ISOLATION OF BRAIN TUBULIN BY AFFINITY CHROMATOGRAPHY

Norman D. Hinman, Janet L. Morgan
Nicholas W. Seeds and John R. Cann

Department of Biophysics and Genetics
University of Colorado Medical Center
4200 East Ninth Avenue
Denver, Colorado 80220

Received April 12,1973

SUMMARY

An affinity chromatographic procedure for the isolation of tubulin from brain is described. The yield is good and the method rapid and gentle. Criteria of purity are colchicine binding, SDS acrylamide gel electrophoresis, and velocity sedimentation.

INTRODUCTION

Microtubules play an essential role in cell structure and several cellular functions (1). The subunit protein of microtubules, tubulin, interacts specifically with certain nucleotides and alkaloid drugs; however, under most conditions tubulin shows a half-life of only several hours for its ability to bind these molecules. Until now tubulin has been isolated from brain by a somewhat lengthy procedure involving several purification steps (2). In this report we describe a new preparation procedure in which affinity chromatography is used to isolate tubulin from brain in a single purification step. This procedure centers around the specific affinity of the protein for colchicine (2); but, because colchicine itself lacks reactive functional groups required for covalent attachment to a matrix, the affinity support is prepared by reacting a mixture of deacetylcolchicine and isodeacetylcolchicine with cyanogen bromide activated Sepharose 4B. When a clarified brain homogenate is passed through a chromatographic column packed with the affinity support, the tubulin is selectively adsorbed to the bed from which it is then eluted in a purified state with a low ionic strength buffer solution.

MATERIALS AND METHODS

Preparation of Affinity Support. A mixture of deacetylcolchicine and isodeacetylcolchicine was prepared from commerical colchicine (Sigma) as described by Wilson et al. (3). The mp and UV spectrum of the crystalline intermediate, trimethylcolchicinic acid, agreed with the literature. The mixture of isomers showed two spots on thin layer chromatography with R_f-values essentially the same as literature values. When the isomers were separated (3) for identification, their R_f-values and UV spectra agreed with the literature.

Approximately 0.86 mmoles of mixed isomers was dissolved in 10 ml of 0.1M sodium phosphate buffer (pH 10.0) and mixed with 10 ml (wet volume) of activated Sepharose (4) and reacted overnight at 4°. The affinity support was then removed by filtration and washed with large volumes of 2.0M NaCl and water. The filtrate was readjusted to pH 10.0 with 0.1M tribasic sodium phosphate and allowed to react with more activated Sepharose. This procedure was repeated a third time. The affinity support contained 6 µmoles of ligand/gm of Sepharose (wet weight). Although no more than 30 ml of affinity support was made from a single ligand preparation, it is likely that the coupling process could be repeated many more times to produce more material.

The first preparation of affinity support often gave tubulin that was less pure than obtained using the second or third batch, suggesting that possible minor contaminants leading to non-specific protein adsorbtion were removed from the ligand solution in the first step. Thus only the second and third preparations of affinity support were used in the experiments described.

Attached ligand was determined as follows: 0.2 gm of support material in 2 ml of 6 N HCl was warmed along with its Sepharose control at about 70° for a few minutes in order to dissolve the ligand-Sepharose beads, and then diluted 10-fold. The ligand-Sepharose solution showed a strong absorption band at 350 mµ, while the control solution showed only a small background. The amount of bound ligand was calculated by comparing the corrected absorbance with that of a standard solution of colchicine in 0.6 N HCl.

