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Characterization of Post-translational Modifications of Brain Tubulin by Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry: Direct One-Step Analysis of a Limited Subtilisin Digest

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Matrix-assisted ultraviolet laser desorption/ionization (MALDI) mass spectrometry was used to investigate the molecular masses and heterogeneity patterns caused by post-translational modifications in tubulin from porcine brain. Direct analysis of the limited digest with subtilisin shows that the molecular masses of the majority of the carboxyterminal fragments are below 2 kDa, while the truncated tubulin subunits have lost approximately the same mass. The results confirm the cleavage sites previously postulated for this protease. The mass information on the peptides allows the degree of polyglutamylation to be measured directly and shows that molecules with two glutamyl residues in the side chain are the most abundant species. In addition it identifies the degree of tyrosination of α tubulin. This one-step monitoring of a complex digest provides information equivalent to that obtainable from the purified components, while the amount of material required is reduced by three orders of magnitude when compared to previous studies. MALDI spectra partially resolve the α and β subunits of the highly homogeneous tubulin from turkey erythrocytes, which lacks polyglutamylation but does not separate α and β subunits from the heterogeneous brain tubulin. Post-translational modifications of the brain tubulin result in shifting peaks to higher molecular masses, in broadening of the peaks, and in loss of resolution.

Methods for the investigation of post-translational modifications based on mass spectrometry are becoming increasingly important (1,2). In contrast to the usually rough estimations of molecular mass by most conventional biochemical methods, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[1] allows the precise determination of molecular mass, while the peak widths measured as full width at half-maximum (FWHM) value can be used to indicate molecular heterogeneity (3). MALDI-MS has proved a powerful tool with which to elucidate post-translational modifications such as glycosylation (4–6) and phosphorylation (5,7,8). It has also been used to establish the location of disulfide bridges (5). The extreme sensitivity of MALDI-MS (9) as well as the ability to analyze mixtures (10–12) means that only small amounts of material are required and time-consuming purification steps can be omitted. In this report we focus on post-translational polyglutamylation, a modification so far documented only for brain tubulin (13–16).

The αβ tubulin heterodimer, the major component of microtubules, displays high molecular heterogeneity in adult brain tissue. Both polypeptides are encoded by multigene families (17–19). Additional heterogeneity results from a variety of post-translational modifications of both the α and β tubulins. Tubulin isolated from porcine brain is particularly well characterized. The carboxyterminal residue of α tubulin goes through cycles of detyrosination and tyrosination (20–22), while the penultimate glutamic acid residue of these tubulin species can be partially removed (14,16,23). In addition the ε amino group of Lys 40 of brain α tubulin is partially acetylated (24). Class III β tubulin is phosphorylated at Ser 444 (15,25). Polyglutamylation is a further post-translational modification found in all major tubulins of

[1] Abbreviations used: MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; FWHM, full width at half maximum; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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adult brain. The side chain locates to Glu 445 of α tubulin, Glu 435 of class II β tubulin (14,16) and Glu 438 of class III β tubulin (15). Mass spectrometry shows that between 1 and 7 side-chain residues are added by polyglutamylation. Tubulin from avian erythrocytes is a much more homogeneous preparation than porcine brain tubulin since it contains only a single α and β isotype, and there are fewer post-translational modifications. They involve a substoichiometric phosphorylation of Ser 441 of β tubulin and the complete detyrosination of α tubulin without a loss of the preceding glutamic acid residue (26).

To investigate the modification located at the carboxy-terminal end, brain tubulin can be truncated by a mild treatment with subtilisin. Although the carboxy-terminal fragments removed by subtilisin were originally assumed to have a molecular mass of about 4 kDa (27), recent peptide sequence analysis and Fast Atom Bombardment-MS studies (13–16) indicate a size of only 1.6 kDa.

Here we have analyzed mass spectra of native tubulin for post-translational modifications by comparing MALDI-mass spectra of tubulin preparations from porcine brain and turkey erythrocytes. We have then studied polyglutamylation in class II β tubulin from brain and tyrosination and glutamylation in α tubulin from brain. MALDI-MS of mixtures allowed a direct examination of the carboxy-terminal fragments of brain tubulin released by subtilisin without the need for conventional purification steps. Thus the amount of protein needed to detect polyglutamylation is reduced to a few micrograms. Finally, the mixture analysis also provides the molecular mass of the truncated tubulin (48.8 kDa), providing independent evidence that the fragments removed by subtilisin are around 1.6 kDa.

