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Proteomic Analysis of Protein Phosphorylation and Ubiquitination in Alzheimer's Disease

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Summary

Posttranslational modifications such as phosphorylation and ubiquitination serve, independently or together, as gatekeepers of protein transport and turnover in normal and disease physiologies. Aberrant protein phosphorylation is one of the defining pathological hallmarks of more than 20 different neurodegenerative disorders, including Alzheimer's disease (AD). The disruption of the phosphorylation of neurotransmitter receptors has been implicated as one of the causal factors of impaired memory function in AD (1–3). Another feature of AD is the aberrant accumulation of proteins that are normally degraded by the ubiquitin proteasome system upon being conjugated to ubiquitin. Thus, elucidating the protein targets of phosphorylation and ubiquitination that can serve as AD biomarkers will aid in the development of effective therapeutic approaches to the treatment of AD. This chapter provides details pertaining to the qualitative and quantitative liquid chromatography tandem mass spectrometry-based analysis of an affinity purified, phosphorylated, and ubiquitinated protein, paired-helical filament tau.

Keywords

Alzheimer's disease (AD); Phosphorylation; Ubiquitin; Tau; Liquid chromatography tandem mass spectrometry (LC-MS/MS); Selected reaction monitoring (SRM)

1. Introduction

Phosphorylation and ubiquitination are two of the many posttranslational modifications of the microtubule-associated protein tau that are critical to the molecular pathogenesis of neurodegeneration in Alzheimer's disease (AD) (4–13). A distinct hierarchical pattern of tau phosphorylation has been shown to correlate with the progression of AD neuropathology (14, 15). Paired helical filaments (PHFs) of hyperphosphorylated tau aggregates comprise the degradation-resistant core of intraneuronal neurofibrillary tangles in AD and other tauopathies. The accumulation of degradation-resistant PHF tau is linked to the impaired function of the ubiquitin proteasome system (16). Although PHF tau is ubiquitinated, it is not subsequently degraded by the proteasome system (6, 17–19). Hence, the determination of the precise phosphorylation and ubiquitination sites of tau that specifically correlate with the progression of AD could serve as effective biomarkers.

The identification of phosphorylation sites typically relies upon the use of antibodies, thereby limiting the investigator's ability to comprehensively evaluate all possible

phosphorylation sites of a protein of interest. Similarly, the detection of ubiquitinated proteins has conventionally been achieved with antibody-based detection methods, the use of which provides information about the general ubiquitination status of a given protein, but not the specific site(s) of ubiquitin conjugation. Here we describe a sensitive and quantitative method employing liquid chromatography tandem mass spectrometry (LC-MS/MS) to evaluate tau phosphorylation and ubiquitination. Although the details of the methods described herein pertain to the analysis of tau, these methods can be applied to the analysis of any phosphorylated and/or ubiquitinated protein.

2. Materials

2.1. SDS-PAGE and in-Gel Trypsin Digestion

1. Precast Tris-glycine 4–20% polyacrylamide gel (Bio-Rad).
2. Running buffer (10×): 250 mM Tris-HCl, 1.92 M glycine, 1% (w/v) SDS. Store at room temperature.
3. Sample buffer (4×): 250 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS (w/v), 200 mM DTT, 0.008% bromophenol blue. Store at –20°C (*see* Note 1).
4. Precision plus dual color standard (Bio-Rad).
5. Scalpels (*see* Note 2).
6. Gel-loading pipette tips.
7. Microcentrifuge tubes rinsed with 50% methanol.
8. Coomassie blue stain or Silver Stain (Silver Quest Silver Staining Kit, Invitrogen) (*see* Note 3).
9. Water (Burdick and Jackson, HPLC grade).
10. Digestion buffer: 50 mM Ammonium bicarbonate (Sigma) made from 100 mM Ammonium bicarbonate stock solution. Check to ensure pH is between 7.5 and 9.0.
11. Acetonitrile (Burdick and Jackson, HPLC grade).
12. Methanol (Burdick and Jackson, HPLC grade).
13. TCEP (“Bond-breaker neutral pH TCEP solution”, Pierce/ Thermo Fisher Scientific): Prepare 10 mM solution in 100 mM ammonium bicarbonate. Prepare 10 mM solution fresh.
14. Iodoacetamide (Sigma): 55 mM prepared in 100 mM ammonium bicarbonate. Prepare fresh.

