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AMINO ACID COMPOSITION AND PROTEOLYTIC GENERATED DOMAINS OF HIGHER PLANT TUBULIN

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Summary : The molecular architecture of tubulin from higher plant remains unknown. In this report we have made an attempt to identify higher plant tubulin domains using total and limited proteolysis of Haemanthus endosperm tubulin. The tubulin was previously purified and characterized (Picquot and Lambert 1988). The amino acid composition revealed a high content of basic residues, such as arginine and lysine. Tubulin domains were probed by tryptic and chymotryptic cleavage and analyzed by immunoblotting using specific monoclonal antibodies against alpha or beta subunits. These data shed light on specific properties of the higher plant tubulin.

Tubulin is one of the most conserved protein during evolution. This protein consists of two similar monomers, alpha and beta each having a molecular weight of 50 kDa. Alpha-beta dimers polymerise into microtubules. In the living cell, microtubules are involved in chromosome transport during cell division, cell wall formation in higher plants, and intracytoplasmic movement of organelles as reviewed in **(1)**. Plant tubulin is distinguished from neurotubulin in its alkaloïd binding characteristics **(2)**. Plant microtubules are resistant to colchicine and vinblastine while animal microtubules are very sensitive **(3)**. This indicates important physiological variability between animal and plant tubulins. Data concerning plant tubulin are still incomplete mainly due to difficulties encoutered in its isolation. Almost no report is yet available concerning the amino acid composition and molecular architecture of higher plant tubulin. It is in this background that we present here results on amino acid composition of higher plant tubulin. We used Haemanthus (Monocotyledons) endosperm tubulin, purified from young fruits as described previously **(4)**. This protein **(4)** is highly homologous to other higher plant tubulin such as the tubulin obtained from coleoptyls **(5)** or derived from plant cell culture **(2,5)**.

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This manuscript deals with three essential analyses : (I) the determination of amino acid composition, (II) total cleavage by trypsin followed by analysis of the amino acid content of the tryptic peptides, and (III) a combination of limited proteolysis (using trypsin and chymotrypsin) and immunoblotting (using monoclonal antibodies specific for alpha or beta subunit). This allowed us to study the domain structure of endosperm tubulin with due comparison with neurotubulin.

MATERIALS AND METHODS

Tubulin extraction

Tubulin was extracted from endosperm cells of Haemanthus Katherinae Bak. and Clivia nobilis Lindl., as described previously **(4)**. The crude extract (100,000g supernatant) is fractionnated by ion-exchange chromatography on DEAE Sephadex A50. The tubulin fraction is then concentrated by ammonium sulfate precipitation. After dialysis and centrifugation of the dialysate, tubulin dimers are able to polymerise into microtubules in vitro, using Taxol as stabilizing agent.

Total tryptic proteolysis

The plant endosperm tubuline (alpha - beta dimers) was cleaved by trypsin. For this, the protein was reduced, aminoethylated, and citraconylated using the methods described in **(6)** and **(7)** which were modified and adapted to microquantities. The so modified protein was desalted by gel filtration on Sephadex G25 with 0.1 M ammonium bicarbonate as eluent and recovered by lyophilisation. The modified tubulin was then subjected to tryptic hydrolysis in 0.1 M ammonium bicarbonate at 37°C for 3 hours. The tubulin/trypsin ratio was 50:1 (w/w). The action of protease was stopped by freeze-drying of sample. The tryptic peptides were separated, after decitraconylation on a HPLC apparatus using a reverse phase column (Waters C18 u-Bondapack 3.9 X 300 mm, at a flow rate of 1.2 ml/min) in an acetonitryl gradient (0-60%). Because of the large number of tryptic fragments obtained, a second separation was performed on the same column in an isocratic gradient.

Amino-acid composition

The amino-acid composition was performed on a HPLC apparatus, using precolumn derivatization procedure with phenylisothiocyanate. Tryptic peptides or endosperm tubulin (alpha-beta dimers) were previously hydrolysed with HCl 5.7 N at 110°C. In the case of endosperm tubulin, cystein was not determined, and results were expressed as residues per dimer.

