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Acyl-biotinyl Exchange Chemistry and Mass Spectrometry-Based Analysis of Palmitoylation Sites of In Vitro Palmitoylated Rat Brain Tubulin

Zhiqiang Zhao · Junjie Hou · Zhensheng Xie · Jianwei Deng · Xiaoming Wang · Danfang Chen · Fuquan Yang · Weimin Gong

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Abstract Research has shown that the palmitoyl group of α-tubulin mediates the hydrophobic interaction between microtubules and intracellular membranes and that palmitoylated tubulin plays a role in signal transduction. There are 20 cysteine residues per α/β tubulin heterodimer. C376 of α-tubulin was reported to be predominantly palmitoylated and C20, C213 and C305 of α-tubulin were palmitoylated at lower levels. The previous method used for the analysis of the palmitoylation sites on α-tubulin was based on ^{3}H-labeling, enzymolysis, purification and sequencing. This approach, although efficient, is laborious. Mass spectrometry (MS), especially tandem MS, has been shown to be a successful method for identification of various post-translational modifications of proteins. We report here a convenient MS-based method to comprehensively analyze the palmitoylation sites of the α/β tubulin heterodimer. Acyl-biotinyl exchange chemistry and streptavidin agarose affinity purification were applied to enrich palmitoylated peptides from tubulin. After nano-LC-MS/MS analysis, database searching and manual analysis of the spectra revealed that 11 cysteine residues of the α/β tubulin heterodimer were palmitoylated.

Keywords Acyl-biotinyl exchange · Mass spectrometry · Tubulin · Palmitoylation site

Abbreviations

HPDP-biotin	N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide
LTQ	Linear ion trap quadrupole
LC	Liquid chromatography
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
NEM	N-ethylmaleimide
PMSF	Phenylmethanesulfonyl fluoride
TFA	Trifluoroacetic acid
ESI	Electrospray ionization

1 Introduction

Microtubules are a main component of the cytoskeleton and carry out a variety of essential cellular functions, including structural support, localization of organelles, segregation of chromosomes and intracellular trafficking. Microtubules are biopolymers of α/β heterodimers that associate head-to-tail and laterally to form hollow tubes [17]. Individual microtubules are assigned to specific functions through two known mechanisms: differential expression of the tubulin isotypes within a cell and reversible post-translational

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modifications of tubulin, including acetylation, polyglycylation and polyglutamylation, tyrosination/detyrosination, phosphorylation and palmitoylation [20]. Previous studies have shown that palmitoyl groups account for at least one type of hydrophobic interaction between tubulin and the intracellular membranes. By interacting with membranes, palmitoylated tubulin in microtubules may retain vesicles and organelles at particular positions [1]. Direct incorporation of palmitoylated tubulin into the plasma membrane of PC12 cells indicates palmitoylation is important for the insertion of tubulin linkers into membranes [21, 23, 24]. In addition, it has been proposed that membrane-associated tubulin plays a role in signal transduction for a number of systems, including lymphocyte activation [8] and regulation of G protein activities [10, 11]. Tubulin palmitoylation was found predominantly on C376 of α-tubulin. The in vivo palmitoylation level was reduced by 60% after this cysteine was mutated to serine in the budding yeast *Saccharomyces cerevisiae* [2]. Ozols et al. developed a [^{3}H]-palmitate labeling method to identify the palmitoylation sites of tubulin [9]. Briefly, purified porcine brain α-tubulin containing [^{3}H]-palmitate was cleaved with cyanogen bromide and then digested with trypsin and Lys-C protease. The ^{3}H-labeled peptides were purified using gel filtration and then subjected to gas/liquid-phase sequencing. The results demonstrated that C376 was the primary site for palmitoylation compared to residues C20, C213 and C305. However, the [^{3}H]-palmitate labeling-based approach is tedious. MS-based techniques have been developed as a very efficient tool for deciphering the post-translational modifications of proteins [6, 15, 19, 25]. Roth et al. developed an MS-based proteomic approach for the identification of palmitoylated proteins [13, 18]. However, tubulin, a known palmitoylated protein, was not identified in their studies. In this study, we report the first comprehensive mapping of in vitro palmitoylated tubulin purified from rat brain tissues using acyl-biotinyl exchange chemistry and LC-MS/MS.

