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Mass Spectrometric Quantitation of Tubulin Acetylation from Pepsin-Digested Rat Brain Tissue Using a Novel Stable-Isotope Standard and Capture by Anti-Peptide Antibody (SISCAPA) Method

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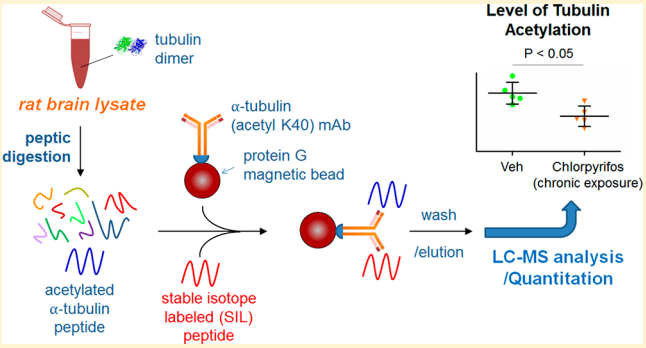
Supporting Information

ABSTRACT: Acetylation of α-tubulin at Lys-40 is a potential biomarker for cognitive deficits in various neurological disorders. However, this key post-translational modification (PTM) has not been previously studied with mass spectrometry, due to the inadequate distribution of tryptic cleavage sites. Following peptic digestion, a surrogate sequence containing this key PTM site was identified and was found to be stable and quantitatively reproducible. A highly sensitive and specific SISCAPA−LC−MS method for quantitating rat brain tubulin acetylation was developed, validated, and applied, and only required a small amount of tissue (2.2 mg). This workflow includes peptic digestion, stable-isotope dilution, capture with antiacetylated peptide antibody bound on protein G beads, and quantitation using LC−MS. The method allowed a lower limit of quantitation at 2.50 pmol/mg and provided a linear range of 2.50−62.50 pmol/mg. Selectivity, intra and interday precision and accuracy were also validated. This method has been successfully applied in a preclinical study of organophosphate neurotoxicity, and we found that chronic exposure to chlorpyrifos led to a significant and persistent inhibition of brain tubulin acetylation.



The tubulin protein superfamily includes α-, β-, γ-, Δ-, δ-, ε-, and ζ-tubulins.[1] Heterodimers of α- and β-tubulin polymerize into microtubules (MTs) accompanying GTP hydrolysis. In neurons, MTs are "railways" of the axonal transport system responsible for the transport of various cargoes including lipids, mitochondria, synaptic vesicles, receptor proteins, mRNAs, and other macromolecules through the cytoplasm of a neuron's axon. In the system, the motor protein kinesin delivers from the cell body to the synaptic terminal, and dynein delivers in the opposite direction.[2] Tubulin receives a host of post-translational modifications (PTMs) in the nervous system, including acetylation,[3] detyrosination,[4] Δ2 modification,[5] polyglutamylation,[6] and polyglycylation.[7] Among them, tubulin acetylation occurs at Lys-40 on the lumen surface and is preserved in α-tubulin isotypes. It is mediated with α-tubulin acetyltransferase 1 (ATAT1)/MEC-17 from inside out, and its deacetylation is mediated with histone deacetylase 6 (HDAC6) and NAD-dependent deacetylase sirtuin-2 (SIRT2).[8,9] Lys-40 acetylation is associated with dendrite growth, MT stabilization, and binding with motor proteins,[10] and experiments have shown that kinesin-1 can only bind to MTs when Lys-40 of α-tubulin was acetylated.[10]

Decreases in tubulin acetylation have been observed in several neurodegenerative diseases (e.g., Huntington's disease,[11] Parkinson's disease,[12] and Alzheimer's disease[13]), and they have also been associated with the neurotoxicity of different toxicants (e.g., organophosphates[14] and viral protein gp120[15]), in the absence of alterations in total α-tubulin[11,12,15] or total tubulin levels.[14] In patients with Huntington's disease (HD), tubulin acetylation decreases were concomitant with the impaired transport of MT-dependent vesicles containing brain-derived neurotrophic factor (BDNF). The transport defects could be rescued by increasing tubulin acetylation using an HDAC inhibitor trichostatin A (TSA). This increased acetylation enhanced the recruitment of motor proteins including kinesin-1 and dynein.[11] Similarly, diisopropyl fluorophosphate (DFP), an organophosphate compound, was observed to cause a decline in neuronal tubulin acetylation after

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in vitro incubation. This alternation, coupled with deficits in MT dynamics, mitochondrial transport, and dopamine release, were found to be correctable or improved by tubacin, a drug that inhibits HDAC6.[14] Despite different mechanisms and symptoms related to above neurological dysfunctions and diseases, there is a correlation of impaired cognitive processes including attention, learning, memory, and other domains of cognition with impaired tubulin acetylation and axonal transport.[2,11,14−17] Additionally, alterations in tubulin acetylation have also been observed in erythrocytes from diabetic patients[18] and leukemia cells,[19] allowing this PTM to potentially serve as a biomarker in the blood.

In consideration of tubulin acetylation as a promising biomarker for cognitive deficits, it is necessary to have analytical methods to measure and evaluate this key PTM. Western blot is most commonly applied for detecting tubulin acetylation.[20,21] A quantitative method was validated using chemically acetylated tubulin as the standard, and the percentages of acetylated tubulin relative to total tubulin were estimated in three different cell lines (0−7%). However, the potential cross-reactivity of the antibody with other proteins or other acetylated lysines on tubulin[20,22] may complicate the result, and Western blot analysis only provides semiquantitative results. Immuno-mass spectrometry has been proven as a reliable technique for the targeted quantitation of proteins in biological matrices, combining advantages of antibody specificity and mass spectrometry selectivity.[23,24] Currently, no mass-spectrometry-based assay has been reported for the quantitation of tubulin acetylation. This is predominantly due to the difficulty of identifying a surrogate peptide containing this key PTM subsequent to nearly exclusive use of trypsin, whereas there is a lack of cleavage sites near α-tubulin Lys-40. In this current work, such a peptide was first identified using mass spectrometry after peptic digestion. Peptic digestion also allowed identification of a new organophosphorylation site at Lys-40 after the incubation of tubulin with chlorpyrifos oxon (CPO). To absolutely quantitate Lys-40-acetylated α-tubulin in rat brain tissue, we developed and validated a novel stable-isotope standard and capture by anti-peptide antibody (SISCAPA) method to enrich the target peptide and the isotope-labeled internal standard (ISTD) from pepsin-digested brain tissue lysate, followed by mass spectrometry detection. This method has been successfully applied to facilitate the mechanistic study of organophosphate neurotoxicity, and we observed that chronic treatment with chlorpyrifos led to persistent decreases in tubulin acetylation in rat brain tissue.

