imidazole containing β-tubulin. The *numbers to the left side* indicate the molecular weight (in kilodaltons) of markers

Optimum conditions for tubulin polymerization

Polymerization started immediately after the addition of GTP and taxol, and maximum polymerization was achieved after 20-min incubation, after which polymerization rapidly leveled off and then remained constant. Tubulin isotypes did not show any polymerization. Heterodimers without GTP increased absorbance at 350 nm but TEM analysis reveled that it was aggregation and not polymerization (data not shown). Optimum GTP and glycerol concentrations for maximal assembly were 2.5 mM and 5%, respectively (Fig. 3a, b). Taxol increased polymerization, with maximum polymerization observed at 100 μM taxol (Fig. 3c). Tubulin assembly was decreased at temperatures <37°C, with 56% assembly at 25°C and virtually disassembly at 4°C (Fig. 3d). The net polymerizing system showed a slow nucleation rate at 25°C. Stable assembly was recorded during spectroscopy at 350 nm for the initial 25-min of incubation at 42°C (data now shown); however, it linearly decreased by 20% with further incubation (Fig. 3d). The formation of long microtubule-like structures was markedly induced when temperature increased from 25°C to 37°C (data not shown).

Polymerization of *P. capsici* tubulin was reduced by 78% and 20% in the presence of 50 μM colchicine and 1 mM $CaCl_2$, respectively. Although turbidity was increased in the presence of colchicine, a hyperbolic curve was not achieved (Fig. 4a). The Cc of tubulin polymerization was determined by measuring the D_{350} as a function of the initial tubulin concentration. Assembly buffer components were used at optimum concentrations for this study. A value of 0.12 mg/ml was obtained as the Cc of *P. capsici* tubulin (Fig. 4b). The TEM analysis of polymerization with 2.5 mM GTP showed microtubules; however, numbers of microtubules were less with more abnormal structures like sheets and coils (data not shown). Taxol at 20–100 μM concentration promoted the assembly of tubulin into microtubules (Fig. 5a, b); however,

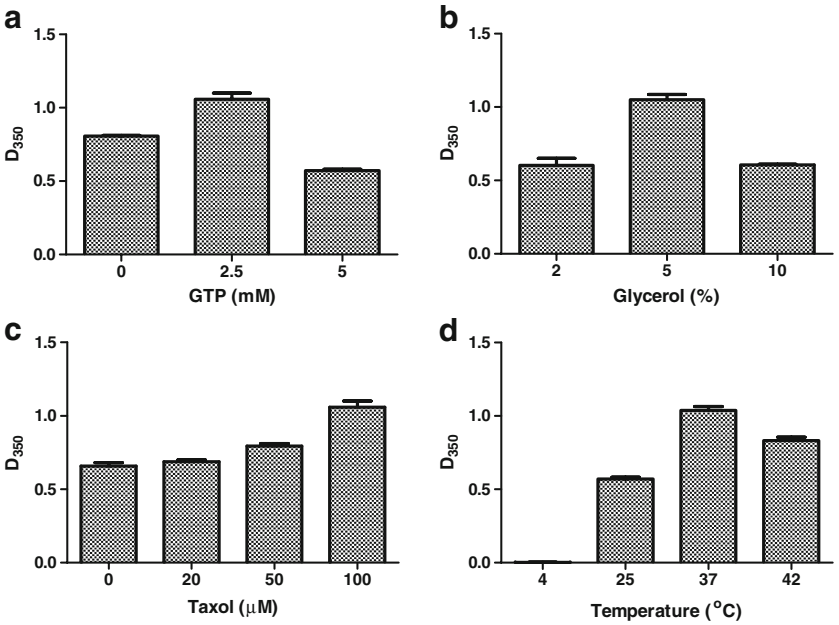

Fig. 3 Polymerization of *P. capsici* tubulin. Polymerization was performed at 37°C using different concentrations of **a** GTP, **b** glycerol, **c** taxol, and **d** at various temperatures. Polymerization studies in **a**, **b**, and **d** contain 100 μM taxol

