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SEPARATION OF TUBULIN SUBUNITS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

When properly solubilized with trifluoroacetic acid (TFA), α- and β-tubulin subunits from a variety of sources may be resolved at high yield by reversed-phase high-performance liquid chromatography (HPLC), using a Waters μBondapak C_{18} column and simple linear aqueous acetonitrile gradients containing TFA. The tubulin subunits are typically the most non-polar proteins present, with the β-tubulin subunit eluting before the α. Column temperatures above ambient improve both the resolution and the yield; less polar solvent systems do not. Tubulins not freely soluble in aqueous TFA may be solubilized in 6 *M* guanidine–hydrochloric acid with no change in retention time. Other columns with shorter carbon chain lengths and larger pore size produce a single, unresolved tubulin peak. Reversed-phase HPLC analysis provides an independent comparative evaluation of organelle-specific tubulins, with characteristic retention time differences observed between homologous ciliary and flagellar outer doublet tubulin subunits and also between them and their cytoplasmic counterparts.

INTRODUCTION

Tubulin, the major structural protein that forms microtubules, is a heterodimer of two distinct but related subunits[1–3]. Named originally with reference to their relative electrophoretic migration on sodium dodecylsulfate (SDS)-polyacrylamide gels, α- and β-tubulin have essentially the same molecular weight but bind SDS differentially. The relative migration rates are a function of the alkyl chain composition of commercial SDS and also depend on whether urea is present during electrophoresis[4,5]. Some striking examples of tubulin variants detectable by SDS-polyacrylamide gel electrophoresis (PAGE) have been reported, for example a testis-specific β-tubulin has been detected in *Drosophila*[6] while a chick erythrocyte β-tubulin is easily separable from its brain tubulin counterpart[7]. In both cases, these represent distinct gene products. Numerous isoelectric variants also have been reported, for example seventeen distinct brain tubulins can be resolved[8] and the specific acetylation of cytoplasmic α-tubulin, leading to a distinct isotype, occurs before incorporation of the tubulin into *Chlamydomonas* flagella[9]. Differences in hydrophobicity between the two tubulin subunits serve as a basis for their separation in Triton–acid–urea gels while

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charge differences allow further isotype separation[10]. On the other hand, homologous tubulin subunits isolated from sea urchin sperm flagella, embryonic cilia, and mitotic cytoplasm are not distinguishable by SDS-PAGE, isoelectric focussing, or Triton–acid–urea gels. However, two-dimensional peptide mapping of these same tubulin subunits, obtained by morphologic fractionation and electrophoretic purification, shows major organelle-specific differences, not easily interpretable in terms of single post-translational modifications[11]. Supporting this observation of organelle-specific tubulins in sea urchin is the fact that nearly a dozen genes for each tubulin subunit have been documented[12].

The presence of local regions of difference among various organellar tubulin subunits, mainly in the form of highly conserved hydrophobic substitutions[11], led to the present study in which reversed-phase high-performance liquid chromatography (RP-HPLC) was explored as a method for resolving α- and β-tubulin quickly and in high yield, with the additional hope of detecting organelle-specific chain types. The general methodology was suggested by the successful resolution of a hemoglobin variant containing the silent, neutral substitution of alanine for valine[13]. A brief report of this approach applied to sea urchin tubulins has appeared in abstract form[14].

EXPERIMENTAL

Protein fractions

Sea urchin sperm flagella and the outer doublet microtubule and central pair fractions thereof, embryonic cilia, and egg cytoplasmic tubulin–vinblastine crystals were prepared as described previously[11,15], using *Strongylocentrotus droebachiensis* (Maine) and *Tripneustes gratilla* (Hawaii). Scallop sperm flagella, the outer doublet and central pair fractions thereof, and gill cilia were prepared by the methods of Linck[16] and Stephens[17], using *Aequipecten irradians* (Cape Cod) and *Placopecten magellanicus* (Maine). The flagellar and ciliary axonemes, fractions thereof, or tubulin-vinblastine crystals were either used directly or stored at −20°C in glycerol–water (50:50) containing 1.5 m*M* magnesium chloride, and 15 m*M* Tris–HCl (pH 8.0).

Sea urchin cytoplasmic tubulin was also prepared from unfertilized eggs of *S. droebachiensis* by two cycles of *in vitro* polymerization, using the controlled pH and 0°–24°C temperature method of Suprenant and Marsh[18]. Elasmobranch tubulin was prepared by two cycles of *in vitro* polymerization from brains of the skate *Raja erinacea* using the method of Langford[19]. Recycled bovine brain tubulin was obtained from Dr. Mitchison. Egg and brain tubulins were frozen in liquid nitrogen as high speed pellets and stored at −80°C until use.

Before use, axonemes in glycerol were recovered by centrifugation after 1:10 dilution with 10 m*M* Tris–HCl (pH 8.0). In some cases, glycerol was dialysed away from glycerinated fractions using 1 m*M* Tris–HCl (pH 8.0), 0.1 m*M* EDTA, and 0.25% mercaptoethanol. Freshly isolated axoneme and outer doublet microtubule pellets or fresh or frozen cytoplasmic tubulin pellets were suspended in distilled water. In all cases, trifluoroacetic acid (TFA) was added to a final concentration of 0.2–0.3%. Some samples were solubilized by dialysis against or suspension in 6 *M* guanidine–HCl, 10 m*M* dithiothreitol (DTT), and 0.2% TFA. The samples were centrifuged at 45 000 *g* for 15 min before use. Additional details concerning solubilization are found in the Discussion section.

Reversed-phase HPLC

A Waters Assoc. high-performance liquid chromatography system consisted of two Model 510 pumps, U6K manual sample injector, Model 721 programmable system controller, Model 441 UV (215 nm) monitor, Model 730 data module, and a column heater. The major column used was a Waters μBondapak C_{18} (30 × 0.39 cm; 125 Å pore size; 10 μm particle size) equipped with a guard column of the same material. Also used were Beckman Ultrasphere Octyl (C_8, 25 × 0.46 cm, 80 Å pore size, 5 μm particle size) and Ultrapore RPSC (C_3, 7.5 × 0.46 cm, 300 Å pore size, 5 μm particle size) columns. The solvents routinely used were 0.1% aq. TFA–acetonitrile (70:30) (solvent A) and 0.1% aq. TFA–acetonitrile (40:60) (solvent B). Runs were generally done at 40°C. To dissociate aggregated material from the column, 1.0 ml of dimethylsulfoxide (DMSO) was injected onto the column[20], followed by an acetonitrile gradient. Columns were considered clean when no "ghost" bands were detected. Fractions were collected at 1- or 2-min intervals and then evaporated to dryness in a Savant Instruments "Speed-Vac" vacuum centrifuge concentrator equipped with both a water aspirator unit and a high vacuum cold trap.