EXPERIMENTAL PROCEDURES

Materials. Acetyl-α-tubulin (Lys-40) monoclonal antibodies: mouse monoclonal antibody (mAb) [6-11B-1] was acquired from Novus Biologicals (Littleton, CO), and rabbit mAb [D20G3] was purchased from Cell Signaling Technology (Boston, MA). Protein G magnetic beads were obtained from Biorad (Hercules, CA). Whole rat brains (Wistar Hannover) were purchased from Bioreclamation IVT (Westbury, NY). Zeba spin desalting columns (7K MWCO, 0.5 mL) and Dulbecco's phosphate-buffered saline (PBS) (calcium- and magnesium-free) were purchased from Thermo Fisher Scientific (Waltham, MA). Pure tubulin (bovine) was purchased from Cytoskeleton (Denver, CO). Modified porcine pepsin was purchased from Princeton Separations (Adelphia, NJ). The protease inhibitors trichostatin A (TSA), phenylmethylsulfonyl fluoride (PMSF), and 1,10-phenanthroline were purchased from Cayman Chemical (Ann Arbor, MI), Research Products International (Mt Prospect, IL), and Oakwood Chemical (Estill, SC), respectively. Fmoc amino acids and resins for solid phase peptide synthesis, including Fmoc−Leu−OH ($^{13}C_6$,^{15}N) and Fmoc−Lys(Ac)−OH, were purchased from ChemPep (Wellington, FL). 2-(*N*-Morpholino)ethanesulfonic acid (MES) anhydrous was purchased from Amresco (Solon, OH). Glycine, tris(hydroxymethyl)aminomethane, ethylenediaminetetraacetic acid (EDTA), magnesium chloride, dithiothreitol (DTT), hydrochloric acid, and LC−MS grade solvents including formic acid, trifluoroacetic acid, acetonitrile, and water were purchased from Sigma−Aldrich (St. Louis, MO).

Solid Phase Peptide Synthesis. The peptides YCLEHGIQPDGQMPSDKTIGGGDDSF (Lys-40 peptide), YCLEHGIQPDGQMPSDK$_{(ac)}$TIGGGDDSF (PTM peptide for Lys-40-acetylated α-tubulin), ELYCLEHGIQPDGQMPSDK$_{(ac)}$TIGGGDDSFNT (extended PTM peptide), and YCL*EHGIQPDGQMPSDK$_{(ac)}$TIGGGDDSF (L* = 7 Da mass shift, stable-isotope-labeled peptide, SIL peptide) were synthesized using Fmoc solid phase peptide synthesis with an AAPPTec Focus XC automated peptide synthesizer (Louisville, KY). Peptides were purified using an Agilent Polaris 5 C18-A preparative column (150 × 21.2 mm, 5 μm) on the Shimadzu LC-20AT HPLC system with a SPD-20A UV−vis detector (Kyoto, Japan).

LC−MS/MS Conditions. Mobile phase A was water containing 0.01% (v/v) formic acid, and mobile phase B was acetonitrile. A Halo peptide ES-C18 column (100 × 4.6 mm, 2.7 μm) by Advanced Materials Technology (Wilmington, DE) coupled with a C-18 guard column (4.0 × 2.0 mm) by Phenomenex (Torrance, CA) was used to separate the peptides. Different LC−MS instrumentation and parameters were applied for peptide sequencing and quantitation. For peptide sequencing, a Waters UPLC system (Milford, MA) coupled to a Waters SYNAPT G2 Q-TOF mass spectrometer with an ESI source (Milford, MA) was operated for LC−MS/MS analysis. Peptides were separated using a gradient method, with a 0.3 mL/min flow rate (minute, % mobile phase B): (0, 5), (60, 50), (60.01, 95), (67.50, 95); the injection volume was 15 μL, and the run time for each injection was 75 min. The MS settings for the SYNAPT G2 mass spectrometer were: capillary voltage: 2.00 kV, sample cone voltage: 35 V, extraction cone voltage: 4.0 V, source temperature: 120 °C, desolvation temperature: 500 °C, and desolvation gas: 500 L/h. Data were acquired with the data-dependent acquisition (DDA) function: 1 s MS survey scan in the m/z range of 300−1900 was followed by MS/MS scans of up to 3 ions, when intensity rose above 1500 counts/s.

The MS/MS scan was acquired over the m/z range of 100−1900, with a 2 s scan rate, and was switched to an MS survey scan after 3 scans. The trap collision energy was set using charge state recognition, applying the default files for 1−4 positive charge states. For peptide quantitation, an Agilent 1100 binary pump HPLC system (Santa Clara, CA) interfaced to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer with an ESI source (Milford, MA) was operated for LC−MS analysis. The capillary voltage was 3.80 kV, the cone voltage was 32 V, the extractor voltage was 1, and the RF lens voltage was 2.0 V. The source temperature was 120 °C, the desolvation gas flow rate was 500 L/h, and the desolvation temperature was 500 °C. Peptides were separated using a gradient method, with a 0.3 mL/min flow rate, (minute, % mobile phase B): (0, 25), (15, 40), (15.01, 95), (22.50, 95), the