MATERIALS AND METHODS

Protein Purification

Microtubule protein was isolated from adult porcine brain (28) and associated proteins were removed by phosphocellulose chromatography (29). Tubulin from the marginal band of turkey erythrocytes was purified following standard procedures (26,30) and dialyzed against water. A modified SDS/PAGE system was used to maximize the separation of α and β tubulins (14,31). Protein concentrations were determined according to Bradford (32).

Limited Proteolysis

Limited proteolysis of pig brain tubulin with subtilisin/Carlsberg (Sigma) at 0.5% by mass was performed at 30°C for 40 min (14,33). Digests were stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 0.2 m*M* and the digest was then cooled on ice. Aliquots were analyzed using the modified SDS/PAGE system (14). Digest mixtures were dialyzed against water or against 10 m*M* 4-ethylmorpholine–acetate buffer, pH 6.8. Limited proteolysis of porcine brain tubulin was also performed in 10 m*M* 4-ethylmorpholine–acetate buffer at 30°C for 40 min. Proteolysis was stopped as above.

Purification of the Carboxy-Terminal Peptides of Brain β Tubulin Isolated from Porcine Brain

Peptides were isolated (14,26) and used for MALDI-MS without further separation.

Sample Preparation for Mass Spectrometric Analysis

Brain or erythrocyte tubulin was digested against water or 10 m*M* 4-ethylmorpholine–acetate buffer, pH 6.8. Digest mixtures were treated as above. For MALDI-MS analysis tubulin concentrations were adjusted to 7–8 μM by the addition of 0.1% trifluoroacetic acid.

trans-Sinapinic acid was used as uv-absorbing matrix at 337 nm. Measurements in the mass range below 5 kDa used a solution of 100 mM sinapinic acid in 0.1% trifluoroacetic acid:acetonitrile (1:1). Measurements of native tubulin, and its subtilisin derivative, used a saturated solution of *trans*-sinapinic acid in acetonitrile:0.1% trifluoroacetic acid (1:2). For analysis by MALDI-MS the protein solutions were mixed with the same volume of matrix solution (for concentrations see above). One microliter of the sample was spotted on a stainless steel probe tip and dried at room temperature.

Mass Spectrometric Analysis

Measurements were performed using a Bruker REFLEX MALD/TOF mass spectrometer. This time-of-flight instrument is fitted with a reflection and dual microchannel plate detectors for high-resolution analysis in the linear and in the reflected modes. Ions formed by laser desorption at 337 nm (N_2 laser, 3 ns pulse width, 10^7 to 10^8 W cm^{-2}, 0.2 mm^2 spot) were recorded at an acceleration voltage of 10–35 kV.

For measurements in the mass range above 5 kDa, acceleration voltages of 35 kV were used in the positive and negative ion mode. Measurements in the mass range below 5 kDa used an acceleration voltage of 20 kV in the positive and negative ion mode. Both the reflected and the linear ion spectra proved useful for analysis of the carboxy-terminal peptides. In the high mass range above 20 kDa only measurements in the linear mode provided spectra of good quality. In the reflected mode intensities were too low and the resolution was poor due to large-scale metastable degradation of the polypeptides. Carbonic anhydrase from bovine erythrocytes (Sigma, C-2273) and BSA (Sigma, A-4378) were used for internal and external calibration of the mass range above 5 kDa. Insulin from bovine pancreas and a syn-

thetic peptide were used to calibrate the mass range below 5 kDa. To increase the accuracy with which the molecular masses of tubulin and its derivatives could be determined, results from several independent measurements of the singly and doubly charged molecular ions were averaged (34).

RESULTS AND DISCUSSION

Analysis of Intact Tubulin from Porcine Brain and Turkey Erythrocytes

Initially we determined the molecular mass of tubulin isolated from adult porcine brain. Tubulin is a noncovalently linked $\alpha\beta$ heterodimer (35–37), and thus each subunit should generate a molecular peak due to the denaturing effect of the matrix containing 33% acetonitrile. The molecular masses calculated from sequence data (36,37) of the α and β subunits are 50,068 and 49,861 Da, respectively. The MALDI-mass spectrum does not resolve the signals of the two subunits and shows instead only a single peak (Fig. 1a). The average maximum molecular mass was 50,445 ± 50 Da. This value deviates from the expected maximum at 49,964 Da of an unresolved peak of both subunits by some 480 Da. This mass increment can only be explained by post-translational modifications, since mass assignment accuracies typical for this method are very high (0.1 to 0.2% for biomolecules with molecular masses above 20 kDa (2)).

