

10-12-2016

# Purification of 26S Proteasomes and Their Subcomplexes from Plants

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## Recommended Citation

Marshall, Richard S.; Gemperline, David C.; and Vierstra, Richard D., "Purification of 26S Proteasomes and Their Subcomplexes from Plants" (2016). *Biology Faculty Publications & Presentations*. 124.  
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# Chapter 24

## Purification of 26S Proteasomes and Their Sub-complexes From Plants

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### Abstract

The 26S proteasome is a highly dynamic, multi-subunit, ATP-dependent protease that plays a central role in cellular housekeeping and many aspects of plant growth and development by degrading aberrant polypeptides and key cellular regulators that are first modified by ubiquitin. Although the 26S proteasome was originally enriched from plants over thirty years ago, only recently have significant advances been made in our ability to isolate and study the plant particle. Here, we describe two robust methods for purifying the 26S proteasome and its sub-complexes from *Arabidopsis thaliana*; one that involves conventional chromatography techniques to isolate the complex from wild-type plants, and another that employs the genetic replacement of individual subunits with epitope-tagged variants combined with affinity purification. In addition to these purification protocols, we describe methods commonly used to analyze the activity and composition of the complex.

**Key words** Affinity purification; *Arabidopsis*; Core protease; Proteasome; Proteolysis; Regulatory particle; Ubiquitin.

## 1 Introduction

Selective proteolysis in plants plays a critical role in both regulating growth and development, and maintaining cellular homeostasis [1-4]. One of the principle pathways for protein degradation in plants and other eukaryotes is the ubiquitin-26S proteasome system (UPS), which involves the covalent attachment of polyubiquitin chains to target proteins followed by their recognition and degradation by the 26S proteasome, an exquisitely designed proteolytic machine [2, 5, 6]. The UPS is highly conserved across all eukaryotes; it was first elucidated by elegant work in rabbit reticulocyte lysates [7-11], and was subsequently identified in other animals, yeast and higher plants [12-17]. Ubiquitin conjugation to target proteins is accomplished through a highly polymorphic, ATP-dependent cascade involving the sequential action of three enzyme classes, termed the E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases [2, 3, 18]. Selectivity in ubiquitylation is driven by the E3 family, which has dramatically expanded during plant evolution to include well over a thousand variants in *Arabidopsis thaliana* and other plant species [19, 20]. Through this myriad of E3s combined with the 26S proteasome, plants precisely control the levels of many key intracellular regulators that impact most, if not all, aspects of plant biology [2, 21].

The 26S proteasome is a 2.5 MDa particle located in the cytosol and nucleus of eukaryotic cells. It is composed of two functionally distinct sub-complexes; the 20S core protease (CP) that houses the proteolytic active sites, and the 19S regulatory particle (RP) that recognizes appropriate substrates (Figures 1A and 1B; [5, 6, 22-24]). The CP has a barrel shape generated by four stacked hetero-heptameric rings, which contain seven  $\alpha$ -subunits or seven  $\beta$ -subunits (termed PAA-PAG and PBA-PBG, respectively, in *Arabidopsis*) in an  $\alpha_{1-7}/\beta_{1-7}/\beta_{1-7}/\alpha_{1-7}$  configuration. Upon assembly, a central chamber is formed at the  $\beta$ -ring interface that houses six peptidase catalytic sites provided by the  $\beta 1$  (PBA),  $\beta 2$  (PBB), and  $\beta 5$  (PBE) subunits [25, 26]. The active sites involve a catalytic triad, one residue of which is an N-terminal threonine that becomes exposed during CP assembly. Collectively these peptidases can cleave a broad range of protein sequences [25, 27]. The  $\alpha$ -rings create two antechambers with narrow opposing axial pores that are gated by extensions at the N-terminus of several subunits [28, 29]. Through this distinctive architecture, the CP acts as a self-compartmentalized protease that will only degrade polypeptides that are deliberately recognized, unfolded, and imported into the  $\beta$ -ring chamber.

