Journal of Chromatography, 525 (1990) 71–84
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5048

ISOLATION OF MAMMALIAN BRAIN TUBULIN BY AMINO-ACTIVATED GEL CHROMATOGRAPHY

ERNEST LACEY* and KARON L. SNOWDON

McMaster Laboratory, CSIRO Division of Animal Health, Private Bag No 1, PO Glebe, N.S W. 2037 (Australia)

(First received April 5th, 1989; revised manuscript received September 25th, 1989)

SUMMARY

In the present study, we report the isolation of the acidic structural protein tubulin using a number of amino-activated gels. Crude 100 000 *g* supernatant derived from sheep brain was applied to gels activated with either aminohexyl, aminoethyl, argininyl, diethylaminoethyl, lysinyl and polylysinyl residues and eluted with three distinct sequential buffer changes (pH 6.5): (i) 0.025–0.4 *M* morpholinoethanesulphonic acid; (ii) 0.076 and 0 379 *M* ammonium sulphate in 0.025 *M* morpholinoethanesulphonic acid; and (iii) 0.8 *M* sodium chloride in 0.025 *M* morpholinoethanesulphonic acid Tubulin was recovered from all columns in an enriched form. However, the elution profile and purity, as judged by [^{3}H]colchicine binding and electrophoresis, varied with the ligand. Hydrophobic gels, such as diethylaminoethyl and aminohexyl, required elution with high-ionic-strength buffers (0.8 *M* sodium chloride) and significant inhibition of [^{3}H]colchicine activity resulted. This problem was avoided with the hydrophilic ligands such as arginine, polylysine and aminoethyl. Manipulation of elution conditions enabled complete elution of tubulin from arginine-activated gels in 2.5% ammonium sulphate without detectable losses of [^{3}H]colchicine binding activity and with purity comparable to that achieved using diethylamino-ethyl Sephacel.

INTRODUCTION

The isolation of tubulin from mammalian brain is currently based on three properties of this protein: (i) temperature-dependent polymerisation–depolymerisation of the tubulin–microtubule equilibrium; (ii) an affinity for ligands, such as colchicine (CLC), vinblastine, tubulin antibodies or lactoperoxidase; and (iii) an ionic interaction with basic (or cationic) residues [1–6].

0378-4347/90/$03.50

Of these approaches, only the latter has been shown to be capable of isolating tubulin from both neural and non-neural sources. Despite this versatility, the use of ionic interactions in isolation is almost exclusively limited to one amino-activated moiety, diethylaminoethyl (DEAE), with elution of tubulin achieved by high salt concentrations.

In the present study, we have examined the utility of ionic interaction for purifying tubulin from sheep brain by varying the ligand and elution conditions. The selection of amino ligands for isolation of tubulin was based on the hypothesis that by using more polar amino residues it may be feasible to achieve a higher degree of selectivity in the ionic interaction and allow dissociation by washing with ammonium ions as a selective counter-ion. Seven commercially available activated gels were selected: aminohexyl, aminoethyl, arginyl, DEAE, lysyl and polylysyl moieties. Two lysyl moieties with either a free ω- or α-amino group were examined.

EXPERIMENTAL

Materials

Sepharose 4B coupled with aminohexyl, arginine and lysine residues and DEAE-Sephacel containing 6–10, 14–20, 4–5 and 95–135 μmol ligand per ml of packed gel, respectively, were obtained from Pharmacia (Sydney, Australia). Guanosine triphosphate (GTP), morpholinoethanesulphonic acid (MES), ethylglycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), Norit A animal charcoal, bovine serum albumin (BSA) and aminoethyl- and poly-L-lysine-linked agaroses (24.9 μmol/ml and 0.5–1.0 mg/ml, respectively) were obtained from Sigma (St. Louis, MO, U.S.A.). Magnesium sulphate ($MgSO_4$), sodium hydroxide (NaOH), sodium chloride (NaCl) and ammonium sulphate ($(NH_4)_2SO_4$) were obtained from H.L.S. Scientific (Sydney, Australia) and were of analytical-reagent grade. All buffers were prepared from glass-distilled water and the pH was adjusted to 6.5 with 10 *M* NaOH.

[^{3}H]CLC, labelled in the A ring (Radiochemical Centre, Amersham, U.K.), was evaporated to dryness under nitrogen, diluted with unlabelled CLC and reconstituted in 0.025 *M* MES buffer to 2000 μM (specific activity = 0.188 GBq/mol). The solution was stored at 4 °C in the dark and diluted to 10 μM prior to use with 0.025 *M* MES buffer (pH 6.5).

Isolation procedure

Crude sheep brain supernatant was prepared by centrifugation (100 000 *g* for 1 h at 4 °C) of 1 g/ml homogenates in 0.025 *M* MES buffer (pH 6.5) containing 1 m*M* GTP, 0.5 m*M* $MgSO_4$ and 1 m*M* EGTA. The supernatant (10 mg crude protein) was applied to a 2-ml gel packed in a 5-ml plastic disposable syringe (plugged with glass wool and extensively pre-washed with buffer). After

adsorption, the column was eluted sequentially with three 4-ml fractions of 0.025 *M* (fractions 1–3), 0.1 *M* (fractions 4–6), 0.2 *M* (fractions 7–9) and 0.5 *M* (fractions 10–12) MES buffers, then 0.076 *M* (fractions 13–15) and 0.379 *M* (fractions 16–18) $(NH_4)_2SO_4$ in 0.025 *M* MES buffer (corresponding to 1 and 5% $(NH_4)_2SO_4$ solutions, respectively) and finally with 0.8 *M* NaCl in 0.025 *M* MES buffer (fractions 19–21). The pH of all buffers was 6.5. Modifications of these sequences to optimise elution of tubulin from arginine-activated columns are described in the text. Protein concentration and [^{3}H]CLC binding were determined on all crude supernatant and column fractions.

