Phytochemistry, 1976, Vol. 15, pp. 1837–1839. Pergamon Press. Printed in England.

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.

Abstract—Colchicine binds to a protein fraction isolated from *Phaseolus aureus*. A protein with characteristics similar to calf brain tubulin, in terms of its MW, elution properties from DEAE cellulose, precipitation by Ca^{2+} ions and Chlorpromazine was detected in whole cell supernatants. This protein consisted of two monomeric subunits with MWs of 56000 and 53000. This protein, tentatively identified as tubulin, was compared by cyanogen bromide peptide mapping with calf brain tubulin.

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[^{3}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 $(NH_4)_2SO_4$ precipitation showed a small barely visible component with a MW of 55000 when examined on SDS polyacrylamide gels (Fig. 1h). 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. 1g). 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% $(NH_4)_2SO_4$ 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. 1e) [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 (0.02%) for the percentage of tubulin present in relation to total soluble protein (see Table 1). Qualitatively similar results were achieved utilizing Chlorpromazine 2×10^{-3} M [10] (Fig. 1f) and by adding 20% ethanol followed by the precipitation of the resolubilized pellet with 20 mM Ca^{2+} (Fig. 1c). Both of these procedures precipitated less material than 20 mM $CaCl_2$ and were marred by the tendency of the proteins to form insoluble aggregates. Vinblastine at a concn of 2×10^{-3} was totally non-specific at 37° and 0° (100000 *g* pellet).

On co-electrophoresis with the fraction shown in Fig. 1e, brain tubulin had a mobility which was identical with the peak marked with the arrow, now tentatively identified as plant tubulin.

Colchicine binding. The colchicine binding reaction was initiated prior to fractionation on DEAE-cellulose to minimize denaturation of the colchicine binding site [11]. The majority of the radioactivity was eluted at a salt concn of 0.1 M NaCl, and represents unbound colchicine[^{3}H] (Fig. 2). The second peak of activity was eluted by ionic conditions identical with those required to release colchicine[^{3}H] bound brain tubulin, and contains a major component with a MW of 55000 (Fig. 1d). The sp. act. of the colchicine binding to the protein peak was 14800 cpm/mg protein.

Preparative SDS polyacrylamide gel electrophoresis. 20 mM $CaCl_2$ precipitates were subjected to preparative electrophoresis. The protein isolated by this procedure alternately ran as a single band with a MW of 55000 (Fig. 1a), or occasionally as a closely spaced doublet with apparent MW of 56000 and 53000 on a second thin gel. The α or trailing band stained more heavily as reported for other tubulins [12].

Cyanogen bromide peptide maps. Calf brain and plant tubulin obtained by preparative electrophoresis, were digested with CNBR and analyzed on SDS gels. Preliminary results indicate that the peptide maps of the two tubulins have marked differences, although both samples possess a number of peptides that co-migrate. In all experiments there was a complete disappearance of the brain tubulin band. The tubulin isolated from plants demonstrated incomplete degradation (at 1 mg/ml) with

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.

