Chapter 15

Global Analysis of Ubiquitination

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Abstract

The covalent attachment of the small protein ubiquitin to other proteins is known to control a host of biological pathways and is emerging as an important regulatory factor in various processes specific to the nervous system. Ubiquitination is also tightly linked to most neurodegenerative disorders. A quantitative, proteome-wide view of the dynamic changes in ubiquitin modification associated with neuronal activity states and various stages of neurodegenerative disorders is therefore desired. Advances in quantitative mass spectrometry and the development of new biological tools make these approaches feasible for many laboratories. We describe here a combination of SILAC-based (stable isotope labeling by amino acids in cell culture) quantitative mass spectrometry and tandem-affinity purification to detect system-wide changes in ubiquitination and ubiquitin chain topologies that will be useful to probe the role of ubiquitin in the nervous system.

Key words: Ubiquitination, SILAC, HB-tag, Tandem-affinity purification, Ubiquitin-chain topology, Mass spectrometry

1. Introduction

We are only beginning to understand the role of the ubiquitin/proteasome system in processes such as nervous system development, neuronal plasticity, or neurodegenerative disorders (1–3). Nevertheless, the importance of protein modification with ubiquitin is evident, and proteome-wide approaches describing dynamic changes in ubiquitination profiles promise to advance our understanding of the role of the ubiquitin/proteasome system in neuronal processes.

Ubiquitin, a 76 amino acid protein, is highly conserved among Eukaryotes and is best known for its function in labeling other proteins for degradation by the 26S proteasome. Ubiquitination, the covalent attachment of ubiquitin to other

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proteins, has also nonproteolytic functions such as direct modulation of protein activity or protein localization, which is often caused by changes in protein interaction partners (4). Ubiquitination involves a cascade of reactions that are catalyzed by the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin ligases) enzymes, and results in the formation of an isopeptide bond between the carboxyl-terminus of ubiquitin and typically the ε-amino-group of a lysine residue in substrate proteins (Fig. 1). Generally, we distinguish between mono, multi, and polyubiquitinated substrates (Fig. 1). While the former two describe the linkage of single ubiquitin molecules to one or more lysine residues in substrates, polyubiquitination involves the formation of ubiquitin chains (5). They are formed through isopeptide linkages between one out of seven different lysine residues in a substrate-anchored ubiquitin and the carboxyl-terminus of a new ubiquitin moiety. Depending on the specific lysine residue used for ubiquitin chain linkages, different chain topologies with distinct signaling functions are formed (6). Different ubiquitin chain topologies as well as precise ubiquitination sites in substrates can be detected using the mass spectrometric analysis we describe below (Table 1). The basis for this is a 114-Da mass shift due to the C-terminal diglycine motif from ubiquitin that remains linked to ubiquitin-acceptor residues (usually lysines) after trypsin digestion (7, 8).

Like other posttranslational modifications, ubiquitination is a reversible modification due to the function of ubiquitin carboxyl-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs). The balance between ubiquitination, deubiquitination, and degradation forms the basis for the highly dynamic character of the ubiquitin proteome (9).

