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Structure of tubulin C-terminal domain obtained by subtilisin treatment

The major α and β tubulin isotypes from pig brain are glutamylated

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Limited subtilisin digestion of the tubulin α, β heterodimer has been used in this work to reduce the total number of tubulin isotypes from 20 for native to 9 for subtilisin-cleaved tubulin. This indicates that the major part of tubulin heterogeneity is located at the C-terminus of the molecule. The C-terminal peptides of both α and β subunits of tubulin were purified by anion-exchange HPLC. Combined use of Edman degradation chemistry and mass spectrometry on the isolated peptides shows that subtilisin cleavage occurs at position Asp-438 and His-406 of α and Gln-433 and His-396 of β tubulin chains. Quantitative analysis of our data show that cleavage at positions His-406 (α) and His-396 (β) occurs with a low efficiency and indicates that the major isotypes of pig brain tubulin are modified by sequential attachment of 1 to 5 glutamic acid residues at positions Glu-445 or -435 of α and β tubulin, respectively

Glutamylation; Mass spectrometry; Post-translational modification; Subtilisin, Tubulin

1. INTRODUCTION

Brain tubulin exhibits a high degree of heterogeneity. This heterogeneity is due to differential expression of a small mutigene family [1] and to posttranslational modifications of several isogenes products. α tubulin is reported to undergo acetylation of Lys-40 [2–4], cyclic removal and addition at the C-terminus of a tyrosine residue [5–6] and polyglutamylation of Glu-445 [7–8]. Specific β tubulin isotypes have been described to be phosphorylated [9–11] and polyglutamylated [12,13].

All of the known mammalian α and β tubulins, apart from their C-termini, are quite similar [14,15]. Comparison of the amino acid sequences of vertebrate α or β tubulin isotypes shows that the C-termini of both α and β chains are composed of two subdomains. α (amino acid residues 402–439) and β (406–430) are conserved among tubulin isoforms, while α (440–451) and β (431–445) that distinguishes the isotypes classes from each other [16] are hypervariable regions [17].

Limited subtilisin treatment of the tubulin dimer releases the highly acidic C-terminal tail from both subunits [18–24]. The cleaved protein, named S-tubulin, has an enhanced ability to assemble into microtubule-related structures [20,25,26] probably because of the decrease of electrostatic repulsion between tubulin molecules. While there is general agreement that the C-terminal fragments of tubulin cleaved by subtilisin are spontaneously released from the protein, the exact site of subtilisin cleavage is controversial. Serrano et al. [19–21,25] and Maccioni et al. [22] have indicated the subtilisin cleavage sites to be located between residues 407–417 on both α and β subunits, i.e. 30 to 45 residues from the C-terminus, while Bhattacharyya et al. [27], De la Viña et al. [23], Paschal et al. [24] and Melki et al. [28] supported the view that the site of subtilisin cleavage is located between 5 and 15 residues from the C-terminus.

In order to gain information on the substructure of the tubulin C-terminal regulatory domain, we have used limited subtilisin digestion of the protein to generate the C-terminal peptides of both α and β subunits of tubulin. We found that cleavage of tubulin heterodimers reduces the number of α and β tubulin isotypes from about 20 for native tubulin to about 9 for S-tubulin, in agreement with Lobert and Correia [29]. The C-terminal peptides were purified by HPLC. Peptide sequencing and mass spectrometry indicate the subtilisin cleavage sites to be at Asp-438 and His-406 in α and Gln-433 and His-396 in β, and show that the major isotypes of pig brain tubulin (i.e. class Iβ-α- and II-β-tubulin, Little and Seehaus [14]) are modified by sequential attachment of 1 to

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Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing, PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

5 glutamic acid residues to the γ carboxyl group of Glu-445 or -435 of α and β tubulin, respectively.

2. MATERIALS AND METHODS

2.1. *Chemicals*

MES (2-(*N*-morpholino) ethanesulfonic acid) was purchased from Calbiochem. EGTA (ethylene glycol bis (β-aminoethyl ether)-*N*,*N*',*N*'-tetraacetic acid, sodium dodecyl sulfate (SDS), subtilisin Carlsberg (P 5380) were from Sigma. Guanosine 5'-triphosphate (GTP) came from Boehringer. Acrylamide and all other gel electrophoresis reagents came from Bio-Rad. Acetonitrile was from Baker. Taxol purified from *Taxus baccata* [30] was a gift from Dr. D. Guenard (ICSN, Gif-sur-Yvette, France). All other chemicals were analytical grade from Prolabo and Merck.