SDS-PAGE

The discontinuous, SDS-containing system of Laemmli[21] was used with 1.5 mm thick × 10 cm long slab gels having a 5 to 15% acrylamide gradient. The SDS was obtained from Sigma and was chosen for its ability to resolve tubulin subunits maximally. Gels were equilibrium-stained with Coomassie Blue (Serva) by the method of Fairbanks *et al.*[22].

RESULTS

Systematic variations

Primary separation conditions. Sea urchin sperm flagellar tubulin, representing approximately two-thirds of the protein in the organelle, is freely soluble below pH 2.5[23]. When sea urchin axonemes are suspended in 0.2–0.3% aq. TFA, more than 85% of the total protein is rapidly solubilized; higher TFA generally decreases the degree of solubilization. Observations in the analytical ultracentrifuge indicate a major peak sedimenting at 2–3 S, characteristic of monomeric tubulin (data not shown).

With these facts in mind, the total protein from *S. droebachiensis* flagellar 9 + 2 axonemes, solubilized in 0.3% aq. TFA, was injected onto a C_{18} column and eluted with a linear gradient of 0 to 60% acetonitrile in 0.2% aq. TFA. Fig. 1 represents an initial survey to effect an RP-HPLC separation of the tubulin subunits. Minor proteins begin to elute when the solvent composition exceeds 30% acetonitrile. Near the end of the gradient (>45% acetonitrile), two major equimolar peaks elute at 45.2 and 46.9 min. Using raw figures from the integrator, these together account for 71% of the total, almost exactly as expected for tubulin. SDS-PAGE identifies these peaks as tubulin, the first being the β and the second the α subunit (see below). In the example shown, direct protein measurement demonstrates that 78% of the injected protein is recovered in the eluate.

Optimization and solvent system variations. By stepping the gradient up to 30% acetonitrile in 10 min, creating a more shallow linear gradient for protein elution, the tubulin subunits are further separated, eluting at 40.8 and 43.7 min. Using such

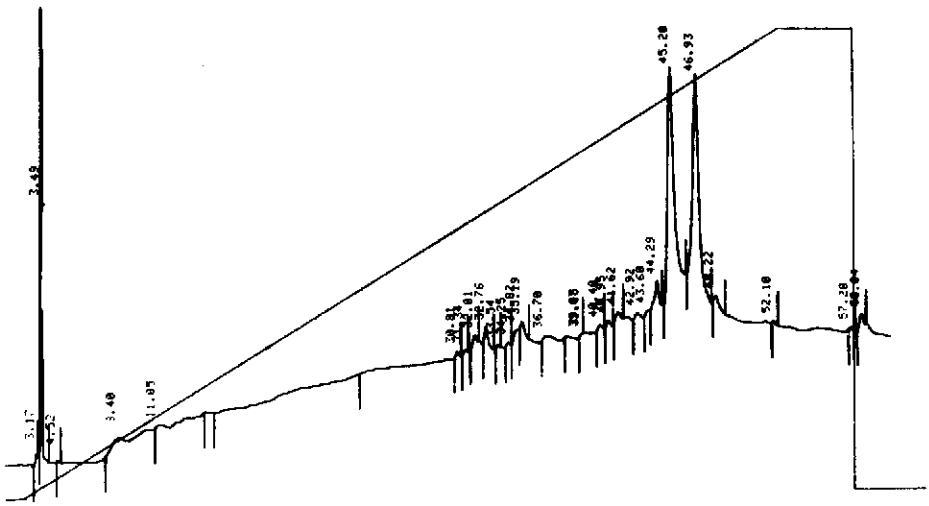

Fig. 1. Elution of sea urchin (*S. droebachiensis*) flagellar tubulin on a μBondapak C_{18} column using a linear 0 to 60% acetonitrile in 0.2% aq. TFA gradient in 50 min, constant 60% acetonitrile for 10 min, 1.0 ml/min, 40°C. Full-scale = 0.2 optical density. The two equal peaks eluting at 45.2 and 46.9 min are, respectively, β- and α-tubulin (25 μg total protein).

a gradient profile as a standard for comparison, the effects of ternary solvent composition on subunit separation and yield may be evaluated. For example, Power *et al.*[20] explored continuously-variable selective elution, based on acetonitrile–propanol ratios, to improve the resolution of subunits of yeast cytochrome *c* oxidase. Similar to the results of Power *et al.*[20], the presence of 2-propanol initially results in longer protein retention on the column, the tubulins eluting in 44.1 and 46.1 min with 25% 2-propanol. As the amount of 2-propanol is increased further, retention times become earlier than with pure acetonitrile (39.7 and 41.3 min at 50%; 32.0 and 33.3 min at 75%). Although the yield of tubulin approaches 100% with 75% 2-propanol, the resolution is decreased. With pure 2-propanol, the subunits are barely resolved at 27.3 and 27.9 min. Since possible ion-pair binding differences between the tubulin subunits might influence retention differentially, a 1:1 mixture of triethylamine (TEA) and TFA, at 0.1% each, was also tested (*cf.* ref. 20). This modification results in even less separation between the tubulin subunits, although tubulin yield is typically >90% over the entire acetonitrile–2-propanol range.

Various minor proteins shift systematically with respect to the tubulin subunits and hence their resolution can be improved with these variations. However, it is evident that the simple water–acetonitrile–TFA system can produce optimal separation of tubulin subunits. All further comparisons were made using it, with the trivial modifications that the TFA concentration was reduced to 0.1% to minimize solvent absorbance and the gradient was stepped rapidly from either 0 or 30% acetonitrile to various intermediate levels to generate final linear elution profiles of variable slope. As an example of these various principles, Fig. 2A illustrates a shallow, stepped linear gradient, designed both to separate the tubulins from one another and to maximize their separation from the minor proteins associated with the sperm flagellar axoneme.