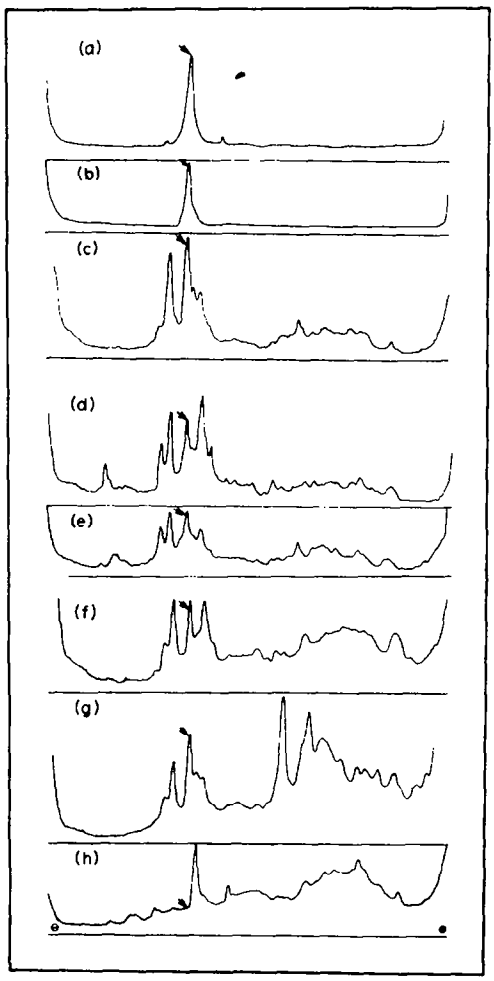

Fig. 1. Densitometer tracings of electrophoresis patterns [13]. The arrow marks the mobility of calf brain tubulin (MW 55000). (a) Tubulin isolated from *P. aureus* by preparative electrophoresis, (b) calf brain tubulin, (c) sequential selective precipitation with $(NH_4)_2SO_4$, 20% ethanol and 20 mM $CaCl_2$, (d) same as (c) except precipitated only with 20 mM $CaCl_2$, and then fractionated on DEAE cellulose, (e) 20 mM $CaCl_2$ ppt, (f) Chlorpromazine ppt, (g) $(NH_4)_2SO_4$ pellet, fractionated on DEAE-cellulose, (H) $(NH_4)_2SO_4$ pellet, 35–50% saturation.

Table 1. Fractionation of extract of *Phaseolus aureus*

Procedure	Volume (ml)	Total protein (mg)
10 l. of sprouts homogenized in buffer. Supernatant after 30 min 30000 *g* (2×)	1770	50×10^3
$(NH4)_2SO_4$ precipitation at 35–50% saturation. Resolubilized in Tris–HCl buffer	1000	5.9×10^3
$CaCl_2$ precipitation, 20 mM resolubilized in EGTA buffer	500	200
Supernatant after 10 min 30000 *g*	500	120

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 (10 l.) were homogenized at 0–4° in 0.19 M NaPPi buffer, containing 9.5 mM $MgCl_2$, 0.9 M sucrose and 0.4 M GTP, pH 7.4 (1 l. loosely packed sprouts: 50 ml buffer) until cell breakage was judged to be greater than 90% by phase contrast microscopy. Eventual pH of the homogenate was 6.5 ± 0.1. Homogenate was centrifuged 2× at 30000 *g* for 20 min and pellets discarded. The soluble protein was fractionated by addition of solid $(NH_4)_2SO_4$. The fraction precipitating between 35–50% saturation was redissolved in 1 l. 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 Ca^{2+} by the gradual addition of solid

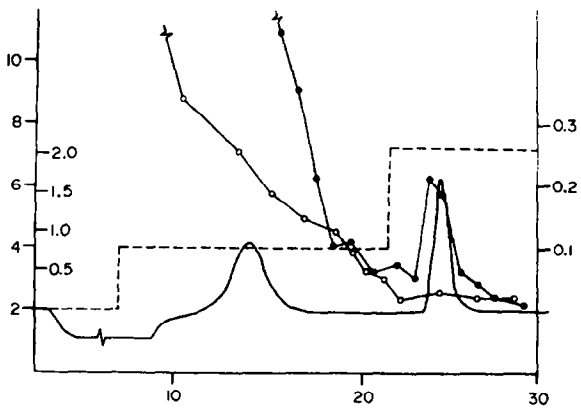

Fig. 2. *Abscissa*: Fraction no.; *ordinate* (*left*) (*outer*) cpm × 10^{-2}/aliquot: (*inner*) OD 280 nm; (*right*) N NaCl. Chromatography of soluble 20 mM $CaCl_2$ ppt on DEAE cellulose. Protein soln was incubated with either unlabelled colchicine (4×10^{-4} M) (—○—○—) followed by the addition of colchicine-[^{3}H] or with colchicine-[^{3}H] only under the same conditions (—●—●—), prior to application to the DEAE column (1 × 12 cm). The column was eluted first with PPMg–GTP buffer containing 0.1 M NaCl and finally with 0.26 M NaCl buffer (----).