2 Materials and Methods

2.1 Materials

NEM, HPDP–biotin and streptavidin agarose were obtained from Pierce (Rockford, IS, USA). Palmitoyl CoA, hydroxylamine, TFA, PMSF, pepstatin, leupeptin, antipain and chymostatin were purchased from Sigma (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile was obtained from J. T. Baker (Phillipsburg, PA, USA). All other chemicals were of analytical grade.

2.2 Purification of Tubulin from Rat Brain Tissue

Twenty Sprague-Dawley rats were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Rat brain tissues were taken out immediately and cleaned with kitchen paper to remove blood clots. The purification procedure was conducted as described by Castoldi et al. [3]. After two cycles of polymerization-depolymerization, the purified tubulin was analyzed using 10% SDS gels stained with Coomassie blue.

2.3 In Vitro Palmitoylation of Tubulin

Autopalmitoylation of tubulin was conducted as described by Wolff et al. [22]. Briefly, palmitoyl CoA was dissolved at a concentration of 1 mM in 50 μL of buffer (100 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$ and 0.03% Triton X-100), and the purified tubulin was then added to a final concentration of 20 μM. The sample solution was incubated at 37 °C for 3 h. After the reaction, two volumes of ice-cold acetone were added to precipitate the palmitoylated tubulin. The pellet was collected by centrifugation (3,000 rpm, 10 min) and air-dried.

2.4 Acyl-biotinyl Exchange and Enrichment of Palmitoylated Peptides

Figure 1 shows the protocol for the enrichment of palmitoylated peptides and MS analysis. First, 300 μL of SDS buffer (4% SDS, 50 mM Tris, pH 7.4 and 5 mM EDTA) containing 10 mM NEM was added to the palmitoylated tubulin pellet, and the mixture was incubated for 10 min at 37 °C with occasional agitation to dissolve the pellet. Then 900 μL of buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM NEM, 0.2% Triton X-100, 1 mM PMSF and 0.25 μg/mL each of pepstatin, leupeptin, antipain and chymostatin) was added to the sample solution and the mixture was incubated overnight at 4 °C with gentle rocking. Unreacted NEM was removed by three sequential precipitations with ice-cold acetone as described above. The tubulin pellet was collected by centrifugation (3,000 rpm, 10 min), dissolved in 240 μL of SDS buffer (4% SDS, 50 mM Tris, pH 7.4 and 5 mM EDTA) and incubated at 37 °C for 10 min. At this point, the sample was divided into two equal portions (+HAM sample and −HAM sample) in 1.5 ml screw-cap centrifuge tubes. The +HAM sample was diluted fivefold with 480 μL of the hydroxylamine-containing (+HAM) buffer (0.7 M hydroxylamine, pH 7.4, 1 mM HPDP-biotin, 0.2% Triton X-100, 1 mM PMSF and 0.25 μg/mL each of pepstatin, leupeptin, antipain and chymostatin). For the −HAM sample, 480 μL of −HAM buffer (50 mM Tris-HCl, pH 7.4, 1 mM HPDP-biotin, 0.2% Triton X-100, 1 mM PMSF and 0.25 μg/mL each of

pepstatin, leupeptin, antipain and chymostatin) was used. The two sample solutions were incubated at room temperature for 1 h with end-over-end rotation. After the reaction, the tubulin was precipitated three times using ice-cold acetone. Each resulting pellet was dissolved in 120 μL of SDS buffer (4% SDS, 50 mM Tris, pH 7.4 and 5 mM EDTA), diluted with 480 μL of low-HPDP-biotin buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2 mM HPDP-biotin, 0.2% Triton X-100, 1 mM PMSF and 0.25 μg/mL each of pepstatin, leupeptin, antipain and chymostatin) and then incubated at room temperature for 1 h with end-over-end rotation. To remove unreacted HPDP-biotin, both samples were precipitated three times using ice-cold acetone. Each pellet was dissolved in 1.5 mL of incubation buffer (50 mM Tris-HCl, pH 7.7, 1 mM EDTA and 0.4% Triton X-100). Twenty micrograms of sequencing grade modified trypsin was added to each sample solution and incubated at 37 °C overnight. To terminate proteolysis, PMSF was added to a final concentration of 1 mM.