2.2. *Tubulin purification*

Pure tubulin was prepared from fresh pig brain by three assembly–disassembly cycles according to Shelanski et al. [31], followed by phosphocellulose (Whatman P 11) chromatography [32]. Tubulin was concentrated by ultrafiltration and stored at −80°C in MG buffer (0.05 M MES, pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 3.4 M glycerol and 200 μM GTP) at a concentration of 7–10 mg/ml. The concentration of tubulin was determined spectrophotometrically using an extinction coefficient of 1.2 mg cm^2 at 277 nm [33] and a molar mass of 100 kDa [34].

2.3. *Cleavage of the C-terminal region of tubulin*

Dimeric tubulin (20 μM) in M buffer (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM GTP), was digested at 30°C by subtilisin (Carlsberg) 20 μg/ml for various times. The cleavage reaction was terminated by addition of 4 mM phenylmethylsulfonyl fluoride (PMSF), freshly prepared in dimethyl sulfoxide. Each sample was supplemented with 6 mM $MgCl_2$ and 40 μM Taxol. Microtubules were pelleted at 300,000 × *g*, 37°C for 15 min. The supernatant containing unpolymerized tubulin (≤1 μM), inactived subtilisin and the polypeptides released by subtilisin was stored at −20°C until used.

2.4. *SDS-polyacrylamide gel electrophoresis and isoelectric focusing*

SDS-PAGE was performed using the method of Laemmli [35] with the modifications described by Melki et al. [28] in order to increase the separation of the α and β subunits of tubulin. IEF was carried out in cylindrical gels and the presence of 9.5 M urea, as described by Wolff et al. [36], except that pH 5.0–5.5 ampholytes (Serva) were used. Gels were stained with 0.2% Coomassie brilliant blue R-250 in 5% acetic acid and 50% ethanol in water.

2.5. *HPLC separation*

Purification of the C-terminal acidic peptides was performed by anion exchange chromatography followed by reverse-phase chromatography on a Waters (Millipore Inc.) HPLC apparatus. The elution of the peptides was monitored at 214 nm.

The DEAE column (DEAE 5PW, Protein Pack, 7.5 mm × 7.5 cm, Waters) was eluted with a linear gradient from 0 to 100% B_1 in 60 min at a flow rate of 1 ml/min. Buffer A_1 was 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and buffer B_1 was 20 mM Tris-HCl, pH 8.0, 500 mM NaCl. The peptides eluted from the DEAE column were further purified by reverse-phase HPLC.

The reverse-phase column (RP18, sphéri-5, 220 × 2.1 mm, 5 μm, Brownlee) was eluted at 15°C at a flow rate of 200 μl/min. After injection of the fractions collected from the DEAE column the solvent B_2 was maintained at 1% for 10 min, then the following gradient was performed: the concentration of solvent B_2 was increased first from 1 to 5% in 1 min, and then from 5 to 80% in 75 min. Solvent A_2 was deionized water acidified to pH 3.0 with trifluoroacetic acid, and solvent B_2 was 80% acetonitrile in water acidified to pH 3.0 with trifluoroacetic acid.

2.6. *Amino acid sequencing*

Peptides were sequenced by automated Edman degradation using a 470A gas-liquid amino acid sequenator (Applied Biosystems) with the modification described by Le Caer and Rossier [37] and further confirmed for β tubulin C-terminal peptides on a 477A pulse-liquid Sequanator (Applied Biosystems).

2.7. *Mass spectrometry*

A ZAB-HS double focusing mass spectrometer (VG Analytical, Manchester, UK) was used. Fast atom bombardment spectra were generated by a 8 keV xenon atom beam. The matrix (1 μl) was a 1:1 mixture of glycerol and thioglycerol. Cesium iodide clusters were used for mass calibration. The spectra resulted from averaging 10 scans/spectra (mass range, 600–2,500 Da) with a resolution set to 2,000. Methylation of free carboxylic groups was performed by reacting the peptides with 3–10 μl of a hydrochloric acid solution in methanol prepared at 0°C by mixing 300 μl of acetyl chloride with 700 μl of methanol 1 h prior to use. Methylation was complete after 15–30 min incubation at room temperature. One microliter of this solution was mixed with 1 μl of the matrix directly on the target and analyzed immediately. About 50–200 pmol of each peptide was used in each experiment.

3. RESULTS

3.1. *Effect of subtilisin cleavage on tubulin heterogeneity*

Purified native or subtilisin-cleaved tubulin was subjected to high-resolution isoelectric focusing. The IEF patterns of native or subtilisin-digested tubulin purified by a cycle of polymerization–depolymerization are displayed in Fig. 1. Comparison of the two patterns shows that the total number of α and β tubulin isotypes is reduced from about 20 for native tubulin to about 9 for subtilisin-treated tubulin, four of which are major bands. This indicates that tubulin heterogeneity is mainly restricted to the highly acidic C-terminal region of α and β tubulin, since subtilisin cleavage sites are located within the C-terminal portion of the molecules.