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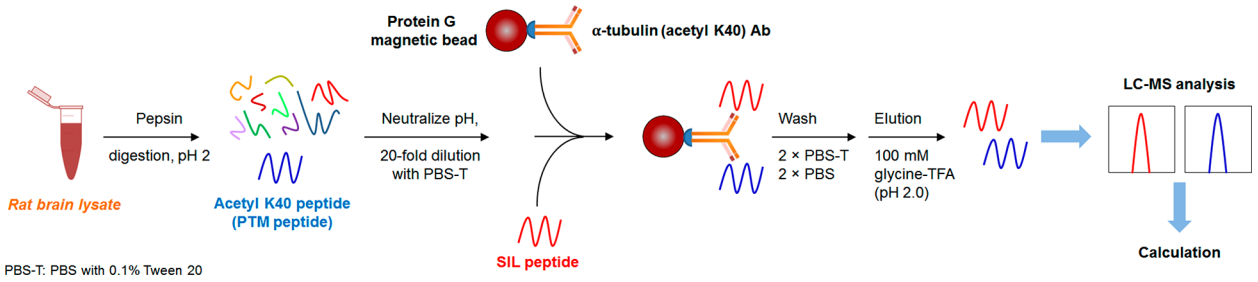

Figure 1. Schematic of the SISCAPA–LC–MS assay for the quantitation of Lys-40-acetylated α-tubulin from rat brain lysate.

injection volume was 30 μL, and the run time for each injection was 30 min. The selected ion recording (SIR) function was applied to monitor precursor ions m/z 1406.0 and 1409.5 (mass window = 1.0 Da) at the retention time of 7.5 min for the PTM peptide and the SIL peptide, respectively. Data were processed with Waters software including a ProteinLynx Global Server (PLGS) 2.4 and Masslynx 4.1 (Beverly, MA).

Tissue Extract. One volume of rat brain tissue was mixed with 1.5 volumes of MES buffer (100 mM MES, 1 mM magnesium chloride, 1 mM EDTA, pH 6.8) supplemented with the enzyme inhibitor cocktail (1 mM PMSF, 1 mM 1,10-phenanthroline, and 5 μM of the HDAC inhibitor trichostatin A, TSA). Brain homogenate was prepared on ice using a tissue grinder. Brain homogenate was frozen at −80 °C for 30 min and thawed at room temperature. The homogenate was sonicated five times for 10 s with 30 s rest intervals on melted ice. The sample was centrifuged at 30 000g (Beckman TL 120.2) at 4 °C for 15 min to remove cell debris, and the supernatant was centrifuged again at 100 000g (Beckman TL 120.2) at 4 °C for 1 h. The final supernatant was collected as brain tissue lysate. The protein concentration was determined with a NanoDrop Lite Spectrophotometer by Thermo Fisher Scientific (Waltham, MA).

Immunoenrichment of the Acetylated Peptide. Brain tissue lysate was mixed with urea to a concentration of 2 M and acidified to pH 2.0 with hydrochloric acid. Total proteins were digested with pepsin (enzyme/total protein = 1:2000) at 37 °C for 18 h. The SISCAPA procedures were based on the immunoprecipitation (IP) protocol by Biorad SureBeads magnetic beads with a minor modification: a 5 μL aliquot of peptic digest (equal to 2.2 mg of brain tissue) was neutralized with 1 M sodium hydroxide, combined with 95 μL of phosphate-buffered saline supplemented with 0.1% Tween 20 (PBS-T), fresh inhibitor cocktail, and 11.4 pmol of SIL peptide. Each 100 μL suspension of protein G magnetic beads was washed four times with 500 μL of PBS-T, before incubation with 2 μL of antibody at 4 °C with constant shaking for 90 min. Antibody-bound magnetic beads were washed three times with 500 μL of PBS-T, before incubation, with 100 μL of diluted brain tissue lysate at 4 °C with constant shaking for 6 h. After magnetization of the beads, the supernatant was discarded, and beads were washed twice with ice-cold PBS-T and PBS, respectively. Enriched peptides were eluted with 40 μL of 100 mM glycine/TFA (pH 2.0) supplemented with 120 pmol of extended peptide at room temperature for 5 min. A summary of the SISCAPA workflow is shown in Figure 1.

Method Validation. The same protocol for brain tissue lysate preparation was used to prepare a blank matrix for acetylated tubulin, except that TSA, an HDAC6 inhibitor, was absent. Selectivity, linearity, intra and interday precision, and accuracy tests were conducted to validate the method. Each test was conducted using 100 μL of 20-fold diluted blank matrix (equal to 2.2 mg of brain tissue) spiked with 11.4 pmol of SIL peptide and the corresponding amount of PTM peptide. The selectivity (n = 3) was tested by comparing the chromatograms of analytes in blank brain tissue lysate, with analytes at LLOQ (2.50 pmol/mg). The linearity was validated with calibration standard samples over the range of 2.50–62.50 pmol/mg (2.50, 6.25, 12.50, 15.00, 25.00, 37.50, and 62.50 pmol/mg) with ISTD (5.00 pmol/mg). Calibration curves were made from peak area ratios between the PTM and the ISTD against their nominal concentration ratios. The intraday (n = 5) and interday (n = 15) precision and accuracy were tested by QC samples at 2.50, 7.50, 15.00, and 37.50 pmol/mg.

Animal Study. Wistar Hannover rats were subcutaneously (s.c.) administered with 18 mg/kg chlorpyrifos in peanut oil every other day for 30 days with a 45 day washout period. Peanut oil with 3% DMSO was administered (s.c.) to rats as the control. All procedures employed in this study were reviewed and approved by the Augusta University Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised in 1996. Rats were anesthetized with isoflurane. The whole brain of the sacrificed animal was removed, washed in PBS, and kept frozen at −80 °C until analysis. Brain tissue lysate was prepared, and sample preparation was done with the immunoenrichment method. The concentration of acetylated tubulin was calculated, and a student t test was conducted to compare the difference between the control and the chlorpyrifos treatment group.

