Cloning, Purification, and Polymerization of Capsicum annuum Recombinant α and β Tubulin

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α and β tubulin genes were cloned from the Capsicum annuum leaves using rapid amplification of cDNA ends (RACE)-PCR. Nucleotide sequence analysis revealed that 1,353 bp Capsicum annuum α/β-tubulin (CAnm α/β-TUB) encodes a protein of 450 amino acids (aa) each. The recombinant α/β tubulin was overexpressed mainly as an inclusion body in Escherichia coli BL21 (DE3), upon induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and its content was as high as 50% of the total protein content. Effective fusion protein purification and refolding are described. The average yields of α and β tubulin were 2.0 and 1.3 mg/l of culture respectively. The apparent molecular weight of each tubulin was estimated to be 55 kDa by SDS-polyacrylamide gel electrophoresis (PAGE). The tubulin monomers were found to be assembly competent using a standard dimerization assay, and also retained antigenicity with anti-His/T7 antibodies. The purified tubulins were polymerized to microtubule-like structures in the presence of 2 mM guanosine 5'-triphosphate (GTP).

Key words: Capsicum annuum; dimerization; microtubule; pepper; rapid amplification of cDNA ends (RACE)-PCR

The basic building block of microtubules is tubulin, a heterodimeric protein consisting of a non-covalent interaction of two related approximately 50 kDa polypeptides known as α- and β-tubulin. Tubulin dimers polymerize in a head-to-tail manner to form 4 to 5 nm linear protofilaments, typically 13 of which are laterally associated in the wall of the 24-nm diameter microtubule.1) During the plant cell cycle, microtubules are reversibly polymerized to form functionally distinct arrays that mediate sub-cellular motility phenomena and provide cytosolic organization. They are involved in many essential functions in plant cells, including cellular morphogenesis, organelle and vesicle transport, chromosome segregation, cell wall formation, and cell division.1,2) Phytophthora blight is caused by the oomycete pathogen P. capsici. It is a devastating disease of pepper (C. annuum) in South Korea and worldwide. Microtubules are a known target of fungicides such as the benzimidazoles. Since some fungicides and herbicides display unacceptable phytotoxicity,3,4) it is important that any antimiotic compound for plant pathogenic fungi should be non-phytotoxic.

Several methods have been developed to isolate native tubulin from plant material,5-7) but lower solubility is a major concern in large-scale applications. The purpose of this study was to overcome the difficulties in preparing sufficient quantities of plant tubulin by purification of recombinant α and β tubulin of C. annuum (CAnm α-TUB/β-TUB). Dimerization of tubulin and the formation of microtubule-like structures were studied. The present work provides information on tubulin of the Solanaceae family, promoting structural and functional research into plant tubulin. In future, the C. annuum microtubule will be used as a control in screening of antimiotic compounds against P. capsici.

Materials and Methods

Plant material, strain, plasmid, and enzymes. C. annuum fresh leaves were obtained from a local farm
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Using oligo(dT) Ver.3.0. The second strand of cDNA was synthesized reverse transcribed using Takara RNA PCR kit (AMV), leaves (ground in liquid nitrogen) using an Easy blue/C212 RNA was isolated from 100 mg of ends (RACE) methods were followed. For this, total cDNA, the RT-PCR and rapid amplification of cDNA ends (RACE) methods were accomplished using BigDyeTM terminator. The reaction product was purified using ethanol precipitation, and sequenced with an automatic sequencer 3730xl (Macrogen, Seoul, South Korea). The α and β tubulin inserts were then cloned into the SalI/NotI and NdeI/XhoI restriction sites (underlined). The total RNA isolated as described above was reverse transcribed using these two primers. The sequencing of both the strands was accomplished using BigDyeTM terminator. The reaction product was purified using ethanol precipitation, and sequenced with an automatic sequencer 3730xl (Macrogen, Seoul, South Korea). The α and β tubulin inserts were then cloned into the SalI/NotI and NdeI/XhoI sites of pET28a (Novagen), respectively, producing fusion proteins with N-terminal hexahistidine tags.

