Pak. J. Bot., 16(2): 101-107, 1984.

TUBULIN SYNTHESIS DURING SPERMATOGENESIS IN THE *FERN ANEMIA PHYLLITIDIS:* DEMONSTRATION WITH ANTITUBULIN ANTIBODIES.[1]

JAVED IQBAL* AND HELMUT SCHRAUDOLF

Department of Biology II, Ulm University,
D-7900 Ulm, West Germany.

Abstract

The presence of tubulin in 10 days old reproductive prothalli of the fern *Anemia phyllitidis* has been demonstrated with antitubulin antibodies. The immuno-precipitated protein migrates as a single band on SDS-polyacrylamide gel and has molecular weight of 55000 Daltons, which is characteristic for tubulin from most animal and plant sources.

Introduction

Microtubules are filamentous components of the eukaryotic cells that are involved in several functions including mitosis, cytoskeleton organisation, ciliary and flagellar movement and intracellular transport and secretion (Hepler & Palevitz, 1974). Tubulin, a dimer of alpha and beta chains, each of about 55000 Daltons appears to be the major structural component of microtubules (Shelanski, *et al.,* 1973).

Tubulin synthesis has been shown to be inducible during cilia or flagella formation/regeneration in several eukaryotes (Bird & Zimmermann, 1980; Gealt & Weeks, 1980; Lefebvre, *et al.,* 1980; Silflow & Rosenbaum, 1981; Weeks & Collis, 1976). Comparatively very little work on biosynthesis of tubulin during any growth phase has been done in plants. There are a few reports where tubulin has been either purified (Luduena *et al.,* 1980; Mizuno, *et al.,* 1981) or characterised by colchicine binding in extracts of plants (Hart & Sabnis, 1973, 1976 a,b; Rubin & Cousins, 1976). In the present paper

[1] Part of a post-doctoral work. Fellowship awarded to the first author by Alexander von Humboldt-Stiftung, West Germany.

* Department of Botany, Punjab University, New Campus, Lahore, Pakistan.

we report the presence of tubulin by immuno-precipitation with anti-tubulin antibodies in 10 days old reproductive prothalli in the fern *Anemia phyllitidis*. This is a stage of development where spermatogenesis is in progress and spermatozoids which have a cluster of flagella all around are about to be released. This is the time in development when on *a priori* basis increased synthesis of tubulin should be observed.

Material and Methods

Spores of *Anemia phyllitidis* were sown and grown upto 10 days in mineral salt medium (Mohr, 1956) with gibberellic acid (5×10^{-5} g/ml) and 2.0 μCi of a complete ^{14}C-amino acid mixture (Specific activity 57 mCi/m atom carbon; Radiochemical Centre, Amersham) in a total of 20 ml of growth medium in conical flask under complete sterile conditions. Growth conditions were similar as reported earlier (Schraudolf, 1966).

(a) *Extraction of proteins and preparation for gel electrophoresis:* For extraction of soluble proteins 4, 6 and 10 days old reproductive prothalli were homogenised in 0.1 M Tris-HCl extraction buffer, pH 7.6; 5 mM mercaptoethanol (ratio of buffer to material 3:1). Following homogenization the sample was centrifuged at 6000 rpm. for 20 min. at 4°C. Proteins were precipitated with ice cold trichloroacetic acid (10%, wt/vol). The precipitated proteins were washed 3-4 times with cold acetone and dried under stream of nitrogen gas. A part of dried proteins was dissolved in electrophoresis sample buffer (10% SDS 30 ml, 0.5 M Tris-HCl, pH 6.8-12.5 ml, beta mercaptoethanol 5.0 ml, water 42.5 ml, 10% glycerol 10.0 ml, and 0.05% Bromophenol blue), sonically oscillated for complete dissolution and treated for 3 min. in a boiling water bath. The protein content of the sample was determined by the method of Lowry *et al.*, (1951) and adjusted to approximately 20 μg/10 μl.

(b) *Preparation and purification of tubulin:* Tubulin was isolated by the method as described by Shelanski *et al.*, (1973). The pig brain was homogenised in the reassembly buffer of Weisenberg (1972); 0.1 M MES (2-[N-Morpholino]-ethane sulphonic acid), 1 mM EDTA, 1 mM GTP, 0.5 mM Mg Cl_2, pH 6.4; and the homogenate was centrifuged at 100,000 x g for 1 hr. To the supernatant an equal volume of 8 M glycerol in reassembly buffer was added and the mixture was incubated at 37°C for 30 min. The reassembled tubulin was collected by centrifugation at 100,000 x g for one hr at 25°C, and dissociated in the cold reassembly buffer at 0°C for 30 min. These procedures were repeated three times. The tubulin was stored at −70°C after two cycles of polymerisation, the third assembly cycle was performed prior to phospho-cellulose chromatography.