CPO-Labeled Peptic Peptide from Pure Tubulin. A volume of 100 μL of 2 mg/mL purified bovine tubulin dissolved in 50 mM Tris HCl (pH 8.0) was treated with 1 mM CPO at room temperature for 6 h. The labeling was repeated overnight with the same amount of CPO. Free CPO was removed from the buffer with the desalting column. The protein was acidified to pH 2.0 with hydrochloride, followed by pepsin digestion and peptide sequencing, using the corresponding methods described above.

RESULTS AND DISCUSSION

Identification of the Surrogate Peptide. Trypsin is the most common protease in proteomic studies, known for its cleavage efficiency, specificity, and reproducibility on the C-terminus of Lys and Arg residues. However, trypsin has certain limitations. Membrane proteins and tightly folded proteins often resist trypsin digestion,[25] and the inadequate distribution of trypsin cleavage sites in certain proteins generates either too long (>20) or too short (<5) peptides, which are challenging for mass spectrometry analysis. Various types of PTMs on

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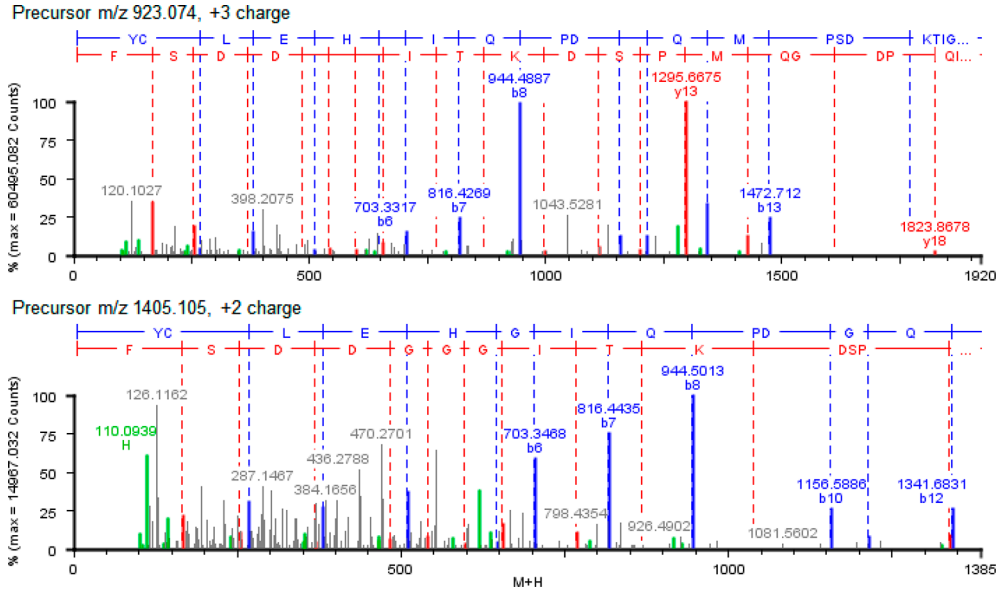

Figure 2. MS/MS fragmentation spectra of the YCLEHGIQPDGQMPSDK$_{40}$TIGGGDDSF peptide (upper) and the YCLEHGIQPDGQMPSDK$_{40(ac)}$TIGGGDDSF peptide (lower). The spectra were acquired using a Waters SYNAPT G2 Q-TOF mass spectrometer.

Table 1. Identification of the YCLEHGIQPDGQMPSDK$_{40}$TIGGGDDSF Peptide (Upper) and YCLEHGIQPDGQMPSDK$_{40(ac)}$TIGGGDDSF Peptide (Lower) with ProteinLynx Global Server (PLGS) 2.4

m/z	charge	peak mW	peptide mW	delta (Da)	delta (ppm)	log likelihood	sequence	PTM	RT (min)
923.074	3	2766.199	2766.190	0.009	3.18	64.87	YCLEHGIQPDGQMPSDK$_{40}$TIGGGDDSF		26.93
1405.105	2	2808.213	2808.201	0.012	4.27	28.78	YCLEHGIQPDGQMPSDK$_{40}$TIGGGDDSF	acetyl K$_{40}$	28.80

trypsin cleavage sites pose another problem because even a small PTM like lysine acetylation limits trypsin access and resists digestion.[26] In the vicinity of α-tubulin Lys-40, there is only Arg-2 and Lys-60. Even with the assumption that no cleavage is missed, a very long peptide with 38 or 58 amino acid residues, when Lys-40 residue is free or acetylated, would be generated by trypsin, beyond the fragmentation and sequencing capability of common mass spectrometry instrumentation. We tested tryptic digestion of purified tubulin and unexpectedly failed to identify a peptide containing Lys-40.

Pepsin is an alternative protease when trypsin is not informative. Pepsin requires a low pH (1.0−3.0) and predominantly cleaves on the C-terminus of aromatic and hydrophobic amino acid residues (Phe, Leu, Tyr, and Trp), not overlapping with trypsin. At low pH, proteins can further denature, increasing pepsin access, making pepsin an ideal protease for proteins with high hydrophobicity and tight conformations. The fact that pepsin is active at low pH (2.5) and temperature (4 °C) is also essential for hydrogen/deuterium exchange experiments.[27] From the peptic digest of pure bovine tubulin, through data-dependent acquisition (DDA) mode, we identified a peptide sequence containing Lys-40: YCLEHGIQPDGQMPSDK$_{40}$TIGGGDDSF (Lys-40 peptide), and the same sequence for acetylated α-tubulin (PTM peptide) was also identified after immnuoenrichment (Figure 2 and Table 1). The most abundant charge state for the Lys-40 peptide was +3, while +2 was observed for the PTM peptide due to acetylation on the positively charged Lys. This sequence is conserved in all α-tubulin chains expressed in human, rat, and bovine brains including α1A-, α1B-, α1C-, and α4A-tubulin,[28] but not in α8-tubulin whose sequence is divergent and lacks Lys-40. With the assumption that the ratio of α-tubulin to β-tubulin is 1:1 in purified bovine tubulin and α8-tubulin and other tubulin isotypes are negligible in the brain,[29] the pepsin digest efficiency was calculated with ratios of peak areas for the Lys-40 peptide between the synthetic source and the peptic digest of 1, 10, and 100 μg/mL total bovine tubulin diluted with 1 mg/mL lysozyme. The digestion efficiency was close to 50% across all tested concentrations, and the RSDs are all within 10% (Table 2), demonstrating the high efficiency and reproducibility of peptic digestion for this peptide sequence. In this project, other advantages for peptic digestion included: pepsin as an aspartic acid protease is not affected by inhibitors in the preparation of brain tissue lysate including serine protease inhibitors, cystein protease inhibitors, and metallo protease inhibitors; the acidic pH for peptic digestion prevents the degradation of the peptide, and Cys and Met residues in the sequence, which are prone to oxidation at higher pH values, were protected.

