Chapter 4

Measuring the Enzyme Activity of Arabidopsis Deubiquitylating Enzymes

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Abstract

Deubiquitylating enzymes, or DUBs, are important regulators of ubiquitin homeostasis and substrate stability, though the molecular mechanisms of most of the DUBs in plants are not yet understood. As different ubiquitin chain types are implicated in different biological pathways, it is important to analyze the enzyme characteristic for studying a DUB. Quantitative analysis of DUB activity is also important to determine enzyme kinetics and the influence of DUB binding proteins on the enzyme activity. Here, we show methods to analyze DUB activity using immunodetection, Coomassie Brilliant Blue staining, and fluorescence measurement that can be useful for understanding the basic characteristic of DUBs.

Key words Deubiquitinating enzymes, DUB assay, K48-linked ubiquitin chains, K63-linked ubiquitin chains

1 Introduction

Ubiquitylation is a reversible posttranslational modification that is key to various cellular processes in almost all physiological pathways of plants [1]. It must be strictly controlled and regulated at multiple steps during these processes. The attachment of ubiquitin to the target proteins is carried out by the sequential activities of the ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s), and ubiquitin ligases (E3s) [2]. The ubiquitylation status of the substrate proteins is also controlled by the activity of deubiquitylating enzymes (DUBs: also deubiquitinating enzymes or deubiquitinases) that can deconjugate ubiquitin or ubiquitin-like proteins from their substrates [3].

The Arabidopsis genome codes for around 50 DUBs [4], though for most of these the exact molecular and biological functions are not yet understood. DUBs remove covalently attached ubiquitin molecules from substrates or hydrolyze the peptide bond between ubiquitin molecules. DUBs can play multiple roles in cellular processes. Firstly, they are essential for the posttranslational

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activation of ubiquitin molecules. Secondly, they are also responsible for the recycling of ubiquitin molecules by removing them from the substrates prior to degradation. Thirdly, DUBs can also actively regulate the stability of ubiquitylated proteins by deubiquitylating them before they are recognized by the degradation machinery. Finally, since the attachment of ubiquitin can affect the binding affinity of the ubiquitylated protein to other proteins, DUBs can also influence protein–protein interaction. Eukaryotes have five DUB families that can be classified according to the difference in their catalytic domains [5]: the ubiquitin-specific proteases (UBPs or USPs), the ubiquitin C-terminal hydrolases (UCHs), the ovarian tumor proteases (OTUs), the Machado–Joseph domain (MJD) or Josephine domain proteases, and the JAB1/MPN/MOV34 (JAMM) proteases. Except DUBs of the JAMM family that are zinc-dependent metalloproteases, all other DUBs are cysteine proteases.

Monoubiquitylation as well as seven different ubiquitin chain linkages (K6-, K11-, K27-, K29-, K33-, K48-, and K63-linkages) are found in vivo [6], indicating that all chain types can have biological significance. In addition, linear or mixed ubiquitin chains also have been shown to have important biological functions [7, 8]. Due to their distinct topology, different ubiquitin linkages can be recognized by different set of proteins and thereby can be involved in different pathways. With the now available information about chain-type specificity of human DUBs, highly specific DUBs can also be used as tools to identify ubiquitin chain types of a ubiquitylated protein [9].

Since substrate identification of DUBs is not trivial, identification of interactors of DUBs and analysis of the enzymatic characteristics are crucial to determine the pathway a given DUB might be involved. In vitro assay for studying DUB activities are therefore useful tools to analyze ubiquitin chain-type specificities of DUBs and also to examine whether interacting proteins can influence DUB activity. The availability of various types of commercial ubiquitin chains enables quantitative and reproducible assays with simple equipment. Fluorescence- or luminescence-based substrates also offer possibility of determining the enzyme kinetics. In this chapter, we describe immunoblot-, Coomassie Brilliant Blue (CBB) staining-, and fluorescence-based analysis of DUB activity. We show the example with the Arabidopsis DUB AMSH3, which is a conserved DUB implicated in intracellular protein trafficking [10, 11].