$CaCl_2$. The suspension was allowed to ppt. for 30 min with stirring and centrifuged at 30000 *g* for 15 min. The supernatant was discarded and the pellet redissolved in 500 ml of 50 mM NaPPi buffer, pH 7, containing 2.5 mM $MgCl_2$ 0.1 mM GTP (PPMg–GTP) [5] salted out with $(NH_4)_2SO_4$ and centrifuged. The supernatant was discarded and the pellet was used for further purification. In other expts the 35–50% $(NH_4)_2SO_4$ pellet was redissolved in a small vol of PPMg–GTP buffer, centrifuged briefly, and either (1) applied directly to a DEAE cellulose column (2 × 40 cm) and fractionated with a stepwise NaCl gradient [5], or (2) precipitated by the gradual addition of Chlorpromazine to a final concn of 2×10^{-3} M [10] for 30 min and centrifuged for 15 min at 20000 *g* (the solubilized pellet was saved for electrophoresis), or (3) precipitated by the gradual addition of EtOH to a final concn of 20% for 15 min and then centrifuged at 20000 *g* for 15 min. Supernatant was discarded and the pellet redissolved in 10 mM Tris–HCl, pH 7.5. After brief centrifugation to remove insoluble material the protein soln was precipitated by the addition of 20 mM $CaCl_2$. Vinblastine precipitation was according to the procedure of [16] with the following exceptions. Tissue was homogenized in 75 mM M Mg–acetate buffer containing 0.9 M sucrose and 37.5 mM Tris–HCl, pH 7.5.

Colchicine binding. The colchicine binding assay for microtubule protein was adapted from previous studies [17] and involved preincubation with colchicine[^{3}H] (4 Ci/mmol) prior to application to a DEAE-cellulose column. In the assay procedure 0.5 ml of protein soln (Ca^{2+} ppt in NaPPi–GTP buffer) containing 0.5 mg of protein was added to 4.5 ml warm binding buffer (WB) and incubated with 40 μl colchicine[^{3}H] for 1.25 hr at 37°. The reaction was stopped by the addition of 50 ml WB buffer containing 4×10^{-4} M unlabeled colchicine at 0° and the sample was applied to a 1 × 12 cm DEAE-cellulose column. Aliquots were diluted with 10 ml Aquafluor (New England Nuclear) and used for liquid scintillation counting.

Polyacrylamide gel electrophoresis. Quantitation of individual bands was done according to ref. [9]. MWs were determined according to the technique of ref. [18]. The gel system used was that of ref. [19].

Preparative polyacrylamide gel electrophoresis. Preparative gels (16 × 14 × 0.22 cm) were made as described above except that polymerization was initiated by the addition of twice the concn of *N, 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 lyophylized 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

1. Ledbetter, M. and Porter, K. (1964) *Science* **144**, 872.
2. Cohen, C., Harrison, S. C. and Stephens, R. E. (1971) *J. Mol. Biol.* **59**, 375.
3. Hart, J. W. and Sabnis, D. D. (1973) *Planta* **109**, 147.
4. Burns, R. G. (1973) *Exp. Cell Res.* **81**, 285.
5. Eipper, B. A. (1972) *Proc. Natl Acad. Sci. U.S.* **69**, 2283.
6. Weisenberg, R. C. and Timasheff, S. N. (1970) *Biochemistry* **9**, 4110.
7. Bhattacharyya, B. and Wolff, J. (1974) *Biochemistry* **13**, 2364.
8. Wilson, L., Bryan, J., Ruby, A. and Mazia, D. (1970) *Proc. Natl Acad. Sci. U.S.* **66**, 807.
9. Kahn, R. and Rubin, R. W. (1975) *Anal. Biochem.* **67**, 347.
10. McGuire, J., Quinn, P. and Knutton, S. (1974) *J. Cell Biol.* **63**, 433 (Abstr.).
11. Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M. and Creswell, K. M. (1974) *Fed. Proc.* **33**, 158.
12. Bibring, T. and Baxandall, J. (1974) *Exp. Cell Res.* **86**, 120.
13. Ludena, R. F. and Woodward, D. O. (1973) *Proc. Natl Acad. Sci. U.S.* **70**, 3594.
14. Feit, H., Slusarek, L. and Shelanski, M. L. (1971) *Proc. Natl Acad. Sci. U.S.* **68**, 2028.
15. Shein-Neiss, Nardi, R. V., Gealt, M. A. and Morris, N. R. (1976) *Biochem. Biophys. Res. Commun.* **689**, 285.
16. Raff, R. A. and Kaumeyer, J. F. (1973) *J. Develop. Biol.* **32**, 309.
17. Weisenberg, R. C., Borisy, G. G. and Taylor, E. W. (1968) *Biochemistry* **7**, 4466.
18. Weber, K. and Osborn, J. (1969) *J. Biol. Chem.* **233**, 4406.
19. Laemmli, U. K. (1970) *Nature* **227**, 680.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.