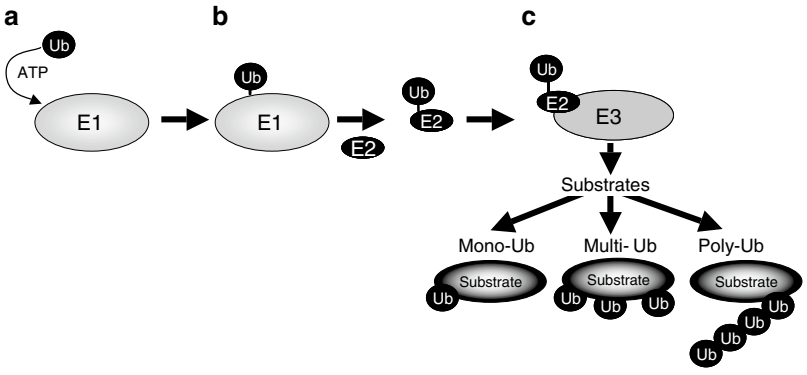

Fig. 1. The three steps of the ubiquitination cascade: (**a**) Ubiquitin is activated by an E1 ubiquitin-activating enzyme (two E1 enzymes are known). (**b**) Transfer of ubiquitin from E1 to an ubiquitin-conjugating enzyme E2 (over 30 different E2 enzymes are known). (**c**) An Isopeptide bond between a substrate lysine and the C-terminal glycine of ubiquitin is created (hundreds of E3 ubiquitin-ligases are known). Substrates can either be mono, multi, or polyubiquitinated. *Ub* ubiquitin.

Table 1
Tryptic signature fragments of ubiquitin chain linkage types (human)

Linkage type	Sequence	Precursor MH^+(Da)
K6	MQIFV**K**(GlyGly)TLTG**K**	1379.772
K11	TLTG**K**(GlyGly)TITLEVEPSDTIENV**K**	2402.266
K27	TITLEVEPSDTIENV**K**(GlyGly)A**K**	2101.102
K29	A**K**(GlyGly)IQD**K**	816.457
K33	IQD**K**(GlyGly)EGIPPDQQR	1637.824
K48	LIFAG**K**(GlyGly)QLEDGR	1460.786
K63	TLSDYNIQ**K**(GlyGly)ESTLHLVLR	2244.198

Precursor masses contain the additional 114.04293 Da for the (GlyGly) remnant originating from ubiquitination of the lysine. Masses can differ due to additional modifications, miss cleavages or SILAC labeling, etc.

The complexity of nervous system function and neurodegeneration requires quantitative, system-level approaches for description of the role of the ubiquitin/proteasome in these processes. Such approaches have been developed for yeast, mammalian cells, and mice (8, 10–14). Typically tagged ubiquitin is expressed in cells, and ubiquitinated proteins are purified based on the affinity tag fused to ubiquitin (15). One important aspect in the analyses of ubiquitinated proteins is that purification is performed under completely denaturing conditions to disrupt noncovalent protein interactions. The denaturing purification conditions avoid copurification of proteins associated with ubiquitinated proteins and thus not only reduce sample complexity but also allow identification of ubiquitination substrates by protein ID without the inefficient detection of ubiquitin attachment sites.

We describe here one of the strategies that has been applied to the quantitative detection of changes in proteome-wide ubiquitination profiles. The protocol presents a simple and efficient method to purify and analyze ubiquitinated proteins under fully denaturing conditions using the histidine-biotin (HB) tandem-affinity tag (11, 14, 16). Ubiquitin is fused to the HB-tag consisting of an RGS-hexahistidine motive followed by a bacterially derived biotinylation signal (Fig. 2). The biotinylation signal induces the attachment of biotin to a specific lysine residue in the tag in vivo (17). Cells use HB-ubiquitin like the endogenous ubiquitin in protein ubiquitination, and ubiquitinated proteins can be sequentially purified by Ni^{2+}-chelate chromatography and binding to streptavidin sepharose (Fig. 2). Importantly, both purification steps tolerate highly denaturing conditions such as 8 M urea or 6 M guanidinium (14, 16, 18). The high-affinity