The CP is capped at one or both ends by the RP, which sits on top of the axial pores. The RP provides activities for recognition of ubiquitylated proteins, substrate unfolding and import, and release of the ubiquitin moieties before substrate degradation. Its binding to the CP is stabilized by ATP, which is thus a necessary ingredient for purifying intact 26S proteasomes. The RP itself consists of two sub-complexes; the base, which contains a hexameric ring of AAA-ATPases (RPT1-6) plus two non-ATPase subunits, RPN1 and RPN2; and the lid, which is composed of an additional 11 non-ATPase

subunits, RPN3, RPN5-13 and DSS1/SEM1 (Figures 1B and 1C; [5, 6, 30-32]. This lid/base demarcation was first revealed by the absence of lid subunits in proteasomes isolated from a *Arpn10* yeast deletion strain, and it was hence thought that RPN10 helps enforce binding of the lid to the base [33]. However, more recent structural studies have demonstrated that RPN10 has a more indirect stabilizing role via its interaction with RPN9 [22]. The ring of RPT subunits in the base promotes substrate unfolding through ATP hydrolysis, and gates the  $\alpha$ -ring axial pores through repositioning of the CP  $\alpha$ -subunit extensions [34-36]. The N-terminal regions from proximal RPT pairs intertwine to create three spokes onto which most RPN subunits are scaffolded (Figure 1C; [37]). The RPN6 subunit acts as a molecular clamp to tether the RP onto the CP [38], while RPN11 is a metalloprotease that uses a zinc-coordinated active site to release the ubiquitin moieties isopeptide-linked to substrates [39, 40]. Through RPN11 and other loosely associated deubiquitylating enzymes such as UBP6/USP14 [41, 42], bound ubiquitins are actively recycled.

Substrate selection by the 26S proteasome is dictated by several ubiquitin receptors intrinsic to the RP lid, including RPN10, RPN13, and DSS1/SEM1 [5, 43-47], and possibly RPN1 in the base [48]. RPN10 binds ubiquitin via defined ubiquitin-interacting motifs (UIMs), of which yeast, human and *Arabidopsis* RPN10 contain 1, 2 and 3 in tandem, respectively [5, 43-45, 49]. By contrast, RPN13 binds ubiquitin via a pleckstrin-like receptor for ubiquitin (PRU) domain, which is structurally distinct from UIMs but binds to the same hydrophobic patch on ubiquitin [50, 51]. More recently, DSS1/SEM1 was also found to be a proteasomal ubiquitin receptor [47]. It had previously resisted identification due to both its small size, which prevented visualization by standard protein stains following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and its paucity of lysine and arginine residues, which complicated detection by conventional mass spectrometric methods. Only with the use of top-down mass spectrometry of 26S proteasome complexes was DSS1/SEM1 first detected in intact 26S proteasomes from *Arabidopsis* [32].

In addition to these core ubiquitin receptors, there are several extra-proteasomal ubiquitin-binding proteins that shuttle ubiquitylated cargo to the RP. They work by virtue of ubiquitin-associated (UBA) domains that bind ubiquitin, combined with a ubiquitin-like (UBL) domain that interacts with the intrinsic ubiquitin receptors such as RPN10. Important shuttle factors in plants include RAD23, DSK2, and DDI1 [5, 44, 45, 52], though many other ubiquitin-binding proteins are known in other species [53]. Numerous other factors also associate sub-stoichiometrically with the mature CP and RP sub-complexes, including deubiquitylating enzymes, several E3 ligases and protein kinases, and a collection of protein folding chaperones [31, 54-56].

Not surprisingly given its intricate architecture, construction of the 26S proteasome requires a large collection of assembly factors that work in synchrony. Included are chaperones required for the

correctly ordered assembly of the  $\alpha$ - and  $\beta$ -rings of the CP and the RPT ring of the RP, which in yeast involve the Pba1/2 and Pba3/4 heterodimers for the CP [57-59], and Nas2, Nas6, Hsm3 and Rpn14 for the RP [59-62]. Additional chaperones then mediate assembly of the final particle. UMP1 is required to connect the two  $\alpha/\beta$  half-barrels to generate the complete CP. Once its job is finished UMP1 is degraded, thus becoming the first proteolytic substrate of the fully assembled CP [63]. ECM29 stabilizes the association of assembled CP and RP and provides a final quality control checkpoint for mature 26S proteasomes [56, 64]. Lastly, in some situations, the RP is replaced entirely by alternate capping particles such as PA200 (also known as Blm10) or CDC48 [31, 65, 66]. The functions of these caps are not yet clear, but recent proposals for PA200 have it participating in 26S proteasome assembly, helping shuttle proteasomes into the nucleus, and/or generating a ubiquitin-independent proteasome containing CP and PA200 only [67-69].