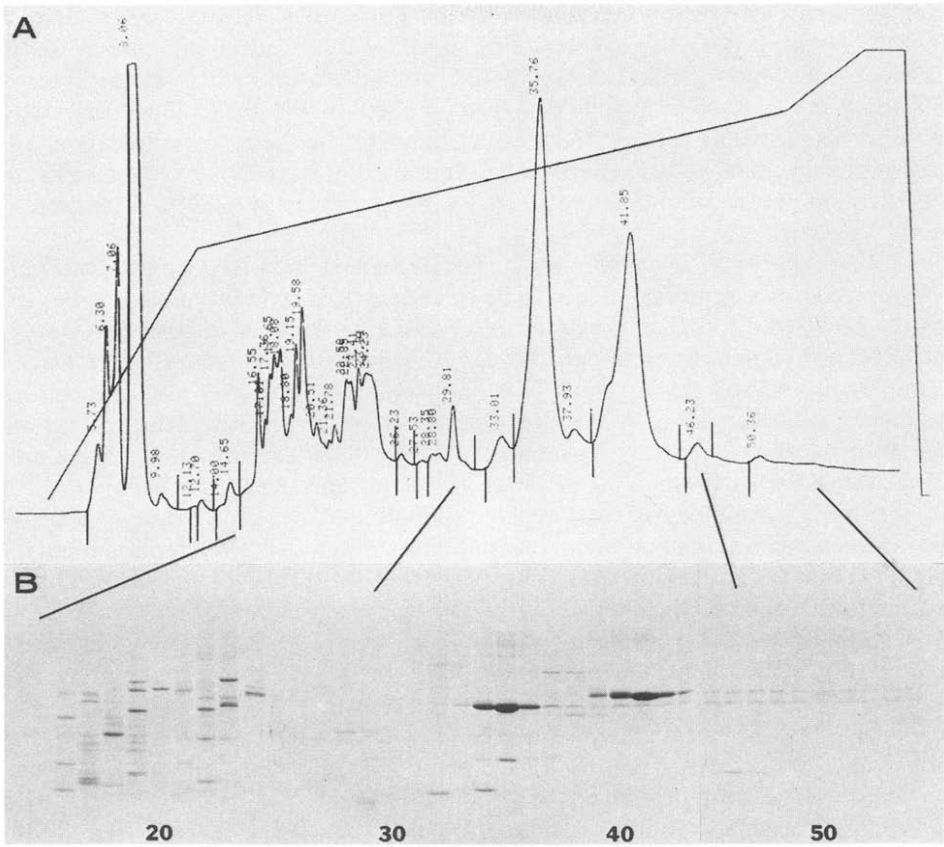

Fig. 2. High-resolution separation of sea urchin outer doublet tubulin. (A) HPLC profile: solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA; gradient: 0 to 55% B in 10 min, 55 to 85% B in 40 min, 85 to 100% B in 8 min, 100% B for 3 min, 1.0 ml/min. Full-scale = 0.5 O.D. (B) SDS-PAGE analysis: forty 1-min fractions from 15 to 55 min.

SDS-PAGE (Fig. 2B) indicates that the tubulin subunits, separated by over 6 min, each can be obtained in 1-ml fractions at >90% purity, >60% yield, all within 45 min. In fact, one can halve the running time, at the same flow-rate, with only a slight increase in tubulin trailing but a yield of >90%.

Effects of reduction and alkylation. Alkylation of the cysteines of sea urchin flagellar tubulin subunits (7 in β-, 9 in α-tubulin[11]) by reaction with iodoacetic acid generates the carboxymethyl derivative which interacts even more anomalously with SDS to produce striking subunit separations. To determine if carboxymethylation of tubulin would improve subunit resolution by HPLC, axonemal proteins were reduced and alkylated, dissolved in 0.2% aq. TFA, and analyzed in parallel with underivatized samples. After reduction and alkylation, both subunits elute earlier; the β subunit by 14.6% and the α by 6.8%. The consequence of this is that the time interval between the two peaks increases by nearly 50%. In parallel, the tubulins from scallop (*A. irradians*) cilia were similarly derivatized. In this case the β- and α-tubulin subunits (which

average eleven cysteines apiece[17]) elute 12.7 and 4.3% earlier, respectively, resulting in an 89% increase in peak-to-peak separation time. Each derivatized subunit is rendered relatively more hydrophilic but not by an amount related to its cysteine content. Offsetting the increase in relative resolution is the fact that the yield of derivatized tubulins in aqueous TFA is generally compromised by the chemical modification. The yield of carboxy-methylated sea urchin flagellar tubulins is relatively unaffected by the modification but the yield of scallop ciliary tubulins, normally >80%, is reduced by more than half.

Solubilization in guanidine–HCl. The reduction and alkylation procedure typically renders tubulin insoluble in water since denaturing conditions need to be used for the reduction step. Total solubilization in 0.2% aq. TFA is not always easily accomplished. Similarly, although the underivatized tubulins studied here as test examples are soluble in aqueous TFA, not all native tubulins are (see below). A number of workers have found that highly associating proteins will chromatograph well after solubilization in 6 *M* guanidine–HCl, perhaps the most striking example being the RP-HPLC analysis of total ribosomal proteins by Ferris *et al.*[24]. Consequently, guanidine–HCl was used to solubilize total axonemal proteins and also the reduced and alkylated tubulins. Under all conditions tested, the retention times for tubulin subunits dissolved in 6 *M* guanidine–HCl, derivatized or unmodified, were indistinguishable from their respective counterparts solubilized directly with TFA. Most minor proteins were also unaffected both in terms of retention times and relative amount (Fig. 3). Solubilization in guanidine–HCl will increase the yield of reduced and alkylated tubulins when these are poorly soluble in aqueous TFA alone, but guanidine–HCl solubilization will also generally decrease the yield of the unmodified protein such that comparable final yields are obtained for both, probably reflecting simple irreversible denaturation on the column irrespective of derivatization. The yield of sea urchin flagellar tubulin solubilized in guanidine–HCl is generally reduced to <50% while that of scallop ciliary tubulin is relatively unaffected, *i.e.* the converse of the carboxymethylation results noted above.

Temperature effects. To explore the effects of column temperature on tubulin subunit resolution and yield, the relatively temperature-sensitive flagellar tubulin from the sea urchin *S. droebachiensis* was compared with the more stable ciliary tubulin from the bay scallop *A. irradians*. The results are given in Table I. Increased temperature results in shorter overall retention times but an increase in both resolution and yield up to 50°C in the case of sea urchin tubulin and 60°C in bay scallop, above which both resolution and yield deteriorate and trailing increases. These apparent temperature optima are each 25–30°C above the maximum temperature at which these organisms can survive and approximate the temperature above which the respective tubulins will denature at neutrality.