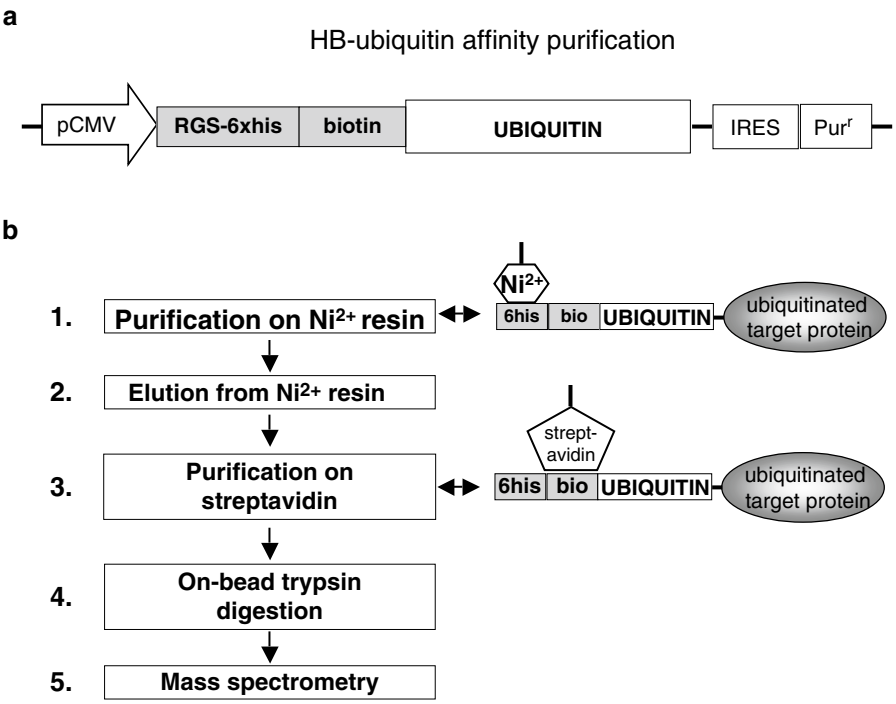

Fig. 2. Tandem-affinity purification of HB-tagged ubiquitin. (**a**) The HB-ubiquitin-tag consists of an RGS-hexahistidine motif (RGS6xHis) followed by a bacterially derived in vivo biotinylation signaling peptide (biotin). The biotinylation signal peptide induces attachment of biotin in vivo and allows purification of HB- ubiquitinated proteins on streptavidin resins. HB-ubiquitin is expressed in the retroviral vector (pQCXIP). Expression is driven by the CMV promoter, and selection of clones with high expression levels is facilitated by an internal ribosomal entry side (IRES) controlling translation of the puromycin selection marker. (**b**) Flow diagram of the two-step purification process. Ubiquitinated proteins are sequentially purified on Ni^{2+} sepharose and streptavidin sepharose. Both purification steps are performed under fully denaturing conditions to minimize background binding. Because of the irreversible nature of the streptavidin-biotin interaction, "on-bead" tryptic digestion is used to elute peptides from the streptavidin sepharose for mass spectrometric identification.

interaction between the biotin attached to the HB-tag and streptavidin allows exceptionally stringent purification conditions, but prevents efficient elution (19). "On-bead" tryptic digest is thus used to release peptides from the streptavidin sepharose beads for analysis by mass spectrometry (14, 16).

Tandem-affinity purification of ubiquitinated proteins is an effective approach to identify ubiquitin profiles. However, detection of system-wide changes in ubiquitination requires quantitative mass spectrometric strategies. We have used stable isotope labeling with amino acids in cell culture (SILAC) (20–23) (Fig. 3) for quantitative detection of changes in global ubiquitin profiles and changes in ubiquitin-chain topologies in response to various conditions (11). The SILAC approach is ideal for this purpose as it lets the investigator combine the different samples before purification to ensure absolutely identical conditions during the entire process.