Even before the realization that the 26S proteasome is a protease, sub-particles of the complexes were described. The first reports of proteasomes used avian erythroblast preparations enriched by differential ultracentrifugation followed by fractionation through a sucrose gradient [70]. These 20S fractions isolated in the absence of added ATP were found to inhibit mRNA translation in a cell-free system, leading to early proposals that the identified complex repressed gene expression through a cryptic ribonuclease activity. This led to the particle initially being named the 'prosome' [70, 71]. Subsequent analyses of these preparations by SDS-PAGE and electron microscopy revealed the signature ladder of  $\alpha$ - and  $\beta$ -subunits at 20-35 kDa, as well as their barrel-like architecture (Figures 2A and 2B; [70-72]). Purification of the 20S fraction from HeLa cells followed by SDS-PAGE also gave rise to this stereotypical protein banding pattern and shape [70], and this was followed shortly thereafter by the first description of plant prosomes, purified from tobacco leaf extracts using similar sedimentation protocols in ATP-free buffers [71]. In these later cases, the purified preparations had strong peptidase activity but little to no RNase activity, thus leading to the conclusion that the CP is actually a protease. Once its true function in protein turnover was confirmed, the moniker for the particle was changed to 'proteasome' [73].

Subsequently, the 20S particle was purified from other plant tissues, including dry pea seeds, potato tubers, mung bean seedlings, and leaves from both spinach and wheat [74-77]. These purifications were typically performed using sequential anion exchange and size-exclusion chromatography steps in the absence of ATP, hence only the CP was isolated. Their remarkable similarity in protein composition and structure, as observed by SDS-PAGE and electron microscopy, respectively, coupled with the fact that several of the plant subunits cross-reacted with antibodies against their yeast, human, rat and *Xenopus* counterparts, strongly implied that the CP was conserved and widely distributed among eukaryotes [74].

The complete 26S proteasome (i.e. the CP capped at one or both ends by the RP) was subsequently discovered by the purification of ubiquitin conjugate-degrading activity from rabbit reticulocytes [78]. While it had been well established that major catabolic processes in animal cells involved the ATP-dependent proteolysis of selective substrates [7], the enzyme(s) responsible for this activity had yet to be identified. Taking advantage of the new ability to synthesize ubiquitylated substrates such as <sup>125</sup>I-labelled ubiquitin-lysozyme conjugates [79], a protocol was developed to purify the responsible ATP-dependent protease. Through a series of anion exchange and size exclusion chromatography steps followed by glycerol gradient sedimentation, all of which were performed in ATP-containing buffers, the responsible activity was isolated [78, 80]. The active enzyme turned out to be the 20S proteasome (i.e. the CP) along with a number of additional polypeptides which together formed a 26S particle, thus providing the first direct link between ubiquitylation and a protease [80-82]. SDS-PAGE analysis of these preparations identified a host of new polypeptides in the 35-100 kDa range in addition to the known CP subunits, which were later shown to comprise a second stable complex, the RP. Shortly thereafter, the RP was demonstrated to have ATPase activities attributable to the RPT subunits, which help in substrate unfolding and maintaining CP-RP association [83]. Electron microscopic images of the full 26S particle then revealed its diagnostic quaternary structure in which the CP is capped by one or two RPs which sit over the axial pores for substrate entry (Figure 2C; [84, 85]).