3.2. *Optimization of subtilisin digestion of tubulin*

To isolate the C-terminal peptides from pig brain tubulin which is a mixture of 4 isotypes classes [17], and identify the modification that account for their heterogeneity, the whole mixture of tubulin isotypes from pig brain was treated with subtilisin. The time course of tubulin digestion at 30°C in buffer M with 1% w/w subtilisin is shown in Fig. 2. Subtilisin digestion of tubulin proceeds very rapidly resulting in complete disappearance of intact α and β chains and concomitant accumulation of shorter polypeptides corresponding to partially digested subunits denoted α_s and β_s, respectively.

As reported previously [27,28] the β subunit is cleaved at a 3-fold faster rate than the α subunit on dimeric tubulin. Furthermore, incubation of tubulin with subtilisin for more than 5 min resulted in the gradual appearance of 2 fragments of about 35 and 22 kDa. Subtilisin attacks preferentially the bonds adjacent to Asp, Glu, Ala, Gly and Val residues. This broad specificity may result in cleavage of the tubulin molecule at different sites. To avoid subsequent degradation of tubulin C-terminal fragments released in the medium, we chose to

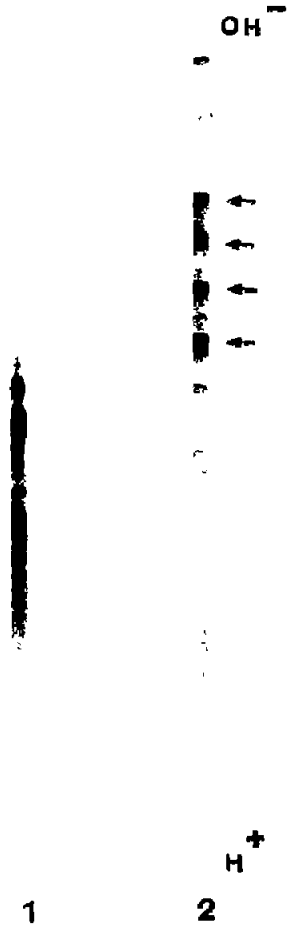


Fig. 1. Isoelectric focusing gels of native and subtilisin-cleaved tubulin. Pure pig brain tubulin (lane 1) and tubulin treated by subtilisin for 30 min (lane 2) were subjected to IEF. The gels were stained with Coomassie blue. The arrows indicate the most prominent isotypes. The basic and acidic ends of the gels are at the top and bottom of the gels, respectively.

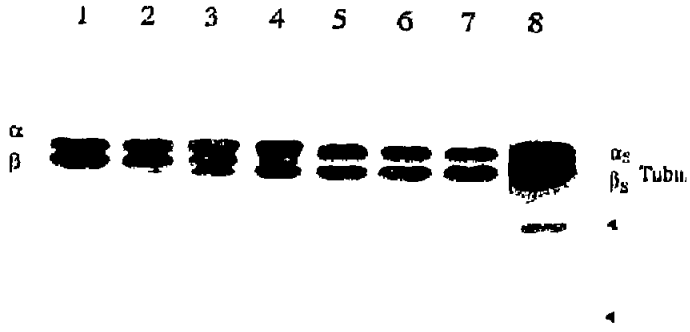


Fig. 2. Time course of tubulin digestion by subtilisin. Tubulin mg/ml) in buffer M containing 1 mM GTP was incubated at 30°C w 1% w/w subtilisin (Carlsberg). Aliquots were removed at timed int vals, subtilisin was inactivated by addition of PMSF, and samp processed for denaturating SDS electrophoresis. Pure tubulin, trea in exactly the same way, except that addition of subtilisin was omitt is in lane 1. Time points are 0.5, 1, 2, 5, 10, 15 min for respectively la 2 to 7. A twofold higher amount of tubulin treated for 15 min subtilisin was loaded in lane 8 in order to visualize minor cleav products. Closed arrowheads indicate degradation fragments of bulin.

treat tubulin with subtilisin for 5 min. Under our experimental conditions, as shown in Fig. 2 lane 5, at 5 min nearly 100% and at least 70% of respectively β and α tubulin C-termini have been cleaved off.