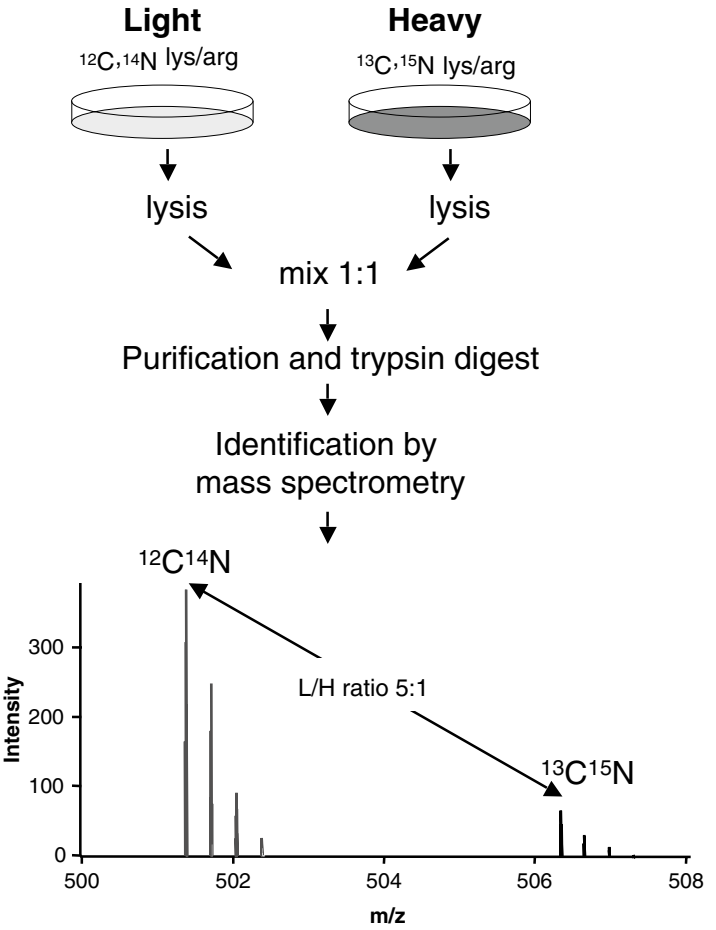

Fig. 3. SILAC strategy for quantitative comparison of proteome-wide ubiquitination profiles. The "light" (^{12}C, ^{14}N arginine and lysine growth medium) and "heavy" (^{13}C, ^{15}N arginine and lysine growth medium) samples are grown under two different conditions according to the experimental plan. Equal amounts of total cell lysates from both samples are mixed and purified under denaturing conditions. Purified proteins are digested with trypsin and prepared for analysis by mass spectrometry. Peptides with identical sequences from the labeled and unlabeled sample are detected as pairs, because $^{13}C^{15}N$-arg/lys incorporation results in a defined mass shift. The peak heights of the SILAC pairs are used to calculate the light/heavy (L/H) ratios, which are equivalent to relative abundance changes of the peptide/protein (*SILAC* stable isotope labeling by amino acids in cell culture). See also Note 1.

Protein ubiquitination has gained significant attention as a posttranslational modification that controls protein abundance, activity, and localization. Recent advances in affinity purification strategies and quantitative mass spectrometry have allowed proteome-wide descriptions of the dynamics of the ubiquitin/proteasome system. We hope that the protocol we describe here will contribute to our understanding of the role of ubiquitination in normal and pathogenic processes of the nervous system.

2. Materials

2.1. Mammalian Cell Culture and Lysis

Quantitative analysis of ubiquitination profile changes is described using the SILAC procedure. For nonquantitative applications, standard growth media can be used. The described purification protocol is identical for both the SILAC strategy and the nonquantitative approach.

1a. *Nonquantitative analysis*: Dulbeco's Modified Eagle's Medium (DMEM; Mediatech, Herndon, VA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO, Bethesda, MA, USA), 1% penicillin/streptomycin (GIBCO, Bethesda, MA, USA).

1b. *SILAC-based quantitative experiments*: SILAC DMEM medium lacking lysine and arginine (Thermo Scientific, Rockford, IL) supplemented with 10% (v/v) dialyzed fetal bovine serum (FBS, GIBCO, Bethesda, MA, USA), 1% penicillin/streptomycin (GIBCO, Bethesda, MA, USA). The heavy medium is supplemented with 0.028 mg/mL $^{13}C_6{}^{15}N_4$ arginine, and 0.073 mg/mL $^{13}C_6{}^{15}N_2$ lysine (isotopic purity >98 atom %) (Cambridge Isotope Labeling, Andover, MA). The light medium contains the same amount of $^{12}C^{14}N$ arginine and $^{12}C^{14}N$ lysine (Sigma Co., St Louis, MO, USA). For triple-labeled SILAC experiments see Note 1.