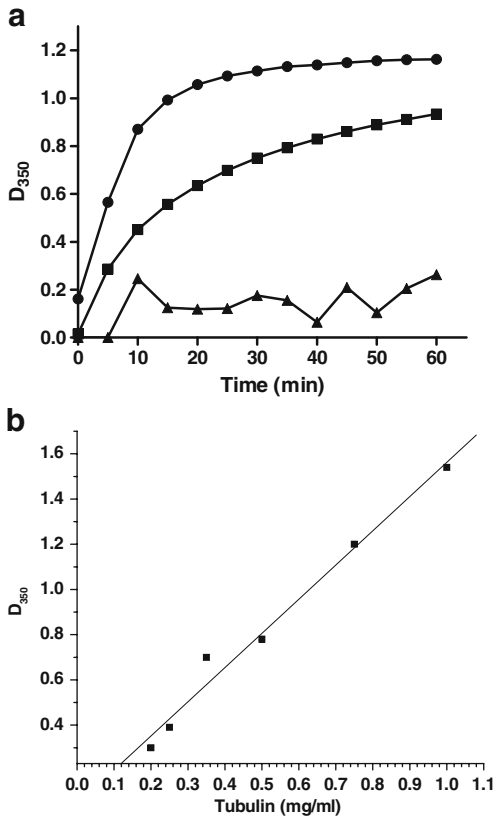

Fig. 4 **a** Polymerization of *P. capsici* tubulin performed at 37°C in the presence of 50 μM colchicine (*filled triangle*), 1 mM $CaCl_2$ (*filled square*), and without colchicine or $CaCl_2$ (*filled circle*). **b** Critical concentration for polymerization of *P. capsici* tubulin. Tubulin (0.2–1.0 mg/ml) polymerized at 37°C as described in the "Materials and methods". D_{350} at saturation of polymerization (60 min) was plotted as a function of tubulin concentration

large sheets also remained to some extent at 20 μM taxol (Fig. 5a). The final dimethyl sulfoxide (*v*/*v*) concentration in all polymerization reactions was 5%. Tubulin assembly was increased by 19% at 5% DMSO concentration as compared to 0.5% DMSO (*v*/*v*) (data not shown).

Binding of benomyl to tubulin sulfhydryl groups

Cysteine species analysis by DiANNA 1.1 revealed that *P. capsici* α- and β-tubulin contained 10 and 12 cysteines, respectively, whereas 20 cysteines are free in the sulfhydryl state (data not shown). The sulfhydryl-specific reagent, DTNB, was used to determine the accessibility of cysteine residues of tubulin for modification by benomyl binding. Figure 6a depicts a comparison of the reaction of 1.0 μM purified *P. capsici* tubulin (80 mM pipes [pH 7.4], 1 mM EGTA, 1 mM $MgCl_2$ at 37°C) with DTNB (1 mM), at a molar ratio (reagent/SH) of 50:1 in the presence or absence of benomyl. The reaction was >93% complete for DTNB without benomyl in 35 min, reacting 18.66±0.13 cysteine residues per tubulin dimer. There were approximately two cysteine equivalents of tubulin remaining which became instantly accessible in 4 M urea. Benomyl significantly reduced the number of cysteine residues accessible to DTNB; there were 14.45±0.4 and 12.43±0.12 residues accessible per tubulin dimer in the presence of 50 and 200 μM benomyl, respectively. The difference in the number of modified cysteine residues in the absence and presence of 50 μM benomyl was 4.21 and that of 200 μM benomyl was 6.23 ($P<0.001$, Tukey–Kramer multiple comparisons test, one-way analysis of variance).

