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In vitro Reconstitution Assays of *Arabidopsis* 20S Proteasome

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[Abstract] The majority of cellular proteins are degraded by the 26S proteasome in eukaryotes. However, intrinsically disordered proteins (IDPs), which contain large portions of unstructured regions and are inherently unstable, are degraded via the ubiquitin-independent 20S proteasome. Emerging evidence indicates that plant IDP homeostasis may also be controlled by the 20S proteasome. Relatively little is known about the specific functions of the 20S proteasome and the regulatory mechanisms of IDP degradation in plants compared to other species because there is a lack of systematic protocols for *in vitro* assembly of this complex to perform *in vitro* degradation assays. Here, we present a detailed protocol of *in vitro* reconstitution assay of the 20S proteasome in *Arabidopsis* by modifying previously reported methods. The main strategy to obtain the 20S core proteasome here is to strip away the 19S regulatory subunits from the 26S proteasome. The protocol has two major parts: 1) Affinity purification of 20S proteasomes from stable transgenic lines expressing epitope-tagged PAG1, an essential component of the 20S proteasome (Procedures A-D) and 2) an *in vitro* 20S proteasome degradation assay (Procedure E). We anticipate that these protocols will provide simple and effective approaches to study *in vitro* degradation by the 20S proteasome and advance the study of protein metabolism in plants.

Keywords: 26S proteasome, 20S proteasome, Ubiquitin-independent, Intrinsically disordered proteins (IDPs), Protein degradation

[Background] In eukaryotes, protein degradation is carried out by the proteasome. The integrative 26S proteasome is comprised of two sub particles: one or two terminal 19S regulatory particle(s) (RP), which serve as a proteasome activator; and the 20S core proteasome (CP), which degrades proteins. Most eukaryotic proteins are polyubiquitinated and channeled into 26S proteasome for degradation. In contrast, proteins that contain intrinsically disordered regions have been found to be directly destroyed by the ubiquitin-independent 20S proteasome (Ben-Nissan *et al.*, 2014). Methods to purify and assembly the 20S proteasome *in vitro* are well established for mammalian cells and yeast. This has led to an understanding and appreciation of the numerous ways by which IDPs interact with the 20S proteasome (Leggett *et al.*, 2005). However, to date a detailed and efficient protocol has not been reported for the purification of the 20S proteasome from plants. Book *et al.* (2010) developed an affinity-based strategy to effectively isolate the 26S proteasome from *Arabidopsis*. In their approach, PAG1, one of the 14 core proteasome polypeptides, is epitope-tagged and immunoprecipitated with epitope-specific antibodies such that the 26S proteasome is recovered. From purification studies of the proteasome complex, there

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are two important components to monitor during the purification scheme: ATP amount and salt concentration (Verma *et al.*, 2000; Leggett *et al.*, 2002). It is known that the integrity of the RP-CP complex relies on ATP, and the abundance of RP subunits is substantially reduced if all purification steps do not include ATP. Similarly, RP subunit abundance is reduced when immunoprecipitates (IPs) are washed with a high salt buffer (800 mM NaCl) before elution (Book *et al.*, 2010). Based on previous methods, we designed a simple approach to specifically isolate the 20S proteasome by immunoprecipitating PAG1 complexes from total protein extracts of stable transgenic lines expressing *gPAG1-Flag-4Myc* (*gPAG1-FM*) under its native promoter. In our protocol, the protein extracts are not supplied with ATP, and IPs are washed with a buffer containing 800 mM NaCl, as this stringent condition has been reported to strip the 19S regulatory subunits away from the 20S core proteasome. For *in vitro* protein degradation assays, we modified a protocol from yeast work with the 20S proteasome (Hsieh *et al.*, 2015). Taken together, this protocol is easy to follow and can provide an effective strategy to study degradation of IDPs in plants. We hope that this protocol will advance research in protein metabolism and regulation in plants.

Materials and Reagents

1. 15 ml conical tube
2. 2 ml Eppendorf tube
3. 1.5 ml Eppendorf tube
4. Pipette tips
5. 96-well Plate (Thermo Scientific, catalog number: 249935)
6. 10-day-old P_{PAG1}-*gPAG1-FM* transgenic seedlings
7. Liquid nitrogen
8. Tris Base (Fisher Scientific, catalog number: BP152-10)
9. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L3771)
10. NaCl (Fisher Scientific, catalog number: BP358-10)
11. $MgCl_2$ (Sigma-Aldrich, catalog number: M9272)
12. EDTA (Fisher Scientific, catalog number: BP120-1)
13. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 43817)
14. Glycerol (Sigma-Aldrich, catalog number: V900122)
15. PMSF (Sigma-Aldrich, catalog number: 78830)
16. Miracloth (Calbiochem, catalog number: 475855)
17. Anti-FLAG® M2 magnetic beads (Sigma-Aldrich, catalog number: M8823)
18. 3× FLAG peptide (DYKDDDDK) (Sigma-Aldrich, catalog number: F4799)
19. Anti-Flag (Sigma-Aldrich, catalog number: F1804)
20. Anti-Myc (Sigma-Aldrich, catalog number: C3956)
21. Anti-SE (Agrisera, catalog number: AS09 532A)
22. DMSO (Sigma-Aldrich, catalog number: D4540)