All isolations were repeated at least twice. Except for the arginine column, elution behaviour was highly reproducible and data from only one elution are presented.

[^{3}H]Colchicine binding assay

Column fractions, crude supernatant and 0.025 *M* MES buffer (blank) (90 μl) were incubated with 10 μM [^{3}H]CLC solution (10 μl) for 1 h at 37 °C in a shaking water-bath. Binding was terminated by 5 min incubation with 0.5 ml of a 2 mg/ml suspension of charcoal in 1% BSA. After precipitation of charcoal by centrifugation at 10 000 *g* for 10 min, bound radioactivity in the supernatant was determined by liquid scintillation spectroscopy as previously described [7]. [^{3}H]CLC binding (pmol per assay) was calculated by subtracting background activity in blank samples, adjusting for sample volume counted and converting to picomoles [7].

The percentage of [^{3}H]CLC bound in each fraction was calculated as the ratio:

$$\frac{[^3\text{H}]\text{CLC bound (pmol per assay) in each fraction}}{\text{total } [^3\text{H}]\text{CLC bound (pmol per assay) for all fractions}}$$

Purification factors were derived from the quotient of the [^{3}H]CLC binding (pmol/mg of protein) for each fraction and the [^{3}H]CLC bound (pmol/mg) in the crude supernatant. Purification factors were not derived for fractions containing less than 0.05 mg/ml protein.

Protein determination

Protein concentrations were determined using commercially available Coomassie blue dye binding reagent (Bio-Rad, Sydney, Australia) according to the method of Bradford [8]. BSA was used to derive the standard curve.

Protein concentrations of column eluates were determined over a range of absorbance values of 0.06–0.6 absorbance units (A.U.) corresponding to BSA concentrations of 5–55 μg per assay. The standard curve was linear within this range. Where absorbances obtained were above 0.6 A.U., the solution was reassayed using an appropriately diluted aliquot. The minimum detection limit for the Coomassie blue assay used in this study was 0.025 mg/ml, derived from

an absorbance reading of 0.060 A.U. from a 200-μl aliquot of crude supernatant mixed with 5 ml of a 1:5 dilution of the dye concentrate.

The proportion of protein eluted was calculated from the following equation:

$$\text{Percentage protein} = \frac{\text{quantity of protein in each fraction} \times 100}{\text{quantity of total eluted protein}}$$

where quantity of total eluted protein is the sum of the protein content of all fractions. Protein recovery is expressed as a percentage of the protein applied to the column.

Note: It has been recently reported that the tubulin estimated using the Coomassie blue assay according to Bradford [8] is 2.05 times lower than that obtained using the Lowry method [9], a result confirmed in our laboratory [10]. This difference has not been taken into consideration in this study; therefore the quoted percentages of tubulin in fractions will be approximately two times lower than that quoted by researchers using the Lowry assay.

Electrophoresis

Molecular mass standards (phosphorylase B, M_r 94 000; BSA, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 000; and α-lactalbumin, M_r 14 100) obtained as a lyophilised mixture (Pharmacia) and dissolved in water (1 ml), pure tubulin (0.2 mg/ml) and column fractions were diluted 1:4 with 0.125 *M* Tris (pH 6.8) containing 10% glycerol, 5% mercaptoethanol, 2.5% sodium dodecyl sulphate (SDS) and 0.1% bromophenol blue and heated at 90°C for 2 min. The samples (25–150 μl) were applied to 16×18×0.15 cm gels, containing a 3% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide resolving gel, and electrophoresed for 5 h at 22–25 A constant current. The gels were stained for 2 h with Coomassie blue R250 in water containing 25% isopropanol and 10% glacial acetic acid and destained by repeated changes of water containing 7% glacial acetic acid and 5% methanol.

RESULTS

[^{3}H]Colchicine binding assay

[^{3}H]CLC binding was linear up to 0.3 mg/ml crude protein corresponding to the binding of 55 pmol per assay. Above this concentration, pronounced negative deviation was observed. Samples binding more than 45 pmol per assay were diluted and re-assayed.

Tubulin interacts with [^{3}H]CLC to form an essentially irreversible complex with molar stoichiometry. Under the assay conditions used, a stoichiometry of 0.7 mol [^{3}H]CLC per mol of tubulin was established based on Coomassie blue estimation of tubulin [8]. This is within the range of reported values (0.2–0.9 mol [^{3}H]CLC per mol of tubulin) [11].