¹Any variation of the standard Laemmli buffer will work. β -mercaptoethanol, a typical component of Laemmli buffers that is included to reduce protein disulfide bonds, is a common source of keratin contamination. We, therefore, prepare our sample buffer with the reducing agent DTT instead.

²The use of scalpels as opposed to razor blades is preferred so as to minimize the chances of gloves making contact with the gel when excising gel bands and cutting the bands into smaller pieces. Avoid using latex gloves as these are a common source of keratin contamination.

³There are alternative commercially available mass spectrometry compatible silver staining kits. Mass spectrometry compatible silver staining methods are those that do not involve the use of glutaraldehyde to fix the proteins in the gel.

15. TPCK-treated trypsin (Promega) (*see* Note 4).
16. Formic acid (Sigma).

2.2. In-Solution Trypsin Digestion

1. Digestion buffer: 100 mM ammonium bicarbonate (Sigma) in water; pH between 7.5 and 9.0 is acceptable.
2. Centrifugal filter devices: (Microcon) for buffer exchange and sample concentration.
3. Desalting spin columns: (Pierce/Thermo Fisher Scientific).
4. Methanol: HPLC grade.
5. TCEP: ("Bond-breaker neutral pH TCEP solution", Pierce/ Thermo Fisher Scientific).
6. Iodoacetamide (Sigma): 1 M stock prepared in 100 mM ammonium bicarbonate; freshly prepared.
7. DTT (Sigma): 1 M stock prepared in 100 mM ammonium bicarbonate. Stock solution can be stored in aliquots at -20°C .
8. TPCK-treated trypsin: (Promega).
9. Glacial acetic acid: HPLC grade.

2.3. Immobilized Metal Affinity Chromatography for Phosphopeptide Isolation

1. Phosphopeptide isolation kit (Pierce/Thermo Fisher Scientific).
2. Water (Burdick and Jackson, HPLC grade).
3. Ammonium bicarbonate (Sigma). Prepare 0.1 M stock in water and ensure pH is in the range of 8.7–9.1.
4. Acetonitrile (Burdick and Jackson, HPLC grade).
5. Glacial acetic acid, HPLC grade.

2.4. Liquid Chromatography

1. Solvent "A" (aqueous buffer): 2% acetonitrile (Burdick and Jackson – HPLC grade), 0.1% formic acid (Fluka) in water (Burdick and Jackson – HPLC grade).
2. Solvent "B" (organic buffer): 95% acetonitrile (Burdick and Jackson – HPLC grade), 0.1% formic acid (Fluka) in water (Burdick and Jackson – HPLC grade).
3. C18 reversed phase microcapillary column: 15 cm length, 75 μm i.d., 5 μm particles with 300 Å pores (Micro-Tech Scientific).
4. XTreme Simple LC system (Micro-Tech Scientific).

⁴TPCK-trypsin from any other commercial source may also be used. TPCK treatment of trypsin inactivates chymotryptic activity and modification by reductive methylation or acetylation prevents autolytic activity.

2.5. Mass Spectrometry

1. LTQ linear ion trap mass spectrometer equipped with a nano-spray ion source (Thermo Electron).
2. PicoTipTM spray tips: uncoated, 360 µm fused silica tubing o.d., 20 µm tip o.d., 10 µm tip i.d. (New Objective).
3. Ultra high-grade purity helium.

2.6. Mass Spectrometry Data Analysis

1. XcaliburTM for mass spectrometer instrument control and data analysis (Thermo Electron) (*see* Note 5).
2. Bioworks software using the SEQUEST algorithm (Thermo Electron).