2. Antibiotics according to selection marker.

3. 10× PBS buffer: 0.58 M Na_2HPO_4, 0.17 M NaH_2PO_4, 0.68 M NaCl, pH 7.4.

4. 27 G needles to shear DNA (Becton Dickinson, NJ, USA).

5. Sonicator (VWR, West Chester, Pennsylvania, USA).

6. 10 μM MG132 (American Peptide, Sunnyvale, CA) dissolved in DMSO, keep frozen at –20°C.

2.2. Purification of HB-Ubiquitinated Proteins

Check the pH of all buffers before use and readjust accordingly.

1. *Buffer A-8:* 8 M Urea, 300 mM NaCl, 50 mM sodium phosphate buffer pH 8.0 (0.68 mL of 0.5 M NaH_2PO_4 and 9.32 mL of 0.5 M Na_2HPO_4 in 100 mL), 0.5% (v/v) Nonidet P-40, pH 8.0.

2. *Buffer A-6.3:* 8 M Urea, 300 mM NaCl, 50 mM sodium phosphate buffer pH 6.3 (8.22 mL of 0.5 M NaH_2PO_4 and 1.78 mL of 0.5 M Na_2HPO_4 in 100 mL), 0.5% (v/v) Nonidet P-40, pH 6.3.

3. *Buffer A-6.3-imidazole:* same as buffer A-6.3, but also containing 10 mM Imidazol, pH 6.3.

4. *Buffer B:* 8 M Urea, 200 mM NaCl, 50 mM sodium phosphate buffer pH (9.21 mL of 0.5 M NaH_2PO_4 and 0.79 mL

of 0.5 M Na_2HPO_4 in 100 mL), 2% (w/v) SDS, 10 mM EDTA, 100 mM Tris, pH 4.3.

5. *Buffer C:* 8 M Urea, 0.2 M NaCl, 0.2% (w/v) SDS, 100 mM Tris, pH 8.0.
6. *Buffer D:* 8 M Urea, 0.2 M NaCl, 100 mM Tris, pH 8.0.
7. Ni^{2+} Sepharose™ 6 Fast Flow beads (GE Healthcare).
8. Immobilized Streptavidin beads (Pierce, Rockford, IL, USA).
9. Poly-Prep® Chromatography Columns (Bio-Rad, Hercules, CA, USA).
10. 25 mM NH_4HCO_3 buffer, pH 8.0.
11. HPLC-grade H_2O.
12. PMSF, 0.5 M solution in isopropanol, stored at 4°C.

2.3. Western Blot Analysis

1. 4× SDS sample buffer: 250 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 300 mM DTT, 30% (v/v) glycerol, 0.02% (w/v) bromophenol blue.
2. Antibodies for detection of the HB-tag: RGS-His Antibody (Catalog number 34610; Qiagen, Valencia, CA, USA), 1:2,000 in blocking buffer, or Horseradish Peroxidase-Conjugated Streptavidin (Catalog number PI21126; Fisher, Pittsburgh, PA, USA), 1:10,000 in TBS-T buffer.
3. 10× TBS-T (Tris-buffered saline with Tween 20), 10× stock: 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4, 1% (v/v) Tween 20.
4. Blocking buffer: 5% (w/v) nonfat dry milk in 1× TBS-T.