Table 2. Pepsin Digestion Efficiency (%, n = 3) for the Generation of the Lys-40 Peptide (YCLEHGIQPDGQMPSDKTIGGGDDSF) from Purified Bovine Tubulin

	1 μg/mL	10 μg/mL	100 μg/mL
digest efficiency	45.4 ± 1.9	53.2 ± 5.2	48.9 ± 2.4
RSD (%)	4.2	9.8	4.9

Pepsin has a similar digestion speed as trypsin. Its applicability in proteomics research has been under appreciated mostly due to its lower specificity (compared to trypsin), which is pH-dependent and allows many missed cleavages. From our

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observation for tubulin, most of the peptic cleavages occurred at the preferred sites, but there were many missed cleavages as well. However, the weaknesses of pepsin can be overcome by maintaining strict digestion conditions and with the advances in proteomic search engines. The reproducibility of pepsin digestion was assessed using the large protein phosphorylase b. Although many nonreproducible peptides were generated in replicate digestions, highly reproducible peptides achieved 85% sequence coverage. More importantly, peptic peptides were found quantitatively reproducible, and the peak areas for reproducible peptic peptides showed an average %RSD of 5.4%.[30] By combining this fact with our data, we hope to raise more awareness of the quantitative reproducibility of peptic digest, its complementarity with tryptic digest, and potential in targeted protein quantitation.

Method Development. SISCAPA is a method for targeted protein quantitation from biological samples. Unlike other IP-MS-based assays, SISCAPA uses an anti-peptide antibody rather than an anti-protein antibody, and proteolytic digestion is done before rather than after immunoenriching the target. The target peptide serves as a surrogate for the protein, and a synthetic isotope-labeled version of the peptide (SIL peptide) is spiked to the digest serving as an internal standard (ISTD). These two peptides are indistinguishably enriched through sample preparation and have the same ionization and fragmentation efficiency in mass spectrometry, but they can be separated according to their mass-to-charge ratios. The amount of target protein is calculated with the amount of ISTD, signal ratio between the surrogate peptide and the ISTD, and the digestion efficiency.[31]

SISCAPA is selected for the quantitation of α-tubulin acetylation in brain tissue because tubulin binds with multiple types of proteins including microtubule-associated proteins (MAPs) and motor proteins, which would interfere with immunoassays and increase matrix complexity, while immunopurification from the proteolytic digest can eliminate these interactions. SISCAPA also separates the target peptide from other tubulin peptides, further decreasing matrix effects for mass spectrometry analysis. The anti-peptide antibody is essential for the success of SISCAPA. Two antibodies are commercially available in immunoassays for Lys-40-acetylated α-tubulin (Western Blot, ELISA, etc.): mouse mAb 6-11B-1 and rabbit mAb D20G3. The epitope for 6-11B-1 is located on the α3 isoform of Chlamydomonas axonemal α-tubulin, within four residues of acetylated Lys-40.[32,33] This epitope is completely contained in the PTM peptide sequence, permitting mAb 6-11B-1 to act as an anti-peptide antibody for the PTM peptide.

The majority of mass-spectrometry-based protein quantitation is conducted using multiple reaction monitoring (MRM) methods. MRM detects an ion transition from a precursor ion (an ionized peptide) to a fragment ion, providing good selectivity and specificity. Peptide length is one of the key criteria for the selection of a signature peptide, and an ideal peptide should have 8−22 amino acids.[34] Large peptides are not preferred because they tend to generate many fragment ions with similar intensities instead of a few predominant ones, normally resulting in low MRM signals.[35] The transition from the ionized Lys-40 peptide to the most intense fragment ion (m/z 923.7 → m/z 945.1, b8) was tested using the synthetic source. Both the peak area and signal-to-noise ratio (S/N) are much weaker than collected from selected ion recording (SIR) of the precursor ion (Figure S1); therefore, a SIR function was applied for detecting the target peptide.

There is a Cys and a Met residue in the PTM peptide, which may oxidize in sample preparation procedures. The stability of the peptide was tested by overnight incubation in digestion and immunoprecipitation buffers at the corresponding temperatures. The stability was demonstrated as the percentage relative to the time-zero sample. In Figure 3, no degradation

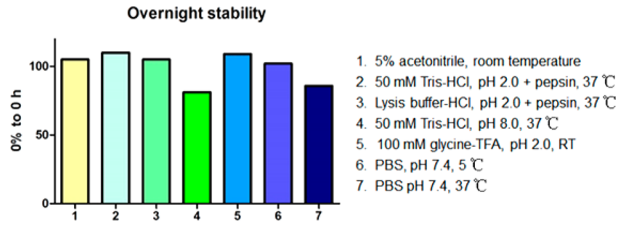

Figure 3. Overnight stability of the peptide YCLEHGIQPDGQMPS-DK$_{40(ac)}$TIGGGDDSF (3.56 μM) in different solutions.

was observed in 5% acetonitrile, pepsin digestion buffers (50 mM Tris HCl, pH 2, with pepsin, 37 °C and lysis buffer, pH 2.0, with pepsin, 37 °C), binding/washing buffer (PBS, pH 7.4, 5 °C), or elution buffer (100 mM glycine/TFA, pH 2.0, room temperature). Moderate degradation was observed at a neutral to mildly basic pH and a high temperature (PBS, pH 7.4, 37 °C and 50 mM Tris HCl, pH 8.0, 37 °C), which were not applied in this assay.