3.3. *Purification of the peptides generated by subtilisin treatment of tubulin*

To isolate tubulin C-terminal fragments released in the medium after subtilisin digestion, pure tubulin solution was supplemented with one molar equivalent of taxol after arrest of subtilisin cleavage by PMSF. Microtubules were then sedimented at 300,000 × g for 15 min. The supernatant containing the peptides from digested tubulin was loaded on an anion exchange column (DEAE). The optical density recording at 214 nm of the eluted material is shown in Fig. 3B. In a control experiment, the supernatant of native tubulin was loaded on the HPLC column and eluted under the same conditions as for subtilisin-treated tubulin (Fig. 3A). Comparison of the elution profiles of native and subtilisin-cleaved tubulin indicates that the peaks eluting between 30 and 42 min contain peptides released after subtilisin treatment. They were collected separately, were denot from 1 to 11 and further purified on a reverse-phase C HPLC column. The elution profiles of DEAE fractic 2, 4, 5 and 7, are shown in Fig. 4. Samples from ea reverse-phase HPLC fraction were used to determ the amino acid sequence of the peptides by automa Edman degradation and to characterize their molecu mass by fast atom bombardment mass spectrometry

3.4. *Identification of the peptides obtained by subtili digestion of tubulin*

Partial N-terminal amino acid sequencing of the covered peptides gave the following sequen GEFEEEEGED---, GXFEEEEGED--, WYTGEG SVEGEGXEEG--- and WYVGEG--- (where X ir cates a cycle in which no PTH-amino acid was tected). Eddé et al. [7] showed that this gap is the pected result for a glutamate residue that is glut ylated on its side chain. These sequences correspon β 434–443, β 397–402, α 439–448 and α 407–412 am acid sequences as determined by Krauhs et al. [34] Ponstingl et al. [38] except that we found a glutan residue in position 440 and a glycine residue in posit 441, while glycine is in position 440 and glutamic in position 441 in the published sequence of tubuli chain. It was checked by amino acid sequencing of synthetic dodecapeptides reproducing the inverted p lished sequence of Krauhs et al. [34] and the seque determined in this work, that our result is not due t artifact.

Our data indicate that the C-terminal part of both α and β chains of tubulin are cleaved by subtilisin at positions. Cleavage points are Asp-438 and His-40 α and Gln-433 and His-396 of β tubulin. Howeve

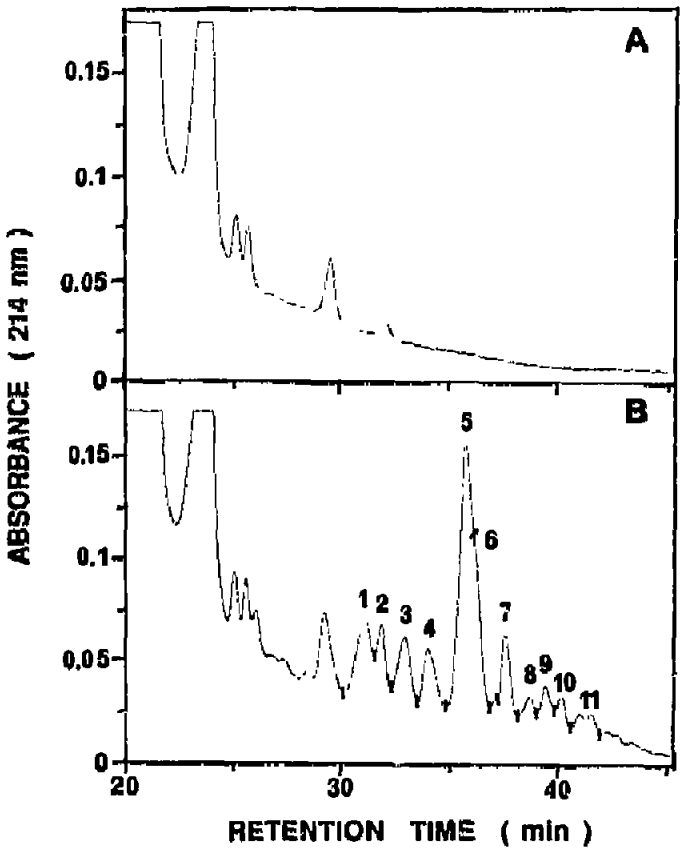


Fig 3 Purification by DEAE HPLC of tubulin C-terminal peptides obtained after subtilisin treatment. Pure pig brain tubulin (20 μM) was treated with subtilisin as described in the methods section. After arrest of subtilisin digestion by addition of 4 mM phenylmethylsulfonyl fluoride, tubulin was polymerized in the presence of 6 mM $MgCl_2$ and 40 μM Taxol and the resulting microtubules were pelleted at 300,000 $\times g$, 37°C for 15 min. The supernatant containing unpolymerized tubulin, inactivated subtilisin and the proteolytic digestion products was injected on an anion exchange column (DEAE 5PW, Waters). The supernatant of pure tubulin incubated under the same conditions in the absence of subtilisin and assembled as described above was used as standard. Separation of the acidic peptides was performed as described in methods section. Elution profiles of the supernatant of native tubulin and of tubulin cleaved with subtilisin for 5 min are shown in panels A and B, respectively. Comparison of these two elution profiles show that peptides eluted between 30 and 45 min are generated by subtilisin treatment. The acidic peptides denoted 1 to 11 were collected separately for further purification on a reversed phase column