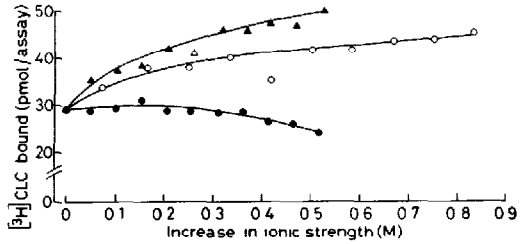


Fig. 1. Effect of increasing ionic strength of MES (●), NaCl in 0.025 *M* MES (○) and $(NH_4)_2SO_4$ in 0.025 *M* MES (▲) buffers on [^{3}H]CLC binding to crude sheep brain tubulin. A 30-μl volume of 100 000 *g* sheep brain supernatant (10 mg/ml) was incubated with 10 μl of [^{3}H]CLC (10 μM) with increasing proportions of either 0.75 *M* MES, 1.2 *M* NaCl in 0.025 *M* MES or 10% $(NH_4)_2SO_4$ in 0.025 *M* MES at pH 6.5 to a final volume of 140 μl. After incubation at 37°C for 1 h in a shaking water-bath, the binding was terminated by the addition of 0.5 ml of a 2 mg/ml charcoal suspension in 1% BSA followed by a 10-min incubation. The sample was centrifuged at 10 000 *g* (10 min) and the supernatant sampled (0.4 ml), mixed with Biofluor (5 ml) and counted. All data represent the mean duplicate determinations.

As assays were performed directly on column eluates, the effect of buffer variation on [^{3}H]CLC binding was examined (Fig. 1). Incubation of crude supernatant with either 0.38 *M* $(NH_4)_2SO_4$ or 0.8 *M* NaCl solutions increased [^{3}H]CLC binding by 59 and 56%, respectively. MES buffer inhibited binding in a concentration-dependent manner at concentrations higher than 0.3 *M*, with 14.5% inhibition occurring at 0.5 *M* MES.

The reversibility of these effects was examined by storing crude supernatant for 1 h in 0.025 *M* MES, 0.5 *M* MES, 0.8 *M* NaCl and 0.38 *M* $(NH_4)_2SO_4$ followed by ten-fold dilution with 0.025 *M* MES prior to [^{3}H]CLC binding. The observed binding results were 118 (100%), 114, 88 and 140 pmol/mg for 0.025 *M* MES, 0.5 *M* MES, 0.8 *M* NaCl and 0.38 *M* $(NH_4)_2SO_4$, respectively. The inhibition of binding apparent in 0.5 *M* MES (Fig. 1) appears to be reversible with no significant difference between 0.5 and 0.025 *M* MES solutions after storage and subsequent dilution. However, [^{3}H]CLC binding to tubulin stored in 0.8 *M* NaCl then diluted has been reduced by 25%. The increased [^{3}H]CLC binding observed after storage and dilution of 0.38 *M* $(NH_4)_2SO_4$ crude supernatant (19%) is directly attributable to the final $(NH_4)_2SO_4$ molarity in the assay (0.038 *M*). By interpolation in Fig. 1, this is estimated at 20%.

Purification of tubulin

Of the applied crude supernatant, 62% was eluted from aminohexyl-activated gel with MES buffers with no [^{3}H]CLC binding observed in any fraction (Fig. 2a). Elution with $(NH_4)_2SO_4$ removed a further 27% of the non-[^{3}H]CLC binding proteins. On elution with 0.8 *M* NaCl, 0.82 mg of protein was recovered distributed over fractions 19–21. These fractions bound [^{3}H]CLC, with a mean