2 Materials

2.1 Recombinant GST-Tagged DUB Purification from Bacteria

1. Buffer A: 50 mM Tris–HCl, 100 mM NaCl, 10% (w/v) glycerol. Adjust the pH to 7.5, cool the buffer down to 4 °C overnight, then readjust the pH again to pH 7.5. Store at 4 °C.
2. Buffer A supplemented with 0.2% (v/v) Triton X-100 and 1× complete EDTA-free protease inhibitor (Roche), prepare directly before use.

3. Buffer A supplemented with 1 mM dithiotreitol (DTT), prepare directly before use.
4. Ultrasonic homogenizer.
5. Refrigerated centrifuge for 50 ml tubes.
6. Refrigerated table top centrifuge for 1.5 ml tubes.
7. GST purification matrix (e.g., Glutathione Sepharose 4B from GE Healthcare).
8. Mini-spin columns, e.g., Mini Bio-Spin Chromatography Columns (Bio-Rad).
   (a) 40 mM reduced glutathion, in case GST-fusion proteins will be eluted with the tag.
   (b) PreScission protease (GE Healthcare), in case the expression vector contains a PreScission protease recognition site (such as the pGEX-6P-series from GE Healthcare) (*see* **Note 1**).
9. Protein molecular weight standards.

2.2 Deubiquitylation Assay (DUB Assay)

1. DUB Assay Buffer: 50 mM Tris–HCl pH 7.2 (*see* **Note 2**), 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT. Prepare directly before use or store at −20 °C.
2. (a) Reaction substrate: In vitro ubiquitylated T7-Sic1^{PY} (Ubn-T7-Sic1^{PY}) prepared using the ubiquitylating enzymes E1(Uba1), E2 (Ubc4), and E3(Rsp5) [12].
   (b) Reaction substrate: Commercially available di- or polyubiquitin (Ub_{2-7}) chains (e.g., from Enzo Life Sciences) (*see* **Note 3**).
3. Heating block.
4. 4× NuPAGE SDS Sample Buffer: 564 mM Tris base, 416 mM Tris hydrochloride, 8% (w/v) SDS, 40% (w/v) glycerol, 2.04 mM EDTA, 0.88 mM SERVA Blue G250, 0.70 mM Phenol Red. Store at −20 °C or room temperature.

2.3 Gradient Gel Electrophoresis

1. NuPAGE 4–12% Bis-Tris gel (Thermo Fisher Scientific, *see* **Note 4**).
2. 20× MES SDS Running Buffer: 1 M MES, 1 M Tris base, 2% (w/v) SDS, 20 mM EDTA. Store the 20× stock at 4 °C. Use the 20× stock to prepare the 1× running buffer before use.
3. Prestained molecular mass marker.
4. Gel apparatus, e.g., XCellSureLock Mini-Cell Electrophoresis System for NuPAGE (Thermo Fisher Scientific).
5. Protein standard markers with known concentration, e.g., BenchMark Protein Ladder (Thermo Fisher Scientific).

2.4 Protein Transfer and Western Blotting

1. 20× NuPAGE Transfer Buffer: 0.5 M Bicine, 0.5 M Bis-Tris (free base), 20 mM EDTA. Store at 4 °C. Prepare 2× transfer buffer with 10% (v/v) methanol before use.

2. Horizontal shaker.
3. Semidry transfer apparatus.
4. Four filter papers (1.5 mm) cut in the size 0.5 cm larger than the protein gel.
5. PVDF membrane or nitrocellulose membrane cut in the size of the protein gel.
6. 100% methanol, in case a PVDF membrane is used.
7. Tris-buffered saline (TBS): 0.5 M Tris–HCl pH 7.5, 1.5 M NaCl, 10 mM $MgCl_2$. Store at room temperature.
8. Tris-buffered saline with Tween-20 (TBST): use 10× TBS stock to prepare 1× working solution. Add Tween-20 to 0.05% (v/v). Store at room temperature.
9. Blocking buffer: 10% (w/v) powdered milk in TBST. Prepare directly before use.
10. Monoclonal anti-ubiquitin (anti-Ub) antibody P4D1 (e.g., from Santa Cruz) (*see* **Note 5**).
11. Anti-mouse HRP-conjugated antibody.
12. Enhanced chemiluminescent (ECL) reagents.
13. Chemiluminescence detection apparatus, e.g., LAS4000 mini system (Fuji Film).