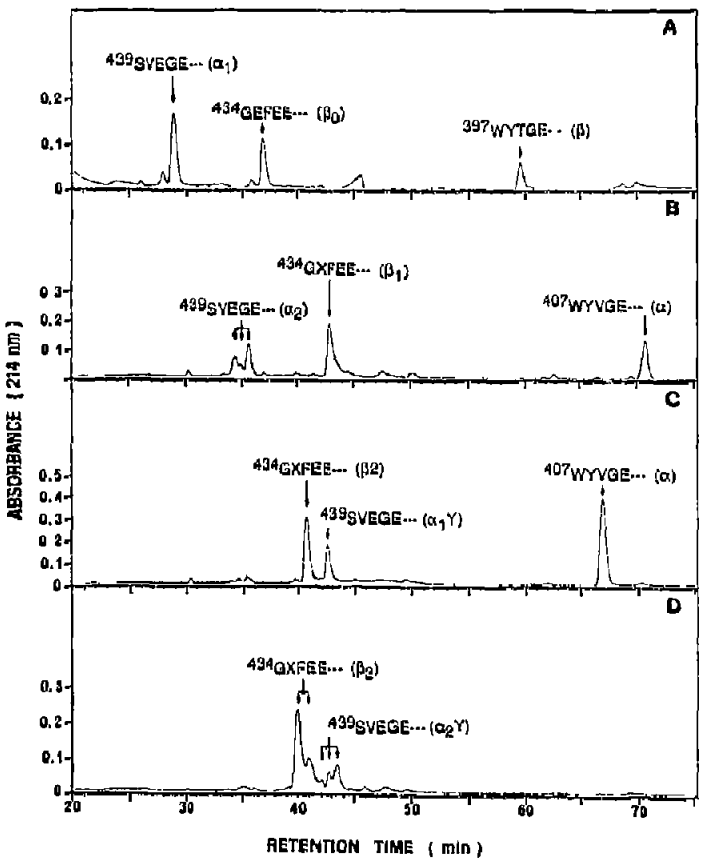
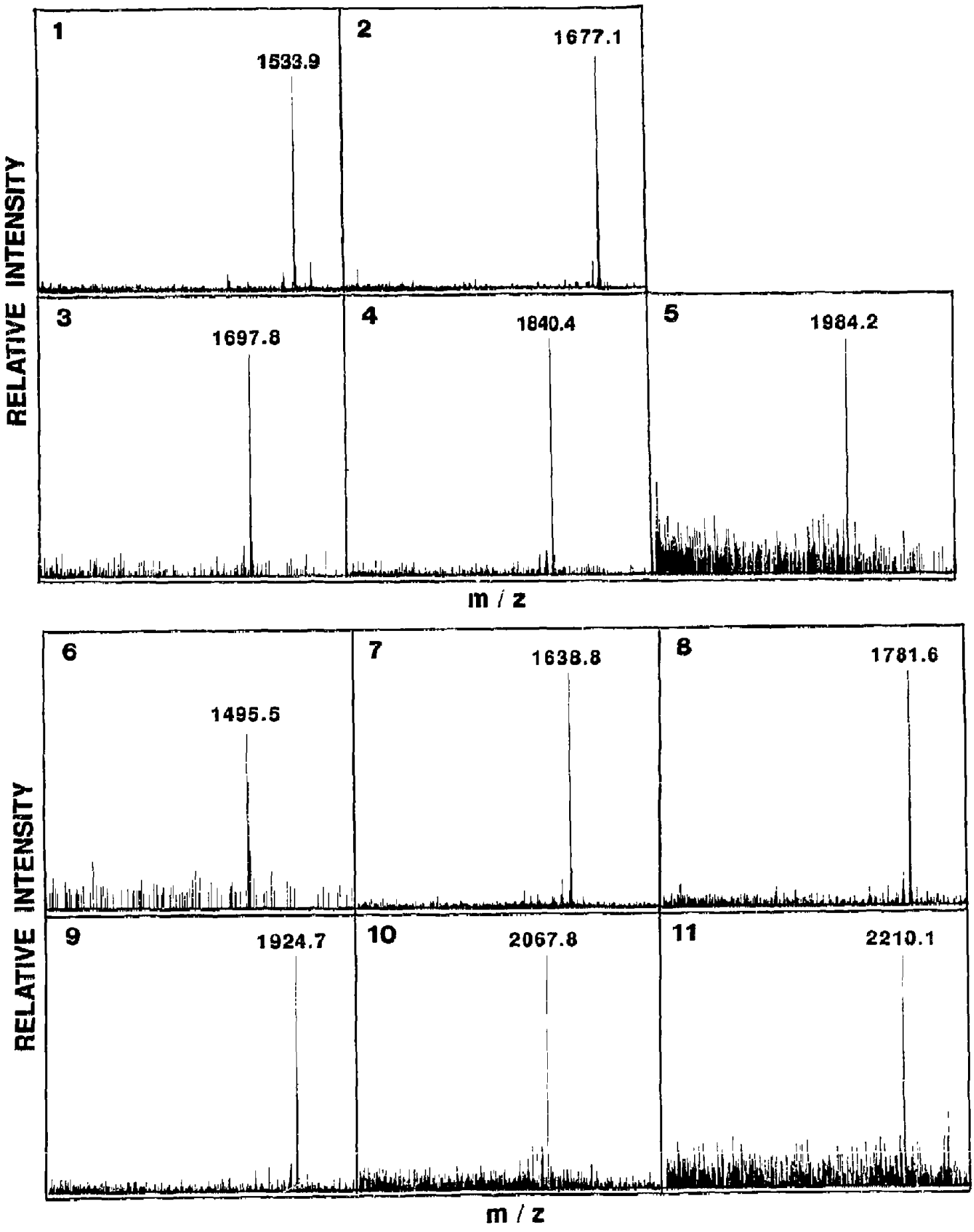