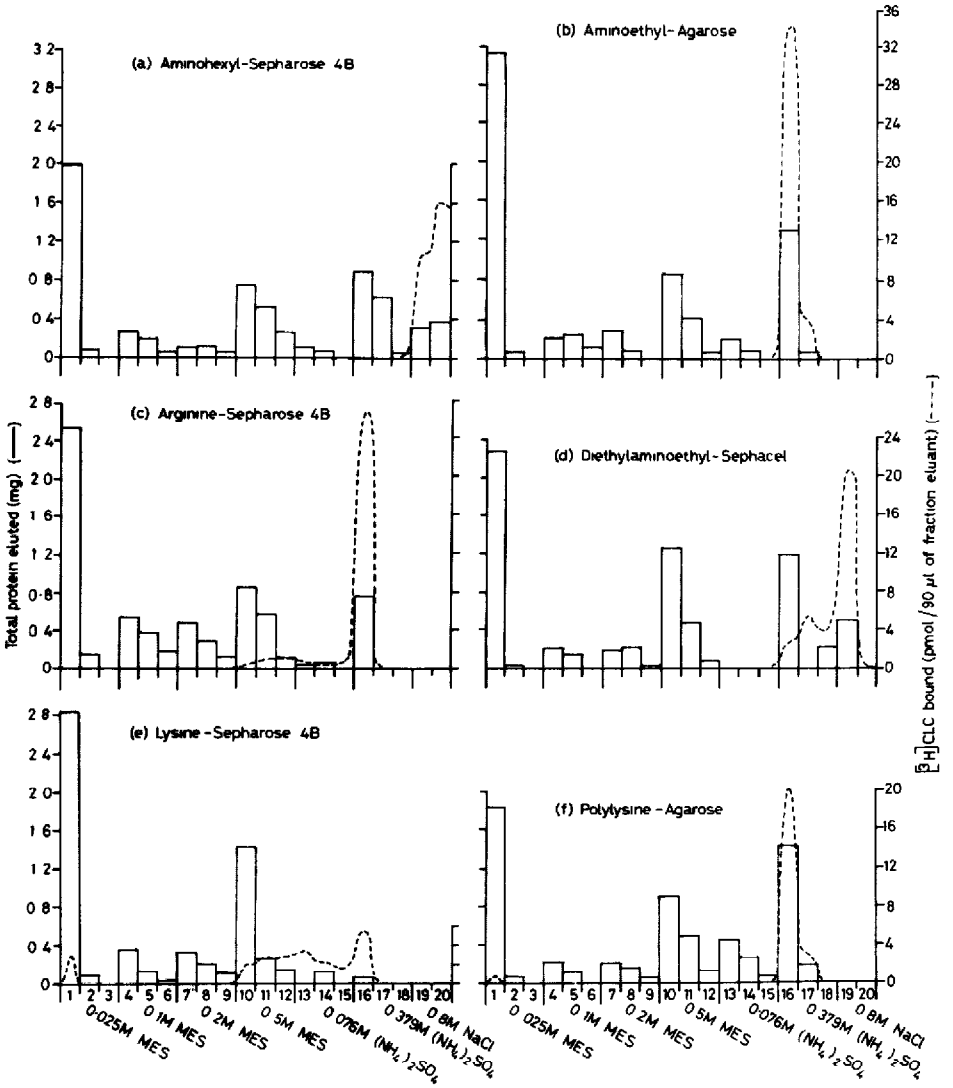


Fig. 2. Purification of sheep brain tubulin using selected amino-activated Sepharose or Agarose gels. Methods for the estimation of protein (—) and [^{3}H]CLC binding (---) are as described in the text.

purification factor of 8.9. By electrophoresis, fractions 20 (Fig. 3c) and 21 (not shown) consisted of tubulin with minor contaminants. Of the applied protein, 83–90% was recovered, with the tubulin fractions constituting 11.6% of the eluted protein and containing 100% of [^{3}H]CLC binding (Table I).

The MES gradient eluted 73% of the applied protein from the DEAE column, with no [^{3}H]CLC binding detected (Fig. 2d). Elution with 5% $(NH_4)_2SO_4$

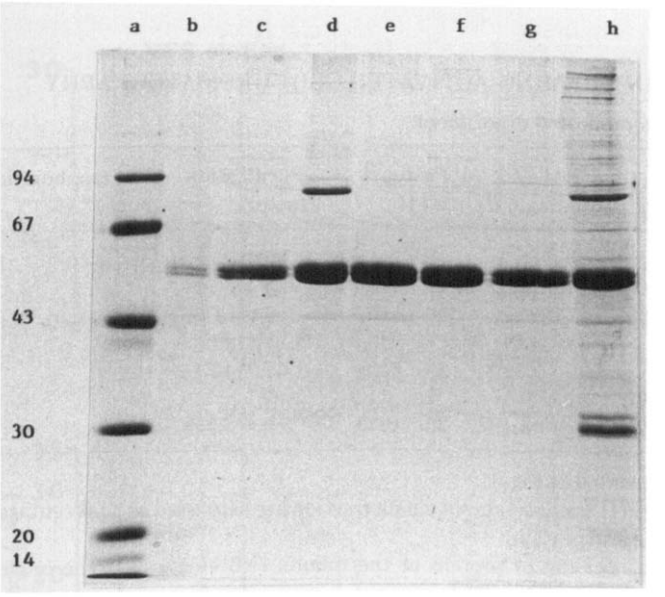


Fig. 3. Electrophoresis of tubulin-containing fractions obtained from eluates of the amino-activated gels described in Fig. 2. (a) Molecular weight standards; (b) sheep brain tubulin obtained by the method of Shelanski et al. [2]; (c) aminohexyl gel, fraction 19 (2 μg); (d) aminoethyl gel, fraction 16 (5 μg); (e) arginine gel, fraction 16 (4 μg); (f) DEAE gel, fraction 19 (2.4 μg); (g) lysine gel, fraction 16 (1 μg); (h) polylysine gel, fraction 16 (5 μg).

removed a further 20% protein, constituting 40% of the total [^{3}H]CLC binding. The residual binding was recovered in 0.8 *M* NaCl fractions in 0.49 mg of protein. Electrophoretic examination indicated tubulin in fractions 17–19 (see Fig. 3f) with contaminating proteins at M_r 30 000, 32 000 and 92 000 in fraction 16. The mean purification factor for fractions 17–19 was 10.3 with protein recoveries of 80–90% (based on two experiments).

The elution profile of crude supernatant from the aminoethyl column was qualitatively similar to that observed with both the aminohexyl and DEAE ligands on elution with MES buffer (Fig. 2b). No [^{3}H]CLC binding was detected in these fractions. Elution with low-ionic-strength NH_4^+ ions removed a further 2% protein. At 0.38 *M* $(NH_4)_2SO_4$ the residual protein (18%) was rapidly eluted, with the majority (>90%) of the [^{3}H]CLC binding being detected in the first fraction (fraction 16). For this ligand, a lower purification factor of 6.6 was observed, consistent with the presence of a single major electrophoretic contaminant at approximately M_r 87 000 (Fig. 3d). Protein recovery from the column was between 86 and 97% of the applied crude supernatant.