To perform affinity purification of the HPDP-biotinylated peptides from the digested tubulin, 0.5 mL of 50% suspension of streptavidin agarose was washed twice by resuspension in 5 mL of ice-cold PBS buffer (0.1 M phosphate, pH 7.2 and 0.15 M NaCl), followed by gravity sedimentation and decanting the supernatant. The washed streptavidin beads were resuspended in 0.25 mL of incubation buffer. To affinity-capture the HPDP-biotinylated peptides, 100 μL of the streptavidin agarose bead suspension was added to each tryptic digest. The mixture was incubated at room temperature with rotation. After 1 h, the beads were pelleted by centrifugation at 2,000 rpm for 10 min and washed four times each with incubation buffer, wash buffer (50 mM Tris-HCl, pH 7.7, 600 mM NaCl, 1 mM EDTA and 0.4% Triton X-100) and bicarbonate buffer (5 mM ammonium bicarbonate in 20% acetonitrile). HPDP-biotinylated peptides were eluted from streptavidin agarose by incubation with 500 μL of 8 M guanidine hydrochloride (pH 1.5) for 30 min with gentle inversion at room temperature. Sample solutions were centrifuged to remove insoluble particulates. The supernatants were diluted 10 times with 0.1% TFA/water.

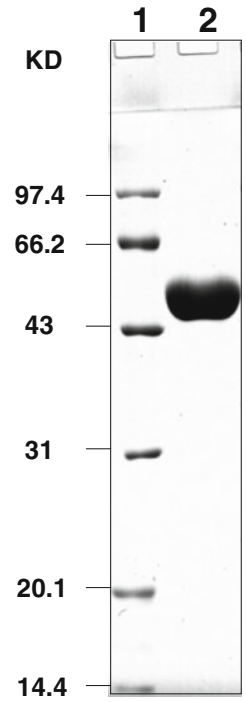

Fig. 2 Purified tubulin was analyzed by SDS polyacrylamide gel electrophoresis. 5 μg purified rat brain tubulin was resolved by 10% SDS gel and stained with Coomassie blue. *Lane 1* Low molecular protein marker (97.4, 66.2, 43, 31, 20.1 and 14.4 kDa); *Lane 2* Purified rat brain tubulin

Two 30 mg portions of C_{18} resin were suspended in methanol and packed into two columns by gravity sedimentation. The packed columns were rinsed with 10 column volumes of 0.1% TFA/acetonitrile and equilibrated with 10 column volumes of 0.1% TFA/water. The +HAM and −HAM samples were loaded respectively onto the two C_{18} columns and desalted using 10 column volumes of 0.1% TFA/water. HPDP-biotinylated peptides were eluted with 1 mL of 60% acetonitrile containing 0.1% TFA and 0.5 mL of 80% acetonitrile containing 0.1% TFA. The eluates of each sample were combined and concentrated to near-dryness in a SpeedVac.

2.5 LC-MS/MS Analysis, Database Search and Manual Assessment of the Spectra

Dried peptide samples were dissolved in 20 μL of 0.1% TFA/water. From that solution, 8 μL was loaded onto a C_{18} trap column by an autosampler and eluted onto a

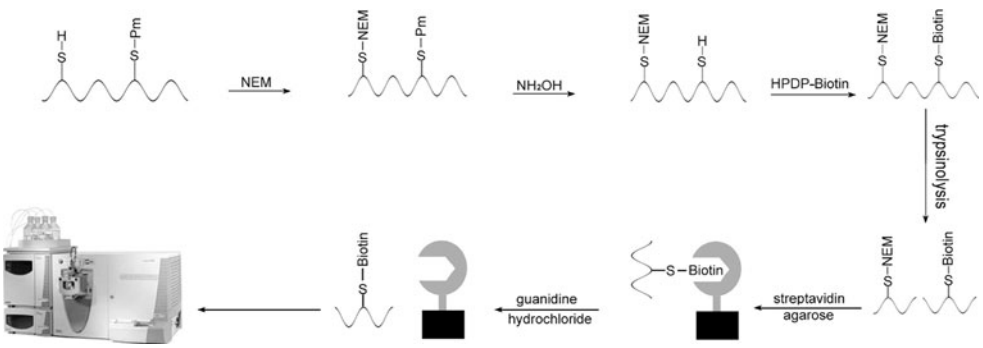

Fig. 1 A schematic depiction of the proteomic acyl-biotinyl exchange method for identification of palmitoyl sites of tubulin. See the text for procedural details