Fig. 4. Reverse-phase HPLC purification of the fractions collected from the DEAE column. Before injection the pH of each sample was brought to 3.0. The fractions collected from the DEAE column were injected on a C18–5 μm reverse-phase column, which was developped as described in section 2. The elution profiles of DEAE fractions 2, 4, 5 and 7 are shown in panels A to D, respectively. Each fraction was collected separately for amino acid sequence determination and mass spectrometry analysis. The N-terminal amino acid sequence of each peptide is given and its nature, deduced by mass spectrometry analysis, is indicated in parentheses. Three peaks (arrows) with identical mass and N-terminal sequence were obtained for biglutamylated ^{439}SVEGEGEEEGEE^{450} α tubulin C-terminal peptide (panel B) and its tyrosinated form (panel D). Two peaks were obtained for the biglutamylated β tubulin C-terminal peptide (^{434}GEFEEEEGEDEA^{445}, panel D). The nature of this apparent heterogeneity is under investigation.

should be noted that preferential cleavage occur at Asp-438 of the α and Gln-433 of the β chains of tubulin molecule. The time course of tubulin C-terminal peptides released during subtilisin treatment was analyzed by anion-exchange chromatography (not shown). At different time intervals between 0 and 15 min, subtilisin treatment was stopped by addition of PMSF and the samples were processed for separation of proteolytic digestion products of tubulin as described in section 2. Comparison of the elution profiles at 1, 2, 5 and 15 minutes clearly indicate synchronous release of peptides WYVGEG--- and SVEGEGXEEG--- from α and

→

Fig 5. Mass spectrometry analysis of carboxy-methylated tubulin C-terminal peptides. Monoisotopic *m/z* values of the protonated molecular ions (MH^+) are indicated. Spectra of α tubulin C-terminal peptides are shown in panels 1 to 5, while panels 6 to 11 correspond to the spectra obtained for β tubulin C-terminal peptides. Mass values at *m/z* = 1533.9 (panel 1) and 1677.1 (panel 2) correspond to the fully carboxy-methylated α tubulin Ser^{439}–Glu^{450} peptides bearing one and two additional glutamyl units, respectively. Mass values at *m/z*= 1697.8 (panel 3), 1840.4 (panel 4) and 1984.2 (panel 5) correspond to fully carboxy-methylated α tubulin Ser^{439}–Tyr^{451} peptides bearing one to three additional glutamyl units, respectively. Mass values at *m/z* =1495.5 (panel 6), 1638.8 (panel 7), 1781.6 (panel 8), 1924.7 (panel 9), 2067.8 (panel 10) and 2210.1 (panel 11) correspond to fully carboxy-methylated β tubulin Gly^{434}–Ala^{445} peptides bearing zero to five additional glutamyl units, respectively.


1
1533.9
2
1677.1
3
1697.8
4
1840.4
5
1984.2
RELATIVE INTENSITY
m / z
6
1495.5
7
1638.8
8
1781.6
9
1924.7
10
2067.8
11
2210.1
RELATIVE INTENSITY
m / z

WYTGEG--- and GXFEEEEGED--- from β tubulin chains.