Similarly to the aminoethyl ligand with polylysine-linked agarose, 97% of the observed [^{3}H]CLC binding was eluted in 5% $(NH_4)_2SO_4$ fractions (Fig. 2f). These fractions constituted an average of 26% of the total protein and

TABLE I

PURIFICATION OF TUBULIN BY AMINO-ACTIVATED GEL CHROMATOGRAPHY

Results in parentheses refer to a replicated experiment.

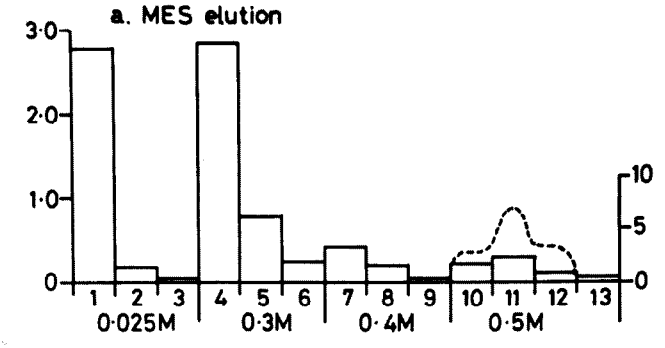
Column[a]	Eluate fraction	[^{3}H]CLC binding (%)	Protein[b] (%)	Purification factor[c]	Electrophoretic purity[d] (%)
Aminohexyl	0.8 *M* NaCl	100 (100)	8.9 (11.6)	8.9 (9.1)	95
Aminoethyl	0.38 *M* $(NH_4)_2SO_4$	100 (100)	19.1 (18.2)	6.5 (6.6)	70
Arginine	0.38 *M* $(NH_4)_2SO_4$	87 (15)	10.9 (5.3)	8.6 (N.D.)[e]	95
DEAE	0.8 *M* NaCl	64 (61)	7.3 (6.3)	10 (14)[f]	98
Lysine	0.5 *M* MES	32 (61)	30 (30)	3.7 (4.7)	–
	0.38 *M* $(NH_4)_2SO_4$	24 (27)	1.2 (1.3)	N.D.[e]	93
Polylysine	0.38 *M* $(NH_4)_2SO_4$	97 (100)	25 (27)	3.8 (5.2)	34

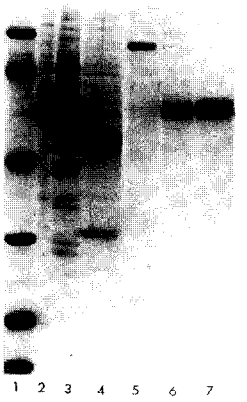
[a]Data derived from columns presented in Fig. 1.
[b][^{3}H]CLC binding and protein in the major tubulin eluate fraction are expressed as a percentage of total eluted [^{3}H]CLC binding and protein.
[c]Ratio of the [^{3}H]CLC binding per mg of protein of the tubulin-rich eluate over the crude supernatant.
[d]Electrophoretic purity as accessed by gel scanning.
[e]Protein concentration was not sufficiently high for accurate estimation of specific purity (i.e. <5 μg).
[f]Purification factor of this eluate is atypically high; in subsequent replication values were always approximately 10. The reason for this discrepancy is unknown.

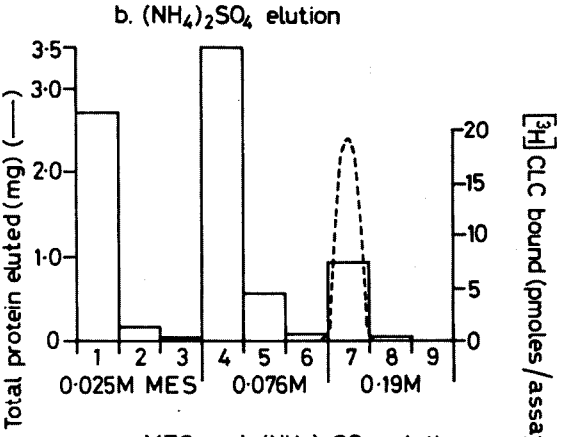
thus a lower purification factor of 5.2. Electrophoresis of fractions 16 (Fig. 3h) and 17 (not shown) showed the presence of tubulin together with substantial quantities of contaminating proteins at M_r 30 000, 32 000, 42 000 and 85 000 and a large number of minor protein bands observed at approximately M_r 38 000, 58 000, 63 000, 95 000 and >96 000 on heavily loaded gels.

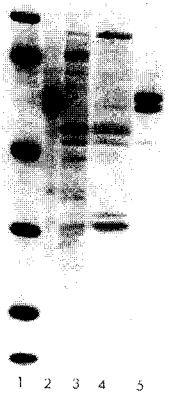
Unlike the polylysine residues, chromatographic separation of crude supernatant using ω-amino-coupled lysine eluted 97% protein in the MES gradient (Fig. 2e) with 61% of the total [^{3}H]CLC binding observed principally in 0.5