2.4. Sample Preparation for MudPIT Analysis

1. 25 mM NH_4HCO_3.
2. 0.4 μg/μL trypsin (Promega) in 1 mM trifluoroacetic acid (TFA) (see Note 2).
3. Trypsin, (Promega, Madison, WI, USA).
4. "Slick Tubes" (Catalog number 16-8110-03P, PGC Scientific, NC, USA).
5. Strong cation exchange PolySULFOETHYL column (The Nest Group, Inc., Southborough, MA, USA).
6. Buffer C18-A: 2% (v/v) acetonitrile (ACN), 98% (v/v) H_2O, 0.1% (v/v) formic acid (FA).
7. Buffer C18-B: 98% (v/v) ACN, 2% (v/v) H_2O, 0.1% (v/v) FA.
8. Buffer SCX-A: 5 mM KH_2PO_4, 30% (v/v) ACN, 0.1% (v/v) FA, adjusted to pH 2.7 with FA.
9. Buffer SCX-B: 5 mM KH_2PO_4, 350 mM KCl, 30% (v/v) ACN, 0.1% (v/v) FA, adjusted to pH 2.7 with FA.
10. 0.1% (v/v) TFA.
11. Vivapure C-18 Microcolumn (Sartorius, Göttingen, Germany).

12. Pepmap C18 capillary column (Dionex, Bannockburn, IL, USA) (length: 15 cm, ID 75 μm), or capillary columns from another vender, or self-packed.
13. 500 fmol/μL BSA tryptic digest (Michrom Bioresources, Auburn, CA, USA).

3. Methods

3.1. Plasmid

The retroviral vector pQCXIP (BD Biosciences) expressing human HB-ubiquitin (11) (Fig. 2) can be used to generate cell lines stably expressing HB-tagged ubiquitin. Viral particles are generated in 293 GP2 packaging cells and used to transduce cells according to standard protocols in order to establish a stable cell line expressing HB-ubiquitin. Expression of HB-ubiquitin should be tested by immunoblotting using the RGS-His antibody, and stable cell lines should be periodically maintained with the appropriate antibiotic selection.

3.2. Growth and Lysis of Mammalian Cells Expressing HB-Ubiquitinated Proteins

1. Grow cells expressing HB-ubiquitin in DMEM (or SILAC DMEM) to about 90% confluency. Use five to ten 150 mm plates. The amount of plates required for the experiment depends on the protein yield of the specific cell line. We recommend growing enough cells to obtain between 10 and 20 mg of total protein.
2. If desired, the proteasome inhibitor MG132 can be used to accumulate ubiquitinated proteins. Cells are incubation at 37°C with 10 μM MG132 dissolved in DMSO for 1.5 h before harvesting.
3. Wash cells on plates twice with 5 mL of ice-cold 1× PBS, pH 7.4.
4. Lyse the cells "on plate" by adding buffer A with 1% PMSF. Pipette several times up and down to make sure that all cells detach and lyse. The lysate gets very viscous due to the presence of chromosomal DNA. Keep the volume as low as possible (e.g., transfer the harvested lysate to the next dish and harvest again). For a total of ten 150 mm plates of cells harvested, use a maximum of 20 mL lysis buffer.
5. Shear the DNA by passing the lysates several times through a 27-G needle, or sonicate on ice for 30 s intervals, until the viscosity is similar to that of water.
6. Centrifuge the lysate at 25,000 × *g* for 30 min at 4°C. Transfer the clarified supernatant to a fresh tube. Measure the protein concentration. For SILAC experiments, mix equal amounts of proteins from the heavy and the light sample. Save 50 μL aliquots of the lysate for analysis.

3.3. Tandem-Affinity Purification of HB-Ubiquitinated Proteins

To distinguish specific ubiquitinated proteins from background proteins, we recommend parallel processing of a sample from cells that do not express HB-ubiquitin. Proteins that are detected in both, the tagged and untagged cell line, are most likely nonspecific background proteins.