Limited proteolytic digestion of plant endosperm tubulin as compared to neurotubulin

Haemanthus endosperm tubulin was digested with trypsin and chymotrypsin at room temperature (20-22°C) for up to 30 min. The reaction was stopped by addition of 2 mM-PMSF. Enzyme/tubulin molar ratio was 1/50, 1/25, 1/12. Similar digestion was done using purified pig brain tubulin (tubulin dimers) obtained by phosphocellulose chromatography (PC-tubulin) according to **(8)**.

Proteolytic fragments were separated by SDS-PAGE using 12.5 % polyacrylamide, 0.1 % SDS gel. After electrophoresis one portion of the gell was silver-stained, the other part was transferred onto nitrocellulose according to **(9)**. The nitrocellulose sheet was soaked in 5 % BSA in TBS, and incubated with monoclonal antibodies to alpha and to beta chickbrain tubulin (Amersham 356 and 357) at a dilution of 1/1000 in TBS/ 0,1 % - BSA buffer.

The first antibody was detected by a second antibody conjugated with colloidal gold, and silver enhanced (Auroprobe BL plus GAM IgG and IntenSE II Kit, Janssen Pharamaceutica).

RESULTS AND DISCUSSION

Amino-acid composition of Haemanthus endosperm tubulin

Haemanthus endosperm tubulin was purified as described in Material and Methods. Purity of the tubulin is shown **Fig. 1.** Results presented here correspond to the total tubulin (alpha + beta) fraction. Attempt to obtain separate alpha and beta subunits by electroelution did not met with success since enough material could not be made available, and therefore analysis of individual alpha, beta amino acid composition could not be possible. As shown **table 1**, the higher plant tubulin is characterized by rich amino acids content as lysine and arginine, and in this respect it is distinct from neurotubulin. Lower content of threonine and methionine was observed in plant tubulin, however, further investigations are needed as these two residues can be underestimated by the technique. The differences that we found between higher plant tubulin and neurotubulin are summarized in **Fig. 2.** These data are the first attempt to study amino-acid composition of higher plant tubulin. On the SDS-PAGE electrophoresis, the higher plant alpha tubulin migrates faster than alpha neurotubulin **(4, 5)**. Furthermore the alpha subunit of plant tubulin also migrates faster than beta subunit of neurotubulin. Such a pattern of electrophoretic mobility may be partly attributed to the high content in basic residues in plant tubulin. As non polar residues are of comparable amount, the

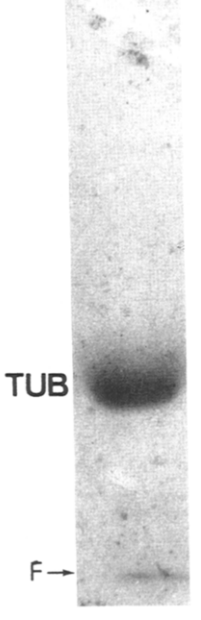

Figure 1 : SDS-PAGE loaded with a large amount (24ug) of plant endosperm tubulin used for amino-acid determination : therefore and are not resolved. The gel was stained with Coomassie blue. No other band than tubulin are detectable. **F** : dye front ; **TUB** : tubulin.

Table 1 : Amino-acid composition of plant endosperm tubulin compared to neurotubulin : the amino-acid composition of pig brain tubulin was deduced form the sequence published by Ponstingl (1981). The relative difference (%) between the two tubulins is calculated for each amino-acid, using pig brain tubulin as a standard (endosperm tubuline - pig brain tubulin)/pig brain tubulin X 100. ASx and GLx represent respectively ASP + ASN and GLU + GLN, which were not differentiated by the technique.

AMINO ACIDS	NUMBER OF RESIDUES		RELATIVE DIFFERENCE (%)
	pig brain tubulin	Haemanthus albumen tubulin	
ASx	92	92	0
GLx	111	97	12,6
SER	50	57	14
GLY	72	85	18
HIS	23	26	13
ARG	43	75	74
THR	59	42	28,8
ALA	65	74	13,8
PRO	40	49	22,5
TYR	35	29	17
VAL	65	57	12,3
MET	28	12	57,4
CYS	20	ND	ND
ILE	45	42	6,6
LEU	63	69	9,5
PHE	43	34	21
TRP	44	ND	ND
LYS	34	57	67,6

increase of basicity could result in enhanced hydrophilic properties of intracellular plant microtubules.