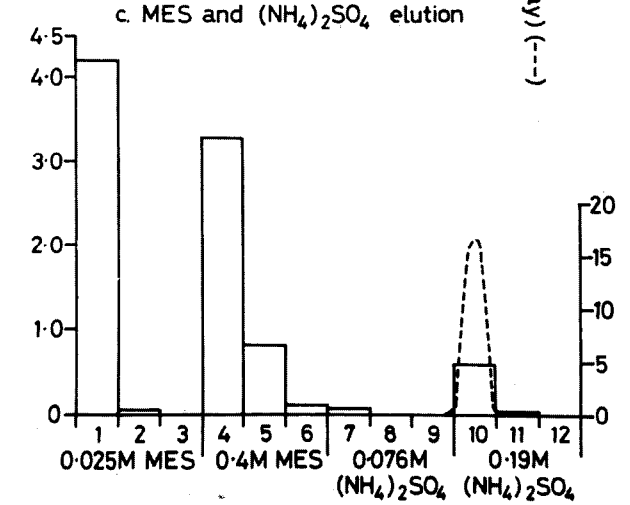
Fig. 4. Effect of variation of MES and $(NH_4)_2SO_4$ buffer concentrations on the elution of tubulin from arginine-activated sepharose. The columns were washed with 0.025 *M* MES (3×4 ml) and then eluted with (a) stepwise MES gradient, (b) stepwise $(NH_4)_2SO_4$ gradient or (c) 0.4 *M* MES followed by a stepwise $(NH_4)_2SO_4$ gradient. Total protein (mg) for each fraction is shown as rectangles while [^{3}H]CLC binding (pmol per 90-μl assay volume) is presented by dashed lines. Electrophoresis of selected fractions is presented adjacent to each elution profile. The lane number, fraction number of quantity of protein (μg) are. (a) (1) M_r standards (from highest to lowest M_r 96 000, 67 000, 43 000, 30 000, 20 000, 14 100); (2) Shelanski et al. [2] two-cycled tubulin, (3) fraction 4 (5 μg); (4) fraction 5 (4.8 μg); (5) fraction 8 (1.6 μg); (6) fraction 10 (1.4 μg); (7) fraction 11 (1.9 μg); (b) (1) M_r standards; (2) Shelanski et al. [2] purified tubulin, (3) fraction 4 (5 μg); (4) fraction 5 (3.5 g); (5) fraction 7 (2 μg); (c) (1) M_r standards, (2) Shelanski et al. [2] purified tubulin; (3) fraction 4 (5 μg), (4) fraction 5 (4.8 μg); (5) fraction 10 (2.5 μg).

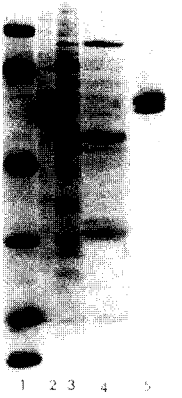

a. MES elution
3·0
2·0
1·0
0
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13
0·025M
0·3M
0·4M
0·5M


1 2 3 4 5 6 7


b. (NH4)2SO4 elution
Total protein eluted (mg) (——)
[3H] CLC bound (pmoles/assay) (---)
3·5
3·0
2·0
1·0
0
20
15
10
5
0
1 2 3 4 5 6 7 8 9
0·025M MES
0·076M
0·19M


1 2 3 4 5


c. MES and (NH4)2SO4 elution
4·5
4·0
3·0
2·0
1·0
0
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12
0·025M MES
0·4M MES
0·076M (NH4)2SO4
0·19M (NH4)2SO4


1 2 3 4 5

M MES fractions. Further elution of [^{3}H]CLC binding protein in $(NH_4)_2SO_4$ fractions occurs probably as a mixed effect of high ionic strength from residual 0.5 *M* MES eluate on the column. At 0.38 *M* $(NH_4)_2SO_4$ a further 27% of the [^{3}H]CLC binding occurs in fraction 16. Protein recovery from the column was 96%.

Despite the major structural changes associated with the coupling of lysine via the α-amino group, no substantial changes in the elution pattern were observed, with 97% of the eluted protein observed in the MES gradient and only 24% of the total [^{3}H]CLC binding detected in 0.38 *M* $(NH_4)_2SO_4$. Electrophoresis of fraction 10–13 (not shown) and 16 (Fig. 3g) showed fractions 12 and 16 to contain essentially pure tubulin with fractions 10 to 11 contaminated with M_r 21 000, 30 000 and 85 000 proteins. Further, no significant differences were observed between α- and ω-amino-coupled lysine ligands for these fractions.

Purification of tubulin using arginine-linked gels gave erratic results. Tubulin was eluted predominantly with either 0.5 *M* MES (fractions 12 and 13) or 0.38 *M* $(NH_4)_2SO_4$ (fraction 16) depending on the particular column. The results for the most successful elution with $(NH_4)_2SO_4$ are presented in Fig. 2c. Although the elution of tubulin varied between replicate columns, similar elution profiles for low-ionic-strengths MES buffers were noted, with an average of 64% of the protein removed with no detectable [^{3}H]CLC binding. Protein eluted at 0.5 *M* MES varied from 21 to 31% with higher protein contents associated with increased [^{3}H]CLC binding (5–85% of the total detected). The 0.38 *M* $(NH_4)_2SO_4$ fractions contained the balance of the protein and [^{3}H]CLC binding with only a minor contribution in the 0.076 *M* fractions. No further [^{3}H]CLC binding was detected at 0.8 *M* NaCl, with a total protein recovery of 80–95% of the applied crude supernatant. Electrophoresis of fraction 16 from all columns showed a tubulin band with minor impurities evident at M_r 42 000 and 85 000 only under conditions of heavy loading (Fig. 3e). Relatively pure tubulin was obtained in fraction 12 after elution with 0.5 *M* MES.