Immunoprecipitation was tested with two commercially available mAbs for Lys-40-acetylated α-tubulin: 6-11B-1 and D20G3. The affinity of these two antibodies with protein A and G magnetic beads was tested, by measuring the recovery of the synthetic PTM peptide from bovine serum albumin in PBS-T. The combination of 6-11B-1 mouse mAb with protein G allowed the highest recovery of the acetylated tubulin peptide and was selected for the SISCAPA assay (Table S1). The recovery was lower than expected and could be related to a combination of antibody−antigen binding affinity, protein A/G-antibody binding affinity, and the antibody/antigen ratio. The immunoprecipitation protocol was adjusted for mass spectrometry analysis. The elution buffer (100 mM glycine/HCl, pH 2.0) was replaced with 100 mM glycine/TFA (pH 2.0) and did not affect the recovery of the immunoprecipitation. Multiple elutions with up to three volumes were tested and did not increase the recovery (data not shown). A high concentration of extended PTM peptide was added to the elution buffer to specifically release the PTM peptide from the capture antibody and reduce adsorption of the target peptide to the autosampler vial. The SISCAPA workflow for the quantitation of Lys-40-acetylated α-tubulin from rat brain lysate is shown in Figure 1.

Method Validation. Method validation was conducted with the synthetic PTM peptide spiked to blank brain tissue lysate (brain tissue lysate depleted with tubulin acetylation), given that the Lys-40 peptide sequence from the peptic digest is quantitatively reproducible. The method was validated with selectivity, linearity, precision, and accuracy. Selectivity (n = 3) was validated by analyzing blank samples and spiked standard samples (2.50 pmol/mg) with ISTD (5.00 pmol/mg), and chromatograms were compared (Figure 4a,b). No significant interferences from blank matrices were observed, demonstrating that acetylated tubulin was depleted in the blank matrix, and the sample preparation and LC−MS method have the

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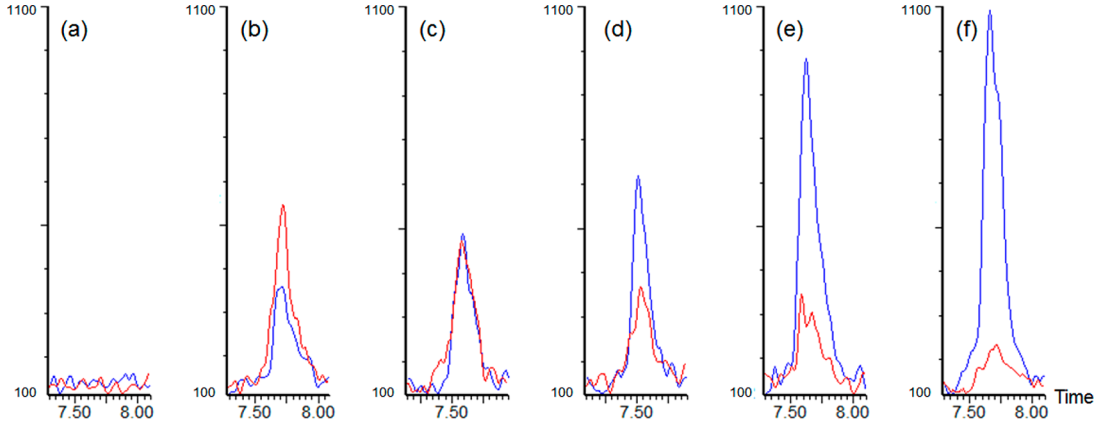

Figure 4. Extracted ion chromatograms for PTM peptide YCLEHGIQPDGQMPSDK$_{40(ac)}$TIGGGDDSF [blue] and SIL peptide YCL*EHGIQPDGQMPSDK$_{40(ac)}$TIGGGDDSF (L* = 7 Da mass shift) [red] from (a) blank brain tissue lysate; calibration standards at (b) 2.50 pmol/mg, (c) 6.25 pmol/mg, (d) 12.50 pmol/mg, (e) 25.00 pmol/mg, and (f) 62.50 pmol/mg of PTM peptide, with 5.00 pmol/mg of SIL peptide. SIR functions were applied for the PTM peptide: mass-to-charge ratio (m/z) 1406.0 and SIL peptide: m/z 1409.5. MS scans were acquired using a Waters Micromass Quattro Micro triple quadrupole mass spectrometer.

necessary selectivity to differentiate and quantitate analytes in brain tissue lysate. The LLOQ of the method was determined as 2.50 pmol/mg, at a signal-to-noise ratio (S/N) > 10 (Figure 4b).

The linearity was tested over the range of 2.50−62.50 pmol/mg with ISTD (5.00 pmol/mg). Signals for the PTM peptide (2.50, 6.25, 12.50, 25.00, and 62.50 pmol/mg) and the SIL peptide are shown in Figure 4b−f. The ISTD signal gradually decreased with an increasing amount of the PTM peptide, reflecting the competition between the target peptide and its isotope-labeled form for antibody capture. The ISTD signal kept the S/N > 10, allowing quantitation of the PTM peptide. The calibration curve, slope, intercept, and R^2 value are shown in Figure 5. The R^2 value was over 0.99, demonstrating a good linearity within the tested range. The intercept on the y-axis was also close to 0. Therefore, we used the following formula to calculate the concentration of acetylated tubulin in the brain tissue: Protein conc (pmol/mg) = SIL conc (pmol/mg) × response ratio × (1/digestion efficiency), response ratio = peak area$_{\text{PTM peptide}}$/peak area$_{\text{SIL peptide}}$, SIL conc = 5.00 pmol/mg.

