Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 818 (2005) 83–87
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

One step flow-through adsorptive purification of tubulin from tissue homogenate

Annamma Anil[a], Reena Pandit[b], Madhavi Indap[b], Arvind Lali[a,*]

[a] *Chemical Engineering Division, Mumbai University Institute of Chemical Technology, Matunga, Mumbai 400019, India*
[b] *Department of Zoology, D.G. Ruparel College, Mahim, Mumbai 400016, India*

Received 16 June 2004; accepted 1 October 2004
Available online 14 November 2004

Abstract

Tubulin, a potential target for anti-cancer drugs, has been purified in one step and obtained as flow-through fraction directly from an extract of a mammalian brain tissue by adsorption chromatography on H-CELBEADS, an indigenously developed rigid, superporous cross-linked cellulose based weakly hydrophobic adsorbent. The fibrous polymerized tubulin mass passed through the H-CELBEADS bed while the associated proteins were separated by adsorption. The final tubulin preparation was obtained free from other proteins as seen on SDS–PAGE. Purified tubulin was obtained in a yield of about 29 mg/100 g brain, and its bioactivity, evaluated through its ability to bind colchicine, was found to be preserved.

Keywords: CELBEADS; Protein purification; Tubulin

1. Introduction

Microtubules constitute one of the major components of the cytoskeleton of eukaryotic cells that are involved, through their unique structure, in many crucial physiological functions like motion, mitosis, and other motility-related phenomena such as the transport of subcellular organelles or changes in cell shape [1,2]. Microtubules are made up of protofilaments that are polymerized form of tubulin, a dimeric protein, along with microtubule associated proteins (MAPs). Thus tubulin is the main constituent of microtubules responsible for maintaining the shape and dynamic equilibrium crucial for the stability of the cytoskeleton [2,3]. As a result, any compound that interacts with tubulin and disrupts its capability to polymerize or depolymerize can be expected to interfere with cell mitosis. Tubulin has been known to interact with certain molecules called tubulin binding agents (TBAs) that target microtubules and lock their non-equilibrium states and thus induce apoptosis in cells [4]. This strategy is being increasingly explored to create a repertoire of potential anti-cancer drugs isolated naturally or synthesized chemically. The success of these methodologies is highly dependent on the supply of pure microtubule preparations that can retain their biological activity for in vitro experiments and can be prepared economically.

The α and β tubulins (55 kDa each) are homologous [5] and form a heterodimer that further polymerizes to form the microtubules. The assembly of tubulin to microtubules in vitro requires the presence of GTP, stabilizing agents, calcium, maintenance of pH and temperature and also the phosphorylation of associated microtubule proteins [6–10]. Tubulin has a unique property of polymerization in vitro at temperatures above 25 °C to form aggregates in the presence of stabilizing agents (viz., glycerol, glutamate, etc.), and depolymerization at 5 °C or lower into a clear solution. Most reported purification strategies exploit this property of tubulin and employ repeated cycles of temperature dependent tubulin polymerization and depolymerization [11] followed by ion-exchange chromatography to remove microtubule associated

* Corresponding author. Tel.: +91 22 24145616x292.
E-mail address: aml@udct.org (A. Lali).

1570-0232/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2004.10.001

proteins called MAPs [12]. There are also reports on affinity interactions of glycolytic enzymes with tubulin [13].

The more often used method of polymerization–depolymerization cycles for purification of tubulin has limitations of being long, laborious and expensive with the purified tubulin fraction being yet contaminated with MAPs as also resulting in loss of bioactivity of the preparation [10,14]. Commercial tubulin preparations, while available at high prices, are unstable which more often necessitates indigenous preparation as and when required. A technique that results in quick and cost effective purification of tubulin can not only facilitate purification in large quantities but will also result in speeding up biomedical research involving tubulin. In this paper, we propose a technique for the isolation of polymerized tubulin in good yield from crude mammalian brain extract using a one step method under temperature independent conditions. The technique involves purification on a chromatography column packed with H-CELBEADS, an indigenously developed rigid, superporous cross-linked cellulose based weakly hydrophobic matrix.