2.5 CBB Staining

1. CBB staining solution: 40% (v/v) ethanol, 7% (v/v) acetic acid, 0.25% (w/v) CBB.
2. Destaining solution: 40% (v/v) ethanol, 7% (v/v) acetic acid.

2.6 Fluorescence-Based DUB Assay

1. TAMRA DUB Buffer: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% (w/v) Pluronic F-127, 1 mM Tris(2-carboxyethyl)phosphine (TCEP). Prepare before use.
2. Reaction substrate: diubiquitin (K63-linked) FRET TAMRA Position 3 (from R&D Systems) (*see* **Note 6**).
3. Reaction plates, e.g., 96-well black plate.
4. Fluorescence plate reader (e.g., Synergy 2 Multi-Mode Microplate Reader from BioTek) with filters for excitation wavelength of 530 nm and emission wave length of 590 nm.

3 Methods

3.1 Recombinant DUB Purification from Bacteria

1. Cool down a refrigerated centrifuge for 50-ml tubes to 4 °C.
2. Add 20 ml Buffer A supplemented with 0.2% (w/v) Triton X-100 and 1× complete EDTA-free protease inhibitor to *E. coli* pellet from 250 ml culture and resuspend pellet.

3. Place the tube in a beaker filled with ice and water and sonicate the sample for 15 min with four cycles at 20% output. Repeat the sonication for another 15 min if necessary.
4. Centrifuge the postsonication solution at 15,000 × *g* for 10 min at 4 °C.
5. Transfer the supernatant into a new 50-ml tube and keep on ice.
6. Take 100 µl of Glutathione Sepharose 4B (75 µl bed volume) with a cut tip and transfer to a 1.5 ml tube (**Note 7**).
7. Add 1 ml buffer A to the beads and centrifuge them for 1 min at 800 × *g* at 4 °C. Remove supernatant. Repeat washing three times.
8. Add 500 µl buffer A to the beads. Transfer the beads with a cut tip to the 50-ml tube containing the protein supernatant.
9. Incubate the protein solution with beads for 2 h at 4 °C with rotation.
10. Centrifuge the protein solution for 3 min at 800 × *g* at 4 °C. Discard supernatant.
11. Add 20 ml buffer A and centrifuge again as above. Repeat washing three times.
12. Discard the washing buffer, leaving ca. 500 µl buffer in the tube. Using a pipette and a tip with a cut-off end, transfer the beads on a mini-spin column.
13. Add 500 µl buffer A containing 0.2% (v/v) Triton to the beads and centrifuge them for 5 s at 800 × *g* at 4 °C. Discard the flow-through. Repeat washing three times.
14. Wash the beads three times with 500 µl buffer A as above.
15. Elute the purified protein with 40 mM reduced glutathione by incubating 10 min at room temperature (*see* **Note 8**). If PreScission protease is used, add 200 µl buffer A containing 1 µl of PreScission protease to the beads and rotate for 16–20 h.
16. Take 6 µl of the purified protein, add 1.5 µl 5× Laemmli buffer and incubate for 5 min at 95 °C. Analyze the purity and concentration of the purified protein on a CBB-stained SDS-PAGE gel using proteins standards.

3.2 Deubiquitylation Assay

1. Set the temperature at 30 °C on a heating block (*see* **Note 9**).
2. Prepare individual reaction tubes for each time point for the experiment and aliquote 3-pmol, 2-pmol, or 8-pmol of recombinant DUB for Ubn-T7-Sic1^{PY} (Immunoblot), polyubiquitin chains (Immunoblot), or diubiquitin (CBB detection), respectively, in the DUB Assay Buffer to make a total volume of 10 µl. Preincubate the tubes for 5–10 min at 30 °C. If DUB inhibitors are to be tested, they can be added to the reaction mixture at this point (*see* **Note 10**).