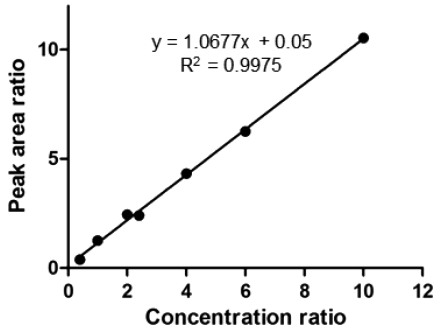

Figure 5. Calibration curve ranging from 2.50 to 62.50 pmol of PTM peptide/mg of brain tissue. The curve was made from peak area ratios between the PTM peptide and the ISTD against their nominal concentration ratios.

The intraday (n = 5) and interday (n = 15) precision and accuracy for the analyte were tested using QC samples at 2.50, 7.50, 15.00, and 37.50 pmol/mg. Precision describes the closeness of a series of measurements from multiple samplings and was represented by relative standard deviation (RSD); accuracy describes the closeness of a measured value to the true value and was represented by relative error (RE). Table 3 shows RSD and RE values of the analyte in all QC samples, which are all below 15% and meet the requirements from Guidance for Industry (bioanalytical method validation) by the US FDA, validating the intra and interday precision and accuracy of this method.

Rat Brain Tubulin Acetylation after Chronic Exposure to Chlorpyrifos. Poisoning by organophosphates poses a serious threat to public health due to their widespread use as agricultural pesticides and is raising concerns due to their easy access by rogue governments and terrorism groups.[36] The mechanism for the acute poisoning is well-known as irreversible inhibition of acetylcholinesterase. However, significant evidence demonstrates that organophosphate exposures resulting in no signs of acute toxicity cause prolonged neurological and neurobehavioral deficits particularly cognitive dysfunction and increase the risk of neurodegenerative diseases.[37−39] Until now, the underlying mechanisms and target proteins have been poorly understood.

This validated SISCAPA−LC−MS assay was applied to analyze brain tissue lysate from rats following repeated

Table 3. Intraday (n = 5) and Interday (n = 15) Precision (RSD) and Accuracy (RE) in Rat Brain Tissue Lysate

nominal conc. (pmol/mg)	intraday measured conc. (pmol/mg)	RSD (%)	RE (%)	interday measured conc. (pmol/mg)	RSD (%)	RE (%)
2.50	2.62 ± 0.13	5.0	4.8	2.66 ± 0.18	6.8	6.4
7.50	7.66 ± 0.59	7.7	2.1	7.81 ± 1.08	13.8	4.1
15.00	12.80 ± 0.77	6.0	−14.7	13.31 ± 1.10	8.3	−11.3
37.50	35.38 ± 3.30	9.3	−5.7	36.01 ± 3.43	9.5	−4.0

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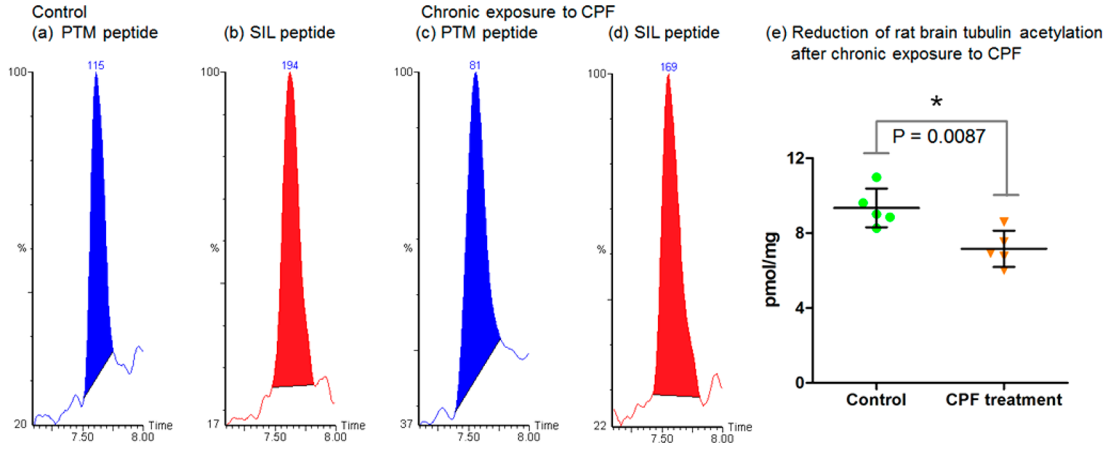

Figure 6. Quantitation of Lys-40-acetylated α-tubulin in rat brain tissue. (a–d) Detection of the PTM and the SIL peptide using LC–MS. Peak areas were labeled. (e) Comparison for tubulin acetylation from control (n = 5, green round) and CPF-treated groups (n = 5, orange triangle). * statistically significant difference (P = 0.0087 < 0.05). MS scans were acquired using a Waters Micromass Quattro Micro triple quadrupole mass spectrometer.

