Chapter 1

Approaches to Determine Protein Ubiquitination Residue Types

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Abstract

Ubiquitination is an important posttranslational modification in eukaryotic organisms and plays a central role in many signaling pathways in plants. Most ubiquitination typically occurs on substrate lysine residues, forming a covalent isopeptide bond. Some recent reports suggested ubiquitin can be attached to non-lysine sites such as serine/threonine, cysteine or the N-terminal methionine, via oxyester or thioester linkages, respectively. In the present protocol, we developed a convenient in vitro assay for investigating ubiquitination on Ser/Thr and Cys residues.

Key words Ubiquitination, Posttranslational modification, Serine/threonine, Cysteine, Hydrolysis

1 Introduction

Ubiquitination is an important posttranslational modification that controls many cellular processes. Many proteins involved in the ubiquitin system play crucial roles in signal transduction and biophysical processes. The effects of ubiquitination on its protein substrates are diverse and influence protein stability and activity, protein–protein interactions, and subcellular localization [1]. Degradation is the usual fate of polyubiquitinated proteins. Ubiquitination is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3), the action of which forms an isopeptide bond between the carboxyl group of the C-terminus of ubiquitin and the ε-amino group of a lysine residue in substrate.

Somewhat surprisingly, many proteins are unstable and rapidly degraded when all lysine residues are mutated to arginine. The RING type E3 ligase mK3 targets the major histocompatibility complex (MHC) class I, an ER-associated degradation (ERAD) substrate, for degradation via ubiquitination of its cytosolic tail [2]. However, a lysine-deficient mutant was also ubiquitinated by mK3. In 2007, the same researcher found that the K-less heavy chain

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(HC) of MHC class I could be degraded, while the KSCT-less HC was stable [3]. Ubiquitination was not influenced by reducing agents, which indicated that the modification was not occurring on a Cys residue, and closer inspection revealed that the MHC class I was ubiquitinated at Lys, Ser, and Thr sites [3]. Similarly, the ERAD substrate NS-1 is ubiquitinated by the HRD1 E3 ligase at Lys and Ser/Thr sites [4], and ubiquitination also occurs on the N-terminal amino of substrates [5] and Cys in substrates [6]. In plants, the $SCF^{TIR1/AFB}$ ubiquitin ligase substrate IAA1 was found to be ubiquitinated on Lys and Ser/Thr residues, which promoted rapid degradation [7]. Ubiquitination can therefore occur on multiple different amino acid residues, which introduces a great deal of complexity and flexibility.

Several methods have been established to identify ubiquitination sites, including mutagenesis followed by degradation assays and LC–MS/MS. However, mutagenesis is laborious if there are numerous Lys or Ser/Thr sites present. Although LC–MS/MS is highly efficient, it is expensive and false positives can be problematic. Biochemical approaches are convenient and can differentiate between ubiquitination on Ser/Thr or Cys in a shorter time. Ubiquitination of Ser/Thr results in a covalent oxyester bond that is sensitive to mild alkaline treatment, while the thioester bond between Cys and the C-terminus of ubiquitin is sensitive to reducing agents, and the isopeptide bond between Lys and ubiquitin is stable under both mild alkaline and reducing conditions [8, 9]. Detection of ubiquitination products following treatment with reducing agents or mild alkaline buffer can therefore determine the site(s) of ubiquitin attachment, and this method was tested in plants.

2 Materials

2.1 Extraction of Plant Proteins

1. 1 M dithiothreitol (DTT): dissolve 1.545 g DTT in 10 ml 0.01 M sodium acetate, filter using a 0.22 μM sterile membrane, aliquot and store at −20 °C (*see* **Note 1**).
2. 100 mM phenylmethanesulfonyl fluoride (PMSF): dissolve 0.174 g PMSF in 10 ml isopropanol, aliquot and store at −20 °C (*see* **Note 1**).
3. Native extraction buffer: 50 mM Tris–MES (pH 8.0), 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, autoclave and store at 4 °C. Add DTT to 1 mM, PMSF to 1 mM, and a protease inhibitor cocktail Complete Mini tablet (Roche) per 10 ml buffer immediately before use (*see* **Note 2**).