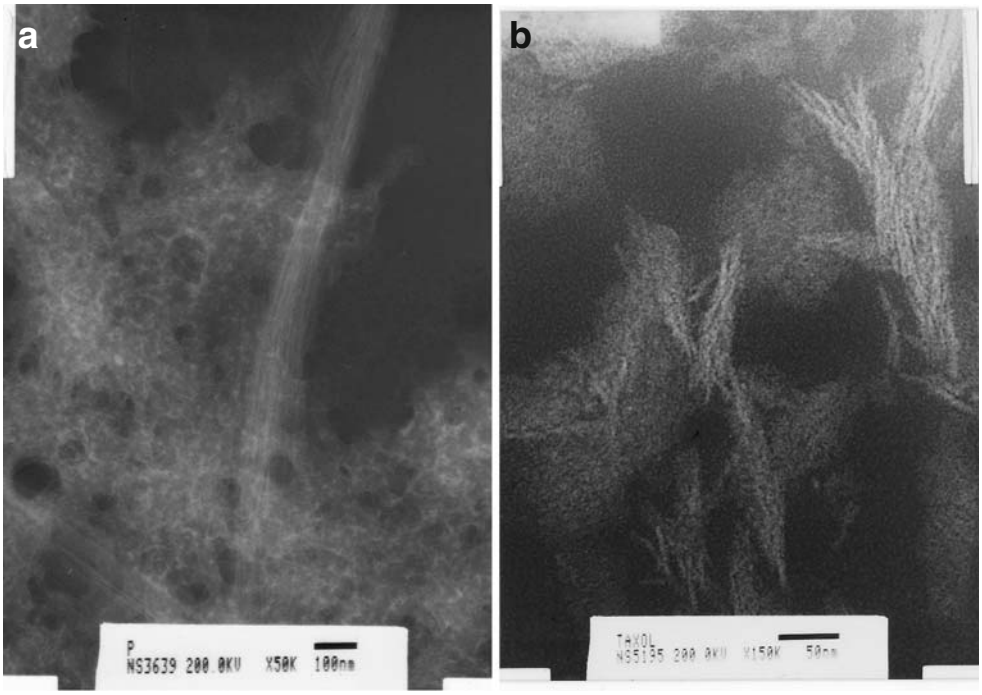

Fig. 5 Electron micrograph of *P. capsici* microtubules. Tubulin was assembled in the presence of 2.5 mM GTP and **a** 20 μM taxol and **b** 100 μM taxol. Polymerization and microscopy conditions were as described in the "Materials and methods"

Titration of tubulin sulfhydryl groups in the presence of taxol, GTP, and colchicine

The results in Fig. 6b show that addition of GTP significantly decreased the rate of reaction of DTNB (curves 4 and 5). There were 16.42±0.1 and 15.81±0.26 cysteine residues accessible per tubulin dimer in the presence of 50 μM and 2.5 mM GTP, respectively. However, 14.64±0.16 cysteine residues were accessible in the presence of 100 μM colchicine (curve 6), completing approximately 73% of the reaction within 35 min. When tubulin was polymerized in the presence of 100 μM taxol and the microtubule pellet was reacted with an excess of DTNB over SH groups, 16.09± 0.14 equivalents of cysteine reacted within 35 min (curve 3). The difference in the number of cysteine residues accessible to DTNB in untreated and treated tubulin heterodimers was statistically significant ($P<0.001$).

Inhibition of tubulin polymerization

The effect of benomyl on the polymerization of purified *P. capsici* tubulin (5.1 μM) was determined by light scattering. As shown in Fig. 7a, benomyl produced a concentration dependent inhibition of the rate and extent of microtubule polymerization. A concentration of 100 μM benomyl decreased the extent of polymerization at 30 min by 16%, while 900 μM benomyl inhibited polymerization by 100% at the same time. The concentration of benomyl at which half-maximal inhibition (IC_{50}) of polymerization occurred was 468±20 μM (Fig. 7b). When tubulin was incubated with 210-O-2 and 210-O-14, the change in the relative fluorescence unit was significantly higher ($P<0.001$) as compared to colchicine effect (Fig. 8).