Culture and isolation of inclusion bodies. The expression plasmids were transformed into E. coli BL21 (DE3) by the heat shock method. The transformed bacteria were grown at 37 °C in LB medium containing 100 µg/ml kanamycin until the A₆₀₀ of the culture reached 0.5. Isopropyl-β-D-thiogalactopyranoside (0.2 mM, IPTG) was added to induce expression of recombinant α/β tubulin at 18 °C overnight. The culture growth was collected by centrifugation and suspended in 40 ml 20 mM Tris buffer, pH 8.0. The cells were disrupted by ultrasonication (Heat Systems, Boston, MA) using 60 cycles, each of 1 s, at 15% output power. Cell-free extract was centrifuged at 23,700 × g for 10 min at 4 °C, and the pellet was resuspended in 40 ml of isolation buffer (20 mM Tris, 300 mM NaCl, and 2% Triton X-100, pH 8.0) for sonication, as described above. The pellet was resuspended in isolation buffer and sonication was repeated twice.

Solubilization of inclusion bodies, refolding, and purification. The pellet obtained after sonication was suspended in 40 ml of binding buffer (6 mM guanidine hydrochloride, 20 mM Tris, 300 mM NaCl, 5 mM imidazole, and 1 mM β-ME, pH 8.0) and incubated at 25 °C with gentle stirring for 30 min. The supernatant was collected by centrifugation at 23,700 × g for 10 min at 4 °C. Undissolved particles were removed by filtration through a 0.45-µm syringe filter (Millipore, Boston, MA).

Supernatant containing α or β tubulin was loaded onto a Ni²⁺ charged sepharose column pre-equilibrated with the binding buffer (flow rate, 0.5 ml/min). The column was washed with binding buffer (3 bed volumes), and then with the washing buffer (6 mM urea, 20 mM Tris, 300 mM NaCl, 20 mM imidazole, and 1 mM β-ME, pH 8.0). The bound protein was refolded on-column using a decreasing gradient of urea (6-0 M), starting with the washing buffer and finishing with the refolding buffer (20 mM Tris, 300 mM NaCl, 20 mM imidazole, and 1 mM β-ME, pH 8.0). After completion of the gradient, washing was continued with two bed volumes of

Cloning of full length α and β tubulins. The 5′ and 3′ RACE products were used to generate primers corresponding to the beginning and the end of the coding region. For α tubulin, the 5′-full primer was 5′-AAGTCGACAAATGAGAATCTTCTATTCACATTTGG-3′ and the 3′-full primer was 5′-AAGTCGACAAATGAGAATCTTCTATTCACATTTGG-3′. These primers contained restriction sites for SalI and NotI (underlined). For β tubulin, the 5′-full primer was 5′-AACATACTATGAGAAGAAATCTTCTACATTCCAAGGAG-3′ and the 3′-full primer was 5′-AACATACTATGAGAAGAAATCTTCTACATTCCAAGGAGG-3′. These primers contained NdeI and XhoI restriction sites (underlined).
refolding buffer. The refolded protein was eluted using elution buffer (20 mM Tris, 300 mM NaCl, 0.5 mM imidazole, and 1 mM β-ME, pH 8.0) with a linear gradient from 0.02 to 0.5 mM imidazole. Fractions containing the target protein were dialyzed against 50 mM Tris (pH 8.0), and concentrated by ultrafiltration using YM10 cut-off membrane (Amicon, Boston, MA). The purity of α and β tubulin was assessed by SDS–PAGE (18) resolved on a 0.75 mm 4% stacking/12% resolving minigel (Hoefer, San Francisco, CA). Proteins were visualized with Coomassie brilliant blue R-250 stain, and sizes were estimated from broad range precision standards (Sigma Aldrich, St. Louis, Missouri).