2. Experimental

2.1. Materials

Tris–HCl buffer, ethylene glycol-0,0′-*bis*(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), magnesium sulfate, guanosine 5′-triphosphate (GTP), glycerol, monosodium glutamate and all other chemicals used were of reagent grade and purchased from S.D. Fine Chem. Ltd., Mumbai, India, and SRL Pvt. Ltd., Mumbai, India. Freshly cut goat brain was obtained from local slaughterhouse in an ice-cold pack. Deionized water was used in all the experimental work.

The adsorbent media used in this work was indigenously developed hydrophobic H-CELBEADS that was prepared using a proprietary procedure which involved cross-linking of a cellulose derivative in a porogen solvent and hardening of the dispersed beads in an aqueous solution. H-CELBEADS, a beaded superporous adsorbent with a distributed surface aromatic hydrophobicity, can be obtained from Resindion Srl, 55 via Roma, Binasco, Milan, Italy. Image analysis of the H-CELBEADS indicated that beads were near spherical in shape (sphericity, ∼0.85) and had a particle diameter between 150 and 200 μm. Porosity analysis by mercury porosimetry gave an average pore radius of 33,000 Å and a pore volume of 60%.

2.2. Preparation of crude tubulin extract

The goat brain maintained under ice-cold conditions (∼4 °C) was cleaned of all the blood residues after the meninges were removed. The chopped brain tissue was then homogenized in a near equal volume of 80 mM Tris–HCl buffer containing 1 mM EGTA and 3 mM $MgSO_4$, at pH 6.8 (buffer A). The homogenate was cold centrifuged at 4 °C at $12{,}000 \times g$ for 45 min to obtain a clear crude extract. The extract was polymerized in the presence of 4 M glycerol and/or 1 M sodium glutamate, and 0.1 mM GTP prepared in buffer A for an hour at a temperature varied in the range of 25–37 °C in a controlled temperature water bath. The turbid solution obtained after the incubation was used for further experiments as the polymerized crude extract. Typically, one brain (about 60 g) yielded 80 ml of the turbid tubulin extract.

2.3. Tubulin purification

The chromatography experimental setup used for the column runs consisted of a 25 mm inner diameter and 250 mm long borosilicate jacketed glass column provided with two 140 mm long BioRad ECONO adapters fitted with 90 μm stainless steel mesh distributors in place of polymeric screens. The column was packed to a bed height of 100 mm. The lower adapter was connected to a BioRad ECONO pump, while the top adapter was connected to an online Hitachi UV–vis 1100 spectrophotometer working at 280 nm, and further to an ISCO Retriever II fraction collector. The absorbance readings at the spectrophotometer were recorded on a continuous chart recorder. All experiments were performed in up-flow mode at linear flow rate of 100 cm/h. The matrix was first equilibrated with 2.5 bed volumes of aqueous equilibrating 80 mM Tris–HCl buffer containing 1 mM EGTA and 3 mM $MgSO_4$, pH 6.8 along with 4 M glycerol or 1 M glutamate and 0.1 mM GTP. The polymerized crude brain extract (80 ml) was then loaded on to the column while the column and feed extract were maintained at a temperature between 25 and 37 °C as desired in the experiment. The flow-through fractions (25 ml each) were collected and analyzed for protein and turbidity following which they were centrifuged at $12{,}000 \times g$ for 30 min at 37 °C. The pellets were de-polymerized at 4 °C in pH 6.8 25 mM Tris–HCl buffer containing 1 mM EGTA and 3 mM $MgSO_4$ to obtain the pure depolymerized tubulin preparation. The column with adsorbed components on the matrix was cleaned-in-place with 0.5 M NaOH, washed sufficiently with de-ionized water, re-equilibrated and re-used without any loss of performance.

2.4. SDS–PAGE gel electrophoresis

Protein analysis in the crude clear extract and the final de-polymerized purified tubulin preparation was done on SDS–PAGE on a vertical Mini-PROTEAN II cell (Bio-Rad, CA, USA) with 12% acrylamide in running gel. The bands were silver stained.