Most of this mass increment can be explained by polyglutamylation (13–16). Although molecular species with oligoglutamyl side chains longer than three residues are not very abundant, up to seven additional glutamic acid residues were found linked to Glu 435 of the major β subunit (14,16) and up to six residues linked to Glu 445 of α tubulin (13,38). Thus polyglutamylation results in a very heterogeneous mass distribution. Different tubulin isotypes (39) and other known post-translational modifications (acetylation of Lys 40 of α tubulin (24), phosphorylation of Ser 444 of β III tubulin (15), and detyrosination (14,20,26) and deglutamylation (23,26) of α tubulin), although substoichiometric, may further increase the heterogeneity seen in the mass distribution. Thus the peak broadening in the mass spectrum seems to be due to the overlapping of several unresolved peaks derived from tubulin species that are differently modified. In fact, the FWHM value of the singly charged molecular peak of porcine brain tubulin (Fig. 1a) amounts to ~2500 Da. This value is too high to be explained by general peak broadening in the high mass range (34).

Figure 1b shows the spectrum of tubulin isolated from turkey erythrocytes. This tubulin, which contains a single α and β species, is the most homogeneous tubulin currently known. The only modifications are the complete detyrosination of the carboxy-terminal end of the α subunit and a 10% phosphorylation of the β subunit (26). This homogeneity has a direct impact on the MALDI-

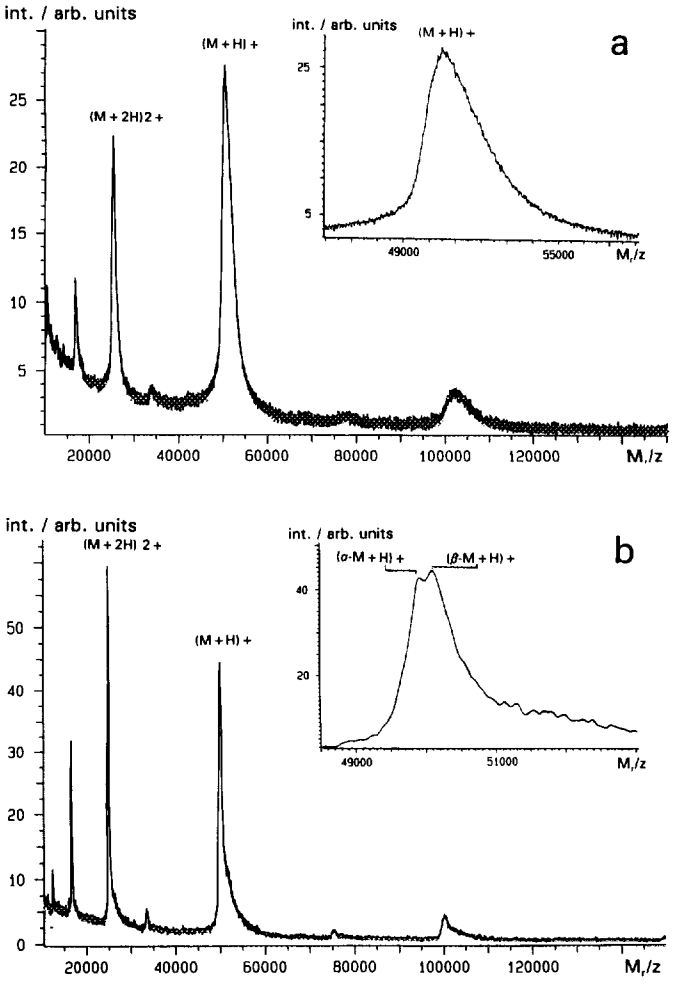

FIG. 1. MALDI-mass spectra of tubulin from adult porcine brain (a) and turkey erythrocytes (b). Experimental details are uv-matrix sinapinic acid, laser wavelength 337 nm. The spectra were recorded in the linear mode. The insets show the region of the singly charged molecular peaks. (a) The signals of the α and β subunits of porcine brain tubulin are not resolved. $(M + H)^+$ and $(M + 2H)^{2+}$ indicate the peak maxima of the singly and doubly charged molecular ions, respectively. $(M + H)^+$ is 50,445 Da. (b) The signals of the singly and doubly charged molecular ions are referred to as $(M + H)^+$ and $(M + 2H)^{2+}$, respectively. Note that the signals generated by the α and β subunits of tubulin from turkey erythrocytes are partly resolved. $(\alpha - M + H)^+$ is 49,925 Da and $(\beta - M + H)^+$ is 50,133 Da.