Assay for Microtubule Protein. The colchicine binding assay for microtubule protein was carried out essentially as described by Weisenberg (2). The assay was optimized for ^{3}H-colchicine (New England Nuclear) concentration, protein concentration and incubation time. Affinity column eluates of 50 µl containing between 20-200 µg protein and 2.4×10^{-5}M ^{3}H-colchicine (550,000 cpm) were incubated at 37°C for 90 minutes. The binding reaction was terminated with 1 ml of ice cold IM buffer (0.01M imidazole Cl (pH 6.8), 0.005M $MgCl_2$) containing 10^{-5}M colchicine, filtered by

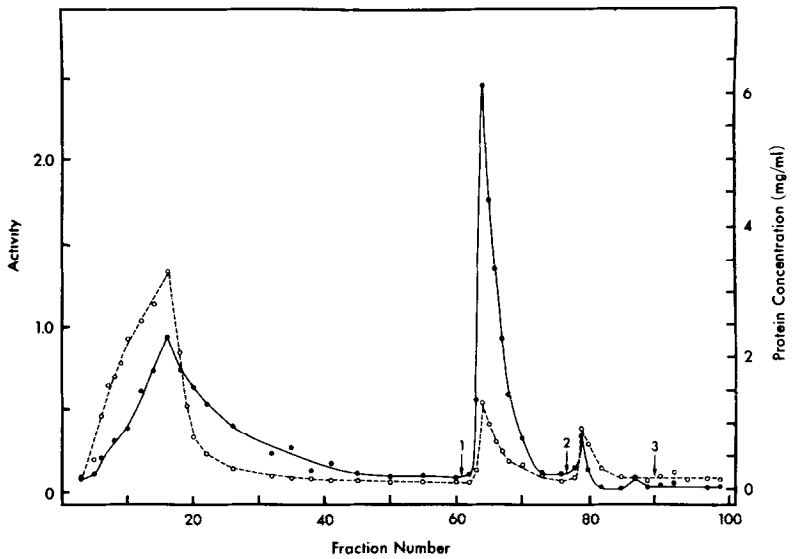

Figure 1. Elution profile obtained by passing 100,000g supernate of mouse brains through a column (3 x 0.5 cm) packed with 0.4 gm of affinity support. The first protein peak represents material not adsorbed to the column; fractions 25-61 contain material removed while the column was being washed; the second protein peak consists of purified tubulin removed with 0.1M NaCl. Buffer solution containing 0.1M NaCl was applied to the column at 1; a 0.5M NaCl solution was applied at 2; and a 1.0M solution was applied at 3. Protein 0-0-0; total colchicine bound (nmoles) per fraction ●-●-●.

gravity through 2 Whatman DE81 (2.5 cm) filter discs and washed under suction with 15 ml of ice cold IM buffer. The filters were homogenized in 3 ml of water and 10 ml of triton-toluene-permafluor (1:2:0.135) and counted at 28% efficiency. The protein concentration of the sample was determined by the method of Lowry et al. (5).

RESULTS AND DISCUSSION

The brains of four adult mice were excised and homogenized in 8 ml of IMGG buffer (0.01 M imidazole Cl, pH 6.8, 0.005 M $MgCl_2$, 0.001 M GTP (Sigma, type II-S), 10% glycerol* (v:v)) and centrifuged at 100,000g for 60 minutes. The 100,000g supernate was applied to a 3 X 0.5 cm column of affinity support (0.4 gm) that had been previously equilibrated with IMGG buffer at 0-4°. This amount of 100,000g supernatant protein saturates the column; saturating conditions are recommended to insure highest purity. After thoroughly washing

*In developing this procedure we found that incorporation of 10% glycerol in the buffer solutions increased the half-life for colchicine binding activity and significantly improved the yield of tubulin.

the column with IMGG buffer, essentially all of the adsorbed tubulin was eluted with the same buffer containing 0.1 M NaCl. Subsequent elution at higher ionic strength removes a small additional amount of protein which is not enriched in tubulin activity.

A typical chromatographic profile is shown in Fig. 1. The first protein peak represents material in the 100,000g supernate which was not adsorbed to the affinity support. Because saturating conditions were used some tubulin is present in this peak. After this has passed through the column, a small amount of protein is continually eluted by the washing solution. The second protein peak with the large coincident activity peak is tubulin removed from the affinity support by 0.1 M NaCl. This material binds 0.55 moles of colchicine per 120,000 gm of protein, which compares very favorably with the values reported by other investigators for highly purified tubulin (2,6). The total colchicine binding activity in this peak represents 34% of the total activity in the original 100,000g supernate. However, by using minimum saturating conditions it is possible to extract all colchicine binding activity from the supernatant. The tubulin peak contained 2 mg of protein, indicating that the affinity support has a maximum tubulin capacity of 5 mg of protein per gm of affinity support. While the foregoing results were obtained with small columns, we have recently used columns holding as much as 2.5 gm of affinity support with comparable results.