Reconstitution of recombinant α and β tubulin in heterodimers. The His tag of alpha tubulin was removed by thrombin action (10 U/mg protein) prior use in dimerization reaction. Recombinant α (T7†His†) and β (His†) tubulin monomers (100 μg/ml) were dimerized on Ni2+ charged sepharose resin in buffer containing 0.1 M Hepes (pH 7.4), 1 mM EDTA, and 1 mM MgCl2 with or without 2 mM GTP, at 25 °C for 20 min (Fig. 1). The washing buffer contained 0.1 M Hepes (pH 7.4), 50 mM imidazole, 1 mM MgCl2, and 1 mM EDTA. The dimerized tubulin was eluted with elution buffer containing 1 M imidazole (the other components were same as in the washing buffer). The inclusion of α and β tubulin components in the dimer was analyzed by western blotting (10) using primary monoclonal anti-his (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-T7 (Chemicon International, Santa Cruz, CA) antibodies. Blotted bands were visualized using goat-anti-rabbit IgG antibodies as secondary antibodies.

Microtubule formation assay. The time course of microtubule assembly was monitored at A\textsubscript{350} (11) using an Optizen 2120 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 MgCl2, and 5% v/v glycerol). The reaction was initiated by adding GTP at a final concentration of 2 or 5 mM. The contents were mixed, and turbidity was measured at 37 °C over a time scale of 95 min, reading at every 5 s.

Transmission electron microscopy. Completely polymerized tubulin mixtures were prepared for transmission electron microscopy (TEM). (12) Briefly, 30 μl of polymerized sample was immediately diluted with 10 μl of 0.4% glutaraldehyde for 1 min at room temperature. Tubulin solution (10 μl) was applied to 200-mesh, copper/formvar coated grids (EMS) for 1 min, washed with two drops of dH\textsubscript{2}O, and stained for 2 min with a drop of 2% uranyl acetate. Excess stain was removed by blotting with filter paper. The samples were air dried and later viewed using a transmission electron microscope (JEM 2010, JEOL, Tokyo).

Results and Discussion

Cloning and sequencing of the α/β tubulin gene

CAnm α/β tubulin cDNAs were isolated by the RT-PCR and RACE methods. Primers corresponding to the 5′- and 3′- ends of the coding region of C. annuum α and β tubulin yielded a product of 1,353 bp (data not shown). The tubulin insert cloned into the pET28a bacterial expression vector provided an N-terminal His-tagged protein, which was purified in one step. The α and β tubulin gene sequences were 1,353 bp long, with G + C contents of 47 and 45% respectively, and were predicted to encode a putative 450 aa protein individually (data not shown). These results were in good agreement with the aa sequences reported for plant tubulins, including Arabidopsis thaliana, (13,14) Glycine max L. (15), and Eleusine indica. (16,17) At the nucleotide level, the CAnm α- and β-TUB genes shared 78–95% and 76–93% homology with coding regions of plant tubulin cDNAs. (18) The genebank accession numbers for the sequences reported in C. annuum are EF495257 (α tubulin) and EF495259 (β tubulin).

Analysis of α and β tubulin sequences

The predicted CAnm α-TUB aa sequence was compared with all isotype tubulin sequences present in the databases. (18) The greatest similarity was found with the α-tubulin family. In particular, the highest match was found with Nicotiana tabacum tubulin (99% identity). α tubulin from other plant sources, such as Zea mays (tubulin α 3-chain), Brassica napus, Oryza sativa, and Eleusine indica, had 92–97% sequence identity with CAnm α-TUB (data not shown). Clustal W alignment (19) of the deduced aa sequence of CAnm α-TUB with some α-tubulin sequences derived from organisms at different evolutionary levels exhibited high degrees of homology (Fig. 2). The aa sequence of CAnm α-TUB had 96, 88, 84, and 78% identity to
A. thaliana, Plasmodium falciparum, Drosophila melanogaster/Xenopus laevis, and Homo sapiens/C11-tubulins respectively. Although the carboxyl termini of C11-tubulins are highly divergent, a carboxyl terminal tyrosine is conserved. CAnm-C11-TUB showed strong homology to C11-tubulins from animals and protists. This homology indicates that the structural tubulin gene predates the plant-animal evolutionary split.