The erratic performance of arginine columns at 0.5 *M* MES and 0.38 *M* $(NH_4)_2SO_4$ was considered to be the result of subtle variations in column performance. However, the electrophoretic distribution of proteins and [^{3}H]CLC at 0.5 *M* MES consistently demonstrated tubulin to be retarded in its elution with respect to the other proteins. To determine whether manipulations of MES or $(NH_4)_2SO_4$ could improve the resolution of these impurities, crude sheep brain supernatant (8.5 mg) was applied to 2 ml arginine-linked Sepharose columns and eluted with either (a) 0.025 *M* MES (fractions 1–3), 0.3 *M* MES (fractions 4–6), 0.4 *M* MES (fractions 7–9) and finally 0.5 *M* MES (fractions 10–13) or (b) 0.025 *M* MES (fractions 1–3), 1% $(NH_4)_2SO_4$ (fractions 4–6) and 2.5% $(NH_4)_2SO_4$ (fractions 7–9) (Fig. 4).

For (a), tubulin was observed in all fractions eluted with 0.5 *M* MES (fractions 10–13, peaking in fraction 11) with no detectable impurities. Electropho-

resis of proteins in fractions 4, 5 (Fig. 4a) and 6 revealed that the major protein bands eluted in 0.3 *M* MES were M_r 42 000, 50 000 and 69 000. In fractions 7 and 8 (Fig. 4a), the M_r 85 000 band was observed.

For (b), elution of the M_r 30 000, 42 000, 50 000, 85 000 and 92 000 protein bands were observed in 0.079 *M* $(NH_4)_2SO_4$ fractions, with greater than 95% of the eluted tubulin observed in fraction 7. Trace quantities of M_r 42 000, 92 000 and 95 000 proteins were observed on heavily loaded gels. Thus, while 0.079 *M* $(NH_4)_2SO_4$ resolved most of the proteins contaminating tubulin, its ability to remove the residual M_r 42 000 and 92 000 bands was inferior to that of 0.4 *M* MES (Fig. 4b).

Optimal purification of sheep brain crude supernatant was achieved with high-ionic-strength MES (0.4 *M*) before elution with 2.5% $(NH_4)_2SO_4$. Under these conditions, tubulin is obtained containing single protein impurities at M_r 95 000, detectable only on overloaded gels (Fig. 4c).

After correction for inhibition and enhancement of [^{3}H]CLC binding in the MES and $(NH_4)_2SO_4$ buffers, respectively, no significant changes in either purification factor or [^{3}H]CLC binding were observed between the three elution sequences. Complete recovery of [^{3}H]CLC binding in either 0.5 *M* MES (Fig. 4a) or 0.19 *M* $(NH_4)_2SO_4$ (Fig. 4b and c) was consistent with the presence of tubulin as shown by gel electrophoresis. Gel scans of fractions 11, 7 and 10 from elutions shown in Fig. 4a, b and c, respectively, were $>98\%$, 95% and $>98\%$ pure tubulin. Reduction of the MES concentration from 0.5 to 0.4 *M* in elutions shown in Fig. 4a and c has resulted in a retarded desorption of tubulin from the matrix, allowing a more complete resolution from contaminating proteins which are eluted at 0.4 *M* MES. The use of 0.4 *M* MES prior to elution of the tubulin not only results in a slight improvement in pority ($>98\%$ compared with 95% for elutions in Fig. 2c and 4b) but more importantly removed the erratic and slow desorption characteristics observed when using 0.5 *M* MES on arginine columns.

DISCUSSION

Tubulin can be readily isolated using a wide range of amino-activated gels; however, the purity achieved and the elution characteristics are highly dependent on the structure of the amino ligand. For a highly acidic protein, tubulin ($pI = 5.0$–6.0), the potential range of amino ligand binding sites is large. The clear differences in the elution profiles of tubulin for each ligand suggest that interactions are occurring at different sites.

Tubulin containing minor impurities was predominantly isolated in the 0.8 *M* NaCl fractions of the aminohexyl and DEAE columns. Thus, while structurally quite distinct, the ligands demonstrate a characteristically tight interaction with tubulin presumably due to the relatively hydrophobic nature of amino residue. The use of the more polar, aminoethyl residue altered the po-

sition of elution with tubulin eluted with 0.38 *M* $(NH_4)_2SO_4$. Although all [^{3}H]CLC binding was obtained by this elution, electrophoresis showed the presence of a major impurity at M_r 87 000.

The arginine moiety linked to the gel via the amino group presents a 5-guanidinopentan-1-oic acid for interaction with tubulin. This ligand, although similar in size to the aminohexyl moiety, possesses amphoteric behaviour. The role of the carboxylate in the potential interaction with tubulin appears to be non-specific since coupling of either the α- or ω-amino groups of lysine to the gel matrix and the consequent change in the relative position of the carboxylate did not affect the chromatography of tubulin. Similar chromatographic distributions of tubulin between the 0.5 *M* MES and 0.38 *M* $(NH_4)_2SO_4$ fractions from arginine and lysine columns suggest that the carboxylate group acts indirectly to reduce the hydrophobicity of the ligand. This suggests that optimal binding to tubulin in less highly hydrophobic and/or acidic regions is occurring such that undesirable carboxylate interactions between the ligand and tubulin can be minimised.