Column variations. Limited by cost, a systematic study of various columns was not attempted but two frequently used and suggested columns were compared with the Waters C_{18} μBondapak. A popular shorter chain length C_8 column, the Beckman Ultrasphere Octyl, was expected to be less retentive. Both columns were run at 25°C, with a 0 to 60% gradient over 1 h. The C_8 column retained flagellar tubulin 27% longer than the μBondapak, the two subunits were not resolved, the single tubulin peak trailed badly, and earlier eluting peaks were less sharp (data not shown). No attempt was made to further optimize conditions. An Altex RPSC column was used to

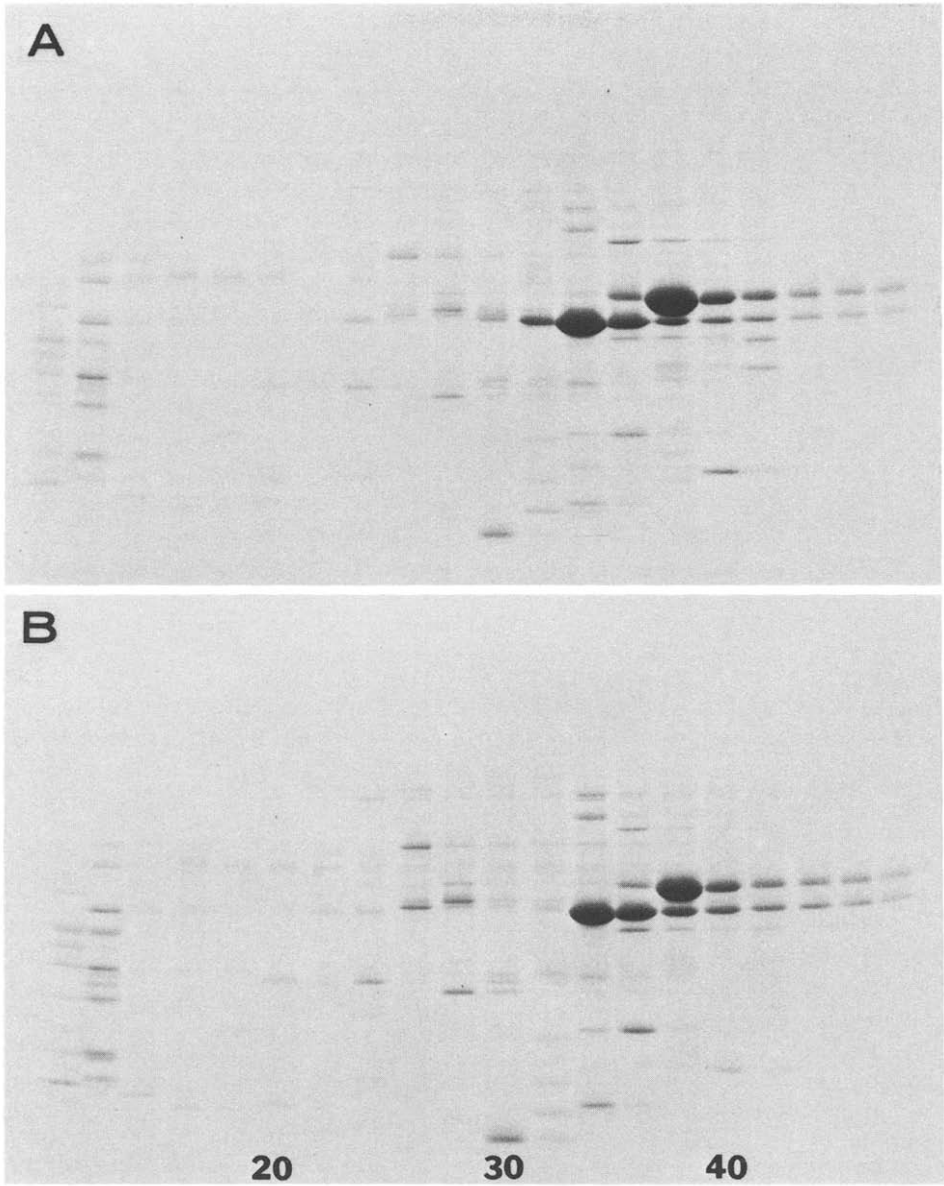

Fig. 3. The effect of guanidine–HCl solubilization on retention and yield. (A) Sea urchin flagellar proteins solubilized with 0.2% aq. TFA alone. Solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA. Gradient: 0 to 20% solvent B in 5 min, 20 to 100% B in 45 min, constant solvent B for 10 min. SDS-PAGE analysis of 2-ml fractions from 10–50 min. (B) Same, but solubilized in an equivalent amount of 6 *M* guanidine–HCl, 10 m*M* dithiothreitol, 0.2% TFA.

advantage over other columns, including the μBondapak, by Ferris *et al.*[24] in their ribosomal protein study cited above. The Ultrapore RPSC column has a substantially shorter chain length (C_3) and larger pore size (300 Å) than the μBondapak. Gradient

TABLE I

EFFECT OF TEMPERATURE ON TUBULIN SUBUNIT RESOLUTION

Solvent A = 30% acetonitrile, solvent B = 60% acetonitrile. Gradient: 0 to 20% B in 5 min, 20 to 100% B in 45 min, constant solvent B for 10 min.

Temperature (°C)	*Retention time*			*Relative tubulin yield (%)*
	α	*β*	*Difference*	
S. droebachiensis flagellar axonemes				
30	36.61	39.83	3.22	83
40	35.36	38.71	3.35	95
50	33.81	37.16	3.35	100
60	32.16	35.46	3.30	99
70	30.71	33.91	3.20	90
A. irradians ciliary axonemes				
30	37.05	39.33	2.28	56
40	36.00	38.40	2.40	71
50	34.36	36.98	2.62	86
60	32.20	34.93	2.73	100
70	30.30	32.88	2.58	85

and flow conditions were designed so that the two columns could be compared directly (Fig. 4). The Ultrapore RPSC yields a very sharp tubulin peak eluting well away from its nearest neighbors but the two subunits are not resolved. In comparison with the μBondapak, there is essentially no trailing. In the example shown, the relative yield of tubulin from the RPSC column operating at 25°C approached 100% while that of the μBondapak was 57% at 25°C and 69% at 40°C.

Separation of characteristic α and β chains in organelle fractions

Flagellar, ciliary, and cytoplasmic tubulins. Using a common gradient profile, the flagellar, ciliary, and cytoplasmic tubulins from two species of sea urchin and the flagellar and ciliary tubulins from two species of scallop were compared. These were chosen since the pairs exist at relative extremes of the environmental temperature range and well-established methods already exist for tubulin isolation and subfractionation. In addition, the subunits of *S. droebachiensis* and *A. irradians* tubulins have been analyzed in terms of peptide maps and amino acid composition[11,17] while equimolar β chains have been identified in flagella of *T. gratilla* and cilia of *A. irradians*[10].