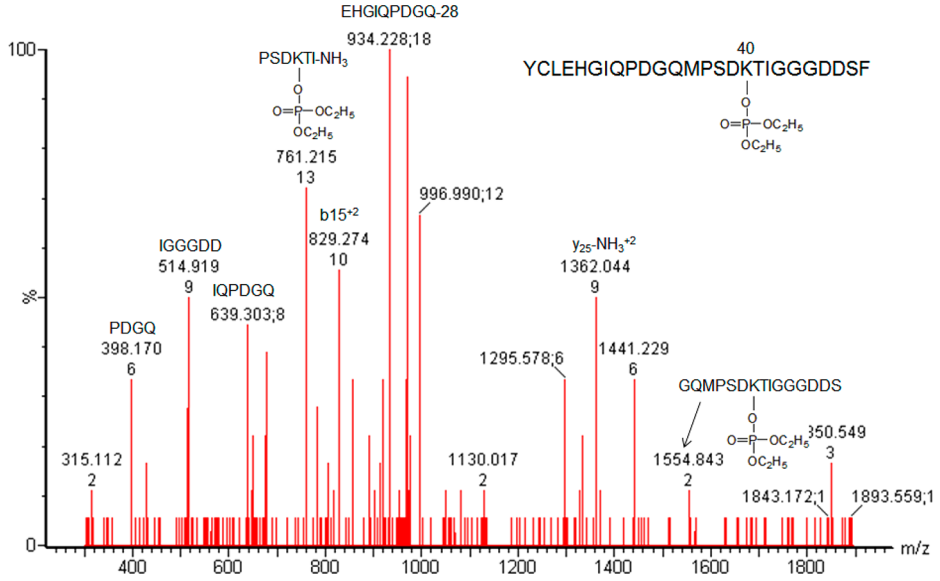

Figure 7. MS/MS fragmentation spectrum of CPO-labeled peptide YCLEHGIQPDGQMPSDK*TIGGGDDSF (* demonstrates a CPO adduction). The precursor ion: m/z of 968.4, +3 charge. The spectrum was acquired using a Waters SYNAPT G2 Q-TOF mass spectrometer.

exposures to chlorpyrifos (CPF), a widely used organophosphate insecticide. The dose for rats (18 mg/kg/every other day for 30 days s.c.) is below the threshold for causing signs of acute toxicity, and it has been previously associated with impairments in axonal transport and cognitive deficits including spatial learning and sustained attention, even after a washout period of 14 to 140 days.[40−42] A 45 day washout period was applied in this experiment before sample collection. After peptic digestion and enriching the PTM peptide as well as spiked ISTD using 6-11B-1 mAb, we detected a lower level of the PTM peptide in the CPF exposure group (Figure 6c) than in the control (Figure 6a), while the alternation of the ISTD signal was minimal (Figure 6b,d). The concentration of Lys-40-acetylated α-tubulin in the brain tissue was calculated using the formula above.

The average level in the control group (n = 5) was 9.35 ± 1.03 pmol/mg and 7.16 ± 0.97 pmol/mg in the CPF exposure group (n = 5). Tubulin acetylation in rat brain was significantly inhibited (P value = 0.0087 < 0.05) after repeated exposure to CPF followed by washout, and the average level decreased by 23.4% when compared to the control (Figure 6e). The results above demonstrated the successful application of this SISCAPA–LC–MS assay in the measurement of tubulin acetylation as a promising biomarker for cognitive deficits. This is the first time that CPF-induced inhibition of tubulin acetylation has been detected, even after long-term, low-level chronic exposure combined with a significant washout period. In consideration of the importance of this key PTM in the axonal transport system, its inhibition may (at least in part) explain the impaired transport of mitochondria, vesicles, and other macromolecules subsequent to chronic exposure to CPF and other organophosphates.[37,43,44] The mechanism underlying CPF-inhibited tubulin acetylation could be associated with organophosphate–tubulin adduction.[45] CPF is metabolized by cytochrome P450s to a potent phosphorylating agent for various proteins,[46,47] chlorpyrifos oxon (CPO).[48] Organophosphorylated tubulin was identified in the brain of mice exposed to CPF or CPO, and disruption of its polymerization

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into microtubules was observed.[49] Mass spectrometry has identified 17 Tyr- and 6 Lys residues on tubulin modified by CPO after in vitro incubation.[46,50] These modifications may inhibit the entry of acetyltransferases into microtubules.[8] With peptic digestion, we have successfully identified CPO adduction on a functional site (Lys-40) of α-tubulin (Figure 7), which could accumulate over repeated exposures to CPF and preserve over the washout period. Therefore, the observed reduction of tubulin acetylation may result from tubulin organophosphorylation, through disruption of tubulin polymerization,[49,51] steric effects for acetylation from inside,[8] or covalent modification of the PTM site.

CONCLUSIONS

α-Tubulin acetylation at Lys-40 is an essential PTM in the axonal transport process, and its reduction is concomitant with cognitive impairments in several unrelated neurodegenerative disorders and diseases. This key PTM has not been previously studied with mass spectrometry due to the lack of common proteolytic cleavage sites in the vicinity of Lys-40. Peptic digestion allowed us to identify a quantitatively reproducible sequence containing this PTM site and a new covalent modification (organophosphorylation) at the same site. Using a commercially available anti-peptide antibody, we developed a novel SISCAPA−LC−MS assay for the absolute quantitation of tubulin acetylation from peptic digest of rat brain tissue. The method had an LLOQ at 2.50 pmol/mg, provided good linearity from 2.50 to 62.50 pmol/mg, and was validated with intra and interday precision and accuracy tests. Quantification of this potential cognition relevant biomarker should facilitate studies of neurodegenerative diseases and neurotoxicity, including the evaluation of pathological progression, investigation of the mechanisms of actions, and development of specific therapeutics especially HDAC6 inhibitors. Also, this bioanalytical method only required 2.2 mg of brain tissue, thus allowing brain-region-specific measurement, considering the heterogeneous distribution of tubulin acetylation and HDAC6 in the prefrontal cortex, hippocampus, cortex, cerebellum and other sections by brain anatomy.[13]

This method has been applied to study how repeated (30 days), low-dose (18 mg/kg every other day) treatment with chlorpyrifos, an organophosphate insecticide, affected rat brain tubulin acetylation. For the first time, we determined that a significant inhibition of tubulin acetylation was related to defects of axonal transport and cognition in experimental animals chronically exposed to an organophosphate, even 45 days after the last exposure. This new observation would help understanding of the toxicological targets of prolonged cognitive impairments in chronic organophosphate poisoning. The mechanisms leading to this inhibition are to be explored but could be associated with organophosphorylations at Lys-40 and multiple other sites in tubulin.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04484.

Figure S1. Detection of the peptide YCLEHGIQPDGQMPSDKTIGGGDDSF (45 μM) using the SIR function (*m*/*z* 923.7, a) and the MRM function (*m*/*z* 923.7 → 945.1, b). Table S1. Comparison of the recovery of 36 pmol of acetylated peptide from 100 μL of 1 mg/mL bovine serum albumin in PBS-T (PDF)

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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