2.2 SDS-PAGE

1. Store 30% acrylamide/bis-acrylamide (29:1) solution (Genestar) at 4 °C.
2. Store *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED; AMRESCO) at 4 °C.

3. 10% ammonium persulfate (APS): dissolve 1 g APS in 10 ml H_2O (10% in W/V) (*see* **Note 3**) and store at 4 °C.
4. 10% SDS: dissolve 100 g in 1 l H_2O and store at room temperature.
5. 1 M Tris–HCl (pH 6.8): dissolve 121.2 g Tris base in 800 ml H_2O, adjust pH to 6.8 with HCl, and adjust volume to 1 l with additional H_2O. Autoclave and store at room temperature.
6. 1.5 M Tris–HCl (pH 8.8): dissolve 181.6 g Tris base in 800 ml H_2O, adjust pH to 8.8 with HCl, and adjust volume to 1 l with additional H_2O. Autoclave and store at room temperature.
7. 10% SDS-PAGE separating gel (5 ml): 1.9 ml H_2O, 1.7 ml 30% acrylamide-bis-acrylamide (29:1), 1.3 ml 1.5 M Tris–HCl (pH 8.8), 0.05 ml 10% SDS, 0.05 ml 10% APS and 0.002 ml TEMED.
8. Stacking gel (3 ml): 2.1 ml H_2O, 0.5 ml 30% acrylamide-bis-acrylamide (29:1), 0.38 ml 1 M Tris–HCl (pH 6.8), 0.03 ml 10% SDS, 0.03 ml 10% APS and 0.002 ml TEMED.

2.3 Immunoblotting Components

1. Running buffer (1 l): dissolve 3.03 g Tris base, 14.4 g glycine and 1 g SDS in 1 l dH_2O.
2. Transfer buffer (1 l): dissolve 3.03 g Tris base and 14.4 g glycine in 800 ml dH_2O, and add 200 ml methanol.
3. 4× SDS loading buffer contained 0.25 M Tris–HCl (pH 6.8), 8% SDS, 40% glycerol, 0.005% bromophenol blue and 20% β-mercaptoethanol.
4. 10× PBS (1 l): 80 g NaCl, 2 g KCl, 35.8 g $Na_2HPO_4{\cdot}12H_2O$, 2.7 g KH_2PO_4, pH 7.4. Dilute to 1× PBS before use.
5. Blocking buffer: 5% skim milk powder in 1× PBS.
6. Antibody dilution buffer: 3% skim milk powder in 1× PBS.
7. Primary antibodies: specific for the epitopes or proteins of interest.
8. Secondary antibodies: goat anti-mouse or goat anti-rabbit (Proteinteach).
9. Horseradish peroxidase (HRP) substrate kit (Millipore).
10. X-ray film.

2.4 Immunoprecipitation

1. Store Protein G Dynabeads (Life Technologies) at 4 °C.
2. MG132 stock solution: MG132 is dissolved in DMSO and 10 mM stock. Aliquot it in small volume and stored at −80 °C (*see* **Note 4**).
3. A 4 °C cold room.
4. Thermo-mixer comfort (Eppendorf) equipped with a constant temperature setting.
5. Amicon Ultra-15 Centricons (Millipore; *see* **Note 5**).

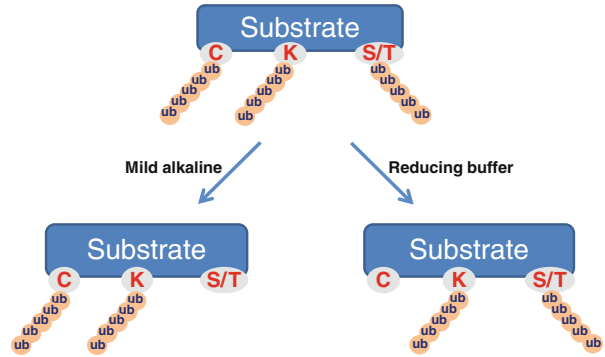

Fig. 1 The schematic diagram of ubiquitination occurring on different amino acids. Ubiquitination occurring on Ser/Thr residues is sensitive to mild alkaline (0.1 M NaOH) treatment, which also abolishes the signal from polyubiquitinated proteins. Ubiquitination occurring on Cys residues is sensitive to reducing agents, which also abolishes the signal from polyubiquitylated proteins