homemade C_{18} column (100 mm × 100 μm) packed with Sunchrom packing material (SP-120-3-ODS-A, 3 μm), followed by nano-LC-ESI-LTQ MS/MS analysis. The LTQ mass spectrometer was operated in the data-dependent mode in which the initial MS scan ranged from 400–2,000 Da. The five most abundant ions were automatically selected for subsequent collision-activated dissociation. All MS/MS data were searched against the Rat International Protein Index (IPI) protein database v.3.51 using the SEQUEST program (Thermo, USA). The search parameters were set as follows: enzyme of trypsin, precursor ion mass tolerance of 2.0 Da and fragment ion mass tolerance of 1.0 Da. The variable modifications were set to oxidation of methionine (Met + 15.99 Da), N-ethylmaleimide on cysteine (Cys + 125.05 Da), O-palmitoylattion on serine (Ser + 238.23), O-palmitoylation on threonine (Thr + 238.23), N-palmitoylation on lysine (Lys + 238.23) and HPDP-biotinylation on cysteine (Cys + 428.19 Da). The search results were filtered with the Xcorr vs. Charge values [Xcorr (+1) > 2.0, Xcorr (+2) > 2.5, Xcorr (+3) > 3.5], and then the spectra of palmitoylated peptides were manually assessed based on the criteria of fragment ion abundance, signal–noise ratios of peaks, the sequential b- or y-ion series with palmitoylation site included and strong proline-directed fragmentation of ions [16].

3 Results and Discussion

3.1 Enrichment and Identification of Palmitoylated Peptides

Figure 1 shows the proteomic strategy we used for the enrichment and analysis of palmitoylated peptides from digested tubulin. The strategy consisted of the following steps: (i) free thiols of tubulin were blocked with NEM; (ii) cysteine-palmitoyl thioester linkages were cleaved with hydroxylamine; (iii) thiols newly liberated by hydroxylamine treatment were labeled with HPDP-biotin; (iv) HPDP-biotinylated peptides of tubulin were affinity-purified using streptavidin agarose after tryptic digestion; (v) the HPDP-biotinylated peptides were analyzed by tandem MS analysis and database searching.

There are three types of protein palmitoylation: (i) S-palmitoylation refers to the incorporation via a thioester bond of a palmitate to cysteine residues; (ii) O-palmitoylation occurs through oxyester attachment of palmitates to serine or threonine residues; (iii) N-palmitoylation was reported in secreted signalling proteins at the N-terminal cysteine residues or at the ε-amino group of lysine residues [14]. Except for S-palmitoylation, N- and O-palmitoylation are hydroxylamine-resistant and can finally be detected by MS/MS. So N-palmitoylation on lysine residues and O-palmitoylation on serine, threonine residues were set as viable modifications during database search.