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23. MG132 (Calbiochem, catalog number: 474787)
24. Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) (Sigma-Aldrich, catalog number: S6510)
25. SilverQuest™ Staining Kit (Invitrogen, catalog number: LC6070)
26. Bradford Reagent (Sigma-Aldrich, catalog number: B6916)
27. 2× SDS-PAGE loading buffer (see Recipes)
28. Extraction buffer (see Recipes)
29. Washing buffer (see Recipes)
30. Reaction Buffer (see Recipes)

Equipment

1. -80 °C freezer
2. Pipettes
3. Centrifuge
4. DynaMag™-2 (Invitrogen, model: 12321D)
5. PolyATtract® System 1000 Magnetic Separation Stand (Promega Corporation, model: Z5410)
6. Rugged Rotator (Glas-Col, model: 099A RD4512)
7. Thermomixer R (Eppendorf, model: 022679810)
8. Microplate spectrophotometer (PerkinElmer, model: VICTOR™ X3)
9. Gel imaging system (Bio-Rad, model: Universal Hood II)
10. Vortex mixer (VWR, model: 945300)

Procedure

Note: Please see Figure 1 for a schematic diagram of the steps described below.

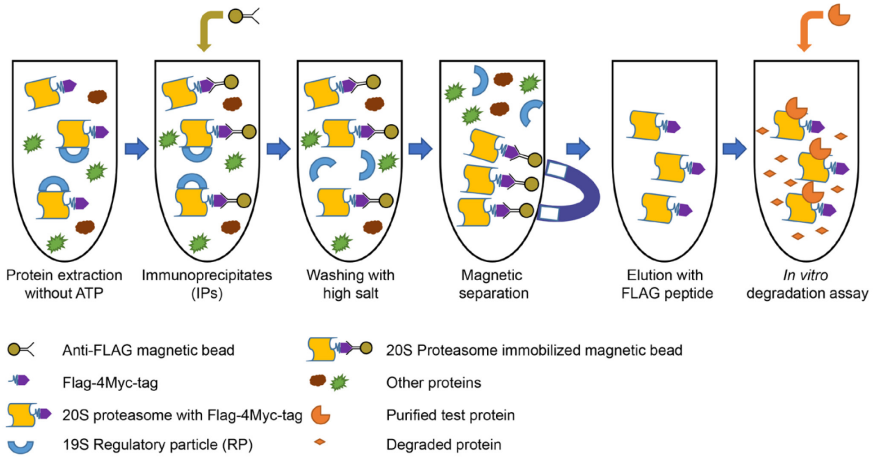

Figure 1. A schematic diagram of procedure

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A. Preparation of 10-day-old P_{PAG1}-*gPAG1-FM* stable transgenic plants (Li *et al*., 2020)

1. Transform a binary vector pBA002a-P_{PAG1}-gPAG1-FM into Col-0 ecotype of *Arabidposis thaliana* by the floral-dip transformation method (Zhang *et al.,* 2006) to generate P_{PAG1}-*gPAG1-FM* stable transgenic plants.
2. Sterilize and place the seeds from the stable transgenic line expressing P_{PAG1}-*gPAG1-FM* on MS medium (Zhang *et al*., 2006) and stratify seeds by keeping them in the dark at 4 °C for 3 days.
3. Germinate seeds and grow the seedlings under a 12 h light-12 h dark cycle at 22 °C for 10 days.
4. Collect 5 g of 10-day-old seedlings, ground to fine powder in liquid nitrogen, and store at -80 °C.