3. Methods

Davies et al. were among the first groups to publish data suggesting that various forms of phosphorylated tau could be utilized as functional AD biomarkers (14, 20–23). Hyperphosphorylated PHF-tau is largely degradation resistant, although it is known to be ubiquitinated. However, the exact temporal correlation between tau phosphorylation, ubiquitination, and neurodegeneration in AD is not known. We have recently applied a series of functional proteomic and LC-MS/MS analyses to determine the extent of phosphorylation and ubiquitination in PHF-tau isolated from human AD brain (24) using an antibody that recognizes a conformational variant of tau that represents an early stage of PHF generation in AD (25, 26). Our data indicate that this conformation of PHF-tau is phosphorylated on at least 30 amino acid residues and is ubiquitinated at Lys-254, Lys-311, and Lys-353. These ubiquitination events occur as a combination of both mono-ubiquitination and poly-ubiquitination.

The methods detailed in this chapter are written based on the analysis of PHF-tau; however, these methods can be extrapolated and utilized for the study of any purified protein that is phosphorylated and/or ubiquitinated. The starting amount of purified PHF-tau used for our studies was 100 µg.

3.1. In-Gel Trypsin Digestion

1. If proteins other than the protein of interest are present in the purified protein sample, it might be desirable to separate the purified protein via SDS-PAGE. Use 50 µg protein for SDS-PAGE analysis. Following electrophoresis, wash the gel three times for 10 min per wash with deionized water to remove any residual SDS.
2. Proceed to Coomassie blue staining or Silver staining. For Silver staining methods, overnight fixation helps to decrease background staining. After staining is complete, wash the gel for 10 min with deionized water.

⁵Xcalibur version 1.4 was used for the studies from which these methods were derived; however, any later version of the software is adequate.

3. Capture an image of the gel. Excise protein band using a clean scalpel and cut into $1 \times 1 \text{ mm}^2$ cubes. Transfer gel pieces to microcentrifuge tube washed with 50% methanol (*see* Note 6). If using Silver stain, destain gel pieces before proceeding to next step.
4. Add 100% methanol for 5 min to dehydrate gel pieces. Add sufficient volume of methanol to cover gel pieces.
5. Remove 100% methanol and add 30% methanol for 5 min to rehydrate gel pieces (*see* Note 7).
6. Remove 30% methanol and wash gel pieces twice for 10 min per wash with water (HPLC-grade).
7. Wash gel pieces three times for 10 min per wash with 30% acetonitrile in 100 mM ammonium bicarbonate.
8. Dry gel pieces in vacuum centrifuge (speed vac) for 15 min. Gel pieces will become opaque when dry.
9. Add 10 mM TCEP (enough volume to cover gel pieces) and let incubate for 1 h at 60°C to reduce protein disulfide bonds.
10. Briefly centrifuge gel pieces and remove liquid.
11. Add 55 mM iodoacetamide (enough volume to cover gel pieces) and let incubate for 45 min at room temperature in the dark to alkylate cysteine residues.
12. Briefly centrifuge gel pieces and remove liquid.
13. Wash gel pieces for 15 min with 100 mM ammonium bicarbonate.
14. Remove liquid. Shrink gel pieces with 100% acetonitrile.
15. Remove liquid. Dry gel pieces in vacuum centrifuge (speed vac) for 15 min. It is critical to ensure that gel pieces are completely dry to facilitate the absorption of trypsin into the gel pieces in the next step.
16. Rehydrate the gel pieces in trypsin solution at 4°C (on ice) for 45 min. Trypsin solution: 1.5 ng/μL trypsin in 50 mM ammonium bicarbonate. Add adequate volume of trypsin solution to completely cover gel pieces.
17. Remove any remaining trypsin solution and add sufficient volume of digestion buffer to completely cover gel pieces.
18. Let incubate at 37°C overnight.
19. Briefly centrifuge gel pieces and transfer supernatant to another microcentrifuge tube washed with 50% methanol.

⁶All steps for in-gel digestion should be performed in a clean laminar flow hood to minimize keratin contamination. Wipe down all surfaces in the hood with 50% methanol prior to beginning any work.

⁷For all steps of the in-gel digestion protocol that entail removing solutions from gel pieces, use a gel-loading pipette tip.