To assess the credibility of the search results, all of the mass spectra of the palmitoylated peptides were manually validated as described in the experimental section. Figure 3 shows an example of spectrum corresponding to the peptide palmitoylated at C376. The efficiency of NEM blockade and HPDP-biotinylation are very important for the subsequent affinity purification and MS analysis. So NEM blockade and HPDP-biotinylation steps were conducted twice respectively. During affinity purification of HPDP-biotinylated peptides, three buffers were used to wash streptavidin agarose as reported [4]. These washes can prevent the nonspecific adsorption of unwanted peptides. Home packed C_{18} column was applied to remove guanidine hydrochloride after the affinity-purified peptides were eluted. If the reactions and washes are incomplete, the false-positive palmitoylation sites will be detected. To minimize the false-positive results, we performed a control experiment (−HAM), in which the sample was not treated with hydroxylamine after the free thiols of tubulin were blocked with NEM. By comparing the spectral counts of the palmitoylated peptides between the +HAM sample and −HAM sample, we could evaluate the degree of credibility of each palmitoylation site. In this study, we defined the credibility of palmitoylation sites by the following criteria: (1) if the spectral count from +HAM sample (+HAM count) was 1, then the corresponding sites were regarded as low credibility; (2) if the ratio of the +HAM counts to −HAM count was below 4, then the sites were regarded as median credibility; (3) if the +HAM count was greater than or equal to 2 and the −HAM count was zero or the ratio of +HAM counts to −HAM counts was greater than or equal to 4, then the sites were regarded as highly credible. Eleven palmitoylaton sites were identified, including nine of high credibility, one of median credibility and one of low credibility (Table 1). Spectral counts of AYHEQLSVAEITNACFEPANQMVK, EIVHLQAGQCG NQIGAK and LTTPTYGDLNHLVSATMSGVTTCLR from −HAM samples were not zero. We think the reason is these peptides with traces of free thiols which are not blocked by NEM may react with HPDP-biotin and finally be detected by MS. Considering that there are several isoforms of α- and β-tubulin, we found that several isoforms of tubulin share the same sequences of the identified peptides (Table 2), except for the TIGGGDDSFTTFFCETGAGK peptide, which is from the tubulin alpha-4A chain. Although the purified tubulin was essentially free from impurities (Fig. 2), it was actually a mixture of several isoforms of α- and β-tubulin. We could not distinguish from which isoforms a certain palmitoylated peptide originated.

Table 1 All the identified plamitoyl-peptides from tubulins

Peptides	Sites location	Spectral counts		Creditability of sites
		+HAM	−HAM	
*K.TIGGGDDSFTTFF**C#**ETGAGK.H*	54(α)	3	0	High
*K.LADQ**C#**TGLQGFLVFHSFGGGTGSGFTSLLMER.L*	129(α)	3	0	High
*K.AYHEQLSVAEITNA**C#**FEPANQMVK.C*	295(α)	6	2	Median
*K.YMAC**C#**LLYR.G*	316(α)	3	0	High
*R.SIQFVDW**C#**PTGFK.V*	347(α)	2	0	High
*R.AV**C#**MLSNTTAIAEAWAR.L*	376(α)	5	0	High
*R.EIVHLQAGQ**C#**GNQIGAK.F*	12(β)	4	1	High
K.ESESCDC#LQGFQLTHSLGGGTGSGMGTLLISK.I	129(β)	4	0	High
*K.LTTPTYGDLNHLVSATMSGVTT**C#**LR.F*	239(β)	5	1	High
*K.NMMAA**C#**DPR.H*	303(β)	2	0	High
*K.TAV**C#**DIPPR.G*	354(β)	1	0	Low