Tubulin domains

In the absence of complete amino-acid sequence data of higher plant tubulin, we have compared neurotubulin and endosperm tubulin after proteolytic cleavage in order to define higher plant tubulin domains. Peptides were generated by tryptic digestion of the total tubulin fraction. After separation

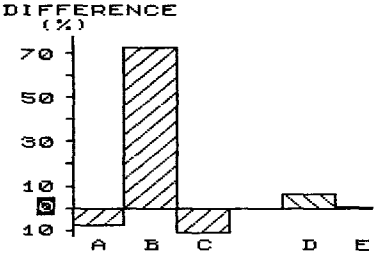

Figure 2 : Relative difference between pig brain tubulin and plant endosperm tubulin for 5 amino-acid families. Difference (%) = ((pig brain tubulin - endosperm tubulin) / pig brain tubulin) X 100. **A** : acidic residues ; **B** : basic residues ; **C** : alcohol residues ; **D** : polar residues ; **E** : non-polar residues. Importance of basic residues was the main difference between the two tubulins.

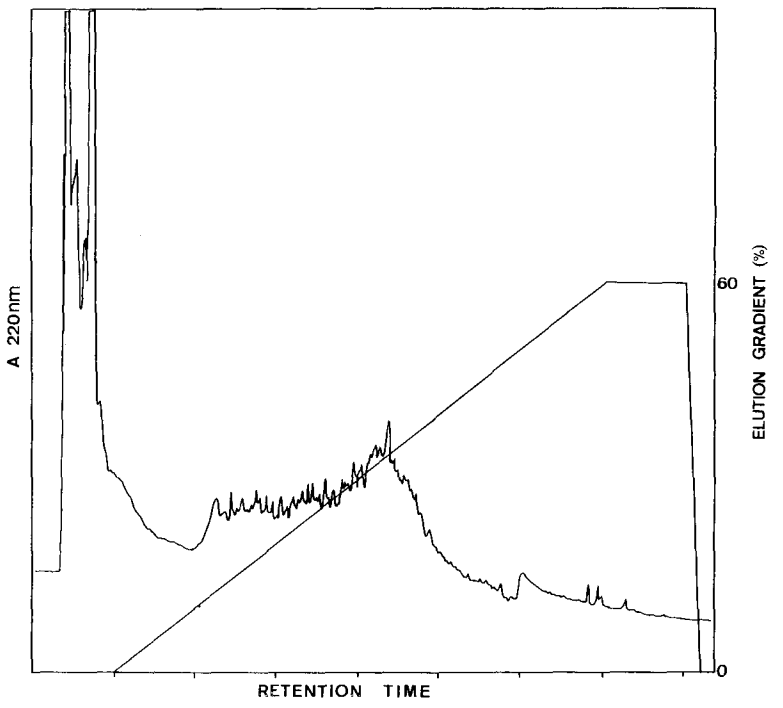

Figure 3 : Peptides of endosperm tubulin obtained by total proteolytic cleavage by trypsin. Separation is processed by HPLC reverse phase chromatography in an acetonitryl gradient (0-60%). Some of the peaks were further purified by reverse phase chromatography, and their amino-acid composition determined. (column : C18 u-Bondapack (Waters) 3.9 X 300 mm, Flow-rate : 1.2 ml/min).

by HPLC (**Fig. 3**), their amino-acid composition was determined. **Fig. 3** indicates a high quantity of peptides that could be related to the elevated level of arginine and lysine residues. Homology with neurotubulin domains was investigated using known neurotubulin sequences **(10,11)**. For each peptide, an homologous domain was searched on neurotubulin using a computer programm set up for this study. Conclusions are given in **Fig. 4.** Most of the homologous domains were found on the beta tubulin subunit, particularly in the regions 50-150 and 250-350 residues.

