ISOLATION OF A TUBULIN-LIKE PROTEIN FROM PHASEOLUS

ROBERT W. RUBIN and ELLIOT H. COUSINS
Department of Biological Structure, University of Miami Medical School, P.O. Box 520875, Biscayne Annex, Miami, FL 33152; Division of Natural Sciences, New College, Sarasota, FL 33578, U.S.A.

(Revised received 8 July 1976)

Key Word Index—Phaseolus aureus; Leguminosae; mung bean; tubulin isolation and comparison.

INTRODUCTION

In plant and animal cells, electron microscopy has demonstrated an apparent structural and morphological conservation of both the diameter and shape of microtubules [1,2]. A recent study demonstrated the presence of colchicine binding activity in extracts of vascular tissues from the plant Heracleum mantegazzianum [3]. However, colchicine could not be shown to bind to post ribosomal supernatants of Zea mays coleoptiles [4]. We now report the presence of colchicine\(^{[\text{H}]}\) binding to a purified protein fraction isolated from Phaseolus aureus, which contains a major component which co-migrates with purified tubulin from brain on polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Fractionation of the whole cell supernatant by \((\text{NH}_4)_2\text{SO}_4\) precipitation showed a small barely visible component with a MW of 55000 when examined on SDS polyacrylamide gels (Fig. 1). During further purification, by ion-exchange chromatography [5], the protein fraction which eluted at 0.2 M NaCl had a prominent band with a MW of 55000 on polyacrylamide gels (Fig. 1a). Further purification utilizing precipitation with 0.03 M CaCl\(_2\) [6], the use of a linear NaCl gradient or gel filtration on G-200 Sephadex [7] resulted in only a slight increase in purity.

The addition of CaCl\(_2\) to a final concn of 20 mM, to a solubilized 35-50% \([\text{NH}_4]\)_2\text{SO}_4 pellet [8], resulted in the formation of a fibrous ppt. Only 60% of the Ca\(^{2+}\) ppt was soluble in 15 mM EGTA buffer. The relative purity of the protein was increased substantially by this procedure (Fig. 1e). The protein continued to precipitate at Ca\(^{2+}\) ion concn of 20-40 mM, in agreement with previously reported results [7,8]. The purity of the ppt. decreased at higher Ca\(^{2+}\) ion concn. Quantitation of the peaks present in the densitometer tracings of the 20 mM Ca\(^{2+}\) ppt. (Fig. 1c) [9] indicates that the component with a MW of 55000 represents 8-10% of the protein entering the gel. It was thus possible to obtain a minimum estimate of purity of 55000 represents 8-10% of the protein entering the protein pep.
significant amounts of undigested tubulin present after 24 hr digestion.

No direct comparisons could be made with previous work [13,14] which examined tubulin CNBR peptides, due to the discrepancies in the gel systems utilized and the failure of these authors to provide MW values. Recent work has demonstrated significant amino acid differences between tubulins isolated from the same species but from different organs [7]. Other workers utilizing plant material have encountered difficulty in obtaining colchicine binding activity and have found this activity to be extremely labile [3,4]. Our study however, clearly demonstrates the presence of colchicine binding proteins in a higher plant. More recently [15] a protein which co-polymerizes with brain tubulin and possesses identical electrophoretic characteristics to mammalian tubulin has been examined from the fungus Aspergillus nidulans. These results are consistent with ours. It would appear that although minor differences exist between tubulins from different sources, the MW, presence of two polypeptides, net charge characteristics and colchicine binding capability have been conserved over a wide phylogenetic spectrum.

**EXPERIMENTAL**

Phaseolus aureus (mung bean) was used in these studies. Seeds (500 g) were allowed to hydrate for 24 hr and then grown in darkness for 4 days. Due to the low protein:mass ratio as compared to animal tissues, a concentrated homogenization buffer system was devised which produced a final 30000 g supernatant of similar ionic content to that found in previously published studies [5]. Sprouts (101) were homogenized at 4°C in 0.19 M NaPO₄ buffer, containing 9.5 mM MgCl₂, 0.9 M sucrose and 0.4 M GTP, pH 7.4 (100 loosely packed sprouts; 50 ml buffer) until cell breakage was judged to be greater than 90% by phase contrast microscopy. Final pH of the homogenate was 6.5 ± 0.1. Homogenate was centrifuged 2 x at 30000 g for 20 min and pellets discarded. The soluble protein was fractionated by addition of solid (NH₄)₂SO₄. The fraction precipitating between 35–50% saturation was redissolved in 1 ml buffer containing 10 mM Tris-HCl, 0.1 mM GTP, pH 7.5 [12]. Insoluble material was removed by centrifugation and the supernatant sol was brought to 20 mM Ca²⁺ by the gradual addition of solid