Central pair tubulin is solubilized by low ionic strength dialysis of flagellar axonemes but dialysis of cilia will solubilize the B-subfibers and one central pair member[11]. In the case of the flagella from the two sea urchin species and the one molluscan species tested, the central pair fraction appears unique in that it yields an early-eluting β subunit and little or no β-tubulin corresponding in elution time to that of the outer doublet. An SDS-PAGE analysis of this is shown in Fig. 5 where the total dialysed sample (A: outer doublet plus central pair) is compared with the central pair fraction (B). Similar results are obtained for the ciliary B-subfiber fraction from both sea urchin species but not for that from scallop cilia.

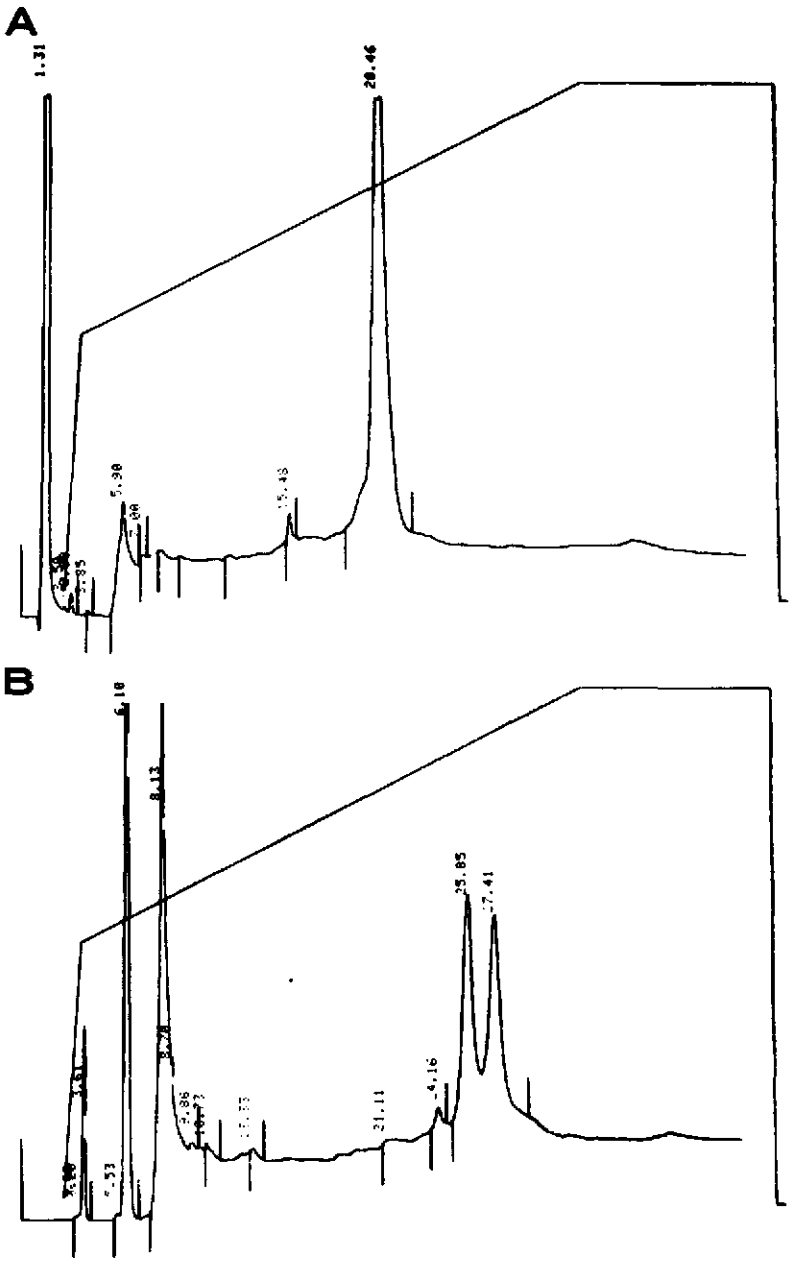

Fig. 4. Direct comparison of Ultrapore RPSC C_3 and μBondapak C_{18} columns. (A) Sea urchin outer doublet tubulin on an Altex Ultrapore RPSC C_3 column. Solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA. Gradient: 0 to 50% solvent B in 1 min, 50 to 100% solvent B in 30 min, constant solvent B for 10 min, 1.0 ml/min, 25°C. Full-scale = 0.2 O.D. (B) Same, but chromatography on a Waters μBondapak C_{18} column, 40°C.