1. Use 70 µL of Ni^{2+} sepharose beads for each 1 mg of protein lysate. Wash the beads 3× with at least 5 bead volumes of buffer A-8 (without PMSF). Pellet the beads by centrifugation at $100 \times g$ for 1 min in a microcentrifuge, and remove the supernatant.
2. Add Ni^{2+} sepharose beads to the lysate, followed by imidazole to a final concentration of 10 mM to reduce nonspecific binding.
3. Incubate on a rocking platform at room temperature for 4 h, or overnight.
4. Pellet the beads by centrifugation at $100 \times g$ for 1 min, and remove the supernatant. Save 50 µL for analysis (Ni-unbound fraction).
5. Wash the Ni^{2+} sepharose beads sequentially with 20 bead volumes of buffer A-8, buffer A-6.3, and buffer A-6.3-imidazole, respectively.
6. Elute HB ubiquitinated proteins 2× with 5 bead volumes of buffer B, make sure that the pH is correctly adjusted. Incubate for at least 10 min at room temperature for each elution step, and pool the eluates. Save 50 µL for analysis (Ni^{2+} eluate).
7. Adjust the pH of the eluate to pH 8.0 (add ~25 µL of 1 M NaOH to each 1 mL eluate).
8. Prepare streptavidin sepharose by washing with 2×3 mL of buffer C. Use 15 µL of beads for each 1 mg protein in the whole cell lysate used in the first step of purification.
9. Incubate the Ni^{2+} sepharose eluate with streptavidin beads in a Poly-Prep chromatography column on a rocking platform overnight at room temperature.
10. Drain the column by gravity. Save 50 µL of the flow-through for analysis (streptavidin unbound fraction).
11. Wash the streptavidin beads (in the Poly-Prep chromatography column) sequentially with 2×25 bead volumes of buffer-C and buffer-D, respectively.
12. Add 3 mL of 25 mM NH_4HCO_3, pH 8.0 and allow the column to drain by gravity.
13. Add 1 mL of 25 mM NH_4HCO_3, pH 8.0 with the Poly-Prep column closed at the bottom, resuspend the beads by pipetting up and down, and transfer the beads to a "slick tube."

14. Collect the beads at the bottom of the tubes by centrifugation at 100 × *g* for 1 min in a microfuge. Carefully discard the supernatant and wash the beads again with 1.5 mL of 25 mM NH_4HCO_3, pH 8.0.
15. Collect the beads at the bottom of the tube as above and add 25 mM NH_4HCO_3 buffer (approximately 50% of the bead volume) in preparation for the trypsin digest (Sect. 15.3.4).

3.4. "On-Bead" Digestion for Mass Spectrometric Analysis

1. Dissolve 20 µg of trypsin in 50 µL of 1 mM TFA in the original glass tube (see Note 2).
2. Incubate the sample (from step 15, Sect. 15.3.3) with trypsin at 37°C for 12–16 h on a rocking platform. Use 1 µg of trypsin for every 2 mg of whole cell lysate used in the first purification step.
3. Carefully collect the supernatant and add FA to a final concentration of 1% (v/v).
4. Reextract tryptic peptides from the beads 2–3× by adding approximately 50% of the bead volume of 25% (v/v) ACN, 0.1% (v/v) FA to the beads.
5. Pool the extracted peptides and concentrate to about 5 µL using a SpeedVac.
6. Add 100 µL of H_2O and concentrate (SpeedVac) to about 5 µL. Repeat this step once more.
7. For a 1D analysis, add 5% (v/v) ACN and 2% (v/v) FA to a final volume of 10 µL, for a 2D analysis to a final volume of 100 µL.
8. Samples can be stored frozen at this step, or used immediately for 1D analysis (Sect. 15.3.6), or further separated on an SCX column for complex peptide mixtures (2D-MS analysis, Sect. 15.3.7).

3.5. Western Blot Analysis of Purification

To analyze the efficiency of the purification, the small samples collected from the different purification steps should be analyzed by immunoblotting.

1. To evaluate the efficiency of binding to Ni^{2+} sepharose, analyze 20 µL of the collected whole cell lysates and 20 µL of the Ni-unbound fraction (step 4, Sect. 15.3.3) by immunoblotting with the anti-RGS-His antibody.
2. To analyze the efficiency of binding to streptavidin, use 10 µL of the Ni^{2+} eluate (step 6, Sect. 15.3.3) and 10 µL of the streptavidin unbound fraction (step 10, Sect. 15.3.3) for immunoblot analysis with the anti-RGS-His antibody, or horseradish peroxidase-conjugated streptavidin (see Note 3).