Table 2 Conservation analysis of palmitoylation sites of tubulin

P Palmitoyl-peptide *S* Protein sequence		IPI accession	Description
P	K.TIGGGDDSFTTFFC#ETGAGK.H		
S	(AA: 31–70) QPDGQMPSDK*TIGGGDDSFTTFF**C**ETGAGK*HVPRAVFVD	IPI00362927.1	Tubulin alpha-4A chain
P	K.LADQC#TGLQGFLVFHSFGGGTGSGFTSLLMER.L		
S	(AA: 121–160) RIRK*LADQ**C**TGLQGFLVFHSFGGGTGSGFTSLLMER*LSVD	IPI00189795.1	Tubulin alpha-1A chain
S	(AA: 121–160) RIRK*LADQ**C**TGLQGFLVFHSFGGGTGSGFTSLLMER*LSVD	IPI00339167.4	Tubulin alpha-1B chain
S	(AA: 121–160) RIRK*LADQ**C**TGLQGFLVFHSFGGGTGSGFTSLLMER*LSVD	IPI00364046.2	Tubulin alpha-1C chain
P	K.AYHEQLSVAEITNAC#FEPANQMVK.C		
S	(AA: 271–310) TYAPVISAEK*AYHEQLSVAEITNA**C**FEPANQMVK*CDPRHG	IPI00189795.1	Tubulin alpha-1A chain
S	(AA: 271–310) TYAPVISAEK*AYHEQLSVAEITNA**C**FEPANQMVK*CDPRHG	IPI00339167.4	Tubulin alpha-1B chain
S	(AA: 271–310) TYAPVISAEK*AYHEQLSVAEITNA**C**FEPANQMVK*CDPRHG	IPI00362927.1	Tubulin alpha-4A chain
S	(AA: 271–310) TYAPVISAEK*AYHEQLSVAEITNA**C**FEPANQMVK*CDPRHG	IPI00471523.1	Tubulin alpha-3 chain
S	(AA: 265–304) HYAPVVSAEK*AYHEQLSVAEITNA**C**FEPANQMVK*CDPHHG	IPI00361239.4	49 kDa protein
P	K.YMACC#LLYR.G		
S	(AA: 301–330) QMVKCDPRHGK*YMAC**C**LLYR*GDVVPKDVNA	IPI00189795.1	Tubulin alpha-1A chain
S	(AA: 301–330) QMVKCDPRHGK*YMAC**C**LLYR*GDVVPKDVNA	IPI00339167.4	Tubulin alpha-1B chain
S	(AA: 301–330) QMVKCDPRHGK*YMAC**C**LLYR*GDVVPKDVNA	IPI00362927.1	Tubulin alpha-4A chain
S	(AA: 301–330) QMVKCDPRHGK*YMAC**C**LLYR*GDVVPKDVNA	IPI00364046.2	Tubulin alpha-1C chain
S	(AA: 244–273) QLVKCDPRLGK*YMAC**C**LLYR*GDVVPKDVNE	IPI00870141.1	Tubulin, alpha-like 3
P	R.SIQFVDWC#PTGFK.V		
S	(AA: 331–360) AIATIKTKR*SIQFVDW**C**PTGFK*VGINYQPP	IPI00339167.4	Tubulin alpha-1B chain
S	(AA: 331–360) AIAAIKTKR*SIQFVDW**C**PTGFK*VGINYQPP	IPI00362927.1	Tubulin alpha-4A chain
S	(AA: 325–354) DIATIKTKR*SIQFVDW**C**PTGFK*VGINYQPP	IPI00361239.4	49 kDa protein
P	R.AVC#MLSNTTAIAEAWAR.L		
S	(AA: 361–400) TVVPGGDLAKVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00189795.1	Tubulin alpha-1A chain
S	(AA: 361–400) TVVPGGDLAKVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00339167.4	Tubulin alpha-1B chain
S	(AA: 361–400) TVVPGGDLAKVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00362927.1	Tubulin alpha-4A chain
S	(AA: 361–400) TVVPGGDLARVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00364046.2	Tubulin alpha-1C chain
S	(AA: 361–400) TVVPGGDLAKVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00464587.1	Tubulin alpha-8 chain
S	(AA: 361–400) TVVPGGDLAKVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00471523.1	Tubulin alpha-3 chain
S	(AA: 222–261) TVVPGGDLAKVQRA*V**C**MLSNTTAIAEAWAR*LDHKFDLMYA	IPI00764107.1	Similar to tubulin alpha-8 chain