3. While preincubating the reaction mixture, prepare the ubiquitin substrates to a concentration of 250 ng/μl in DUB Assay Buffer. Start the reaction by adding the following amount of substrates to the preincubated reaction mixture from **step 2**: (a) 500 ng Ubn-T7-Sic1^{PY}, (b) 250 ng polyubiquitin chains for immunodetection or (c) 1 μg diubiquitin for CBB detection. Incubate in the heating block at 30 °C for the desired amount of time.
4. Terminate the reaction by adding the 4× NuPAGE SDS Sample Buffer and place the tubes on ice.
5. Once all reactions are terminated, incubate samples at 80 °C for 10 min and let cool at room temperature.

3.3 Gradient Gel Electrophoresis

1. Prepare 800 ml 1× running buffer. Unpack the precast NuPAGE 4–12% Bis-Tris Gel, remove the comb and wash the sample loading pockets with distilled water.
2. Assemble the gel apparatus and the gel. Fill the apparatus with 1× running buffer.
3. Load on all of your reaction mixture in each lane.
4. Run the gel at 200 V for 35 min. For the polyubiquitin chains, continue with a NuPAGE transfer and immunoblotting (*see* Subheading 3.4). For diubiquitin, continue with CBB staining (*see* Subheading 3.5) (*see* **Note 11**).

3.4 Protein Transfer and Western Blotting

1. Disassemble the gel plates and incubate the gel for 15 min in the 2× NuPAGE transfer buffer containing 10% (v/v) methanol.
2. Soak the filter papers in the 2× NuPAGE semidry transfer buffer and assemble a stack in the following order (from bottom to top): Two filter papers, PVDF (washed for 30 s in 100% Methanol) or nitrocellulose membrane, gel and two filter papers. Eliminate any air bubbles by rolling a glass tube over the transfer package after adding each filter paper.
3. Transfer the protein to the membrane for 25 min at 15 V.
4. Optionally, boil the membrane in ultrapure water on a heating plate for 10 min (*see* **Note 12**).
5. After the transfer, place the membrane in the blocking buffer and incubate for 15–30 min at room temperature on a shaker.
6. Prepare a 1:1000 dilution of the primary anti-ubiquitin (P4D1) antibody in the blocking buffer.
7. Incubate the primary antibody with the membrane at room temperature with shaking for at least 1 h or overnight at 4 °C.
8. Remove the solution with the primary antibody. Wash the membrane for 15 min with TBST buffer. Repeat the step three times, using fresh TBST buffer each time.
9. Prepare a proper dilution of the secondary antibody in TBST (anti-mouse HRP-conjugated antibody).