2.5. Assay for protein

Total protein concentrations were estimated by the Bradford method [15] using the micro-assay Bio-Rad protein assay procedure and with bovine serum albumin as standard protein.

2.6. Turbidimetry assay

Bioactivity of the purified tubulin was qualitatively tested by checking the inhibition of polymerization of tubulin through colchicine binding [4,8]. Tubulin polymerization was carried out on a Jasco V-530 UV–vis spectrophotometer for the purified tubulin samples. Microtubule assembly was followed by rate of increase in turbidity at 350 nm at 37 °C in 10 s time interval [16]. Each polymerization reaction mixture contained 1 mg/ml of purified tubulin, 1 mM GTP, 80 mM Tris–HCl buffer, 1 mM EGTA and 3 mM $MgSO_4$, pH 6.8 and 1 M glutamate. Same reaction was repeated in the presence of 1 mM of colchicine. The extent of reduction in appearance of turbidity reflected the bioactivity of purified tubulin.

3. Results and discussion

In addition to tubulin, the mammalian brain extract is known to contain proteins that are associated with microtubule formation. These microtubule-associated proteins (MAPs) are closely bound to the tubulin polymers constituting the microtubule and are, therefore, difficult to separate by traditional methods of purification. The most widely used method employs repeated polymerization and depolymerization steps in attempts to exploit the property of tubulin being the only protein capable of polymerization–depolymerization with respect to temperature variation between 37 and 4 °C [16]. In one report, tubulin was purified on a strong anion exchanger after two cycles of assembly–disassembly in an attempt to increase tubulin yields and simplify the purification [17].

There have been some reports whereby attempts have been made to further simplify tubulin purification. Notable among these are those based on affinity interactions with specific tubulin binding agents [13,18,19]. However, this approach though capable of giving pure tubulin in one step, requires preparation of complicated and expensive affinity adsorbents. Rapid purification of tubulin on membrane ion-exchange chromatography has been reported [20]. Ion exchange based purifications of tubulin involve adsorption of tubulin on to the solid matrix (resin or membrane) and subsequent selective elution of the molecule in an ionic buffer of appropriate strength.

For the present work, it was decided to develop a novel 'negative' purification technique that would combine the unique property of temperature induced polymerization of tubulin and the possibility of complete adsorption of all the other soluble contaminants including MAPs from the tissue extract. This was possible if all soluble components from a suspension of polymerized tubulin were adsorbed while the particulate suspension of tubulin would pass unadsorbed through an adsorbent column. For this purpose, an adsorbent matrix was required such that in addition to being capable of adsorbing all soluble components, the packed bed of the chosen adsorbent should remain unclogged in the presence of fine

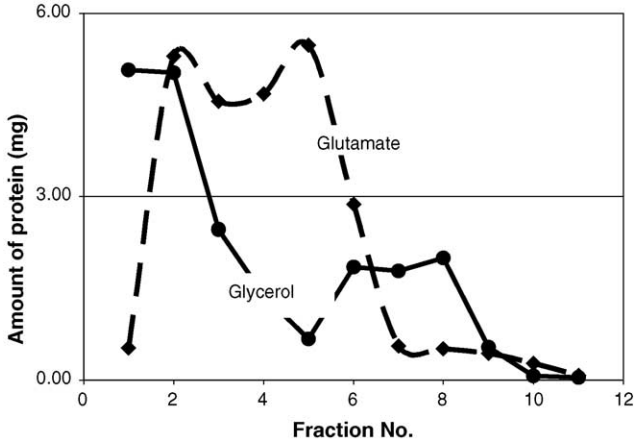

Fig. 1. Protein chromatograms of flow-through fractions of polymerized brain tubulin crude extract on H-CELBEADS equilibrated in 80 mM Tris–HCl buffer, 1 mM EGTA and 3 mM $MgSO_4$, pH 6.8 along with 4 M glycerol/1 M glutamate and 0.1 mM GTP. Linear flow velocity: 100 cm/h.