Table 2 continued

P Palmitoyl-peptide *S* Protein sequence		IPI accession	Description
P	R.EIVHLQAGQC#GNQIGAK.F		
S	(AA: 1–30) MR*EIVHLQAGQ**C**GNQIGAK*FWEVISDEHGI	IPI00400573.1	Tubulin beta-2C chain
S	(AA: 1–30) MR*EIVHLQAGQ**C**GNQIGAK*FWEVISDEHGI	IPI00765366.1	Tubulin, beta 4
P	K.ESESCDC#LQGFQLTHSLGGGTGSGMGTLLISK.I		
S	(AA: 121–160) RK*ESESCD**C**LQGFQLTHSLGGGTGSGMGTLLISK*IREEYP	IPI00475639.4	Tubulin beta-2A chain
S	(AA: 121–160) RK*ESESCD**C**LQGFQLTHSLGGGTGSGMGTLLISK*IREEYP	IPI00655259.1	Tubulin beta-2B chain
P	K.LTTPTYGDLNHLVSATMSGVTTC#LR.F		
S	(AA: 211–250) CFRTLK*LTTPTYGDLNHLVSATMSGVTT**C**LR*FPGQLNADL	IPI00197579.2	Isoform 1 of tubulin beta-5 chain
S	(AA: 211–250) CFRTLK*LTTPTYGDLNHLVSATMSGVTT**C**LR*FPGQLNADL	IPI00475639.4	Tubulin beta-2A chain
S	(AA: 211–250) CFRTLK*LTTPTYGDLNHLVSATMSGVTT**C**LR*FPGQLNADL	IPI00655259.1	Tubulin beta-2B chain
S	(AA: 211–250) CFRTLK*LTTPTYGDLNHLVSATMSGVTT**C**LR*FPGQLNADL	IPI00765366.1	Tubulin, beta 4
P	K.NMMAAC#DPR.H		
S	(AA: 291–320) QQMFDAK*NMMAA**C**DPR*HGRYLTVATVFRGP	IPI00195673.1	Tubulin, beta 6
S	(AA: 291–320) QQVFDAK*NMMAA**C**DPR*HGRYLTVAAVFRGR	IPI00197579.2	Isoform 1 of tubulin beta-5 chain
S	(AA: 291–320) QQMFDAK*NMMAA**C**DPR*HGRYLTVATVFRGR	IPI00362160.1	Tubulin beta-3 chain
S	(AA: 291–320) QQMFDAK*NMMAA**C**DPR*HGRYLTVAAVFRGR	IPI00400573.1	Tubulin beta-2C chain
S	(AA: 291–320) QQMFDSK*NMMAA**C**DPR*HGRYLTVAAIFRGR	IPI00475639.4	Tubulin beta-2A chain
S	(AA: 291–320) QQMFDSK*NMMAA**C**DPR*HGRYLTVAAIFRGR	IPI00655259.1	Tubulin beta-2B chain
S	(AA: 291–320) QQMFDAK*NMMAA**C**DPR*HGRYLTVAAVFRGR	IPI00765366.1	Tubulin, beta 4
S	(AA: 59–88) QQVFDAK*NMMAA**C**DPR*HGRYLTVAAVFRGR	IPI00829443.1	Isoform 2 of tubulin beta-5 chain
P	K.TAVC#DIPPR.G		
S	(AA: 341–370) FVEWIPNNVK*TAV**C**DIPPR*GLKMAVTFIGN	IPI00197579.2	Isoform 1 of tubulin beta-5 chain
S	(AA: 341–370) FVEWIPNNVK*TAV**C**DIPPR*GLKMSATFIGN	IPI00400573.1	Tubulin beta-2C chain
S	(AA: 341–370) FVEWIPNNVK*TAV**C**DIPPR*GLKMSATFIGN	IPI00475639.4	Tubulin beta-2A chain
S	(AA: 341–370) FVEWIPNNVK*TAV**C**DIPPR*GLKMSATFIGN	IPI00655259.1	Tubulin beta-2B chain
S	(AA: 341–370) FVEWIPNNVK*TAV**C**DIPPR*GLKMAATFIGN	IPI00765366.1	Tubulin, beta 4
S	(AA: 109–138) FVEWIPNNVK*TAV**C**DIPPR*GLKMAVTFIGN	IPI00829443.1	Isoform 2 of tubulin beta-5 chain