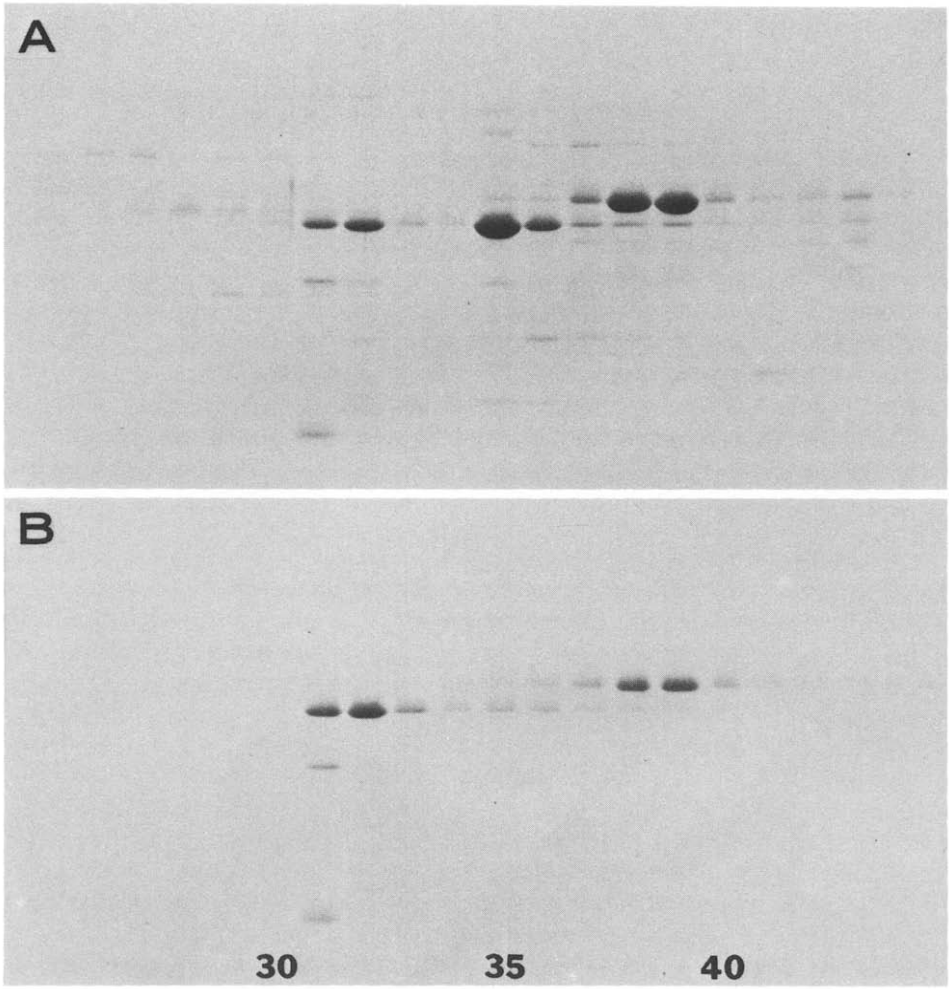

Fig. 5. SDS-PAGE analysis of sea urchin (*S. droebachiensis*) flagellar central pair tubulin fractionation. Gradient as defined in Fig. 3 and Table II. (A) Total axonemal proteins after 48 h dialysis against 1 m*M* Tris–HCl (pH 8), 0.1 m*M* EDTA; injected in aqueous TFA; fractions from 25 to 45 min. (B) The solubilized central pair fraction obtained by pelleting out the outer doublet microtubules from (A); the major β-tubulin elutes at 32 min.

The early-eluting β-tubulin is evidently a conformational variant that results from EDTA dialysis, based upon three independent arguments. First, no comparable amount of an early-eluting β-tubulin is detectable when undialyzed flagellar or ciliary axonemes are analyzed (*cf.* Fig. 3 or Fig. 6A). Second, when sea urchin flagellar central pair tubulin is prepared by direct high-salt extraction[23] or if ciliary B-subfiber is prepared by thermal fractionation[25], the β-tubulin elutes at the same retention time as that from the outer doublet or the A-subfiber, respectively. Finally, when either the central pair or B-subfiber fraction (containing the early-eluting β-tubulin) is injected in 6 *M* guanidine–HCl, only a normally-eluting β chain is seen after renaturation.

Nevertheless, such a variant still has some usefulness for comparative purposes.

Table II summarizes the relative retention times for the major and minor β and α chains for these various invertebrate tubulins and for brain tubulin as well. In addition, selected examples of the basic HPLC data illustrating important variations are shown in Fig. 6. In spite of the wide range of environmental temperatures that these organisms span, there is no clear relationship between growth temperature and tubulin HPLC elution time. The sperm flagella α chains of the cold-water sea urchin or scallop are more hydrophobic (elute later) than their warm-water counterparts but the reverse is true when one compares ciliary α chains. The two sea urchins have nearly identically eluting ciliary or flagellar β chain counterparts while the scallops do not.

In cases where cilia and flagella are compared directly, reproducible organelle-

TABLE II

COMPARATIVE RETENTION TIMES FOR TUBULINS FROM VARIOUS SOURCES

All runs at 40°C, solvent and gradient as in Table I, all intraspecies comparisons were made on the same column with the same solvent batch, values rounded to nearest 0.1 min, replicates reproducible within a S.D. of ± 0.04 min. Numbers in parentheses represent minor components.

	β	β	α
Sea urchin (S. droebachiensis)			
Sperm flagella	–	35.4	38.7
Outer doublet fraction	–	35.4	38.7
Central pair fraction	31.8	(35.4)	38.7
Embryonic cilia	–	35.7/36.2	37.3
A-tubule fraction	–	35.7/36.2	37.3
B-subfiber fraction	31.8	(35.7/36.2)	37.3
Egg cytoplasm	–	36.6	37.5
Sea urchin (T. gratilla)			
Sperm flagella	–	35.4	38.4
Outer doublet fraction	–	35.4	38.4
Central pair fraction	32.0	(35.3)	38.4
Embryonic cilia	–	35.4/36.2	38.3
A-tubule fraction	–	35.4/36.2	38.3
B-subfiber fraction	32.1	(35.5/36.2)	38.3
Egg cytoplasm	–	36.4	38.2
Scallop (P. magellanicus)			
Sperm flagella	–	36.8	39.4
Outer doublet fraction	–	36.7	39.4
Central pair fraction	32.7	(36.6)	39.4
Gill cilia	–	37.3	39.6
A-tubule fraction	–	37.3	39.5
B-tubule fraction	–	37.1	39.6
Scallop (A. irradians)			
Gill cilia	–	36.0	38.8
A-tubule fraction	–	36.1	38.9
B-subfiber fraction	–	36.1	38.8
Skate (*R. erinacea*) brain	–	36.9	37.9