SDS acrylamide gel electrophoresis was used to analyze the protein composition of the 100,000g supernate, the material which passed through the column without being adsorbed, and the tubulin preparation. Scans of typical gels are presented in Fig. 2. Scans A and B reveal that a major component in the 100,000g supernate with an apparent molecular weight similar to tubulin subunits (57,000) is specifically adsorbed by the affinity support. Scan C shows that 90-95% of the protein in the tubulin preparation migrates as a single band. The only other significant band accounts for less than 5% of the total protein and has an apparent molecular weight of 30,000. It is interest-

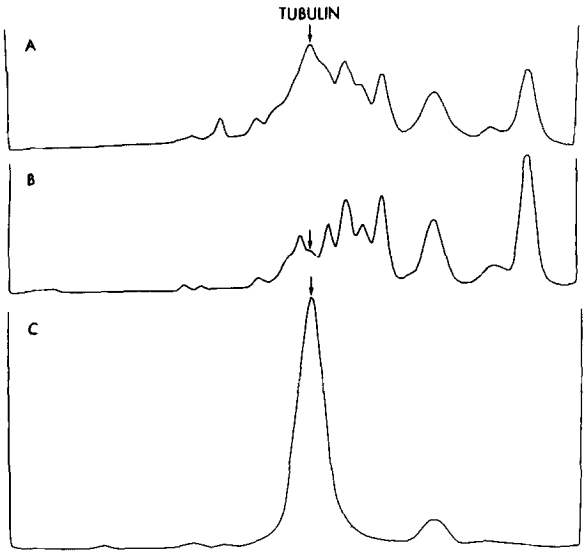

Figure 2. SDS acrylamide gel electrophoretic patterns: A, 100,000g supernate before application to affinity column; B, material in 100,000g supernate which did not adsorb to the column; C, tubulin preparation. Electrophoresis was carried out essentially as described by Maizel (8) in 5% gels using a continuous tris-acetate (pH 9.0) buffer system containing 1% SDS. Samples (40 µg protein) were reduced with 1% β-mercaptoethanol before being applied to the gels. Gels were stained with amido schwartz and scanned at 650 mµ. The anode is to the right.

ing to note that during equilibrium centrifugation of purified tubulin Weisenberg (2) often detected a 30,000 molecular weight component even though the preparation ran as a single band during urea acrylamide gel electrophoresis.

Tubulin prepared by affinity chromatography was also analyzed by velocity sedimentation. The sedimentation patterns for a preparation which had aged for about 6 hrs at 6° and then stored overnight at -10° are displayed in Figs. 3A and 3B. The patterns show two components, and longer times of sedimentation revealed no additional ones. The major component is the slower sedimenting of the two and comprises 85% of the protein. Its $s_{20,w}$ = 5.9S is in good agreement with the published values for tubulin (2,7). The minor component has an $s_{20,w}$ = 20S. In view of the fact that the preparation had aged overnight, it seems likely that this component represents aggregated tubulin. It is well known that upon storage tubulin tends to form aggregates which are usually removed by gel filtration prior to sedimentation analysis (2,7).