suspension of 'polymerized' tubulin. Superporous derivatized CELBEADS, an indigenously developed rigid beaded matrix, when used in a packed bed configuration, has been shown to facilitate through passage of fine particulate matter on account of its large pores [21]. Further, since most proteins have some degree of surface hydrophobicity, an hydrophobic adsorbent appeared a better choice than an ion exchanger. Use of the superporous weakly hydrophobic H-CELBEADS was, therefore, expected to result in the through-flow of the polymerized tubulin (microtubules) while adsorbing and retaining the soluble proteins from the brain extract.

Figs. 1 and 2 are the chromatograms obtained on the H-CELBEADS column when loaded with polymerized brain extract in 1 M glutamate and 4 M glycerol, respectively, in presence of 0.1 mM GTP under conditions mentioned in the methods section. The use of the microtubule stabilizing

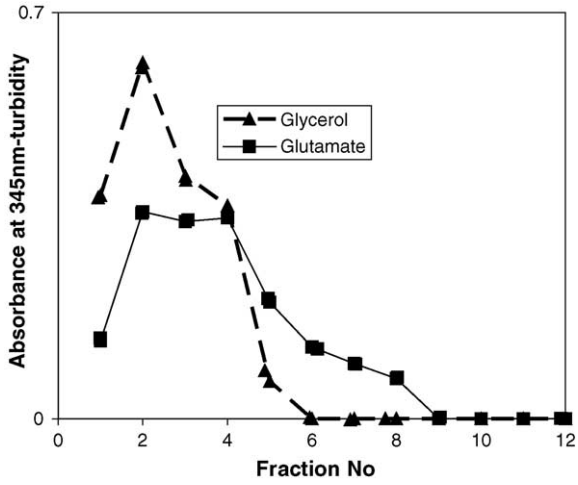

Fig. 2. Turbidity chromatograms of flow-through fractions of polymerized brain tubulin crude extract on H-CELBEADS equilibrated in 80 mM Tris–HCl buffer, 1 mM EGTA and 3 mM $MgSO_4$, pH 6.8 along with 4 M glycerol/1 M glutamate and 0.1 mM GTP. Linear flow velocity: 100 cm/h.

Table 1
Comparison between the yields of de-polymerized purified tubulin with glutamate and glycerol induced 37 °C polymerization of the mammalian brain tissue homogenate as determined by protein analysis

Agent	Protein (mg)			
	Crude	Bound	Flow-through	Depolymerized pellet
Glutamate	184	158.06	25.94	3.112
Glycerol	184	163.05	20.95	7.6

agents like glycerol and glutamate helps in induction of polymerization of the tubulin and also stabilizes the microtubule. Figs. 1 and 2 show that glycerol induced polymerization gave a flow-through fraction with higher turbidity that was also associated with a distinct protein band. On the other hand, glutamate induced polymerized extract gave a broad turbid band associated with two unresolved protein peaks (fractions 1–8). Table 1 gives the comparison of the two runs made with glutamate and glycerol as polymerization promoting agents. While the flow-through protein was larger in case of glutamate, the purified tubulin yield was lower than that obtained with glycerol. This was seen to be the result of the difficulty experienced in de-polymerizing the palletized tubulin form the turbid fractions in case of glutamate assisted polymerization. However, glycerol assisted polymerized extract gave turbid fractions that gave easily soluble pellet and a higher tubulin yield. All further experiments were performed with glycerol as polymerizing agent. Fractions 1–5 thus were collected as tubulin rich fractions (Figs. 1 and 2).