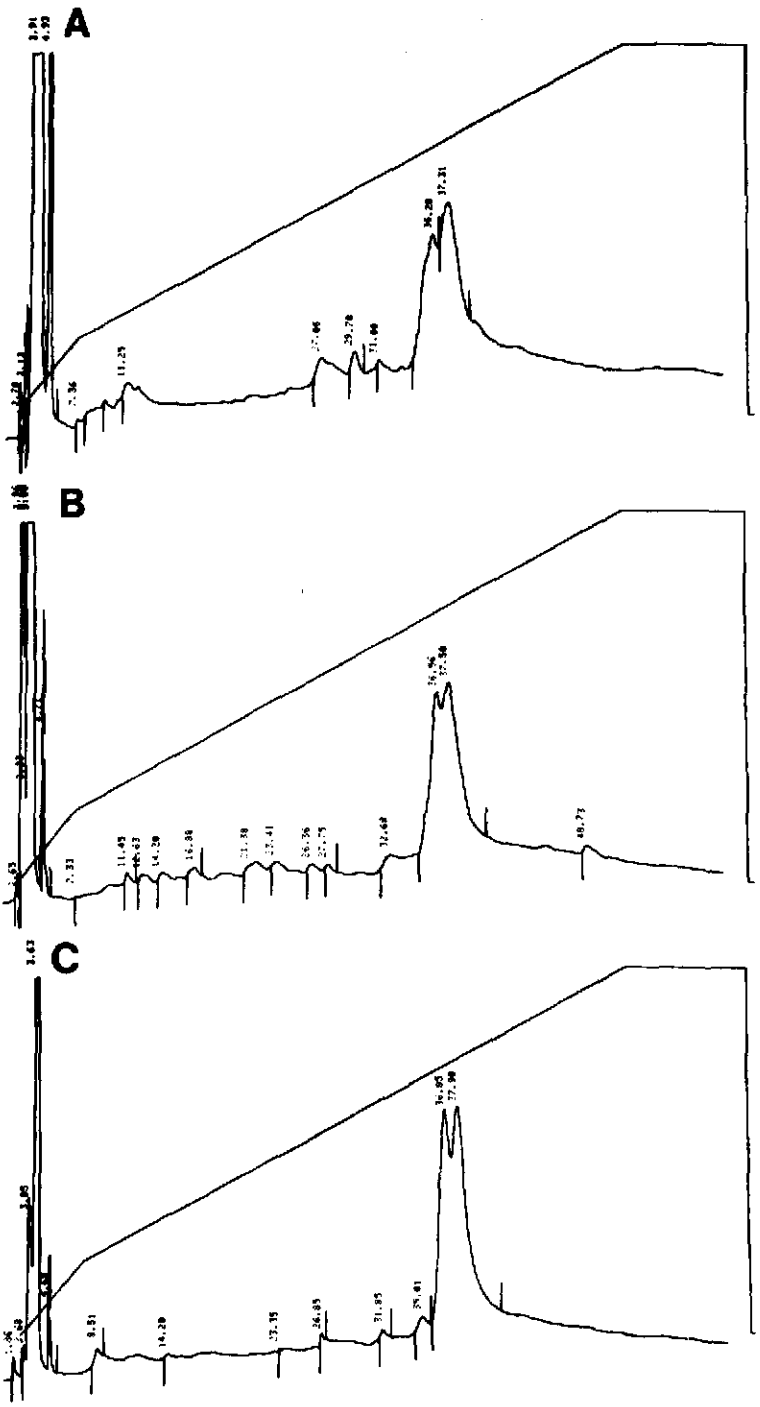

Fig. 6. HPLC profiles of tubulins from ciliary and cytoplasmic microtubules. Gradient as defined in Fig. 3 and Table II. (A) Sea urchin (*S. droebachiensis*) embryonic ciliary tubulin, showing partial resolution of two approximately equimolar β-subunits. Full-scale = 0.1 O.D. (B) Sea urchin (*S. droebachiensis*) egg cytoplasmic tubulin, having closely-eluting β and α subunits (solubilized in 6 *M* guanidine–HCl). Full-scale = 0.2 O.D. (C) Skate (*Raja erinacea*) brain tubulin, also showing closely-eluting β and α subunits. Full-scale = 0.2 O.D.

specific differences are evident. The most striking is seen in the α chains of *S. droebachiensis*. The ciliary β-tubulin in both sea urchin species is evidently comprised of two equimolar subspecies (Fig. 6A), both of which have slightly greater retention times than their single flagellar counterpart. (These cannot correspond to the equimolar electrophoretic β chains described earlier[17] since neither scallop cilia nor *T. gratilla* flagella show the two HPLC β chains whereas they, but not *S. droebachiensis* cilia, have two equimolar electrophoretic β chains). In *P. megellanicus*, the (single) ciliary β chain has a significantly greater retention time than its flagellar counterpart. The early-eluting β chains, discussed above, show no significant differences in retention times when cilia are compared with flagella.

The β chain of sea urchin egg cytoplasmic tubulin elutes later than either of the 9 + 2 counterparts while the α chain elutes earlier than the flagellar subunits and in *T. gratilla* even earlier than the ciliary α chain. Consequently the cytoplasmic tubulins are not well resolved (Fig. 6B). One interesting further difference is that *S. droebachiensis* egg cytoplasmic tubulin is nearly insoluble in 0.2% aq. TFA alone, requiring 6 *M* guanidine–HCl solubilization, whereas the *S. droebachiensis* 9 + 2 tubulins are freely soluble, as is egg cytoplasmic tubulin from *T. gratilla*.

Elasmobranch and mammalian brain tubulins. Skate brain tubulin was found to be very freely soluble in aqueous TFA and served as an early test material for this study. Like the egg cytoplasmic tubulin described above, the tubulin subunits were barely resolved and had retention times comparable to but later than *S. droebachiensis* egg tubulin (Fig. 6C). After publication of an abstract of this basic methodology[14], a number of workers attempted to apply it to the separation of mammalian brain tubulin subunits but with little reported success. The major problem was mainly one protein solubility.

Fig. 7 illustrates the rapid resolution of bovine brain tubulin into single fractions each containing the β and α subunits at better than 80% purity, as judged by SDS-PAGE gel densitometry. Unlike skate brain tubulins, which elute at 18.6 and 19.7 min on this gradient, these comparatively more hydrophilic subunits are well resolved at 14.6 and 17.1 min, with relatively little trailing, considering the load and the speed of the run. This particular sample was prepared by diluting one part of the protein contained in a 0.1 *M* piperazine-N,N′-bis(2-ethane sulfonic acid) repolymerization buffer (at 15 mg/ml) with nine parts of 0.5% aq. TFA. Direct addition of TFA to the highly buffered salt solution results in the irreversible precipitation of tubulin, as does direct dialysis against 0.2% aq. TFA. Unlike the sea urchin cytoplasmic and 9 + 2 tubulins, vertebrate brain tubulin subunits do not chromatograph well after guanidine–HCl solubilization.

DISCUSSION

The RP-HPLC methods presented here are generally useful for rapid separation of the major α- and β-tubulin subunit classes in relatively high yield, although these may co-elute with some minor proteins. In the species studied thus far, β-tubulin always elutes before α-tubulin. The β/α subunit separation is species-specific, particularly evident with brain tubulins, and it is also organelle-specific, notably in cytoplasmic *versus* ciliary *versus* flagellar tubulins, where the degree of separation increases in the order given.