3 Methods

In this chapter, we describe the development of a detailed protocol for determining ubiquitination events on nonlysine residues. Substrate proteins are first obtained from transgenic plants or transient expression in *Nicotiana benthamiana* leaves as described previously [10]. Confirmation of ubiquitination is needed. Treatment with SDS followed by immunoprecipitation helps to exclude ubiquitinated interacting partners. Treatment with reducing agents or mild alkaline then determines if modification has occurred on Cys or Ser/Thr residues, respectively. All procedures should be carried out on ice unless otherwise specified. A schematic diagram is shown in Fig. 1.

3.1 Protein Expression and Extraction

1. Harvest transgenic plants or *N. benthamiana* leaves expressing substrates or control proteins and freeze immediately in liquid nitrogen.
2. Grind the material and resuspend in 1 ml native extraction buffer per 0.4 g powder (*see* **Note 6**).
3. Centrifuge at 14,000 × *g* for 6 min at 4 °C and the supernatant was transferred to a new tube. Repeat this centrifugation for four times and transfer the supernatant each time. Use supernatant in immunoprecipitation assay.

3.2 Immunoprecipitation

1. Filter supernatant using a 0.45 μM sterile membrane to remove debris (*see* **Note 7**).
2. Prepare Protein G Dynabeads by washing three times with ice-cold native buffer.

3. Antibody (10 μg antibody/ml native buffer) is incubated with Protein G Dynabeads in a centrifuge tube at 4 °C for 2 h. Wash the antibody three times with ice-cold 1× PBS and removes all liquid after the final wash.
4. Add 1 ml of supernatant and MG132 to a final concentration of 50 μM (*see* **Note 4**). Shake gently at room temperature for 30 min and at 4 °C for 2 h to bind substrate proteins (*see* **Note 8**).
5. Collect Dynabeads and wash five times with cold 1× PBS (150 mM NaCl was added to PBS; *see* **Note 9**). Use the immunoprecipitated mixture for in vivo ubiquitination assays and western blotting.

3.3 In Vivo Detection of Polyubiquitination

1. Immunoprecipitate substrates and control proteins using specific antibodies as described in Subheading 3.2.
2. Suspend beads with bound proteins in 40–60 μl 10 mM Tris–HCl (pH 6.8) containing 0.5% SDS, and heat at 95 °C for 5 min (*see* **Note 10**). This will remove bound proteins and disrupt protein–protein interactions.
3. Centrifuge at 14,000×*g* for 30 s. Transfer supernatant to a new tube.
4. Dilute supernatant to 1 ml with 1× PBS.
5. Re-immunoprecipitate proteins for 2 h and collect beads as described above.
6. Elute samples using SDS sample buffer at 95 °C for 5 min and separate by SDS-PAGE. Western blotting will be conducted to detect polyubiquitinated substrates.

3.4 Detection of Polyubiquitination on Cys Residues

1. Immunoprecipitate substrate and control proteins as described in Subheading 3.2 and treat with SDS as described in Subheading 3.3.
2. Divide beads into two tubes and adjust volume to 30 μl with 1× PBS.
3. Add 10 μl nonreducing or reducing 4× SDS sample buffer, respectively, and boiled at 95 °C for 5 min.
4. Separate by SDS-PAGE and observe whether ubiquitination is decreased in reducing buffer by western blotting.

3.5 Detection of Polyubiquitination on Ser/Thr Residues

1. Immunoprecipitate substrate and control proteins as described in Subheading 3.2 and treat with SDS as described in Subheading 3.3.
2. Collect beads by centrifugation at 14,000×*g* for 30 s, transfer supernatant to a new tube and adjust the final volume to 40 μl with 10 mM Tris–HCl (pH 6.8).

