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PURIFICATION OF TUBULIN FROM RAT PANCREAS

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Rat pancreas tubulin binds to colchicine derivatives coupled to CNBr-Sepharose 4B and can be eluted stepwise with sodium chloride. The subsequent filtration of the colchicine binding peak on DEAE-Sephadex A50 gives tubulin at least 90-95 % pure as checked by SDS polyacrylamide slab gel electrophoresis.

Microtubules play an essential role in cell structure and several cellular functions (1). In the exocrine pancreas, it has been suggested that microtubules were implicated in the intracellular transport of exportable proteins (2). The subunit protein of microtubules, tubulin, interacts specifically with alkaloid drugs like colchicine or vinblastine. The introduction of radioactive colchicine (3) led to the isolation of "colchicine-binding protein", the soluble receptor for the drug (4). The specificity and the kinetic properties of a protein-colchicine complex in the rat pancreas cytosol has been demonstrated in a previous work (5). In this report, we describe for the first time the purification of rat pancreas tubulin by affinity chromatography on "colchicine" agarose column and subsequent ion exchange chromatography. The two-steps filtration of crude pancreas supernatant solution yield tubulin that is 90-95 % pure by SDS polyacrylamide slab gel electrophoresis.

MATERIAL AND METHODS

Preparation of affinity support

A mixture of deacetylcolchicine (DAC) and isodeacetylcolchicine (IDAC) was prepared from commercial trimethyl colchicinic acid (Sigma) as described by Wilson et al (6). The mixture of isomers showed two spots on thin layer chromatography with Rf-values essentially the same as literature values (TMCA, Rf 0 ; DAC, Rf 0.4 ; IDAC, Rf 0.3).

Abbreviations used : GTP, guanosine-5''-triphosphate, PMSF, phenylmethylsulfonyl fluoride, K = kilodaltons.

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Approximately 0.5 mmole of mixed isomers was dissolved in 20 ml of 0.1 M sodium bicarbonate buffer (pH 8.0) containing 0.5 M sodium chloride and mixed with 20 ml (wet volume) of CNBr-Sepharose 4B (Pharmacia). The mixture reacted during two hours at 20°C, the affinity support was then removed by filtration, excess ligand was washed with coupling buffer, remaining active groups were blocked during one hour with 1.0 M ethanolamine, pH 8.0. Then, the product was washed with three cycles of alternating pH. Each cycle consisted of a wash at pH 4 (0.1 M acetate buffer containing 1 M NaCl) followed by a wash at pH 8.0 (0.1 M Tris buffer containing 1 M NaCl).

Attached ligand was determined as follows : 0.1 gm of support material in 1 ml of 6 N HCl was warmed along with its Sepharose 4B control at about 70°C for a few minutes in order to dissolve the ligand-Sepharose beads, and diluted ten-fold. The ligand-Sepharose solution showed a strong absorption band at 350 nm, 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. The affinity support containing 6 μmole of ligand/gm of Sepharose 4B.

Colchicine binding assay

The binding of [^{3}H] colchicine to tubulin was determined by the adsorption of the tubulin-colchicine complex to DEAE-impregnated filters (7). Reaction mixtures containing 10 mM imidazole (pH 6.8), 5 mM $MgCl_2$ (IM buffer), 0.1 mM GTP, 10-100 μg protein per ml, 0.1 μM [^{3}H] colchicine (Amersham, 7.7 Ci/mmol ; 1 Ci = 3.70 x 10^{10} B_q) were incubated at 37°C during 30 minutes. The filter stack was placed in a counting vial containing 10 ml of Biofluor (NEN) and counted after digestion for 18 h at 20°C in a Intertechnique (Model SL-3000) scintillation spectrophotometer.

Assembly purification

Microtubule protein was also purified from rat brain by polymerization-depolymerization in 100 mM Mes, 0.5 mM $MgCl_2$, EGTA 1 mM, pH 6.6 (Buffer A) according to the method of Shelanski et al (8). The polymerization steps were carried out in Buffer A supplemented with 4 M glycerol and 1 mM GTP (Buffer B). Microtubule pellet was resuspended in 25 mM Mes, 0.125 mM $MgCl_2$, 0.35 M NaCl (Buffer C), cooled at 0°C, and centrifuged at 60,000 xg at 4°C. The supernatant was applied on DEAE-Sephadex A50 column equilibrated with buffer C, microtubule-associated proteins (MAP's) eluted in the same buffer, while tubulin eluted with 0.7 M NaCl. Tubulin was tested for its colchicine-binding activity, desalted by dialysis and lyophilized. Purity of tubulin was checked by SDS polyacrylamide slab gel electrophoresis, rat brain tubulin was utilized as a standard to compare with rat pancreas tubulin.

Crude rat pancreas supernatant

Rat pancreas was minced and homogenized in cold IM buffer supplemented with 1 mM GTP and 0.1 mM PMSF; the homogenate was kept for 15 min on ice, then centrifuged at 100,000 xg for one hour at 4°C in a MSE superspeed 75 centrifuge to obtain the high-speed supernatant (Cytosol). The supernatant solution was used as the source for affinity chromatography.