3.6. Mass Spectrometry

1. Condition the Pepmap C18 column sequentially in buffer C18-B for 30 min, and buffer C18-A for 30 min. Adjust the column flow rate to 250 nL/min.

2. Inject 1 µL of 500 fmol/µL BSA digest and separate the sample at the following gradient using buffers C18-A and C18-B: 0% buffer C18-B for 5 min, 0–35% buffer C18-B for 80 min, 80% buffer C18-B for 5 min, and 0% buffer C18-B for 30 min. Inject 1 µL of 10–50 fmol/µL BSA digest using the same gradient. The column is now ready to be used.
3. The protein digest from step 8, Sect. 15.3.4 can be directly injected onto the column for LC-MS/MS analysis.

3.7. Identification of Proteins by MudPIT Analysis

Peptide separation by ion-exchange chromatography for complex sample mixtures

1. Separate samples (generated at step 8, Sect. 15.3.4) on a strong cation exchange column with the following gradient: 0–5% buffer SCX-B for 2 min, 5–35% buffer SCX-B for 30 min, and 35–100% buffer SCX-B for 10 min. Collect the flow-through and each peak fractions. Collect about 10–20 fractions.
2. Concentrate each fraction to about 5–10 µL in a SpeedVac.
3. Add 180 µL of 0.1% (v/v) TFA to each fraction.
4. Desalt the samples using Vivapure C-18 Microcolumns according to the manufacturer's instructions.
5. After desalting, concentrate samples to 1–2 µL (using a SpeedVac) and add 10 µL of 5% ACN (v/v), 2% (v/v) FA to each sample for LC-MS/MS analysis.

3.8. LC-MS/MS Analysis

1. Each desalted fraction from step 5, Sect. 15.3.7 can be analyzed by LC-MS/MS (as described in Sect. 15.3.6).
2. Automatically submit the acquired LC-MS/MS data to commercially available search engines, such as MASCOT, Protein Prospector, and/or SEQUEST, for database searching, protein identification, and characterization of posttranslational modifications.

4. Notes

1. A triple SILAC procedure can also be used (24). This allows comparison of three different samples. In case of the triple SILAC strategy light growth media ($^{12}C^{14}N$ arginine and $^{12}C^{14}N$ lysine), medium growth media (4,4,5,5-D_4-lysine and $^{13}C_6$-arginine), and heavy growth media ($^{13}C_6{}^{15}N_2$-lysine and $^{13}C_6{}^{15}N_4$-arginine) are used.
2. The amount of trypsin suggested is a rough estimate. To determine the amount of trypsin required more accurately, measure the protein concentration in the Ni^{2+} eluate (step 6,

Sect. 15.3.3) and in the streptavidin unbound fraction (step 10, Sect. 15.3.3). The difference in protein concentration in these two fractions is a good approximation of the amount of protein bound to the streptavidin beads. For every 1 μg of protein bound to the streptavidin beads use between 0.01 and 0.02 μg of trypsin (the ratio of protein/trypsin is between 1/100 and 1/50). Diluted trypsin solution should be prepared immediately before the digestion and can be stored at −20°C.

3. The RGS-His antibody detects HB ubiquitinated proteins and can be used to measure the efficiency of the purification steps. Horseradish peroxidase-conjugated streptavidin can be used to analyze the purification after elution from the Ni^{2+} sepharose. However, it is not very useful for analysis of the first purification step because all eukaryotic cells express between four and six endogenous biotinylated proteins. The endogenous biotinylated proteins are relatively abundant and can complicate interpretation of the Western blot results. However, endogenous biotinylated proteins are lost during the first purification step and horseradish peroxidase-conjugated streptavidin is useful for the detection of HB ubiquitinated proteins after the first purification step, providing important information about the efficiency of biotinylating HB-ubiquitinated target proteins.

Acknowledgments

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