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**Table 1. Fractionation of extract of Phaseolus aureus**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>101 of sprouts homogenized in buffer, Supernatant after 30 min 30000 g (2 x)</td>
<td>1770</td>
<td>50 x 10⁻³</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation at 35–50% saturation. Resolubilized in Tris-HCl buffer</td>
<td>1000</td>
<td>5.9 x 10⁻²</td>
</tr>
<tr>
<td>CaCl₂ precipitation, 20 mM resolubilized in EGTA buffer</td>
<td>500</td>
<td>200</td>
</tr>
<tr>
<td>Supernatant after 10 min 30000 g</td>
<td>500</td>
<td>120</td>
</tr>
</tbody>
</table>

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![Fig. 1. Densitometer tracings of electrophoresis patterns [13].](image)
The arrow marks the mobility of calf brain tubulin (MW 55000). (a) Tubulin isolated from *P. aureus* by preparative electrophoresin, (b) calf brain tubulin, (c) sequential selective precipitation with (NH₄)₂SO₄, 20% ethanol and 20 mM CaCl₂, (d) same as (c) except precipitated only with 20 mM CaCl₂, and then fractionated on DEAE cellulose. (e) 20 mM CaCl₂ ppt, (f) Chlorpromazine ppt, (g) (NH₄)₂SO₄ pellet, fractionated on DEAE-cellulose, (H) (NH₄)₂SO₄ pellet, 35–50% saturation.

![Fig. 2. Abscissa: Fraction no.; ordinate (left) (outer) cpm x 10⁻⁷ aliquot: (inner) OD 280 nm: (right) NaCl. Chromatography of soluble 20 mM CaCl₂ ppt on DEAE cellulose. Protein soln was incubated with either unlabelled colchicine (4 x 10⁻⁷ M; (---)), or with colchicine-[¹H] (open circle), or with colchicine-[¹H] only under the same conditions (---), prior to application to the DEAE column (1 x 12 cm). The column was eluted first with PPMg-GTP buffer containing 0.1 M NaCl and finally with 0.26 M NaCl buffer (-----).](image)
Tubulin-like protein from *Phaseolus*

The suspension was allowed to ppt. for 30 min with stirring and centrifuged at 30,000 g for 15 min. The supernatant was discarded and the pellet redissolved in 500 ml of 50 mM NaPi buffer, pH 7, containing 2.5 mM MgCl₂, 0.1 mM GTP (PPMg-GTP) [5] salted out with (NH₄)₂SO₄ and centrifuged.

The supernatant was discarded and the pellet was used for further purification. In other exps the 35-50% (NH₄)₂SO₄ pellet was redissolved in a small vol of PPMgGTP buffer, centrifuged briefly, and either (1) applied directly to a DEAE cellulose column (2 x 40 cm) and fractionated with a stepwise NaCl gradient [5], or (2) precipitated by the gradual addition of Chlorpromazine to a final concn of 2 x 10⁻⁸ M [10] for 30 min and then centrifuged for 15 min at 20,000 g (the solubi-

Colchicine binding. The colchicine binding assay for microtubule protein was adapted from previous studies [17] and involved preincubation with colchicine[^H] (4 Ci/mmol) prior to application to a DEAE-cellulose column. In the assay procedure 0.5 ml of protein soln (Ca⁺⁺ ppt in NaPi-GTP buffer) containing 0.5 mg of protein was added to 4.5 ml warm binding buffer (WB) and incubated with 40 μl colchicine[^H] for 1.25 hr at 37°. The reaction was stopped by the addition of 50 ml WB buffer containing 4 x 10⁻⁴ M unlabeled colchicine at 0° and the sample was applied to a 1 x 12 cm DEAE-cellulose column. Aliquots were diluted with 10 ml Aquafluor (New England Nuclear) and used for liquid scintillation counting.

Preparative polyacrylamide gel electrophoresis. Preparative gels (16 x 14 x 0.22 cm) were made as described above except that polymerization was initiated by the addition of twice the concn of N, N', N' tetramethylethylenediamine (TEMED) and no stacking gel was used. Protein concns were determined by gel quantitation [9].

Cyanogen bromide cleavage and peptide maps. Cyanogen bromide cleavage was carried out in 70% formic acid [13] and the resulting peptides lyophilized and then solubilized in SDS sample buffer. Protein determinations were made by the method of ref [20]. Calf brain microtubule protein was prepared by the method of ref [5].

REFERENCES