Limited proteolysis

Trypsin and chymotrypsin limited digestion of endosperm tubulin and neurotubulin was performed concomitantly. Results are given in **Fig. 5.** According to Mandelkow et al. **(12)**, peptides were analysed after Western-blotting using monoclonal antibodies to alpha or beta chicken brain tubulin (Amersham 356 and 357) that recognized the C-terminal region. Therefore, detected peptides (**Fig.5**) represent peptides that contain the C-terminal region. Under the same conditions, the higher plant endosperm tubulin is less sensitive than neurotubulin to the proteolytic cleavage, and

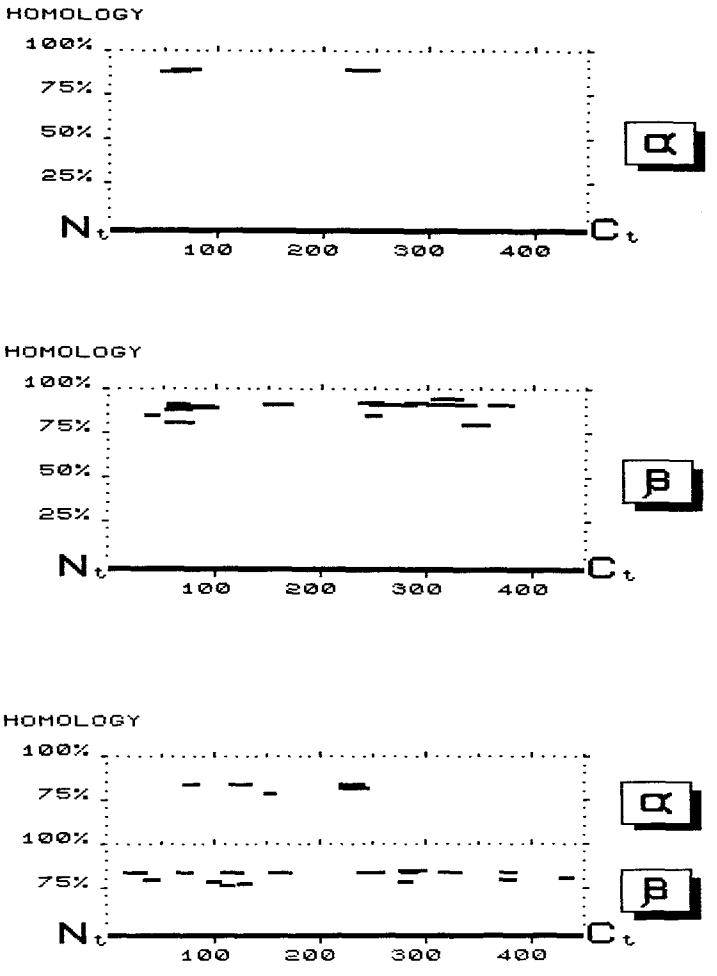

Figure 4 : Domains of pig brain tubulin showing an homology with tryptic peptides of endosperm tubulin : the homology is based on amino-acid composition, as explained in the text. The 2 upper graphs refer to peptides which show an homology restricted to one tubulin subunit, or . The lower graph concerns the peptides which have an homology with both subunits. On each grah, pig brain tubulin is represented as a straight bold line with N- and C- terminals indicated.

some of the peptides are only weakly labelled after immunoblotting. Data are summarized **Fig. 5** and **6.** Increased enzyme/tubulin ratio or incubation time, resulted in progressive loss of these peptides.

The **chymotryptic peptides** that contain the C-terminal region are comparable for neuro and plant tubulins, particularly for the alpha subunit, where similar peptides are found (41, 33 kDa). The plant beta subunit is less sensitive : one 21 kDa neuropeptide is only weakly detected in plant tubulin, while 3 peptides are seen between 33 and 43 kDa in both cases as a result of proteolysis.