B. Affinity purification of 20S proteasomes (Book *et al*., 2010)

1. Re-suspend the 5 g powder sample in 8 ml of extraction buffer.
2. Keep the sample on ice for 8 min. Keeping the sample cold, homogenize it well with a vortex mixer 2-3 times.
3. Centrifuge the fully dissolved protein extract at 4 °C for 15 min at 21,000 × *g* and then filter the protein extract through one layer of pre-wet Miracloth.
4. After filtration, centrifuge the cleared protein extract again at 4 °C for 15 min at 21,000 × *g*.
5. Collect the supernatant from Step B4 in a pre-cooled 15 ml conical tube and keep it on ice.
6. Prepare Anti-FLAG beads for immunoprecipitation during the centrifugation steps. Add 200 µl bed volume Anti-FLAG® M2 magnetic beads (for 5 g plant tissue) into a new 2 ml Eppendorf tube on ice.
7. Wash the magnetic FLAG-beads with 600 µl of 0.1 M glycine HCl (pH 3.0) to remove un-conjugated antibody.
8. Invert the tube gently and leave the mixture in the tube for 1.5 min.
9. Immediately re-equilibrate the Anti-FLAG® M2 magnetic beads with 1 ml buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0).
10. Remove the equilibration buffer, followed by washing the beads with 1 ml extraction buffer three times using DynaMag™-2.
11. Completely remove the extraction buffer and add 200 µl new extraction buffer into the tube to re-suspend the beads.
12. Add 200 µl equilibrated magnetic beads into sample.
13. Rotate the 15 ml tube at 4 °C for 30 min using a rugged rotator.
14. At the end of Step B13, prepare 3× FLAG peptide solution for elution of Flag-4Myc-tagged PAG1 from the Anti-FLAG® M2 magnetic beads.
15. Add 35 µl 3× FLAG elution buffer stock (4 mg/ml) into 245 µl extraction buffer to make a final concentration 500 ng/µl of 3× FLAG peptide. Mix well and put it on ice.
16. After Step B13 is done, load the 15 ml tube on the PolyATtract® System 1000 Magnetic Separation Stand for a few seconds, then slowly pour out the supernatant. Supernatant can be discarded.

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17. Re-suspend the beads with 6 ml washing buffer and transfer all the beads to a new, clean precooled 15 ml tube.
18. Wash the beads with 6 ml washing buffer three times at 4 °C, each time for 5 min using the rugged rotator.
19. After washing three times, add 2 ml of extraction buffer to a 15 ml tube to re-suspend the beads.
20. Transfer all the beads carefully to a clean 2 ml Eppendorf tube.
21. Load the 2 ml tube into DynaMag™-2, and remove the extraction buffer.
22. Add 250 µl 3× FLAG elution buffer into the 2 ml tube, incubate the tube for 30 min at 4°C with 1,200 rpm shaking using Thermomixer R.
23. Load the 2 ml tube into DynaMag™-2. Transfer the eluates to the 1.5 ml Eppendorf tube and store at -80 °C.

C. Silver staining of purified proteasome
1. Add 20 µl 2× SDS-PAGE loading buffer into 20 µl of the eluted sample and boil at 95 °C for 8 min.
2. Load 20 µl eluted sample onto two 10% SDS-PAGE gels and subject to electrophoresis for 2.5 h at 80 V.
3. Stain one of the gels with SilverQuest™ Staining Kit (Figure 2).

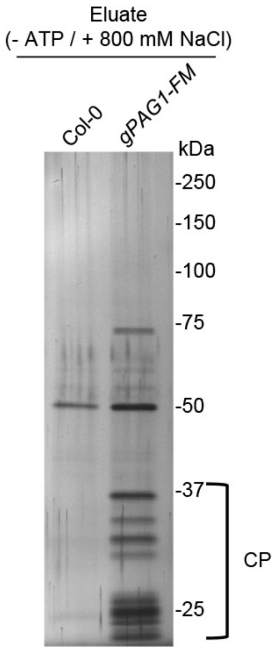

Figure 2. A representative silver-staining image of immunoprecipitated PAG1-FM-containing 20S resolved by SDS-PAGE. The immunoprecipitation was performed using the anti-FLAG® M2 magnetic beads with the protein extracts prepared from P_{PAG1}-*gPAG1-FM* transgenic seedlings (*gPAG1-FM*) or from Col-0 (control), respectively. The bracket indicates subunits of the 20S core proteasome (CP).

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4. Do Western blot analysis for the other gel using anti-Myc or anti-Flag antibodies.
5. Take images of silver staining gel using a gel imaging documentation system.

D. Proteasome activity assay (Yang *et al.*, 2004; Han *et al.*, 2019)
1. Prepare reaction buffer containing 50 μM Suc-LLVY-AMC substrate, which is widely used as a fluorogenic substrate for measuring the chymotrypsin – like activity of the 20S proteasome (Reidlinger *et al*., 1997).
2. Add 10 μl eluted sample into 90 μl reaction Buffer, mix well and add into 96-well Plate.
3. Add 10 μl extraction buffer into 90 μl reaction Buffer as a blank control and repeat each reaction three times.
4. Incubate the reaction mixtures at 37 °C for 20, 40, 60, 80, 100, 120 min.
5. Monitor the fluorescence reading of the released AMC at the indicated times using a Microplate spectrophotometer by fluorescence using 380 nm excitation and 440 nm emission wavelengths.
6. Plot proteasome activity in relative fluorescence units per 1 μl of reaction mixtures, using free AMC as a standard.
   Note: You can also use other representations to display your data, such as relative fluorescence units per 10 μl of eluted sample.