Most reports dealing with tubulin polymerization and depolymerization steps indicate the corresponding temperatures as 37 and 4 °C [5,14,16]. Chromatography experiments using these temperatures gave a tubulin yield of about 5% of the total extract protein amounting to approximately 15 mg/100 g brain. With some of the reports stating maximum obtainable yields in excess of 25 mg/100 g, it was decided to modulate tubulin polymerization through change in polymerization temperature [22]. Fig. 3 demonstrates the observed effect of polymerization temperature on the percentage (of total protein) yield of de-polymerized tubulin. It can be seen that in the range of temperature tested, a temperature of 25 °C

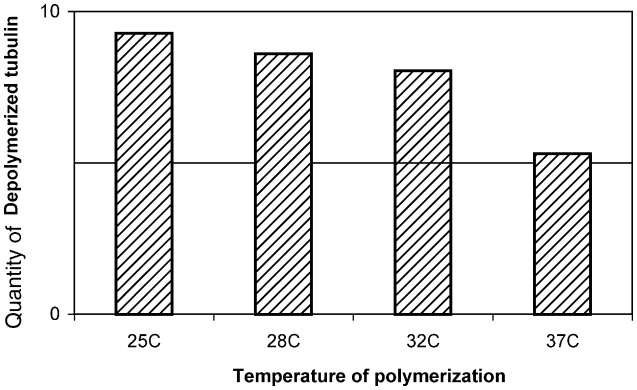

Fig. 3. Effect of temperature of polymerization on the %yield of purified tubulin on total extract protein.

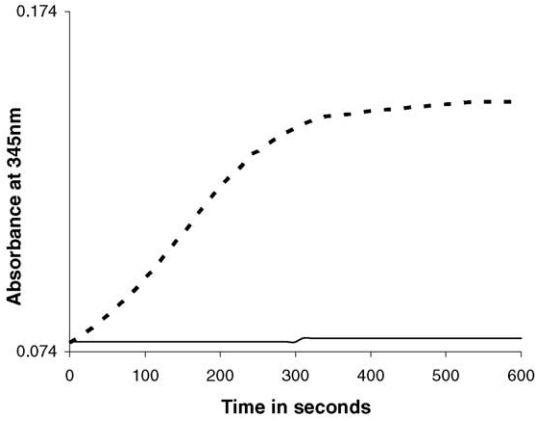

Fig. 4. Microtubule assembly of tubulin (- - -) in absence of colchicine; (—) in presence of colchicine. Solution (1 mg/ml) of depolymerized tubulin in 80 mM Tris–HCl buffer, 1 mM EGTA and 3 mM $MgSO_4$, pH 6.8 with 1 M glutamate and 1 mM GTP incubated at 37 °C.

gave maximum yield (about 29 mg/100 g brain). This can be attributed to two possible reasons. One, the reduction of hydrophobic interaction with temperature between associating tubulin molecules results in less compact aggregates that are easy to 'de-polymerize'. Secondly, a higher temperature may affect the structure of the associated tubulin molecules leading to their inability to dissociate fully later at lower temperatures.

The bioactivity of the purified de-polymerized tubulin preparation was assessed by turbidimetric analysis whereby the capacity of the protein to polymerize in the presence of glutamate was evaluated. Fig. 4 shows the change of turbidity with time in presence and absence of colchicine. It can be seen that while purified tubulin gives an increase in turbidity with time, presence of tubulin binding colchicine does not show any appearance of turbidity.

The SDS–PAGE presented in Fig. 5 demonstrates effectiveness of the single step strategy used for purification of tubulin. It can be noted that the crude extract contains significant amount of contaminating proteins mainly MAPs and Tau proteins in the entire marker molecular weight range

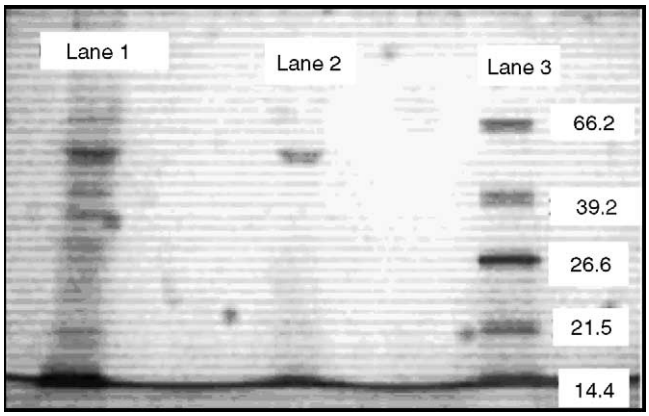

Fig. 5. SDS–PAGE analysis. Lane 1: crude brain tissue homogenate; lane 2: purified tubulin preparation; lane 3: low molecular weight markers.