Discussion

In this study, the amino acid sequences and G+C content of the *P. capsici* tubulin are in good agreement with several reported fungi including *Fusarium moniliforme* (Yan and Dickman 1996), *N. crassa* (Orbach et al. 1986), and *Candida albicans* (Daly et al. 1997). GTP is required at the E-site in order to polymerize tubulin (MacNeal and Purich 1978), but this nucleotide is hydrolyzed and becomes nonexchangeable upon polymerization. The resulting metastable microtubule structure is thought to be stabilized by a cap of remaining GTP-tubulin subunits at the ends, the loss of which results in rapid depolymerization (Mitchison and Kirschner 1984). Given the importance of nucleotide binding in tubulin, residues reported to directly involve in nucleotide binding were compared in *P. capsici* tubulin. Only one semiconserved substitution, α:Ala100, was observed, similar to observations by Lowe et al. (2001), where hydrophobic residues at the intradimer interface are hydrophilic for the dimer–dimer contact (i.e., β:Ile347 [versus α:Cys347], β:Val257 [versus α:Thr257], and α:Ala100 [versus β:Gly100]). Characterization of the β-tubulin genes of benomyl-resistant mutants of various fungi revealed several different point mutations which confer benomyl resistance (Yan and Dickman 1996; review within). Based on a multiple sequence alignment, three benomyl sensitive sites (His6, Glu198, and Arg241) were

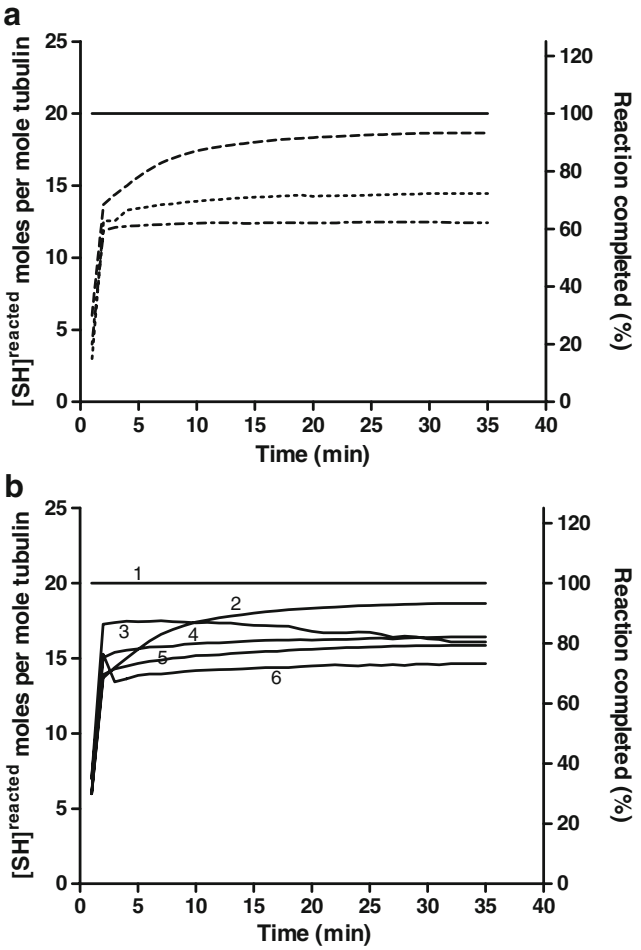

Fig. 6 Reaction kinetics of DTNB with tubulin SH in the presence of benomyl. **a** Tubulin (1 μM) was incubated with 4 M urea (*plane line*), without benomyl (*broken line*), 50 μM benomyl (*dashed line*), and 200 μM benomyl (*broken-dashed line*) at 4°C for 15 min and then treated with 1 mM DTNB. **b** Tubulin (1 μM) was prepared with *curves 1* 4 M urea, *2* untreated protein, *4* 50 μM GTP, *5* 2.5 mM GTP, *6* 100 μM colchicine at 4°C for 15 min and then treated with 1 mM DTNB, and *3* tubulin (1 μM) was incubated with 100 μM taxol at 37°C for 30 min, centrifuged, and microtubules were treated with 1 mM DTNB. The rate and extent of sulfhydryl group modification were monitored by measuring the absorbance at 412 nm from three independent measurements