Tubulin was purified by chromatography on Whatman P-11, phospho-cellulose which had been equilibrated with buffer P (100 mM Pipes (piperazine-N, N′-bis [2-ethanesulfonic acid]; 1,4-Piperazinediethane sulfonic acid), 2 mM EGTA (ethyleneglycol bis [β-aminoethylether] N, N′ tetra acetic acid), 1 mM Mg SO_4, pH 6.5). The reassembled

tubulin was suspended in buffer P at a concentration of not more than 10 mg/ml of protein and chromatographed on phospho-cellulose at 4°C (2-3 mg per ml bed volume). The pure tubulin was eluted from the column with the P-buffer at a flow rate of one bed volume per 3 hr. The fractions containing tubulin were analysed by SDS-acrylamide gel electrophoresis by the method described by Weber & Osborn (1969). The molecular weight of tubulin was estimated from the mobility in the SDS gels. Nuclear and cytoplasmic proteins of Ad^2+ND_2 non disinfectant Hela cells were used as molecular weight marker (these were a kind gift from Prof. Dr. W. Deppert, Biochemistry Department, Ulm University).

(c) *Preparation of anti-tubulin serum:* Antibodies against purified tubulin from step b were obtained by injecting guinea-pig subcutaneously with the tubulin emulsified in Freund's complete adjuvant, followed by intravenous booster injection 4 weeks later. The antiserum was harvested from the ear vein 7 days after the booster injection and stored at −20°C until used. The specificity of the antiserum was demonstrated by electrophoretic pattern of the immuno precipitate.

(d) *Immunoprecipitations:* Protein (20 μg) from step (a) was dissolved in 200 μl of Schwyzer's extraction buffer, 1977; (137 mM NaCl, 20 mM Tris, 1 mM $Mg\ Cl_2$, 1 mM $CaCl_2$, 1% NP 40 (Shell), 10% glycerol, pH 9.0) and incubated at 4°C with 10 μl anti-tubulin serum. After 1 hr 80 μl of settled staphylococcal protein A-sepharose (Pharmacia) swollen in phosphate buffer saline (PBS; 140 mM NaCl 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) was added.

Of the mixture incubated as above, washing and elution of the immunoprecipitate was carried out as described by Schwyzer (1977). The final elute was lyophilised and resuspended in electrophoretic sample buffer.

(e) *Polyacrylamide gel electrophoresis and fluorography:* For electrophoretic separation of soluble proteins from step (a) and resolution of immunoprecipitates band for tubulin from step (d) polyacrylamide gel system of Laemmli (1970) was used. A 20 μg of protein in approximately 10 μl was applied per slot. Electrophoresis was performed at a constant current of 12 mA with 12.5% slab gels of 1 mm thickness for 8 hr. After electrophoresis the gels were prepared for fluorography as described by Bonner & Laskey (1974).

Results and Discussion

Anemia spores germinate 4 days after sowing. The single vegetative apical cell of the sporling transforms into an antheridium by 6th day of sowing, spermatogenesis initiates by 8th day and spermatozoids are formed on/or about 10th day (Schraudolf, 1966).

