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Overexpression, purification, and functional analysis of recombinant human tubulin dimer

Itsushi Minoura [1], You Hachikubo [1], Yoshihiko Yamakita, Hiroko Takazaki, Rie Ayukawa, Seiichi Uchimura, Etsuko Muto *

Laboratory for Molecular Biophysics, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

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ABSTRACT

Microtubules consisting of tubulin dimers play essential roles in various cellular functions. Investigating the structure–function relationship of tubulin dimers requires a method to prepare sufficient quantities of recombinant tubulin. To this end, we simultaneously expressed human α1- and β3-tubulin using a baculovirus-insect cell expression system that enabled the purification of 5 mg recombinant tubulin per litre of cell culture. The purified recombinant human tubulin could be polymerized into microtubules that glide on a kinesin-coated glass surface. The method provides a powerful tool for in vitro functional analyses of microtubules.

1. Introduction

Microtubules are cytoskeletal filaments formed by the spontaneous polymerization of tubulin heterodimers, which are each composed of α- and β-tubulin. These highly conserved intracellular structures play crucial roles in a wide range of cellular functions including cell division, morphogenesis, and intracellular transport; impaired microtubule function leads to various human diseases. For example, mutations in tubulin genes lead to neuronal malformation [1–3], and disarrangement of tau–microtubule complexes may lead to Alzheimer's disease and related tauopathies [4].

To better understand the roles played by microtubules in cells and disease, in vitro functional analyses have been performed using tubulin purified from the mammalian brain. The purification method is well established and provides a biochemically useful amount of tubulin (typically 10–30 mg tubulin per 100 g of wet tissue) [5]. However, such native tubulin comprises more than 24 isoforms due to the multiple tubulin genes involved and various posttranslational modifications (PTMs) [6–8], and this diversity prevents rigorous examination of the structure–function relationship of tubulin.

We therefore need to purify isotypically pure tubulin composed of single α- and β-tubulin polypeptides, ideally with a single, defined PTM state. However, heterologous expression of αβ-tubulin in bacteria has proven to be difficult, likely because bacteria lack the chaperones and cofactors required for tubulin folding and dimerization. Using budding or fission yeast for tubulin expression marked a breakthrough in developing a better purification method [9–12]. These organisms were chosen because (a) as eukaryotes they have the chaperones and cofactors required for proper tubulin assembly [13] and (b) they have only two α-tubulin genes, one of which is nonessential, and one β-tubulin gene. The resultant limited number of tubulin isotypes greatly simplifies the purification of the expressed tubulin.

Although the yeast expression system has proven to be a powerful tool, the method suffers from the following limitations: First, because of the low yield, the method requires large-scale cell cultures, and obtaining 1 mg of pure yeast tubulin typically requires 25–100 g of yeast cells, which means 4–15 litre of yeast culture. Second, the amino-acid sequence divergence between yeast and mammalian tubulin limits analysis of the properties that are specific to mammalian tubulin.

To overcome these difficulties, in this study, we developed a method to overexpress and purify recombinant human tubulin using a baculovirus-insect cell expression system. Our method yielded 5 mg of recombinant tubulin, composed of human α1- and β3-tubulin, per litre of insect-cell culture (~15 g of cells).

Abbreviations: MAP, microtubule-associated protein; WT, wild-type; PTM, posttranslational modification

* Corresponding author. Fax: +81 48 467 7145.
E-mail address: emuto2@brain.riken.jp (E. Muto).
[1] These authors contributed equally to this work.

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The purified tubulin is functional in that it can be polymerized into microtubules that support kinesin motility. The successful preparation of recombinant human tubulin opens the way for future in vitro analyses with broad applications, especially in biomedical research.

2. Materials and methods

2.1. Construction of baculovirus transfer plasmids

Homo sapiens α1-tubulin (TUBA1B; NP_006073) and β3-tubulin (TUBB3; NP_006077) sequences in pUC19 plasmid were custom-synthesized by Medical & Biological Laboratories Co. (Nagoya, Japan). For affinity purification of the protein products, synthetic sequences encoding a glycine-based linker (GGSGG) and a His-tag (HHHHHHHH) were fused to the 3′ end of the α1-tubulin cDNA sequence. Sequences encoding the above glycine-based linker and a FLAG tag (DYKDDDDK) were fused to the 3′ end of the β3-tubulin cDNA sequence. To increase expression levels, an L21 leader sequence [14] was added to each of these sequences just before the start codon. The inserts were cloned into pFastBac Dual vector (Life Technologies, Carlsbad, CA, USA); the α1-tubulin sequence was inserted after the polyhedrin (P_H) promoter by using Spe I and Hind III sites, and the β3-tubulin sequence was inserted after the p10 promoter by using Nhe I and Xho I sites (Fig. 1A).