20. Add 50 mM ammonium bicarbonate to cover gel pieces and incubate at 37°C with shaking for 15 min.
21. Add volume of acetonitrile equal to volume of 50 mM ammonium bicarbonate utilized in **step 20** and incubate at 37°C with shaking for 15 min.
22. Centrifuge gel pieces and add supernatant to the supernatant collected in **step 19**.
23. Extract peptides: Add acetonitrile/5% formic acid (50:50) to gel pieces and incubate at 37°C with shaking for 15 min.
24. Remove supernatant and combine with supernatant collected in **steps 19** and **22**.
25. Repeat **step 23**.
26. Remove supernatant and combine with supernatant collected in **steps 19, 22**, and **24**.
27. Dry down gel extract and reconstitute in volume of liquid chromatography Solvent “A” that is compatible with the volume of the sample loop in the liquid chromatography system that will be utilized to analyze the sample (*see* Note 8).

3.2. In-Solution Trypsin Digestion

1. Use at least 50 µg protein for in-solution digestion. Protein sample should ideally be free of any detergents prior to in-solution digestion and LC-MS/MS (*see* Note 9). Perform buffer exchange with 100 mM ammonium bicarbonate using Microcon centrifugal filter devices. Be aware of the molecular weight cut-off (MWCO) for the filter devices. Optimum volume of the sample following buffer exchange is the volume that yields a protein concentration of ~0.1–1.0 µg/µL.
2. Add methanol to 40% to denature protein (*see* Note 10).
3. If protein does not contain any cysteine residues, skip this step and proceed to **step 4**.
 - a. Add TCEP to 5 mM final concentration to reduce protein disulfide bonds. Vortex and incubate for 30 min at 37°C.
 - b. Alkylate cysteine residues by addition of iodoacetamide to 10 mM final concentration. Vortex and incubate for 1 h at room temperature in the dark.

⁸Digested samples can be desalted using a C18 Zip Tip (Millipore) or any other C18-based spin column, cartridge or tip. However, when analyzing phosphopeptides, it should be noted that phosphate groups generally render peptides more hydrophilic and there is a risk of the phosphopeptides not binding to the C18 resin used for desalting, consequently resulting in sample loss.

⁹Perform acetone precipitation if detergents are present in the protein sample at or above the following concentrations: SDS – 0.05%; Triton X-100, CHAPS, NP-40, Tween 20 and Octyl glucopyranoside – 1%. The procedure for acetone precipitation is as follows: add 4× sample volume of cold acetone (–20°C); vortex and incubate for 1 h at –20°C; centrifuge for 10 min at 12,000 × g; decant supernatant while being careful to not dislodge the protein pellet; resuspend protein pellet in 100 mM ammonium bicarbonate.

¹⁰This method is intended for use with purified protein samples. Thus, the use of stronger denaturants, or acid-labile surfactants should not be required for efficient enzymatic digestion. However, if desired, the enzymatic digest can be performed in the presence of RapiGest™ (Waters), which is a mass spectrometry-compatible acid-labile surfactant that has been demonstrated to generate quantitative and qualitative improvements in peptide and protein identifications. A detailed protocol for in-solution enzymatic digestion using RapiGest™ is available in (29).

- c. Stop alkylation reaction by adding DTT to 20 mM final concentration. Vortex and incubate for 1 h at room temperature.
 - d. Rid samples of any excess iodoacetamide using a protein desalting spin column (Pierce/Thermo Fisher Scientific).
4. Add trypsin to sample at a ratio of 1 trypsin:50 protein. Let incubate for 2 h at 37°C.
5. Repeat **step 4**.
6. Add trypsin to sample at a ratio of 1 trypsin:50 protein and let incubate overnight at 37°C.
7. Stop enzymatic digestion by acidifying with addition of acetic acid to 5% final volume. Dry sample in speed vac.