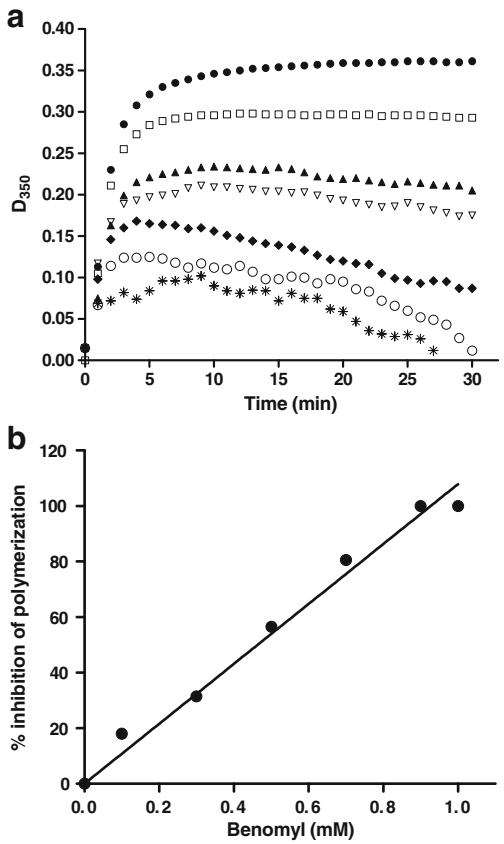

Fig. 7 Inhibition of microtubule assembly by benomyl. **a** Polymerization of tubulin (0.3 mg/ml) in the assembly buffer was measured in the absence (*filled circle*) and presence of 100 (*open square*), 300 (*filled triangle*), 500 (*open down triangle*), 700 (*filled diamond*), 900 (*open circle*), or 1,000 μM (*asterisk*) benomyl. Microtubule assembly was monitored by measuring the increase in absorbance at 350 nm with time. **b** Inhibition of tubulin polymerization at 30 min is plotted as a function of the benomyl concentration

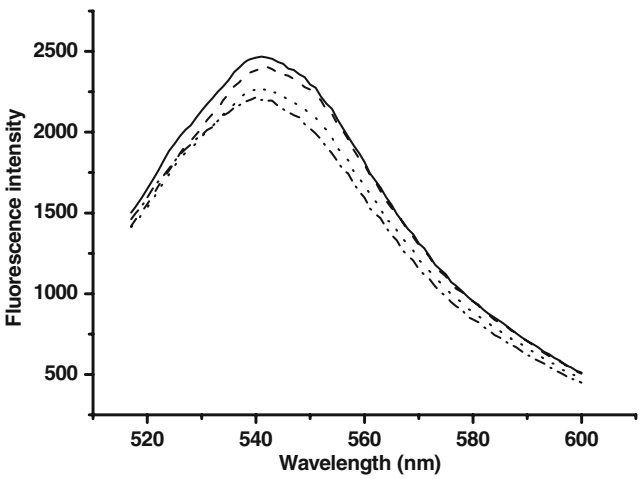

Fig. 8 Fluorescence emission spectra of the *P. capsici* tubulin with and without inhibitors. *Lines* control (*plane*), 4 mM colchicine (*dashed*), 210 O-2 (*dotted*), 210 O-14 (*dash-dotted*)

reported in *P. capsici* β-tubulin when compared to wild type β-tubulin gene in various fungi (Yan and Dickman 1996). The high amino acid identity between *P. capsici* tubulin and tubulins from other *Phytophthora* sp. made *P. capsici* tubulin as a suitable tool for antimicrotubule drug screening against this fungus.