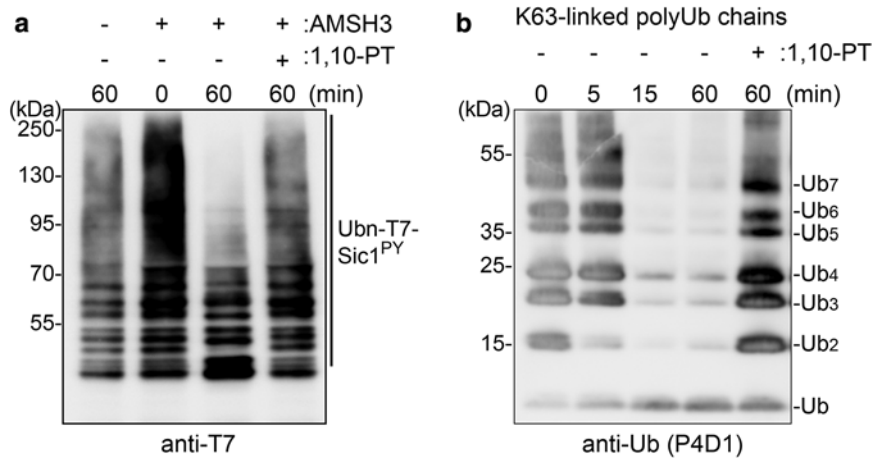

Fig. 1 DUB assay using immunoblot detection. (**a**) DUB assay of AMSH3 using Ubn-Sic1^{PY} as substrate. 1,10-PT was preincubated with AMSH3 for 10 min before the addition of the substrate. The reaction mixture was subjected to SDS-PAGE and immunoblotting with anti-ubiquitin antibody. (**b**) DUB assay using commercial K63-linked polyubiquitin chains. Reactions were terminated at the indicated time points. 1,10-PT was preincubated with AMSH3 for 10 min before the addition of the substrate

10. Incubate secondary antibody with the membrane at room temperature with shaking for at least 45 min or overnight at 4 °C.
11. Wash the membrane as in **step 7**.
12. Take out the membrane from the washing solution and remove excess liquid. Incubate the membrane with the ECL solution (600 µl are sufficient for a 6.5×8.0 cm membrane) for 5 min. Remove excess ECL solution with a paper towel and detect the chemiluminescence. Optimal exposure time varies between experiments. For a typical result of a DUB assay using polyubiquitin chains, *see* Fig. 1a, b.

3.5 CBB Staining

1. After electrophoresis, disassemble the gel plates. Incubate the gel in the destaining solution on a shaker for 15 min at room temperature, in order to remove excess SDS from the gel.
2. Discard the destaining solution. Pour the CBB-staining solution over the gel and gently shake for 60 min at room temperature.
3. Discard the staining solution. Wash the gel gently several times with tap water. Pour the destaining solution over the gel and incubate it on a shaker at room temperature until the gel background is reduced to a satisfactory extend. For a typical result of a DUB assay using diubiquitin, *see* Fig. 2.

3.6 Fluorescence-Based DUB Assay

1. Prepare dilutions of the DUB in the TAMRA DUB buffer, e.g., 0, 2.5, 5, and 10 nM to a total volume of 450 µl.
2. Pipet 100 µl of the reaction mixture into four wells of the reading plate (*see* **Note 13**).

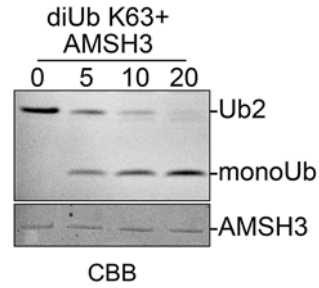

Fig. 2 DUB assay followed by Coomassie Brilliant Blue (CBB) staining. To the reaction tubes containing 8 pmol Arabidopsis AMSH3, 1 μg K63-linked diubiquitin was added and the reaction was conducted for 0, 5, 10, and 20 min. Degradation of diubiquitin and accumulation of monoubiquitin can be observed

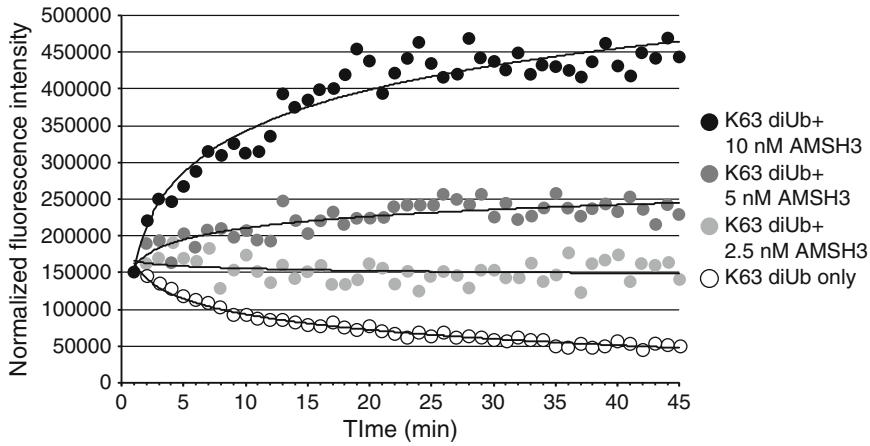

Fig. 3 Fluorescence-based DUB assay with diubiquitin FRET TAMRA. 96-well reaction plate containing 0, 2.5, 5, and 10 nM AMSH3. Reactions was started by addition of 0.2 μM diubiquitin (K63-linked) FRET TAMRA Position 3 and changes in the fluorescence were measured every minute over a time frame of 45 min