spectra. In contrast to the spectrum of brain tubulin, the signals of α and β subunit of erythrocyte tubulin are partly resolved and the FWHM value of the singly charged molecular peak is only about 800 Da (Fig. 1b). The experimental values of the α and β subunits are 49,925 ± 50 and 50,113 ± 50 Da, respectively. These results agree well with the masses calculated from the chicken cDNA sequences (26,40,41) assuming that the α tubulin is completely detyrosinated (26). The comparison of the mass spectra of tubulin from brain and erythrocytes (Fig. 1) shows that MALDI-mass spectroscopy

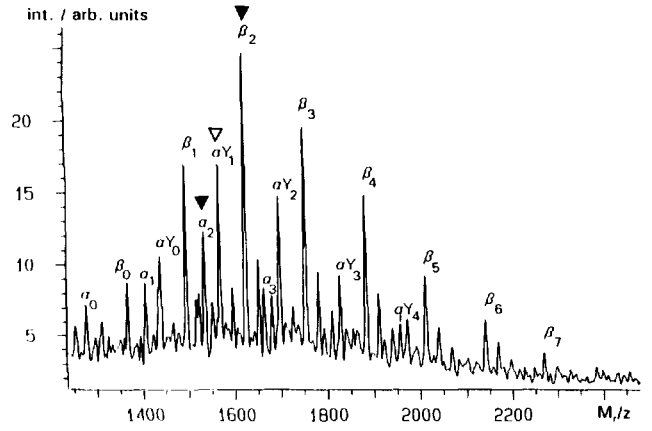

FIG. 2. Reflected MALDI-mass spectrum of the carboxy-terminal peptides released by subtilisin treatment of porcine brain tubulin. The spectrum was taken directly on the complex mixture resulting from the subtilisin treatment. "β" indicates the singly charged peptides released from the β subunits, "αY" and "α" indicate the tyrosinated and detyrosinated peptide species from the α subunits. The most intense peaks of the detyrosinated α peptides and the β peptides correspond to those molecular species which carry two additional glutamic acids. They are labeled by a closed arrowhead. The most intense peak of the tyrosinated α peptides (αY) shows only one additional glutamic acid residue, and is labeled by an open arrowhead. The index system used for the α and β peaks (zero to seven), which corresponds to the number of additional glutamic acid residues, is consistent with the indexing system used in Table 1.

can provide decisive clues concerning the heterogeneity of the sample and the extent of possible post-translational modifications.

Characterization of Polyglutamylation as Post-translational Modification

A detailed direct characterization of native tubulin and its post-translational modifications by MALDI is not possible because of the high molecular mass and the limited resolution in this mass range. Therefore, defined proteolytic fragments resulting from limited protein digestion must be used (34). For tubulin from adult porcine brain, the carboxy-terminal peptides can be removed by a mild treatment with subtilisin (14,16,27).

Figure 2 shows the negative ion spectrum of the peptides released from pig brain tubulin while Table 1 assigns the recorded masses to individual peptides. Polyglutamylated peptides from the β subunit and from both the tyrosinated and detyrosinated forms of the α subunit are observed. The pattern of 129-Da increments, each of which corresponds to a single glutamyl residue, is apparent. Peak intensities of the carboxy-terminal peptides can be used for a semiquantitative analysis of the polyglutamylated species. Figure 2 shows that the molecules with two additional glutamic acid residues are the most abundant species both for the β peptides and the detyrosinated α peptides. In the case of the detyrosinated α