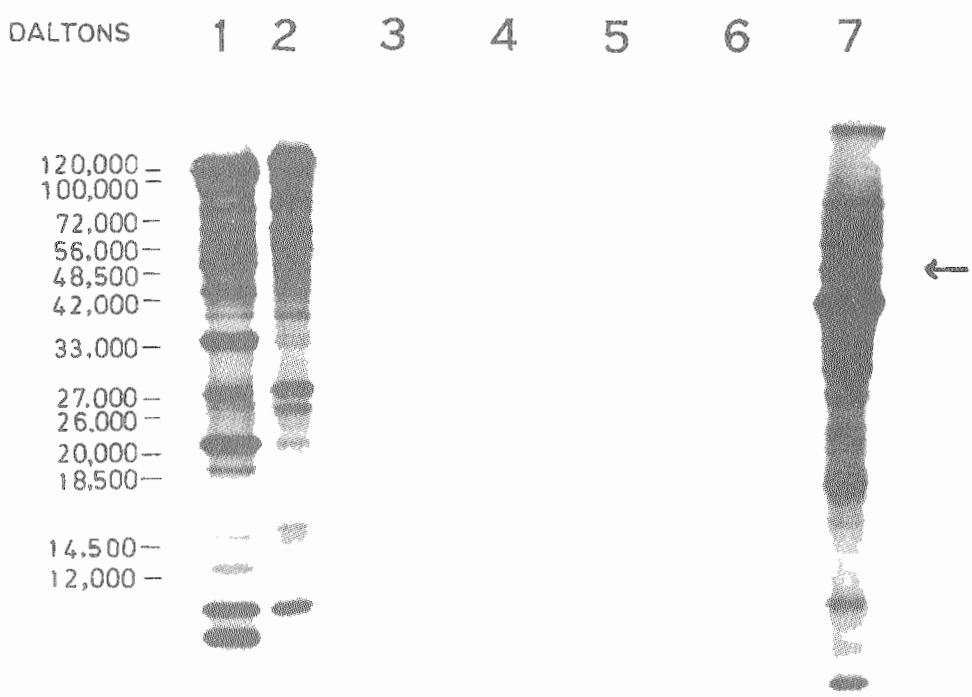

Fig. 1. Immunoprecipitation of *in vivo* synthesized proteins with antibodies specific for tubulin from 4, 6 and 10 days old reproductive prothalli of *Anemia phyllitidis*. The ^{14}C labelled products were analysed by SDS-polyacrylamide gel electrophoresis and fluorography.

Lane 1 & 2 : Marker proteins from Ad^2+ND_2 virus non-disinfectant Hela cells. Lane 1 (high salt nuclear extract); Lane 2 (cytoplasmic extract).

Lane 3 : Protein extract immunoprecipitated with anti-tubulin antibodies 4th day after sowing. No tubulin band.

Lane 4 : Protein extract immunoprecipitated with antitubulin antibodies 6th day after sowing. No tubulin band.

Lane 5 : Protein extract immunoprecipitated without any antibody 10th day after sowing. No tubulin band.

Lane 6 : Protein extract immunoprecipitated with antitubulin antibodies 10th day after sowing. Clear tubulin band visible at 55000 Daltons.

Lane 7 : *In vivo* incorporation of label into proteins 10th day after sowing in reproductive prothalli.

Lane 1 & 2 were exposed for 2 weeks; lane 3, 4 & 5 for three weeks, lane 6 for 2 weeks and lane 7 for 24 hr, respectively.

(i) *Analysis of total extractable proteins:* The *in vivo* labelling of 10 days old reproductive prothalli have shown the synthesis of numerous proteins during spermatogenesis of the fern *Anemia.* About 50 discrete bands of proteins were visualised in the fluorogram (Fig. 1; Lane 7). The predominant protein products ranged in molecular weight from approximately 10,000 to 120,000 Daltons, although minor components of high molecular weight were also detectable. The most strongly labelled proteins are in the range of 33,000 to 100,000 Daltons. Proteins of molecular weight 55,000 (Probably tubulin) showed the most pronounced labelling and appear as a major component at this stage of development (Fig. 1; Lane 7, marked by arrow).

(ii) *Immunoprecipitation of tubulin:* To establish with certainty that the proteins at molecular weight 55,000 may have polypeptides of tubulin, immunoprecipitation with anti-tubulin antibodies was carried out and characterised by SDS polyacrylamide gel electrophoresis. The fluorography of the coomassie blue stained gel showed the identity of the precipitation line. Only one protein band with a molecular weight of 55,000 which is specific for tubulin was observed (Fig. 1; lane 6), thus clearly establishing the presence of tubulin.

To ascertain the most active phase for tubulin synthesis during the development of antheridium and spermatogenesis, protein extracts from just germinated spores (4th day after sowing, with a single apical cell), reproductive prothalli with antheridium initiation (6th day after sowing) and 10 days old reproductive prothalli where spermatogenesis was in progress, were immunoprecipitated with anti-tubulin antibodies. In fluorogram the immunoprecipitate for tubulin was only observed at 10th day of development (Fig. 1). On *a priori* basis this is a stage of development where presence and/or active synthesis of tubulin is expected, as at this stage the formation of spermatozoids is in progress and a pool of tubulin is required for flagellar formation, which are formed in a cluster all around the spermatozoids.