Electrophoresis

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was performed on 7.5 % slab gel with a discontinuous buffer according to Davis (9) with 0.05 M Tris-glycine (pH 9.3), SDS 0.1 %, in the upper chamber, and 10 mM Tris-HCl (pH 8.3) in the lower and 40 mA/slab at 20°C. Slab gels were stained with 0.25 % Coomassie Brilliant Blue in 50 % methanol and 10 % acetic acid and destained in 5 % methanol and 7/5 % acetic acid. Protein samples were denatured by boiling for 5 min. in 2 mM Tris-HCl buffer (pH 8.9),

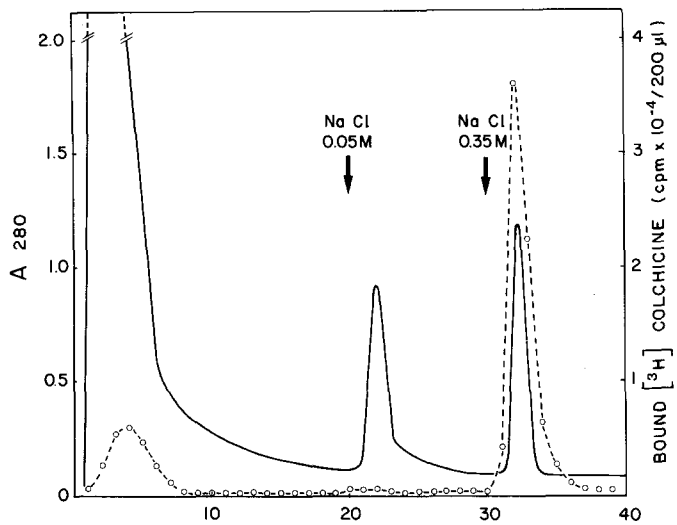

Fig. 1 : Elution profile obtained by passing a 100,00 xg supernate of rat pancreas through a column (4 x 1 cm) packed with 1 gm of affinity support. After sample application, the column was washed with IMG buffer and eluted in this buffer first containing 0.05 M then 0.35 M NaCl. Fractions of 4.0 ml were collected and assayed for colchicine-binding (o---o) and A_{280} (——).

0.45 % SDS, 10 mM dithioerythritol (DTE). Protein determination of samples was determined by the method of Lowry et al. (10) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Colchicine-binding activity was used to identify tubulin binding to the DAC-IDAC affinity and DEAE-Sephadex columns. Rat pancreas supernatant was directly applied to the affinity column and washed with IM buffer supplemented with 0.1 mM GTP (IMG buffer) until no further protein passed through. Proteins which are bound to the matrix by non-specific interactions were eluted with 0.05 M NaCl in IMG buffer, then tubulin was eluted with 0.35 M NaCl in the same buffer. A typical profile is shown in Fig. 1, the

Table 1 : Purification steps of colchicine-binding protein from rat pancreas.

STEP	Vol (ml)	Total protein (mg)	Sp. Act. (cpm/µg)	Yield (%)
1. Soluble supernatant	8	200	8.8	100
2. DAC-Sepharose 4B column (NaCl 0.35 M)	12	5	260	74
3. DEAE-Sephadex A50 column (NaCl 0.70 M)	8	0.5	880	25

first protein peak represents material which was not absorbed to the affinity support. Because saturating conditions were used, some colchicine-binding activity in the peak eluted with 0.35 M NaCl represents 60-80 % of the total activity in the original 100,000 x g supernatant (table 1). In rat pancreas tubulin content is approximately ten-fold lower than in rat brain (data not shown). This affinity chromatography column removes 95 % of the proteins present in the supernatant. However, there are some contaminants that we have tried to remove with a second step of chromatography on DEAE-Sephadex A50. Fractions of the affinity column which have a colchicine-binding activity (0.35 M NaCl peak) were pooled and passed through a DEAE-Sephadex column equilibrated with IMG buffer and 0.35 M NaCl. Subsequent washing with the same buffer eluted the proteins which contamined the tubulin in the first purification step. Then the column was developed by elution with 0.7 M NaCl. The elution profile shown in Fig. 2 indicates that the totality of the [3H] colchicine binding activity eluted with 0.7 M NaCl. The active fractions were pooled, dialyzed against distilled water for 24 hours and lyophilized. A summary of yields and purification of rat pancreas tubulin for the various steps is given in Table 1. We can observe that the tubulin content of rat pancreas was approximately 1 % of the total soluble proteins. The protein eluted from the DEAE-Sephadex column (NaCl 0.7 M peak) has a [^{3}H] colchicine binding specificity activity of 880 cpm/µg which is 100 times greater than that of the supernatant proteins.