TABLE 1

Assignment of the C-Terminal Peptides to Molecular Masses

Peak	Structure	Experimental molecular mass (Da)	Calcd values (Da)
β_0	GEFEEEEGEDEA	1369	1369.3
β_1	GEFEEEEGEDEA | E	1498	1498.4
β_2	GEFEEEEGEDEA | EE	1628	1627.5
β_3	GEFEEEEGEDEA | EEE	1757	1756.6
β_4	GEFEEEEGEDEA | EEEE	1885	1885.7
β_5	GEFEEEEGEDEA | EEEEE	2015	2014.8
β_6	GEFEEEEGEDEA | EEEEEE	2144	2143.9
β_7	GEFEEEEGEDEA | EEEEEEE	2273	2273.0
β_8	GEFEEEEGEDEA | EEEEEEEE	2402	2402.2
α_0	SVEGEGEEEGEE	1279	1279.2
α_1	SVEGEGEEEGEE | E	1408	1408.3
α_2	SVEGEGEEEGEE | EE	1538	1537.4
α_3	SVEGEGEEEGEE | EEE	1666	1666.5
αY_0	SVEGEGEEEGEEY	1442	1442.4
αY_1	SVEGEGEEEGEEY | E	1572	1571.5
αY_2	SVEGEGEEEGEEY | EE	1701	1700.6
αY_3	SVEGEGEEEGEEY | EEE	1830	1829.7
αY_4	SVEGEGEEEGEEY | EEEE	1959	1958.8

Note. Assignment of the experimental molecular masses found in Figs. 2 and 3 to the structures of the C-terminal peptides of brain tubulin. β peptides correspond to residues 434–445 of class II tubulin, α peptides correspond to residues 439–450, and αY-peptides to residues 439–451 of α_1 tubulin (38). The structures are labeled as in Figs. 2 and 3. In the case of the detyrosinated α peptides (α_0–α_3) it was assumed that the peptides retained their full complement of Glu 450. The last column shows the calculated values for the molecular masses of the peptides. The deviations between the experimental and the calculated values are smaller than ±1 Da.

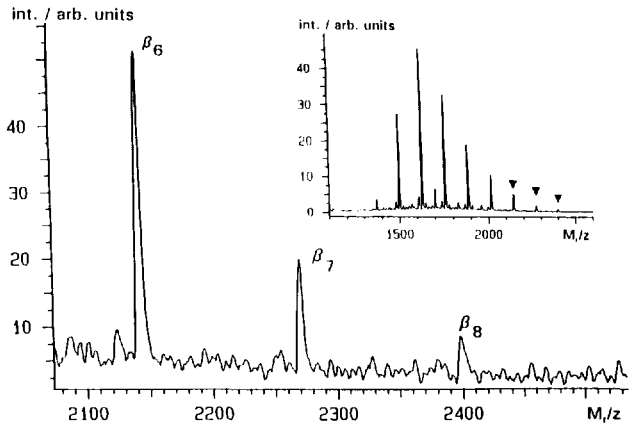

FIG. 3. MALDI-spectrum of the isolated β peptides, to which six to eight additional glutamic acid residues are linked. The spectrum was recorded in the reflected mode. The inset shows the complete peak distribution of the β peptides. The peak of highest intensity corresponds to the species with two additional glutamic acids. The closed arrowheads indicate the species displayed in the main window (β_6 to β_8). The indexing of the β peptides corresponds to the number of additionally attached glutamic acid residues and is consistent with Table 1 and Fig. 2.

peptides the number of side-chain glutamyl residues was calculated assuming that Glu 450 had not been removed (14,23,42). Among the tyrosinated α peptides the species with one additional glutamic acid residue is the most abundant. These results, obtained on the unfractionated digest, are in excellent agreement with earlier investigations in which the peptides were separated and purified (14,26). Figure 2 and Table 1 show that the α and β peptides contain species with up to four and seven side-chain residues, respectively. MALDI-spectra of the mixture of β peptides purified by conventional methods reveal in addition a small amount of a species with eight additional glutamic acid residues (Fig. 3).

Comparison of negative and positive ion spectra of the peptides shows that a better signal to noise ratio can be achieved in the negative ion mode (data not shown). Since the strongly acidic peptides (Table 1) have an increased tendency to release a proton, an increase in sensitivity for the detection of polyglutamylation is observed. In this context the influence of the acidic carboxy-terminal end on the formation of negative ions of intact tubulin was also tested. Compared with the positive ion mode no increase of the signal intensity was observed. Obviously, in relation to the entire tubulin molecules, the acidic carboxy-terminal regions do not seem to dominate the ionization process.