(14–66 kDa). These contaminants are absent from the purified tubulin seen as a single band at molecular weight of about 50 kDa.

The column after obtaining the flow-through fractions was regenerated with 3 bed volumes of 0.5 M NaOH. Extensive water wash to obtain neutral pH and re-equilibration with the working buffer allowed re-use of the adsorbent without any loss of performance.

4. Conclusions

The following conclusions are drawn from the work:

1. The concept of using flow-through chromatography on a superporous hydrophobic adsorbent matrix column to adsorb all contaminants from the mammalian brain extract containing tubulin in polymerized state provides a single step and quick method of obtaining purified tubulin in good yield free from other extract proteins.
2. Polymerization of tubulin in presence of 4 M glycerol results in higher recovery of tubulin than with 1 M sodium glutamate.
3. Lower polymerization temperature of 25 °C than the mostly reported 37 °C, gave a higher yield of purified tubulin.
4. The overall technique of polymerization of tubulin in the clarified crude brain extract in presence of 4 M glycerol and at 25 °C, and subsequent passage of the turbid tubulin extract through a bed of superporous hydrophobic matrix H-CELBEADS results in purification of bioactive tubulin from contaminating proteins. The technique is simple, quick, and cost effective and should go some way in making tubulin easily available for biomedical research.

References

[1] J.A. Snyder, J.R. McIntosh, Annu. Rev. Biochem. 45 (1976) 699.
[2] R.L. Margolis, L. Wilson, Nature (London) 292 (1981) 705.
[3] F. Pirollet, D. Job, E.H. Fischer, R.L. Margolis, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 1560.
[4] E. Hamel, Cell Biochem. Biophys. 38 (2003) 1.
[5] Y. Lynn, Mystery of vital cell protein solved after 30 years, Web page of Berkley National Laboratory California.
[6] R.L. Margolis, L. Wilson, B.I. Kiefer, Nature 272 (1978) 450.
[7] R.D. Sloboda, W.L. Dentler, J.L. Rosenbaum, Biochemistry 15 (1976) 4497.
[8] E. Hamel, Biochem. Biophys. Acta 675 (1981) 226.
[9] M. Castoldi, A.V. Popov, Protein Expr. Purif. 32 (2003) 83.
[10] R.W. Clark, M. Volpi, R.D. Berlin, Biochemistry 27 (1988) 1025.
[11] M.L. Shelenski, F. Gaskin, C.R. Cantor, Proc. Natl. Acad. Sci. U.S.A. 70 (1973) 765.
[12] D.B. Murphy, R.B. Vallee, G.G. Borisy, Biochemistry 16 (1997) 2598.
[13] B. Rousset, J. Wolff, J. Biol. Chem. 255 (1980) 11677.
[14] M.D. Weingarten, M. Suter, D. Littman, M. Krischner, Biochemistry 13 (1974) 5529.
[15] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[16] E. Hamel, C.M. Lin, Biochemistry 23 (1984) 4173.
[17] S. Roychowdhury, F. Gaskin, J. Neurochem. 46 (1986) 1399.
[18] N.D. Hinman, J.L. Morgan, N.W. Seeds, R.J. Cann, Biochem. Biophys. Res. Commun. 52 (1973) 752.
[19] Y. Ikeda, M. Steiner, J. Biol. Chem. 251 (1976) 6135.
[20] R.D. Sloboda, L.M. Belfi, Protein Expr. Purif. 13 (1998) 205.
[21] A. Lali, K. Manudhane, N. Motlekar, P. Karandikar, Indian J. Biochem. Biophys. 39 (2002) 253.
[22] M. Caplow, J. Shanks, R.L. Ruhlen, J. Biol. Chem. 263 (1988) 10344.