As polylysine has an amide 'backbone', the putative ligand is considered as an aminopentyl moiety. Importantly this moiety contains adjacent aminopentyl residues analogous to the conformational flexibility observed in proteins. Polylysine is known to interact with tubulin in vitro and is capable of restoring the ability of tubulin preparations to polymerise [12]. This is thought to be due to the ability of polylysine to interact with tubulin at microtubule associated protein (MAP) binding sites. As MAPs are highly basic proteins, these sites are thought to be located in the glutamate-rich regions of tubulin. Although tubulin is eluted entirely in 0.38 *M* $(NH_4)_2SO_4$ from polylysine columns, the purity of the tubulin preparation is low. This is probably a direct effect of the high conformational flexibility of the ligand enabling the stabilisation of other acidic proteins.

Recently, Kocha et al. [13] reported the role of hydrophobic ligands in the purification of tubulin. They suggest hydrophobicity as the critical element in the purification of tubulin using either CLC-linked gels or non-specific hydrophobic ligands such as phenyl, diphenyl, trimethoxyphenyl, naphthyl and aminohexyl groups. Although our results with both the DEAE and aminohexyl ligands concur with their general conclusions of tight association, this association is clearly not irreversible as suggested by Kocha et al. [13]. Further, in this study we were able to document that isolation can also be achieved by the choice of more polar ligands. This suggests that tubulin also contains a series of hydrophilic sites for ligand binding which can be dissociated for routine purification of native tubulin. These observations are not surprising since such a highly acidic protein could be anticipated to contain a wide diversity of potential binding sites with differing conformational and structural pre-requisites.

The arginine ligand was chosen as the specific ligand for the purification of sheep brain tubulin since the need for high concentrations of NaCl for elution

and its detrimental effect on [^{3}H]CLC binding represent a major disadvantage for both the aminohexyl and DEAE ligands. By varying the elution sequence using 0.4 *M* MES and 2.5 or 5% $(NH_4)_2SO_4$, the contaminating proteins in the initial 0.5 *M* MES fractions can be completely removed with no significant loss of tubulin. This procedure enabled the development of a large-scale preparative elution sequence for tubulin, capable of isolating 500-mg batches of tubulin in under 2 h [10]. This procedure is significantly faster than existing DEAE columns without the added complexity of denaturation of tubulin associated with the use of high salt concentrations.

The present use of NH_4^+ ions for the chromatographic purification of tubulin has not previously been reported and, based on the observed effects of MES and NaCl on [^{3}H]CLC binding, is clearly advantageous. The mechanism of enhancement of [^{3}H]CLC binding in NaCl solutions is associated with irreversible changes within tubulin, an observation recently published by Croom et al. [14], who caution against its use in biochemical studies. Our results support this conclusion. While NH_4^+ ions also enhance binding, the effect appears to be reversible and possibly reflects aspects of the acid–base interactions of MAPs within the glutamate-rich regions of tubulin.

Although only seven amino ligands were examined in this study, it is apparent that the potential exists for further development of other amino-activated gels with differing affinities. This is not only important for the isolation of tubulin but may also be useful for other acidic proteins. Such an approach offers a considerably broader scope for isolation of acidic protein than is currently available with DEAE chromatography.

ACKNOWLEDGEMENT

This work was funded by Grant No. US20P from the Wool Research Trust Fund on the recommendation of the Australian Wool Corporation.

REFERENCES

1 R.F. Luduena, in K. Robserts and J.S. Hyams (Editors), Microtubules, Academic Press, New York, 1979, p. 65.
2 M.L. Shelanski, F. Gaskin and C.R. Cantor, Proc. Natl. Acad. Sci. U.S.A., 70 (1973) 765.
3 N.D. Hinman, J.L. Morgan, N.W. Seeds and J.R. Cann, Biochem. Biophys. Res. Commun., 70 (1973) 752.
4 Y. Ikeda and M. Steiner, J. Biol. Chem., 251 (1976) 6135.
5 B. Rousset and J. Wolff, J. Biol. Chem., 255 (1980) 11677.
6 R.C. Weisenberg, G.G. Borisy and E.W. Taylor, Biochemistry, 7 (1968) 4466.
7 E. Lacey, J.A. Edgar and C.C.J. Culvenor, Biochem. Pharmacol., 36 (1987) 2133.
8 M.M. Bradford, Anal. Biochem., 72 (1976) 248.
9 L.C. Morejohn, T.E. Bureau, L.P. Tocchi and D.E. Fosket, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 1440.
10 E. Lacey, unpublished results.

11 H. Schmitt and D. Atlas, J. Mol. Biol., 102 (1976) 743.
12 H.P. Erickson and W.A. Voter, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 2813.
13 T. Kocha, T. Fukuda, T. Isobe and T. Okuyama, Chem. Pharm. Bull. (Tokyo), 34 (1986) 3791.
14 H.B. Croom, J.J. Correia and R.C. Williams, Arch. Biochem. Biophys., 249 (1986) 397.