The purification procedure reported here is reasonably efficient compared to tubulin purification procedures from *A. nidulans* (2–3 mg/4 l culture; Yoon and Oakley 1995) and *S. cerevisiae* (0.05% of soluble protein; Kilmartin 1981; 5–10 mg/88 l culture; Davis et al. 1993). Overexpression of β-tubulin alone in *S. cerevisiae* was reported to be highly toxic to the cells (Burke et al. 1989; Weinstein

and Solomon 1990). Such toxic effect or susceptibility of tubulin to proteolytic degradation was not observed during individual expression and purification of *P. capsici* recombinant tubulin. Eukaryotic tubulin monomers expressed in *E. coli* were found in inclusion bodies (Hollomon et al. 1998) and also in soluble form (Hollomon et al. 1998; MacDonald et al. 2001), though without any clear reason why a particular state was favored (i.e., fusion partner, *E. coli* strain, or intrinsic nature of tubulin). To prevent aggregation, the rate of protein synthesis can be decreased by lowering the temperature, resulting in an increase in the amount of soluble protein expressed (MacDonald et al. 2001). However, we found no effect of decreasing temperatures on the solubility of *P. capsici* tubulin. To express a protein in a soluble form is not a trivial undertaking, and until a more detailed structural analysis has been made of fungal tubulin in its native form, recombinant soluble form, and recombinant form refolded from inclusion bodies, we will not know whether conclusions drawn from comparisons of in vivo and in vitro biological characteristics are correct.

After dialysis (although it was difficult to know to what extent the protein was already in its correctly folding form), lack of polymerization might be an indication of, among other factors, a misfolded form. A polymerization pattern typical of a fast nucleation rate was observed in *P. capsici* tubulin. Hydrolysis of GTP is believed to provide energy for microtubule polymerization and to induce the instability in the microtubule lattice which subsequently produces depolymerization. In the present study, assembly took place rapidly, suggesting that GTP was readily hydrolyzed. 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 (Li et al. 1996). Five percent glycerol increased the number of microtubules in the sample; however, some sheets and coiled structures were observed (data not shown). Taxol has been used to promote assembly of tubulin into microtubules (Oxberry et al. 2001a). *P. capsici* tubulin required a taxol concentration of 100 μM for maximum microtubule formation, while that concentration was ≥80 μM in *A. nidulans* (Yoon and Oakley 1995). Increasing temperature had a substantial positive effect on tubulin assembly that is similar to observations made with natural partially purified tubulin (Bellocq et al. 1992). Any possible effect of DMSO, used as the benomyl, taxol, or colchicine diluent, on polymerization was ruled out following observation of apparently normal microtubules in the DMSO control (containing 5% DMSO). It has been proposed that $CaCl_2$ promotes microtubule disassembly by binding to free GTP, which prevents it from contributing to polymerization and increases the hydrolysis of stabilizing GTP in the microtubule cap (O'Brien et al. 1997). In this study, Ca^{2+} had a negative effect on normal microtubule structure formation, although not overall polymerization (data not shown).

The binding of benomyl to *P. capsici* 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. It is believed that the benzimidazole drugs bind to the colchicine binding site on the tubulin molecule. It is known that colchicine protects one sulfhydryl group from reaction with other sulfhydryl reagents (Basusarkar et al. 1997). It would have been expected that only one cysteine should be protected due to benomyl; however, there were ~4–6 cysteine residues accessible at 50–200 μM benomyl. There may be more than one site contributing for benomyl binding to *P. capsici* tubulin. Others have suggested that the binding site of benomyl is situated on α-tubulin, on α-tubulin and regulated by the β-subunit, on both α- and β-tubulin, or at the αβ-tubulin interface (Oxberry et al. 2001b; review within). Colchicine protected 4.02 sulfhydryl groups in *P. capsici* tubulin which were higher than the expected number of sulfhydryl groups. However, it is possible that several sulfhydryl groups were partially reacted and this number reflects the sum of partial protection of several groups (Roychowdhury et al. 2000).