3.3. Immobilized Metal Affinity Chromatography for Phosphopeptide Isolation

1. Resuspend dried, digested peptide sample in 50 µL 5% acetic acid in water. Ensure that sample pH is <3.5. If the sample pH is >3.5, a significant amount of nonspecific binding may occur, consequently decreasing the selectivity of the gallium-chelated resin for binding to phosphopeptides.
2. Follow the procedure detailed in the Pierce phosphopeptide isolation kit (product 89853) product information sheet.
3. Do not combine final elution fractions. Dry down elution fractions in speed vac and reconstitute peptides in liquid chromatography Solvent “A”.

3.4. Liquid Chromatography

Utilize an LC method with the following parameters:

1. Flow rate of 0.4 µL/min.
2. 30 min equilibration with Solvent “A”.
3. Sample loading time (load at 95% Solvent “A”) equivalent to the time it would take to load a sample with a volume that is 2.5× the sample loop volume.
4. For the efficient separation of hydrophilic phosphopeptides, utilize the following gradient: 5–25% Solvent “B” in 60 min followed by 25–90% Solvent “B” in 10 min. Otherwise, utilize a gradient of 5–40% Solvent “B” in 60 min followed by 40–90% Solvent “B” in 10 min.
5. Condition column with 90% solvent “B” for 15 min.

3.5. Mass Spectrometry

1. Set spray voltage to 2.0 kV and heated capillary temperature to 200°C.
2. Create a data dependent MS/MS instrument method with the following parameters:
 - a. Full scan range: 400–1,800 m/z.

- b. Five most abundant ions selected for MS/MS fragmentation.
 - c. Dynamic exclusion enabled – exclude ions for 30 s after being detected twice within 30 s (*see* Note 11).
 - d. Minimum MS signal: 500 counts, minimum MS/MS signal: 100 counts.
 - e. Activation time: 30 ms (120 ms for MS/MS/MS methods; *see* **step 3**).
 - f. Collision energy: 35% (24% for MS/MS/MS methods; *see* **step 3**).
3. For the analysis of phosphopeptides, use a data-dependent MS/MS/MS (MS3) method whereby an MS3 scan will be triggered if, among the three most abundant ions in the MS/MS scan, a neutral loss of 98, 49, or 32.7 Da is detected. These neutral loss masses correspond to the loss of phosphoric acid (H₃PO₄) on singly, doubly, and triply charged precursor ions, respectively (*see* Note 12). Figure 1 is an illustration of the neutral loss data-dependent algorithm that is utilized for the identification of phosphopeptides.

3.6. Mass Spectrometry Data Analysis of Protein Phosphorylation and Ubiquitination

1. Conduct a SEQUEST search against the appropriate organism database using a threshold of 100, monoisotopic mass type, and automatic charge state determination. Include the following differential modifications: +57.02 on Cys (if protein was reduced and alkylated using iodoacetamide); +16.0 on Met (oxidation); +80.0 on Ser, Thr, and Tyr (phosphorylation); –18.0 on Ser, Thr (neutral loss of phosphoric acid) (*see* Note 12); +114 on Lys (–GG from the c-terminus of ubiquitin that remains conjugated to a Lys residue of an ubiquitinated protein following trypsin digestion).
2. Manually inspect spectra passing a threshold of cross-correlation vs. charge state values of 1.5 for +1 ions, 2.0 for +2 ions, and 2.5 for +3 ions to verify that all major fragment ions are identified and, in the case of serine and threonine phosphopeptides, that phosphorylated residues that are identified with a differential modification of –18 are from MS3 scans.

3.7. Quantification of Posttranslational Modifications by Selected Reaction Monitoring

3.7.1. Evaluation of External Standard Peptides for Use in SRM Method

1. After performing qualitative LC-MS/MS analysis (*see* **Subheadings 3.4–3.6**) to determine possible phosphorylation and/or ubiquitination sites on the protein of interest, synthesize peptides to be utilized as external standards for performing quantification by selected reaction monitoring (SRM). Adhere to the following

¹¹The optimum duration for dynamic exclusion should be empirically determined based on the average chromatographic peak width. Using a dynamic exclusion duration of 30 s with an LC system that generates peak widths of 1 min permits the acquisition of MS/MS data for a peptide as it begins to elute as a peak, at the apex of the peak and at the peak tail.