So far the characterization of tubulin in plant extracts has been done by colchicine binding. Hart & Sabnis (1973) in *Heracleum mantegazzianum* and later in a variety of plants and their tissues (pea, myrhis, mustard, *Caulerpa* fronds, *Heracleum*) has encountered difficulty in obtaining colchicine binding activity and have found this activity to be extremely labile (Hart & Sabnis, 1976a,b). They ascribed this to the fact that in plants tubulin is present in smaller amounts than in animals and its colchicine activity is much less stable *in vitro.*

Keeping these inherent problems of characterising plant tubulin by colchicine binding, it appears that immunoprecipitation technique utilising anti-tubulin antibodies is more convenient, sensitive and reproducible as is shown by present investigation. Next we aim to look at the sequential appearance of specific messengers for tubulin from *in vitro* translation products of poly $(A)^+$ RNA from reproductive prothalli.

Acknowledgements

One of us (Javed Iqbal) is grateful to Alexander von Humboldt-Stiftung, West Germany, for the second term award. Thanks are also due to Prof. Dr. W. Deppert and Dr. M. Staufenbiel of Biochemistry Department, Ulm University, for their help in the preparation of antitubulin antibodies and laboratory facilities for immunoprecipitation experiments.

References

Bird, R.C. and A.M. Zimmermann. 1980. Induction of tubulin synthesis during cilia regeneration in growing *Tetrahymena. Experimental Cell Research,* 128: 199-205.

Bonner, W.M. and R.A. Laskey. 1974. A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem,* 46: 83-88.

Gealt, M.A. and D.P. Weeks. 1980. Tubulin synthesis in a temperature sensitive mutant of *Chlamydomonas reinhardii. Experimental Cell Research,* 127: 329-339.

Hart, J.W. and D.D. Sabnis. 1973. Colchicine binding protein from phloem and xylem of a higher plant. *Planta,* 109: 147-152.

Hart, J.W. and D.D. Sabnis. 1976 a. Binding of colchicine and lumicolchicine to components in plant extracts. *Phytochemistry,* 15: 1897-1901.

Hart, J.W. and D.D. Sabnis. 1976 b. Colchicine binding activity in extracts of higher plants. *Journal of Experimental Botany,* 27: 1353-1360.

Hepler, P.K. and B.A. Palevitz. 1974. Microtubules and microfilaments. *Ann. Rev. Pl. Physiol.,* 25: 309-362.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London), 227: 680-685.

Lefebvre, P.A., C.D. Silflow, E.D. Wieben and J.L. Rosenbaum. 1980. Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella. *Cell,* 20: 469-477.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem., 193:*
Folin-phenol reagent. *J. Biol. Chem.,* 193: 265-275.

Luduena, R.F., G.M. Myles and T.A. Pfeffer. 1980. Isolation and partial characterisation of flagellar tubulin from *Marsilea vestita. Expt. Cell. Res.,* 130: 455-459.

Mizuno, K., M. Koyama and H. Shibaoka. 1981. Isolation of plant tubulin from Azuki bean epicotyls by ethyl-N-phenylcarbamatesepharose affinity chromatography. *J. Biochem.*, 89: 329-332.

Mohr, H. 1966. Die Abhangigkeit des protonema Wachstums und der Protonema polaritat bei Farnen vom Licht. *Planta*, 47: 127-158.

Rubin, R.W. and E.H. Cousins. 1976. Isolation of a tubulin like protein from *Phaseolus. Phytochemistry*, 15: 1837-1839.

Schraudolf, H. 1966. Die wirkung von Phytohormonen auf Keimung und Entwicklung von Farnprothallien. II. Analyse der Wechselbeziehung zwischen Gibberellinkonzentration, Antheridienbildung and physiologishen Alter der Prothalliumzellen von *Anemia phyllitidis. Planta* (Berl.), 68: 335-352.

Schwyzer, M. 1977. Purification of SV 40 T-antigen by immunoaffinity chromatography on Staphylococcal protein A-sepharose. *Colloq. INSERM*, 69: 63-68.

Shelanski, M.D., F. Gaskin and C.R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Nat. Acad. Sci.* (USA), 70: 765-768.

Silflow, C.D. and J.L. Rosenbaum. 1981. Multiple α and β tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell*, 24: 81-88.

Weber, K. and M. Osborn, 1969. The reliability of molecular weight determinations by dodecylsulphate-polyacrylamide gel electrophoresis. *J. Biol. Chem.*, 244: 4406-4412.

Weeks, D.P. and P.S. Collis, 1976. Induction of microtubule protein synthesis in *Chlamydomonas reinhardii* during flagellar regeneration. *Cell.* 9: 15 27.

Weisenberg, R C. 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. *Science*, 177: 1104-1105.