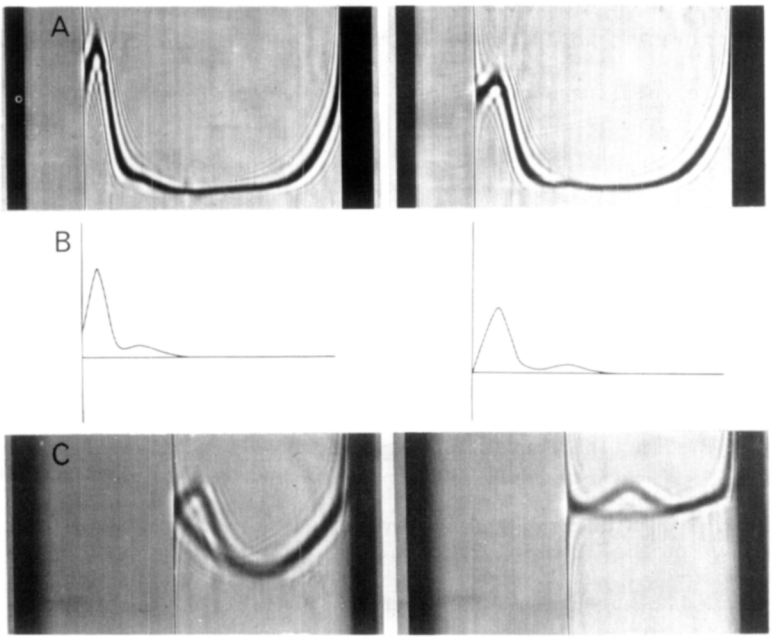

Figure 3. Velocity sedimentation patterns: A, a tubulin preparation aged 6 hrs at 6° and then stored overnight at -10°. Photographs were taken at 28.5 and 49.5 minutes after reaching full speed of 48,000 rpm; bar angle was 50°. Sedimentation was in a 30 mm path length cell at 8°; protein concentration, 1.6 mg per ml; solvent, buffer solution used to elute tubulin from the column. B, patterns shown in A after correction for the curved base line as determined in a separate experiment. C, sedimentation patterns of the same tubulin preparation after concentration and dialysis against tris HCl at pH 8.8, containing 6M guanidine HCl and 1% β-mercaptoethanol; 12 mm path length cell at 21°; patterns obtained 1 hr 46 minutes and 4 hr 37 minutes after reaching full speed of 60,000 rpm; bar angle, 40°; protein concentration 4 mg/ml.

To determine whether or not the 20S component was in fact aggregate, the same preparation was concentrated and dialyzed exhaustively against 0.15M tris HCl (pH 8.8) containing 6M guanidine HCl and 1% β-mercaptoethanol. The resulting patterns (Fig. 3C) show a single component, $s_{20,w}$ = 3.1S, even after prolonged sedimentation; indicating that the faster moving component seen earlier is aggregated tubulin and that our tubulin preparation is homogeneous by this criterium.

Using affinity chromatography a good yield of purified tubulin can be obtained in a rapid and gentle manner. Due to the extreme lability of GTP and colchicine binding activities of the protein, these features are especially advantageous. This method can be used effectively on either a small or large

scale. Furthermore, it would appear suitable for the quantitation of tubulin in cells at different stages of the cell cycle and different growth conditions.

ACKNOWLEDGMENTS

Supported in part by Research Grant 5R01 HL13909-21, USPHS; and Jane Coffin Childs Memorial Fund project #281. N. W. Seeds is a Career Development Awardee of the USPHS (1K04-GM40170), and J. L. Morgan is supported under Training Grant 5T01 GM00781, USPHS. This is publication No. 539 from the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado.

REFERENCES

1. Porter, K. R. In Ciba Foundation Symposium on Principles of Bimolecular Organization, ed. by Wolstenholme, G. E. W. and M. O'Connor, Little Brown, Boston (1966).
2. Weisenberg, R., G. Borisy, and E. Taylor, Biochem., 7, 4466 (1968).
3. Wilson, L., and M. Friedkin, Biochem., 5, 2463 (1966).
4. Cuatrecasas, P., J. Biol. Chem. 245, 3059 (1970).
5. Lowry, O., N. Rosebraugh, A. Farr, and R. Randall, J. Biol. Chem., 193, 265 (1951).
6. Ventilla, M., C. Cantor, and M. Shelanski, Biochem. 11, 1554 (1972).
7. Weisenberg, R., and S. Timasheff, Biochem., 9, 4110 (1970).
8. Maizel, J. V., Jr. In Fundamental Techniques in Virology, ed. Habel, K., and N. P. Salzman, Academic Press, New York (1969).