GTP, required for microtubule assembly under most conditions, is known to interfere with the alkylation of tubulin and cross-linking by bifunctional thiol reagents (Luduena and Roach 1991) and can be photocross-linked to the Cys12 of β-tubulin (Shivanna et al. 1993) as well as covalently cross-linked with cysteine 295 of α-tubulin (Bai et al. 1998). Total two cysteines have been reported to covalently cross-link with GTP in tubulin heterodimer. In this study, there was no significant effect on SH-group accessibility at higher GTP concentrations. Binding of 50 μM GTP to *P. capsici* tubulin protected 2.24 sulfhydryl groups from DTNB and it matches previous reports (Shivanna et al. 1993; Bai et al. 1998). The order of accessibility to a number of cysteines was benomyl > colchicine > GTP > taxol. The time course of the DTNB reaction reveals two populations of SH groups in tubulin: fast and slowly reacting (Britto et al. 2005; Roychowdhury et al. 2000). Fast reacting SH groups are accessible ≤2 min in all experimental conditions (Fig. 6). It is noteworthy that in reacted tubulin, slow phase was admitted immediately after fast phase, as compared to a hyperbolic curve in unreacted tubulin. In the case of taxol- and colchicine-treated tubulin, the number of reacted SH groups decreased during the slow phase of reaction. Although the reason behind this is not clear, the primary product of the reaction of DTNB is itself a reactive intermediate for further attack on another thiolate, forming a protein–protein disulfide bond now lacking the chromophore which could explain

our results (Pryor 1962). During this study, successful polymerization and accessibility of sulfhydryl groups in *P. capsici* tubulin heterodimer resembled previously published reports from other tubulin sources indicates that the *P. capsici* tubulin was folding correctly and can be used for further in vitro screening experiments.

Although microtubules are a known target for fungicides such as benzimidazoles, the only compound previously reported to exhibit strong antimicrotubule activity toward *P. capsici* fungi is the experimental fungicide zarilamide (Young 1991). Benomyl is used extensively as an agricultural fungicide against a wide range of fungal diseases affecting fruit trees and field crops and has been highly useful as a research tool in fungal cell biology (Hoyt et al. 1991). Some fungi, viz., *Schizosaccharomyces pombe* and *S. cerevisiae* are benomyl resistant (Neff et al. 1983; Thomas et al. 1985) and *Phytophthora* sp. is less sensitive to benzimidazole fungicides. However, we found that benomyl inhibits the polymerization of *P. capsici* tubulin, with 50% inhibition (IC_{50}) of polymerization occurring at a benomyl concentration of 468±20 μM, which is lower than values reported for other fungi (Davidse and Flach 1977). Functionality of this recombinant tubulin was conformed to inhibition of polymerization by benomyl. So we used *P. capsici* tubulin for antimicrotubule compound screening and demonstrate that by strategically placing two FRET probes on these two proteins, we are able to monitor the intermolecular co-association by fluorescence quenching between the fluorescence donor and acceptor. The utility of the system is that it should be adaptable to high-throughput screening toward small-molecule antimitotic agent targeting the microtubule structure. Structural elucidation and in vivo antiphytophthora activity of 210-O-2 and 210-O-14 are in progress.

In conclusion, *P. capsici* recombinant α- and β-tubulin produced a microtubule-like structure at an optimized condition. Accessibility of the tubulin cysteine residues was modified in association with benomyl, GTP, taxol, and colchicine binding indicated that these binding sites were folded correctly. Inhibition of recombinant *P. capsici* tubulin polymerization by benomyl indicates that fungicide binding site was folded correctly and *P. capsici* tubulin can be used further for in vitro antimitotic compound screening using FRET technique.

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