¹²Phosphoserine and phosphothreonine residues are converted to dehydroalanine and 2-aminodehydrobutyric acid, respectively, following a neutral loss of phosphoric acid (H₃PO₄) upon collision induced dissociation (CID). Phosphotyrosine residues rarely undergo this neutral loss upon CID.

considerations and guidelines when selecting suitable peptides for quantitative analysis:

- a. Perform a BLAST search to ensure that the peptide selected for quantification is unique to the protein of interest.
 - b. Select peptides with a length of ~ 6–13 amino acid residues. Peptides of this length are efficiently retained on reversed phase microcapillary columns and elute with a peak width that is amenable for quantification.
 - c. If possible, avoid peptides containing reactive and labile residues such as cysteine and methionine that readily undergo oxidation during synthesis and while in typical solvents. Tryptophan is prone to alkylation during synthesis. Such modifications will reduce the actual amount of external standard peptide and will result in erroneous quantification, namely over-estimation.
 - d. Synthesize both the modified (phosphorylated or ubiquitinated) and unmodified versions of the peptide standards to deduce the stoichiometry or extent of posttranslational modification at specific amino acid sites. However, be mindful that sites of posttranslational modification that occur in proximity to the proteolytic site could impede proteolysis.
2. Utilize standard Fmoc chemistry to synthesize the external peptide standards (*see* Note 13).
 3. Verify the stock amount of the synthesized peptide by quantitative amino acid analysis (*see* Note 13).
 4. Assess the purity of the synthesized peptide by reversed phase chromatography (*see* Note 13).
 5. Confirm the identity of the synthesized peptide by infusing the peptide into the mass spectrometer and conducting MS/ MS fragmentation.

3.7.2. SRM Method for Quantification of Post-translational Modifications—

Although triple quadrupole mass spectrometers are ideal for SRM-based quantification, linear ion trap mass spectrometers are practical alternatives (27). A schematic of the SRM method used for quantitative analysis is presented in Fig. 2.

1. Select an external synthetic peptide MS/MS product ion with an m/z greater than that of the precursor ion.
2. Create an SRM instrument method whereby the precursor-to-product ion transition selected in **step 1** is monitored using a scan range that minimizes the duty cycle of the mass spectrometer.
3. Analyze the external peptide standard using the LC method detailed in **Subheading 3.4** (the method can be significantly shortened, so long as the peptide elutes in a distinct peak) and the SRM method created as outlined above.

¹³Most universities have a Molecular Biology Resource Core Facility or a Biopolymer Core Facility that offers these services to investigators for a fee.

4. Utilize Qual Browser (Xcalibur™ software) to display only the scans that contain distinctive product ions for each spectrum and integrate the resultant peaks using the ICIS peak detection algorithm with 15-point Boxcar smoothing.
5. Repeat **steps 3 and 4** using various amounts of external standard peptide spanning at least 1 order of magnitude (e.g., 100 amol, 1 fmol, 10 fmol, 100 fmol, 1 pmol) to calculate an equation for the line of best-fit based on the standard curve of the peak area vs. amount of peptide.
6. Analyze the sample of interest using the SRM method developed in **step 2** above.
7. Repeat **step 4** and determine the amount of the protein of interest in the sample by using the integrated peak area of the monitored peptide and the line of best-fit calculated from the SRM analysis of the external peptide standards. For further information about the effective development and optimization of SRM methods, please refer to (28).

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Fig. 1. Mass spectrometry data-dependent algorithm for the identification of phosphopeptides. **(a)** Mass spectrometer instrument scan cycle. **(b)** Schematic representation of an MS/MS spectrum in which a peptide has undergone a neutral loss of phosphoric acid and the resultant MS/MS/MS spectrum. **(c)** Structure of phosphoserine amino acid residue which, upon a neutral loss of phosphoric acid by a beta-elimination reaction, is converted to dehydroalanine.

Fig. 2.
Selected reaction monitoring (SRM) method used for quantitative mass spectrometry-based protein quantification.