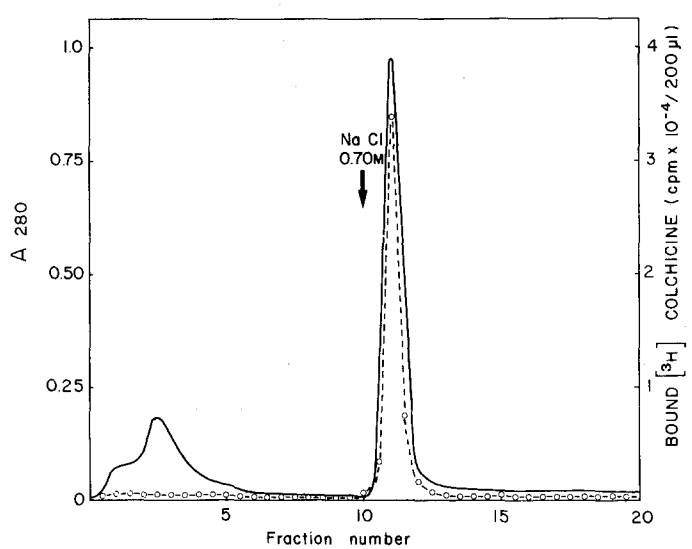

Fig. 2 : Chromatography of the 0.35 M NaCl peak from the affinity support through a column (5 x 1 cm) packed with DEAE-Sephadex A50. The column was washed with IMG buffer containing 0.35 M NaCl and eluted in this buffer with 0.7 M NaCl (final concentration). Fractions of 4.0 ml were collected and assayed for colchicine-binding (o---o) and A_{280} (———).

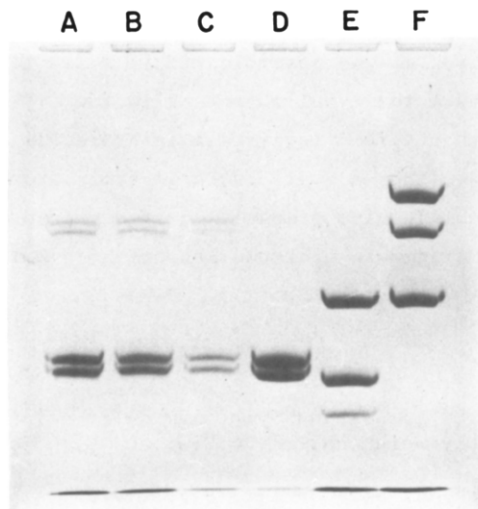

Fig. 3 : Electrophoresis of different tubulin preparations in SDS polyacrylamide slab gel. A,B,C : rat pancreas tubulin obtained after the DEAE-Sephadex A50 column (NaCl 0.7 M peak) ; A and B ; 25 µg ; C : 13 µg ; D : 25 µg of rat brain tubulin obtained with a single cycle of polymerization and subsequent chromatography on DEAE-Sephadex A50 (NaCl 0.7 M peak) ; E and F : molecular weight markers (5 µg of each protein) ; from top to bottom ; E : bovine serum albumin (68 K) ; α-amylase (50 K) ; actin (43 K) ; carbonic anhydrase (29 K) ; F : the same as in E except ; β-galactosidase (116 K) ; phosphorylase b (97.4 K).

SDS polyacrylamide slab gel electrophoresis was used to analyze the protein composition of the material which is eluted from ion-exchange chromatography. The colchicine binding peak obtained with ion exchange chromatography shows a high degree of purification. As shown in Fig. 3, (A, B, C) 90-95 % of the proteins in the tubulin preparation migrates as α and β-tubulin, the two subunits of tubulin. Molecular weight determination with protein markers indicates a value of 55000 and 52000 daltons for α and β-tubulin, these values agree well with those of α and β-tubulin from different sources (this study, 12, 13). The two only significant bands account for less than 5-10 % of the total protein, the minor bands have molecular weights of 100,000 and 30,000 daltons. The latter contaminant is also present in rat brain tubulin (this study, fig. 3D and 14, 15, 16); because pancreas is potentially rich in proteolytic enzymes, we had suggested that this low molecular weight might be the result of some proteolysis along the time of experiment. This hypothesis is ruled out because inclusion of PMSF in the different steps of tubulin purification (Fig. 3C) or ultrafiltration through a XM-50 membrane (Fig. 3B) did not remove this low molecular weight contaminant. The presence of a high molecular weight (MW 100,000) has also been described elsewhere (15), this high molecular weight was still present in SDS-urea polyacrylamide gels (unpublished results), this observation suggested that this protein was not aggregated tubulin.

With the addition of several purification steps (affinity and ion exchange chromatography), it was possible within a day to obtain tubulin from a tissue (pancreas) where the tubulin content is low (1 % of the total soluble protein), ten to twenty-fold lower than in brain (11). This method can be used effectively on either a small or large scale. Furthermore, it would appear suitable for quantitative preparation of rat pancreas tubulin, this protein might be polymerized in microtubules on which different parameters might be studied.

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