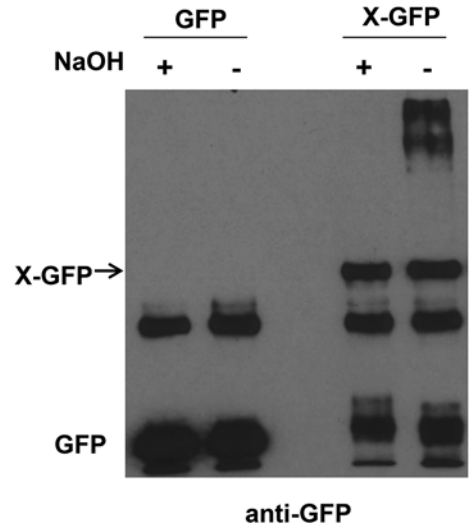

Fig. 2 The ubiquitination on Ser is sensitive to NaOH treatment. The protein X-GFP can be poly-ubiquitinated in vivo. X-GFP is immunoprecipitated and checked for ubiquitination after being treated with NaOH or 1× PBS as described in Subheading 3.5. The polyubiquitin of X-GFP is reduced by treatment with NaOH. GFP is used as a control in this assay

3. Divide supernatant into two equal aliquots and add NaOH to a final concentration of 0.1 M to one aliquot, and 1× PBS to the other aliquot to a final volume of 60 μl.
4. Incubate at 37 °C for 2 h.
5. Dilute to 4 ml with 10 mM Tris–HCl (pH 6.8) in Amicon Ultra-15 Centricons following hydrolysis and concentrate to a volume of 1 ml at 4 °C (*see* **Note 11**).
6. Test the pH using pH indicator paper, stop concentrating when the pH reaches 7–7.5, and repeat **step 5** if the pH is too high (*see* **Note 12**).
7. Re-immunoprecipitate proteins at 4 °C and elute in reducing buffer by boiling at 95 °C for 5 min. Separated by SDS-PAGE. An example was shown in Fig. 2.

3.6 Western Blotting

1. Separate samples by 10% SDS-PAGE at 160 V for 1 h.
2. Transfer to nitrocellulose membrane (in transfer buffer) at 100 V for 75 min.
3. Block membrane with blocking buffer for 1 h at room temperature or overnight at 4 °C.
4. Incubate membrane with primary antibody in antibody dilution buffer for 1 h at room temperature or overnight at 4 °C.
5. Remove antibody solution and wash membrane twice with 0.05% PBST for 15 min (*see* **Note 13**).

6. Incubate membrane with secondary antibody in antibody dilution buffer for 1 h at room temperature.
7. Wash membrane twice with 0.05% PBST for 15 min (*see* **Note 13**).
8. Detect the signal using a Millipore Chemiluminescence HRP Substrate Kit.

4 Notes

1. DTT (1 M) and PMSF (100 mM) stocks are unstable at room temperature or 4 °C and should be stored at –20 °C. Aliquot small volumes to avoid repeated freeze-thawing. Add to solutions immediately before use.
2. For membrane proteins, detergents such as NP-40 should be included to improve protein extraction. NP-40 should be added to native buffer just before use and the amount should be less than 1% in accordance with protein properties.
3. The "H_2O" used in this protocol is ultrapure water with an electrical resistivity of 18 MΩ cm at 25 °C.
4. For unstable substrate proteins, MG132 (or another proteasome inhibitor) should be used to prevent degradation. MG132 should not be freeze-thawed repeatedly and a working concentration of 50–100 μM is recommended.
5. An Amicon Centricon of less than one third of the molecular weight of the target protein should be used, and the centrifugal speed and centrifugal time should be in accordance with the manufacturer's instructions and with protein solubility.
6. The amount of buffer added can be adjusted according to the target protein expression level. The dilution ratio mentioned in the text was determined from empirical results.
7. This step is critical. Removing debris helps to minimize unrelated interacting proteins.
8. Ubiquitinated proteins can be unstable, rapidly degraded and hence difficult to detect. Incubating at room temperature with MG132 likely minimizes degradation and increases the amount of the ubiquitinated form.
9. Adding NaCl to wash buffer can reduce contaminating proteins during immunoprecipitation.
10. Interacting partner proteins may be coimmunoprecipitated. To avoid the false signal from ubiquitinated interacting proteins, beads should be boiled as described to disrupt protein–protein interactions.
11. The volume is dependent on protein solubility.

12. The pH is important and a high pH may affect separation by SDS-PAGE.
13. If a high background occurs during western blotting, add 0.1 % Tween 20 to PBST wash buffers. If the signal is too low, 1× PBS without Tween 20 should be used.

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