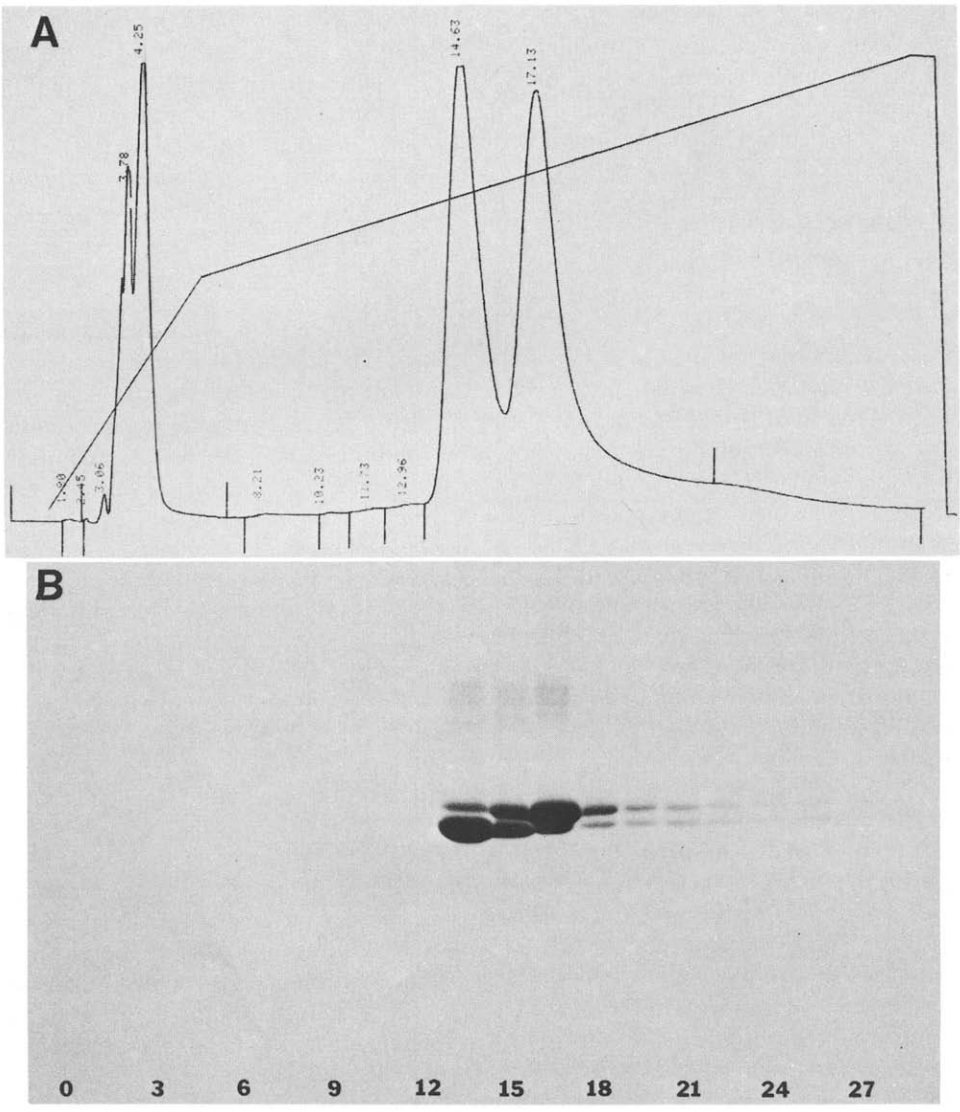

Fig. 7. Rapid elution of bovine brain tubulin subunits. (A) HPLC profile. Solvent A = 30% acetonitrile in 0.1% aq. TFA, solvent B = 60% acetonitrile in 0.1% aq. TFA. Gradient: 0 to 50% in 5 min, 50 to 100% in 25 min, 1.0 ml/min. Full-scale = 1.0 O.D.; sample = 0.4 mg total protein. (B) SDS-PAGE analysis of the entire gradient, 1.5-ml fractions.

An additional aspect of this approach is that it produces the equivalent of a two-dimensional analysis of ciliary or flagella proteins, basically in accord with increasing relative hydrophobicity. The examples here used 20–40 fractions but obviously this can be increased almost without limit. Such an analysis has other limitations, however, since proteins with molecular weights in excess of about 100 000

are either voided from or irreversibly retained by the column. Other proteins may simply be insoluble under the conditions employed.

The key to good resolution and high yield is the initial solubilization to produce fully dissociated tubulin subunits. This is dependent upon complete reduction of –S–S– bridges, the absence of salt and divalent cations, and, of course, the tubulin in question must be soluble in the pH range of 2–2.5. The first conditions are easily met by dialysis of the protein into a weakly Tris-buffered solution (maximum 10 m*M*, pH 8) containing 10 m*M* DTT or 0.1 *M* mercaptoethanol, followed by acidification with TFA. Surprisingly, most 9 + 2 axonemes studied thus far dissolve almost totally when suspended in 0.2–0.3% aq. TFA. On the other hand, some tubulins are simply insoluble in aqueous TFA even if they are initially water soluble, *e.g.* one urchin cytoplasmic tubulin but not the other. Guanidine–HCl solubilization is required in such cases, generally accompanied by some loss of protein on the column.

After HPLC resolution, some tubulins, when pure, self-associate or simply denature and may be quite insoluble after evaporation of TFA–acetonitrile. This problem may be circumvented in many cases by drawing off most of the acetonitrile under aspirator vacuum and then dialyzing the aqueous TFA solution against the desired final buffer or renaturation solution. Alternatively, the dried sample may be dissolved in and renatured from urea or guanidine.

A good example of the application of these facts learned from tubulin separation, after the initial publication of the basic method[14], has been the successful resolution of the tektins, outer doublet microtubule-associated proteins which are extremely insoluble[26]. Derived from sea urchin (*S. purpuratus*) sperm flagella, these require solubilization in 6 *M* guanidine–HCl and DTT prior to HPLC analysis. In this case, >80% yields and essentially complete separation of these three extremely similar proteins is accomplished on a C_{18} column with a shallow, concave gradient of acetonitrile. Attempts to apply this same approach to the tektins from the closely-related sea urchin *S. droebachiensis* studied here requires 9 *M* guanidine–HCl for solubilization and yields poorly-resolved tektins at comparatively low yield. Conversely, the tubulins from *S. purpuratus*, although resolving as well as those from *S. droebachiensis*, chromatograph at considerably lower yields.

Columns which theoretically should bind tubulin less tightly, produce better resolution, and give higher yields, at best do only the latter. O'Hare *et al.*[27] and Pearson and Regnier[28] present arguments for increased resolution and recoveries on shorter chain length, larger pore-size columns. In basic agreement with our own results, Steffensen and Anderson[29] recently compared the relevant column chemistries directly, using water soluble, monomeric proteins both larger and smaller than tubulin subunits, and found that properly end-capped C_{18} columns produced separations and yields comparable to shorter chain length columns, while larger silica pore size increased yield only slightly. The μBondapak C_{18} medium is end-capped and bears a relatively light carbon load. Evidently the unique efficacy of this column is a function of some differential interaction of the C_{18} groups and/or the silica surface with very specific and distinctive hydrophobic surface regions characteristic of the two tubulin subunits.

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