Further analysis of the structure of the recovered peptides was carried out using fast atom bombardment mass spectrometry. As already described [7], a very weak signal was detected when the highly hydrophilic acidic peptides from the C-terminal part of tubulin were analyzed. After methylation of the free carboxyl groups, protonated molecular ions (MH^+) were highly enhanced. This allowed their easy detection. The two peptides with the following N-terminal sequences: ^{407}WYVGEG--- (α) and ^{397}WYTGEG--- (β) gave no interpretable mass spectra, even after carboxyl methylation. This is probably due to their high molecular weight above the practical limit of the mass spectrometer and also to the low amount of material used. The expected theoretical monoisotopic masses of the non-glutamylated forms of these peptides would be 3650.6 and 4335.7 for those cleaved at positions α 438 and β 433, and 5093.1 and 5705.2 for the intact forms. These peptides reacted with DM1A and DM1B monoclonal antibodies (not shown), which are antibodies that react specifically with α 426 to 450 and β 416 to 430 peptides, respectively [39]. This indicates that α tubulin peptides with the N-terminal sequence ^{407}WYVGEG--- are not cleaved at position 438.

The FAB-mass spectra of each of the other carboxyl-methylated peptides are shown in Fig. 5. The ion at mass/charge ratio (m/z) of 1495.5 corresponds to the carboxyl methylated form of the dodecapeptide ^{434}GEFEEEEGEDEA^{445} (1495.5 calculated m/z for $(M+H)^+$), which was denoted β_0. The masses of the other β peptides denoted β_1 to β_5 were 1638.8, 1781.6, 1924.7, 2067.8 and 2210.1, respectively. They are related to each other by an incremental mass shift of 143 Da, i.e. the exact mass of a methylated glutamate (129 + 14 Da), and correspond to the dodecapeptide ^{434}GEFEEEEGEDEA^{445} bearing one to five additional glutamates, respectively. Mass measurements made on the fractions containing the α tubulin C-terminal sequence ^{439}SVEGEGXEEGXX^{450} gave the following mass/charge ratios: 1533.9, 1677.1, 1697.8, 1840.4 and 1984.2 (Fig. 5). The calculated monoisotopic m/z for the $(M+H)^+$ ions mass of totally carboxy-methylated α tubulin C-terminal peptide ^{439}SVEGEGEEEGEE^{450} and of its tyrosinated form are expected to be 1391.5 and 1554.5, respectively. We thus conclude that the ions at m/z of 1533.9 and 1677.1, denoted α_1 and α_2, respectively, correspond to the mono- and bi-glutamylated forms of the detyrosinated peptide, while the ions at m/z of 1697.8, 1840.4 and 1984.2, denoted α_1Y, α_2Y, α_3Y, respectively, correspond to the mono-, bi- and tri-glutamylated forms of the tyrosinated peptides.

4. DISCUSSION

The advantage of enzymatic partial digestion of a protein under non-denaturating and well controlled conditions lies in the fact that endoproteases, especially if they have a narrow specificity, cleave their substrate at a restricted number of sites. This may be useful to obtain structural information such as the identification of regions of a native protein which are relatively unstructured and therefore more sensitive to proteases than compact domains.

Limited subtilisin digestion of tubulin has been a useful tool to probe the role of the highly flexible and acidic C-terminal domain of the protein in the regulation of tubulin assembly. In the present work we have optimized subtilisin treatment of a complete mixture of tubulin isotypes and isoforms from pig brain in order to generate intact C-terminal peptides of tubulin α and β chains. This was achieved by short digestion of tubulin with subtilisin to avoid subsequent degradation in the medium of the released peptides. Anion exchange HPLC was used to isolate the acidic C-terminal peptides of tubulin from the bulk solution containing uncleaved tubulin, GTP and GDP, subtilisin and PMSF. They were purified further by reverse-phase HPLC. All peptides were characterized by peptide sequencing and mass spectrometry.

Our results show that both α and β chains of the major isotypes of pig brain tubulin (Iβ-a and II-b) [14] are glutamylated at position 445 and 435 respectively. Thus the α and β subunits of tubulin are mixtures of non- up to penta-glutamylated forms of each chain. Although we did not obtain, in the present work, evidence for higher degrees of glutamylation, we believe that they occur, in agreement with Redeker et al. [8] and Alexander et al. [12] who showed respectively that class Ib α-tubulin isotype from mice brain and class III β-tubulin isotype from bovine brain are hexaglutamylated. The relative amount of tubulin subunits bearing more than five glutamyl units would represent less than 10% of the bulk mixture of tubulin isoforms, since peptides representing less than 2% of tubulin C-terminal peptides were detected and analyzed (see table I). This view is further supported by IEF analysis of native and subtilisin-treated tubulins. Indeed, glutamylation of tubulin C-terminal region must account for the major part of the observed charge heterogeneity of the molecule. In agreement with Lobert and Correia [29], we find that the number of tubulin isotypes is reduced after subtilisin cleavage from 20 for native tubulin to 9 for S-tubulin, four of which are major products. This observation is what we expect since subtilisin, under the conditions described in section 2, cleaves both a and β subunits of tubulin heterodimer at two positions: Asp-438 and His-406 of α and Gln-433 and His-396 of β, yielding S-tubulin, a mixture of two types of α and β chains that differ by their length and pIs.