Characterization of Brain Tubulin Truncated by Subtilisin

SDS/PAGE analysis of brain tubulin treated with subtilisin shows a shift to lower molecular masses. From this shift it was estimated that fragments with a total mass of about 4 kDa are removed from the carboxy-terminal end of both the α and the β subunit (27). However, sequence studies of the peptides removed by subtilisin (14,16) demonstrate that the major cleavage sites are at position Asp 438 of the α subunit and Gln 433 of the β subunit. While these studies on purified peptides could not exclude that additional peptides released from the carboxy-terminal end had been lost during purification, the direct MALDI analysis of the unfractionated digest (Fig. 2) excludes this possibility. In addition we have also used MALDI-MS to determine the molecular mass of the truncated tubulin molecules in the unfractionated digest (27). Figure 4 shows that the proteolytic treatment shifts the major peak of the high mass range from 50,445 ± 30 to 48,814 Da. Thus the fragments removed by subtilisin are in the range of 1.6 kDa and correspond to the peptides characterized in Table 1. This result fits previous observations which showed that the truncated tubulin molecules have the same amino-terminal sequences as native tubulins (14). Figure 4 also shows the molecular signal of some intact tubulin as a small satellite peak on the high mass flank of the truncated molecules. SDS/PAGE analysis of this digest (data not shown) confirmed that the enzymatic treatment was not yet complete. The mass difference between the intact and the truncated tubulin agrees with the most abundant carboxy-terminal peptides (Table 1, Fig. 2), i.e., the species with two glutamic acid residues in the side chain. The combined results confirm the location of the subtilisin cleavage sites deduced from sequence analysis (14,16) and exclude an additional removal of other peptides.

We also examined a subtilisin digest of erythrocyte tubulin. Here the observation of the high molecular mass

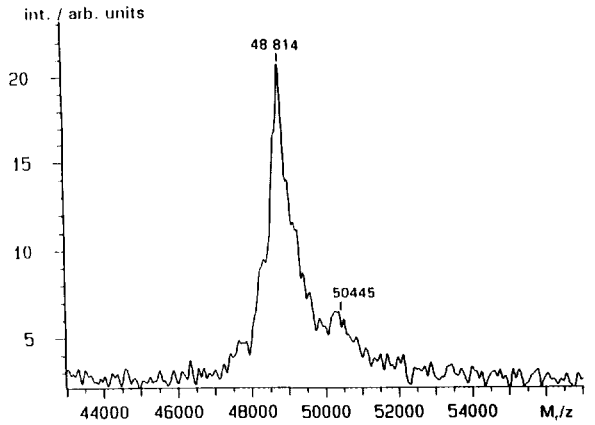

FIG. 4. Linear MALDI-mass spectrum of porcine brain tubulin treated with subtilisin. The part of the mass range where the charged molecular peak of partly proteolyzed tubulin is visible at 48,814 kDa is displayed. The satellite peak on the higher mass flank corresponds to the peak of unproteolyzed tubulin (MA 50,445 Da; see Fig. 1a).

range was not possible since such tubulin preparations are extensively proteolyzed by subtilisin and form a heavy precipitate (26). The supernatant provides a very complex spectrum (data not shown). Although many signals are found, most cannot be assigned to distinct peptides since the cleavage sites of the relatively unspecific protease subtilisin are not known. However, the carboxy-terminal peptides of α and β tubulin are easily found at the predicted molecular masses. No peak pattern with 129-Da increments similar to that seen in spectra of peptides from porcine brain tubulin (see Figs. 2 and 3) is detected. This observation confirms earlier results which concluded from the purified carboxy-terminal peptides that erythrocyte tubulin is not subject to polyglutamylation (26).

In conclusion, our results on brain tubulin show that post-translational modifications can be directly identified in the mixture of peptides resulting from a limited subtilisin treatment. In addition, it was possible to confirm the cleavage site of the protease by determining the molecular mass of the truncated protein. The latter information is not directly available by conventional methods such as SDS/PAGE because of the relatively large experimental error compared to MALDI-MS. It is important to note that the information on the peptides and the truncated protein was obtained in a single analytical step. Thus, the time for analysis is significantly shortened. More importantly, the amount of material needed for the analysis is reduced by three orders of magnitude compared to more conventional studies which were based on the purified peptides (13–16,26).

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