Figure 2 shows the blocks of highly homologous residues. The extremely homologous regions included the diagnostic portion from aa 401 to 409, which is conserved exclusively within the C11-tubulin subclass. 20)

Fig. 2. Homology between C. annuum and Other α-Tubulins. Identical and conserved amino acids are denoted by * and :, respectively. The amino acids highlighted in black/gray are discussed in the text. DROME, D. melanogaster tubulin α-3 chain (P06605); XENLA, X. laevis (P08537); HUMAN, Human alpha 8-tubulin (Q9NY65); ARATH, A. thaliana (P29511); PLAFK, P. falciparum (P14642); CAnm, C. annuum (ABP63320).
The potential GTP-binding site in CAmm α-TUB was deduced by conserved domain search\(^{21}\) and located at residues 140 to 146. The cell attachment sequence, RGD tripeptide, was present at 320 to 322 residues. The characteristic α-tubulin MREC1 domain, which has been hypothesized to be involved in autoregulated post-transcriptional mechanisms\(^{22,23}\) was conserved in CAmm α-TUB at the N-terminal. The lysine residue at position 40, modified in many isotypes of α-tubulin by the addition of an acetyl group, was conserved\(^{24,25}\). The C-terminal domain of the tubulin family, which plays a crucial role in tubulin polymerization and ligand interaction, changes greatly among different organisms and among various isotypes. In CAmm α-TUB, the carboxyl terminus region was not fully conserved from those of other α-tubulins. The tyrosine residue located at the C-terminal end was conserved, indicating that this residue, the usual substrate for polyglycylation and polyglutamyl modification,\(^{26}\) was highly conserved, suggesting that it is an efficient substrate for these post-translational modifications. The BLAST of the CAmm β-TUB aa sequence resulted in 100% sequence identity with Lycopersicon esculentum, and 93–99% with other plant β tubulins, viz., Zea mays (beta-1 chain), E. indica, A. thaliana, Eucalyptus grandis, Solanum tuberosum (beta-2 chain), and N. attenuata. Microtubule-dependent processes in plants and algae are very sensitive to low concentrations of dinitroaniline and phosphoric amide herbicides as compared to these processes in animals.\(^{27,28}\) Hence structural comparisons of plant and animal tubulins should give insight into the relations among diverse groups and into distinct functional properties in these two tubulin groups. Sequence alignment of the deduced aa sequences of the β tubulin cDNAs from different groups is shown in Fig. 3. The mutation sites, such as His6, Glu198, Tyr50, and Arg241 for benzimidazole resistance in fungi and plants,\(^{17,29,30}\) are highly conserved in CAmm β-TUB (Fig. 3). Ala165, a benzimidazole-sensitive site in A. nidulans, is replaced by Leu168 in CAmm β-TUB, similarly observed in E. indica β tubulins.\(^{17}\) Since P. capsici causes Phytophthora blight, a devastating disease of the pepper plant (C. annuum), it is worthwhile to note that β tubulin from P. capsici has 86% sequence identity with CAmm β-TUB, and that mutant sites for benzimidazole resistance are conserved in both. The two positions (Met200 and Thr323) that are not significant for dinitroaniline resistance in E. indica β tubulins\(^{17}\) are conserved in C. annuum and P. capsici β tubulins also (Fig. 3 gray). These analyses are useful during the screening of non-phytotoxic, antimitotic agents for P. capsici.

**Expression and purification of α/β tubulin**

Protein production from inclusion bodies is generally protected from proteolytic degradation, and easily solubilized, and contains almost pure protein in different states of aggregation, in an inactive form.\(^{31}\) In this study, different concentrations of IPTG (0.1 to 1.0 mM) were compared for their effectiveness in inducing maximal expression of α/β tubulin. The optimal concentration was 0.2 mM (data not shown). After 12 h of induction with 0.2 mM IPTG at 18 °C, recombinant plasmids pET28a α- and β-TUB exhibited high levels of the α and β tubulin overexpression respectively in E. coli BL21 (DE3), which accounted for about 50% of total cellular proteins (Fig. 4, lane A1). SDS–PAGE analysis of the soluble and insoluble fractions of the induced E. coli cell extracts indicated that the fusion protein was mostly expressed as insoluble inclusion bodies (data not shown).