2.2. Preparation of recombinant human microtubules

The transfer plasmid was used to generate recombinant baculoviruses using the Bac-to-Bac System (Life Technologies). To express recombinant tubulin, HighFive cells (Life Technologies), grown to a concentration of 2.0–2.5 × 10^6 cells/ml, were infected with the viruses at a multiplicity of infection of 20, and cultured for a further 24–72 h in suspension at 27 °C. Cells were collected (~15 g wet weight from 1 litre of suspension culture) and lysed for 15 min in 90 mL of PMI buffer (0.1 M PIPES, 10 mM $MgSO_4$, 2 mM EGTA, pH 6.8) supplemented with 0.5 M 3-(1-pyridinio)-1-propane sulfonate (NDSB201, Calbiochem, Merck KGaA, Darmstadt, Germany), 1% CHAPS, 5 mM DTT, 1 mM ATP, 1 mM GTP, and protease inhibitors. This and the following steps were performed on ice or at 4 °C unless otherwise stated. The lysate was centrifuged for 25 min at 200,000×*g*. The resultant supernatant was supplemented with 10% (v/v) glycerol and incubated with 30 ml of DEAE-Sepharose resin (DEAE Sepharose Fast Flow, GE Healthcare) for 60 min. After the resin was washed with DEAE wash buffer (0.1 M PIPES, 10 mM $MgSO_4$, 10% glycerol, 60 mM NaCl, 1 mM ATP, 1 mM GTP, and protease inhibitors, pH 6.8) the retained protein was eluted with the same buffer containing 0.4 M NaCl, pH 7.0. To purify recombinant tubulin, this crude solution (~40 ml) was mixed with 4 ml of TALON resin (Clontech, Takara-Bio Inc., Ootsu, Japan) for 30 min. His-tagged tubulin was eluted with PMG buffer (0.1 M PIPES, 5 mM $MgSO_4$, 10% glycerol, pH 7.0) supplemented with 0.3 M NaCl and 0.25 M imidazole. The eluate was diluted with an equal volume of PMI buffer supplemented with 10% glycerol, 1 mM GTP, 2 mM ATP, and 0.04% NP-40, and then mixed with 4 mL of anti-FLAG-tag antibody-conjugated resin (Sigma–Aldrich) for 1 h. FLAG-tagged tubulin was eluted with PMG buffer supplemented with 2 mM EGTA, 0.15 M NaCl, 0.02% NP-40, 1 mM GTP, and 0.2 mg/ml FLAG peptide (Sigma–Aldrich).

The resultant purified tubulin was concentrated to 3–4 mg/ml with an Amicon Ultracel-30 K filter (Millipore, Merck KGaA), centrifuged to remove aggregation, and then polymerized by adding 1–5 μM of taxol (Tocris) at 30 °C for 1 h. The polymerized material was supplemented with 2 mM ATP and 1 M NaCl, and then centrifuged for 12 min at 250,000×*g*. The pellet was suspended in BRB buffer (80 mM PIPES, 5 mM $MgCl_2$, 1 mM EGTA, pH 6.8) containing 1 mM GTP and 0.1 mM taxol at room temperature. The microtubules were stored at 4 °C until use.

Alternatively, tubulin dimer eluted from the FLAG affinity column was concentrated to >5 mg/ml, frozen in liquid N_2 with 30% glycerol, and then stored at −80 °C until use. Upon use, tubulin was thawed and further purified by a cycle of polymerization and depolymerization.

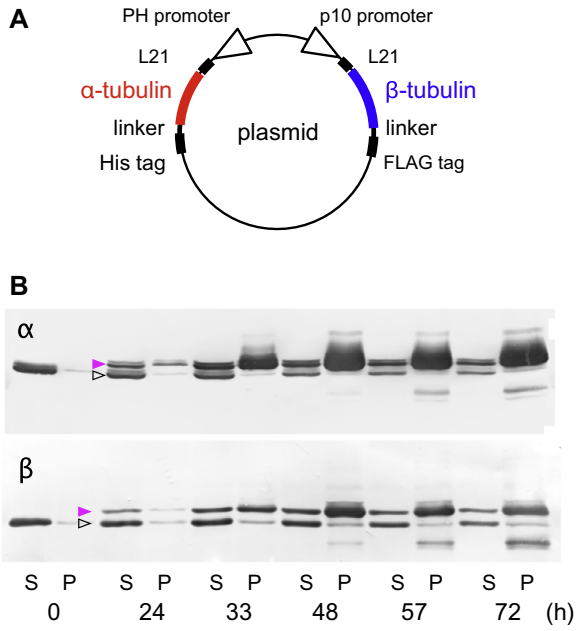

Fig. 1. Expression of recombinant human α1β3-tubulin. (A) Design of the transfer plasmid. (B) Time-course of recombinant tubulin production after viral infection into HighFive cells. Tubulin in the cell lysate supernatant (S) and precipitate (P) was detected by Western blotting using DM1A (anti-α-tubulin) and ab6046 (anti-β-tubulin) antibodies. The recombinant tubulin (magenta arrowheads) migrates slower than the endogenous tubulin (open black arrowheads) in SDS–PAGE, due to the attached tag (see Fig. 2C). Polypeptides were quantified as shown in Fig. S1.

2.3. Preparation of other proteins

Kinesin-1 was purified from pig brain as previously described [15]. Brain tubulin was also purified from pig brain by two cycles of polymerization and depolymerization, as previously described, whereby microtubule-associated proteins (MAPs) were removed by the addition of high-molarity PIPES buffer during the second cycle [16]. To obtain yeast tubulin dimer fused to exactly the same tags as those used for the recombinant human α1β3-tubulin, *Saccharomyces cerevisiae* α-tubulin (*TUB1*) sequence was fused to sequences encoding the glycine-based linker (GGSGG) and His-tag (HHHHHHHH), and *S. cerevisiae* β-tubulin sequence with introduced taxol-binding ability ($TUB2^{tax}$) [17,18] was fused to sequences encoding the glycine-based linker (GGSGG) and FLAG tag (DYKDDDDK). The yeast wild-type (WT) and yeast + tag tubulin dimers were purified using the *S. cerevisiae* expression system [11].

2.4. Mass spectrometry analysis

Mass spectrometry determination of full-length tubulin polypeptides was performed with a quadrupole time-of-flight mass spectrometer (QSTAR; AB/MDS SCIEX). See Supplementary Materials for the detail.