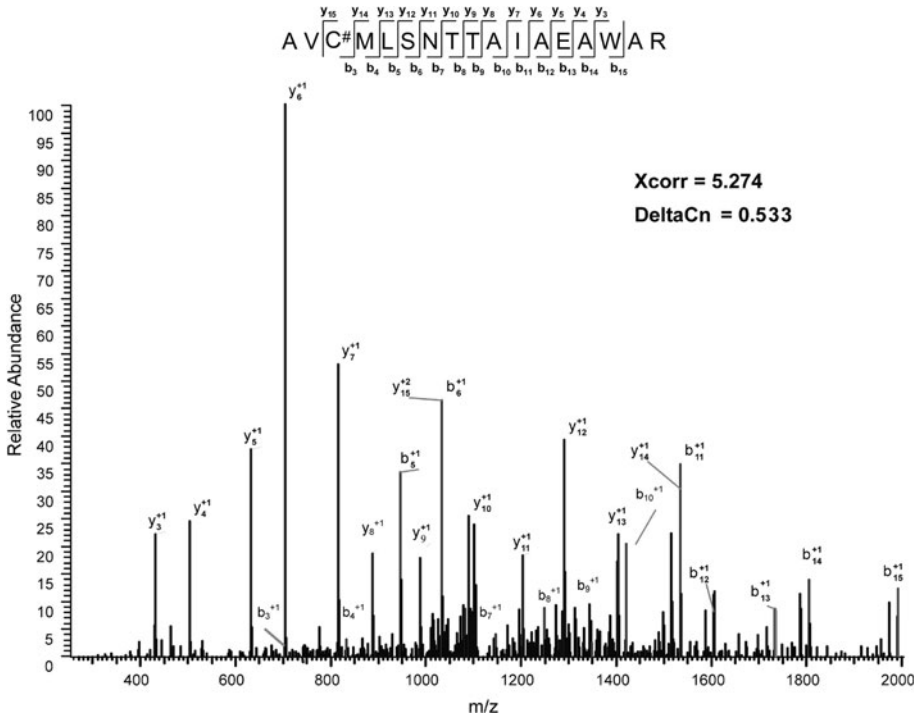

Fig. 3 An example of a MS/MS spectrum acquired by a LTQ linear ion trap mass spectrometer that was assigned to a peptide containing palmitoylated site (C376). Spectrum was generated by collision-induced dissociation in the LTQ linear ion trap. Fragment ions assigned to both y-ions and b-ions were labeled. The palmitoylation site was marked with "#"

Palmitoylations of C20, C213 and C305 of α-tubulin, which were previously reported to be palmitoylated at lower levels [9], were not detected in our study. We think it is likely that the palmitoylation levels of these cysteine residues were too low to be detected by our MS instrument.

Because a marked reduction in polymerization competence of palmitoylated tubulin has been reported [22], it can be concluded the purified tubulin had a low level of palmitoylation because we purified it through two cycles of polymerization-depolymerization. It was necessary to palmitoylate the tubulin in vitro before identification. There are 20 cysteine residues that are potentially available for palmitoylation per tubulin heterodimer. As reported, a maximum of approximately six palmitates are added per heterodimer in 3 h at 37 °C under our native experimental conditions [22]. Because some cysteine residues may only be partially palmitoylated, the number of palmitoylation sites identified in our results was certainly more than six.

3.2 This Method can be Used to Identify Other Palmitoylated Proteins and Palmitoylation Sites as Well

Until now, more than 100 proteins have been reported to be modified with palmitate, a 16-carbon saturated fatty acid. Post-translational modification of proteins by palmitate has multiple effects on protein function. It influences membrane binding and membrane targeting of modified proteins. In particular, many palmitoylated proteins are concentrated in lipid rafts. This concentration is required for efficient signal transduction [12]. Although palmitoylation is a prevalent and important post-translational modification of proteins, its underlying mechanisms remain poorly understood. To gain a better understanding of the role of palmitoylation, palmitoylated proteins and palmitoylation sites must be characterized. Several methods based on fatty acid exchange have been developed for this purpose. A variety of different thiol-specific compounds containing biotin, such as biotin-BMCC, biotin-PEO-maleimide, biotin-PEO-iodoacetamide or biotin-HPDP, have been applied to label the free sulfhydryl groups liberated after cleaving palmitate from palmitoylation sites [5]. Recently, a method to identify palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue was reported [7]. In our protocol, the palmitoyl groups on the cysteine residues of tubulin were exchanged to HPDP-biotin. After tryptic digestion, the HPDP-biotinylated peptides were enriched by streptavidin agarose, released by treatment with guanidine hydrochloride and finally subjected to LC–ESI–MS/MS analysis. The method described here can also be applied to identify this post-translational modification in other biological systems.

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