It is commonly believed that failure of attempts to produce diffracting crystals for 3D structural analysis is due to tubulin heterogeneity. Our data convey the

conclusion that S-tubulin is heterogeneous. The fact that longer treatments of tubulin with subtilisin result in the appearance of degradation products of about 35 and 22 kDa that remain associated with S-tubulin [29] and that S-tubulin assembles with a very low critical concentration into protofilamenteous structures [18,20,25–27] questions the suitability of subtilisin-treated tubulin for successful crystallization experiments. In this respect, homogeneous tubulin produced by overexpression systems should represent a key solution for systematic studies of crystallization conditions.

The relative abundance of the different α and β isoforms was estimated by calculating the amounts of glutamylated C-terminal peptides released by subtilisin treatment. The data are summarized in Table I. Glutamylated α and β chains represent the major forms of tubulin in adult pig brain. The presence of glutamylated α C-terminal peptides, tyrosinated or not, indicate that the two modifications occur independently, in agreement with the data of Eddé et al. [40].

Finally our results reconcile discrepant reports relative to the cleavage site of subtilisin on tubulin; one stating that subtilisin treatment removes a 4 kDa fragment from the C-terminus of tubulin [19,20,22] while several others supporting the view that subtilisin cleavage removes much less than a 4 kDa fragment [23,24,27–29].

Indeed, the present results show that two types of peptides are removed after subtilisin treatment both from the α and β chains of tubulin, yet comparison of the relative abundance of each type of α or β C-terminal peptides clearly indicate that approximately 6% of tubulin molecules are cleaved at position His-406 of α and His-396 of β tubulin chains (Table I). The weak proportion of this latter category of tubulin and the fact that subtilisin cleavage sites have so far been mapped using antibodies directed against the C-terminal portion of the molecule [23,39] or using synthetic peptides reproducing part of the tubulin binding site of MAPs [28] may explain the reason why this population has not been detected so far. Indeed, a 10% variation of the proportion of S-tubulin cleaved at positions Asp-438 on α and Gln-433 on β would not be detected by the highly amplifying Western blot method used in the study of De la Viña et al. [23], nor by the binding assay of Melki et al. [28] since it corresponds in the latter case to the experimental error. In addition one would expect these two forms of S-tubulin to separate on SDS-PAGE and on native gels since they differ by approximately 3 kDa. However the fact that the protein bands corresponding to tubulin are rather diffuse may mask the separation. Consequently, methods such as immunoprecipitation, allowing specific enrichment in S- tubulin cleaved at positions His-406 on α and His-396 on β would be more convenient for such studies.

Tubulin molecule is highly conserved evolutionarily based on sequence data [14]. Combining our data and those of Rudiger et al. [13] on glutamylation of pig brain tubulin with those of bovine class III β tubulin [12] and mice class Ib α tubulin [7] shows that glutamylation occurs in a variable region of α and β tubulin chains. The exact functional role of tubulin glutamylation, which probably occur in many other eukaryotic organisms, is not known. The fact that this modification concerns a region of the molecule that is exposed on the surface of the microtubule lattice [41,42] and that interacts with MAPs [43] suggest that it may modulate the affinity of MAPs toward tubulin, which has consequences on microtubule stabilization and bundling [28]. In order to assess the effect of polyglutamylation on microtubule dynamic instability, it will be necessary to develop methods for purification of individual tubulin isotypes and isoforms.

Finally, the isolation of other glutamylated-tubulin isotypes from other species, and comparison of their primary and tertiary structures may allow the identification of consensus sequences which are recognized by the enzymes responsible of these posttranslational modifications, and contribute to the effort that is made to isolate these enzymes.

Table I

Structure and relative abundance of tubulin C-terminal peptides

Tubulin chain	C-terminal peptides amino acid sequence		DEAE peaks								Total
			1+2	4	5+6	7	8	9	10	11	
β	^{434}GXFEEEEGED...	degree of glutamylation	0	1	2	3	4	4		5	
		relative amounts in %	3.1	5.7	11.2	9.8		16.8		21.6	58.2
	^{397}WYTGE	relative amounts in %	1.1								1.1
α	^{439}SVEGEGXEEG...	degree of glutamylation	1	2	1Y	2Y		3Y	3Y		
		relative amounts in %	4.6	4.5	7.3	3.2			6.1		25.7
	^{407}WYVGE...	relative amounts in %		5.0							5.0

The relative amount of each form of tubulin C-terminal peptides was calculated by dividing the amount of peptide in each fraction by the total sum of tubulin C-terminal peptides purified by reverse-phase HPLC.

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