3. Dilute diubiquitin (K63-linked) FRET TAMRA Position 3 to a concentration of 10 μM in the TAMRA DUB buffer. Using a channel pipette, start the reaction by adding 2 μl of the substrate to have a final concentration of 0.2 μM in the assay.
4. Immediately close the fluorescence plate reader and start the reaction. Measure the changes in fluorescence every minute over a time period typically between 45- and 120 min (ex. 530 nm; em. 590 nm). A typical result of a TAMRA DUB assay is presented in Fig. 3 (*see* **Note 14**).

4 Notes

1. In some cases, fusion of large protein tags such as GST or MBP can affect enzyme activity. In these cases the tags should be cleaved off after purification using proteases such as Thrombin,

Factor Xa, or PreScission protease. PreScission protease has an advantage in that it is active at 4 °C. Moreover, since it is available as a GST-fusion protein, the protease remains on the beads. An untagged DUB can be detected on an immunoblot only when a specific antibody for the DUB is available. In case such antibody is not available, the presence of a tag allows detection of the DUB with a tag-specific antibody or otherwise the amount of the DUB in the reaction should be monitored by gel-staining.

2. The pH of the DUB Assay Buffer may need to be optimized for each DUB as different enzymes may have different optimal pH.
3. All major ubiquitin chain types (K6-, K11-, K27-, K29-, K33-, K48-, K63-linkages) as well as linear di- and tetraubiquitin are commercially available.
4. For Ubn-Sic1^{PY}, a standard 10% SDS-PAGE can also be used. Instead of a NuPAGE gel, self-made gradient gels or other commercially available gradient gels can also be used. However, we had the best experience with NuPAGE-gels for the detection of monoubiquitin by immunoblotting.
5. Other anti-ubiquitin antibodies can also be used.
6. FRET TAMRA diUb substrates are available with different fluorophore positions. It may be necessary to establish the most suitable substrate for your enzyme. We experienced that the attachment of the fluorophore to certain positions interfered with the DUB activity. UB-AMC is also a widely used substrate, but does not convey chain-type specificities.
7. The amount of the beads to be used depends on the volume of the cell culture, expression levels, and solubility of the recombinant protein as well as the binding capacity of the beads. Typically, for purification from a 250 ml culture, 50–100 µl bed volumes of beads are used.
8. Glutathione can be removed from the eluate by dialysis or with desalting columns.
9. Reaction temperature might have to be optimized depending on the origin of the DUB. Activity of some DUBs might be affected by temperature.
10. AMSH DUB activity can be inhibited by 10 mM EDTA, 1 mM 1,10-phenanthroline (1,10-PT), and 5 mM *N*-ethylmaleimide (NEM). Other DUB inhibitors that can be used for in vitro assays include 2 µM Ub-aldehyde or 250 µM *N*,*N*,*N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN).
11. If the quality and amount of the purified DUB is high enough, CBB staining provides faster results than immunoblotting. Silver staining or fluorescent dyes can also be used. In case the results have to be quantified, direct staining of the gel is more precise over immunoblotting.

12. Boiling of the membrane after the transfer may enhance the detection of monoubiquitin, though in an in vitro DUB assay this step could be skipped.
13. Different plate types are suitable for different readers. The reaction volume depends on the plate type. Typically, we recommend a 50–100 µl reaction volume for a 96-well plate. When using smaller volumes, make sure that the reaction mix fully covers the whole area of the bottom of the well.
14. Using different concentrations of fluorescent substrates, Michaelis–Menten kinetics can be determined.

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