E. *In vitro* 20S proteasome degradation assay (Hsieh *et al.*, 2015)
1. Prepare purified proteins for testing.
2. Estimate the concentration of the purified proteasome and test proteins by the Bradford method (Bradford, 1976). (Method: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/b6916bul.pdf).
3. Prepare *in vitro* 20S proteasome-decay reaction mixtures on ice as follows (Table 1):

Table 1. *In vitro* 20S proteasome degradation reaction system

One reaction	Reaction mixtures for five indicated times
150 nM Purified test protein	According to the concentration
10 nM Purified 20S proteasome	According to the concentration
50 mM Tris-HCl (pH 7.5)	10 μl [1 M Tris-HCl (pH 7.5)]
2% DMSO or 50 μM MG132	4 μl (2.5 mM MG132, dissolved in DMSO)
Add H_2O to final volume 40 μl	Add H_2O to final volume 200 μl

Note: The amount of the purified 20S proteasome and test protein in the reaction mixture is only a reference, the reaction condition needs to be optimized for different proteins.

4. Then distribute the mixtures evenly into five PCR or 1.5 ml tubes and incubate the tubes at 22 °C.
5. Stop the reaction by adding 40 μl 2× SDS-PAGE loading buffer at the indicated times (0, 5, 10, 20, 30 min) followed by Western blot analysis (Figure 3).

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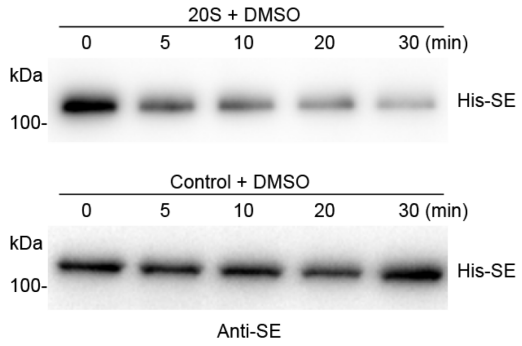

Figure 3. A representative image of *in vitro* 20S proteasome-mediated protein degradation assay. Recombinant 6xHis-SUMO-SE protein was incubated with the PAG1-FM immunoprecipitate from P_{PAG1}-*gPAG1-FM* transgenic plants or control IP from Col-0, respectively. The reaction mixture was stopped at the indicated time intervals. Western blot assay of 6xHis-SE was probed with an anti-SE antibody.

Data analysis

For additional reference images of silver staining and *in vitro* 20S proteasome degradation assay, see Figures 4c and 4d from Li *et al*. (2020), respectively. For additional reference images of the proteasome activity assay, see the Extended Data Figure 7b from Li *et al*. (2020).

Notes

1. In step B, all the buffers and tubes required for purification should be pre-cooled to 4 °C before use.
2. In step B, Miracloth is pre-wet with extraction buffer.
3. In step B, divide the final elution samples into several tubes to avoid freeze-thaw samples in future use, which will effect proteasome activity.
4. In step E, optimize the *in vitro* degradation conditions according to test proteins, including pH, degradation time, degradation temperature and the concentration of the 20S proteasome and test proteins.
5. In step E, prepare and evenly distribute the reaction mixtures quickly and keep on ice. Make sure all time points start the reaction at the same time.

Recipes

1. 2× SDS-PAGE loading buffer
   0.125 mM Tris-HCl, pH 6.8
   20% glycerol
   4% SDS

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0.2 M DTT

0.02% bromophenol blue

2. Extraction buffer

   50 mM Tris-HCl, pH 7.5

   25 mM NaCl

   2 mM $MgCl_2$

   1 mM EDTA

   5% glycerol

   2 mM PMSF (add just before use)

3. Washing buffer

   50 mM Tris-HCl, pH 7.5

   800 mM NaCl

   2 mM $MgCl_2$

   1 mM EDTA

   5% glycerol

   2 mM PMSF (add just before use)

4. Reaction Buffer

   50 mM Tris-HCl, pH 7.5

   25 mM NaCl

   2 mM $MgCl_2$

   1 mM EDTA

   2 mM dithiothreitol (DTT)

   5% glycerol

   50 μM Suc-LLVY-AMC substrate

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Competing interests

The authors declare no competing interests.

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