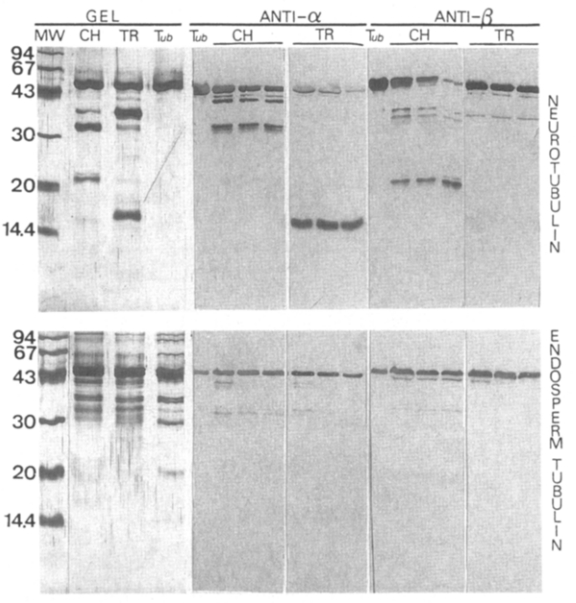

Figure 5 : Partial enzymatic cleavage of plant endosperm tubulin with chymotrypsin and trypsin : comparison with neurotubulin. After SDS-PAGE, part of the gel was stained with silver nitrate (GEL), the remaineder was electrotransfered onto nitrocellulose and probed with antibodies. The peptides containing the C-terminal domain are revealed using monoclonal antibodies specific for alpha (anti-) or (anti-) tubulin subunit. **MW** : molecular weight markers ; **Tub** : non digested tubulin ; **CH** : tubulin hydrolysed with chymotrypsin ; **TR** : tubulin hydrolyzed with trypsin. Neurotubulin and endosperm tubulin are indicated on the right.

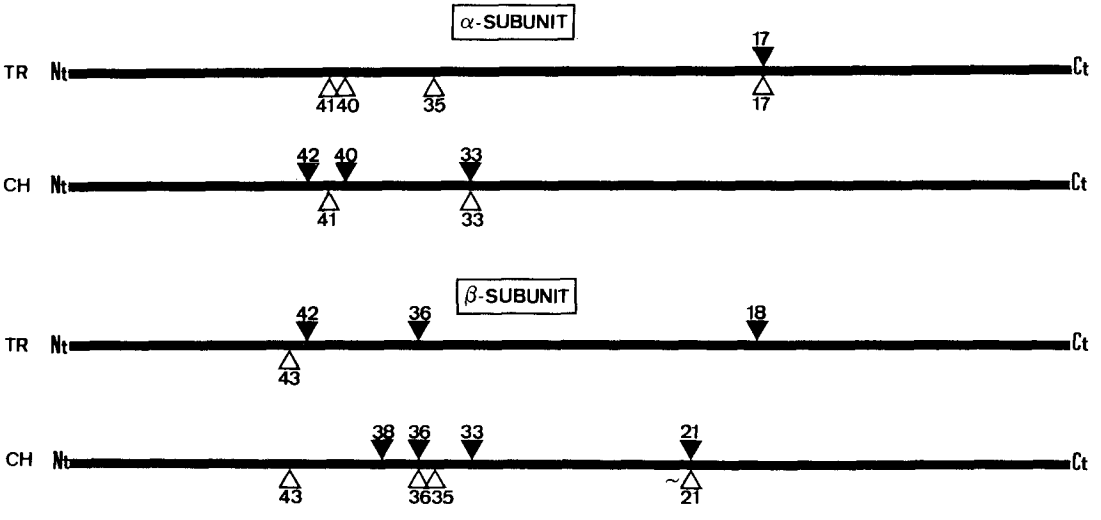

Figure 6 : Chymotrypsin and trypsin cleavage sites as determined by limited proteolysis followed by immunoblotting (the corresponding immuno-blots are shown in **Fig. 5**). **TR** : trypsin cleavage sites ; **CH** : chymotrypsin cleavage sites. : neurotubulin . : endosperm tubulin. For each cleavage site, the molecular weight of the resulting C-terminal peptide is indicated in kDa. Numbers : molecular weight in kDa.

Differences are obtained using **tryptic digestion.** For the alpha neurotubulin only one peptide of 17 kDa was obtained that is hardly detected in plant alpha tubulin, while 3 peptides of higher molecular weight (41, 40 and 35 kDa) are produced from endosperm tubulin. For beta subunits, one fragment only is obtained from plant beta tubulin (42 kDa) and this fragment is found common to neurotubulin, while 2 others (36 and 18 kDa) from neurotubulin are not detected in plant tubulin.

These data indicate that chymotrypsin cleavage sites are mostly common between neurotubulin and Haemanthus endosperm tubulin, while trypsin cleavage sites are highly different. The major drawback is the lower amount of peptide that is obtained with plant tubulin.

These observations provide novel informations on the structure of the higher plant tubulin. As endosperm tubulin was shown to be highly homologous to other plant tubulins **(4)**, the present data point out certain characteristics that could be directly related to specific microtubule functions in the living cell of higher plants.

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