During purification, there was no loss of protein carrying the His6-tag in the flow-through, suggesting protein binding to the nickel resin (data not shown). SDS–PAGE analysis of the fractions showed that elution with ≥ 250 mM imidazole resulted in a single band of about 55 kDa for α (Fig. 4A, lanes 6–12) and for β tubulin (Fig. 4B, lanes 6–10). This process typically yielded 2.0 mg of α and 1.3 mg of pure β tubulin from 1 liter of culture. The procedure proved to be efficient, as reported from other sources such as tubulin from ginkgo pollen, 2 mg from 100 gm of ginkgo pollen,\(^{7}\) and Mimosa pudica, 1.5 mg from 50 g leaves.\(^{60}\) Even though decreasing the temperature has been successful in reducing the tendency of certain proteins to aggregate,\(^{32,33}\) in this case, the content of soluble protein remained unchanged at different induction times (6–15 h), growth phases, and through a temperature range of 30 to 18 °C (data not shown).

**Confirmation of hetero-oligomer formation**

The purified tubulin monomers were made to dimerise, as described in “Materials and Methods” (Fig. 1). When analyzed by western blotting, the bands corresponding to dimeric tubulin reacted with both the anti-α and anti-T7 antibodies (Fig. 5A, B; lanes 3, 5). During the reaction of α and β tubulin in the presence and absence of GTP, the fractions collected after washing of the resin did not show any band for α/β tubulin, indicating affinity binding of tubulin dimer to resin (Fig. 5A, B; lanes 2, 4). Since the elution of α tubulin only (T7’His’\(^{\text{\textsuperscript{+}}}\)) containing the reaction mixture did not show any band (Fig. 5A, B; lane 1), it can be concluded that there was no specific interaction between α tubulin and Ni\(^{2+}\) sepharose, and that α tubulin was dimerized with β tubulin only.

**Polymerization of tubulin**

Continuous changes in \(^{35}\)S were recorded during polymerization of tubulin in the presence of GTP. An obvious increase in absorbance was observed immediately after the addition of GTP, reaching nearly saturation point in 40 min (Fig. 6). An increase in
absorbance was recorded for α tubulin homodimers, but not for β homodimers (data now shown). Such αα- and ββ-homodimers have been reported in recombinant nematode tubulin. Polymerization occurred at both GTP concentrations tested and in the absence of GTP (Fig. 6). There was a significant increase in polymerization at 2 mM GTP concentration. In the absence of GTP, tubulin produced abnormal structures due to aggregation of protein, without resulting in any dominant structure (data not shown).

Fig. 3. The Amino Acid Sequence Alignment of C. annuum β Tubulin and Corresponding Sequences from Other Plants and Fungi. Symbol meanings are as in Fig. 2. The amino acids highlighted in black/gray are discussed in the text. CAnm, C. annuum β tubulin (ABP65322); Atha, A. thaliana (AAA32893); EInd, E. indica (AAD20187); PCap, P. capsicum (ABP65321); ANid, A. nidulans (1312295B); NCra, N. crassa (AAA33617).
and $\beta$ tubulins produced abnormal structures in the absence of GTP. 34) Optimal formation of typical long microtubule-like structures occurred at 1 mg/ml of each tubulin concentration (Fig. 7A, B). This structure resembled previously published normal microtubular structures from other plant sources in terms of appearance and size.6,7)

The correct and efficient refolding of an inclusion body to its active form has been a topic in both basic research and industrial applications. This method gave $\alpha/\beta$ tubulin in correctly folded form since recombinant tubulins formed heterodimers and polymerized to microtubule like structures. In the future, this tubulin from C. annuum can be used as control group for high-throughput screening of some fungicides against P. capsici.

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