The existence of a similar 26S proteasome in plants was initially implied by the detection of an ATP-dependent activity in oat and wheat germ extracts capable of degrading ubiquitylated proteins [86, 87]. This was followed some years later by the first isolation of a complete plant 26S proteasome holocomplex from spinach leaves [88]. As with the mammalian forms, purification was achieved by anion exchange and size exclusion chromatography, followed by glycerol gradient centrifugation, all in the presence of ATP to stabilize the CP-RP association. These spinach preparations were, like their rabbit reticulocyte counterparts, able to rapidly degrade ubiquitylated substrates in an ATP-dependent manner, and further analysis by native-PAGE, SDS-PAGE and electron microscopy revealed the complete subunit composition and “caterpillar-like” structure of the plant particle (Figure 2D; [88]). Similar purifications were successful using rice suspension culture cells and garlic cloves [89, 90], which were accompanied by the first demonstrations that proteasome inhibitors designed for their mammalian counterparts were effective with the plant particles, suggesting very similar enzymatic mechanisms [75, 91].

Despite its prevalence as a genetic model, purification of the 26S proteasome from the flowering plant *Arabidopsis thaliana* was not reported until several years after other plant species [92]. First protocols involved differential PEG precipitation followed by anion exchange and size exclusion chromatography, with the latter exploiting the large size of the holoprotease. More recently, an improved one-step affinity method was developed [31], based on the strategies that had been

successfully employed in yeast [93]. Here, epitope-tagged proteasomes were generated by genetically replacing individual CP or RP subunits with variants bearing N- or C-terminal tags; these tagged particles could then be purified with appropriate affinity matrices. This approach enables rapid and robust purification of the whole 26S proteasome complex when performed in the presence of ATP, or enables purification of the CP or RP sub-particles alone when performed in the absence of ATP, combined with high salt washes [31]. Here, we describe both the conventional chromatography based method as well as the affinity method for purifying 26S proteasomes from *Arabidopsis* seedlings. We also describe several methods for characterizing the purified complexes, including activity assays and both native- and SDS-PAGE analyses.

## 2 Materials

### 2.1 Seed Sterilization, Plant Growth and Tissue Harvesting.

1. Sterile, double-distilled H<sub>2</sub>O.
2. Bleach solution: 10% (v/v) bleach and 0.02% (v/v) Triton X-100.
3. Ethanol solution: 70% (v/v) ethanol.
4. Liquid GM growth medium: 3.2 g/L Gamborg's B5 basal medium with minimal organics, 1% (w/v) sucrose, and 0.05% (w/v) 2-(N-morpholino)ethane sulphonic acid (MES), with the final solution re-adjusted to pH 5.7.
5. 250 mL wide-mouth Erlenmeyer flasks (*see Note 1*).
6. Rotating platform shaker with compatible Erlenmeyer flask mounts.
7. Appropriate lighting apparatus.
8. Preparative centrifuge with fixed angle rotor capable of processing 50 mL sample at 60 x g such as an Allegra<sup>®</sup> X-15R (Beckman-Coulter) with rotor inserts for 50 mL screw top tubes.
9. Additional equipment: aluminum foil, autoclave, balance, 15 mL and 50 mL screw top tubes, liquid nitrogen, paper towels, refrigerator, sterile laminar flow hood, and sterile pipettes.

### 2.2 Conventional Proteasome Purification

1. Protein extraction buffer (Buffer A): 50 mM potassium phosphate, pH 6.0, 2 mM MgCl<sub>2</sub>, 5% (v/v) glycerol, and 20 mM adenosine 5'-triphosphate (ATP; *see Note 2*), supplemented just before use with 5 mM β-mercaptoethanol, 5% (w/v) polyvinylpyrrolidone, 0.6% (w/v) sodium metabisulphite, 2 mM phenylmethylsulphonyl fluoride, and 0.8% (v/v) plant protease inhibitor cocktail, with the final solution re-adjusted to pH 6.0.
2. Cheesecloth and Miracloth.
3. 30 mL high-speed polycarbonate centrifuge tubes, such as Nalgene<sup>™</sup> Oak Ridge (Thermo Scientific).
4. 40% polyethylene glycol (PEG) 8000.

5. 1 M KCl.
6. 6 mL anion exchange chromatography column, such as Uno<sup>®</sup> Q6 (Bio-Rad).
7. 24 mL size exclusion chromatography column, such as Superose<sup>™</sup> HR6 10/30 GL (GE Healthcare Lifesciences).
8. Fast performance liquid chromatography (FPLC) system and fraction collector, such as AKTA<sup>™</sup> (GE Healthcare Lifesciences).
9. Preparative centrifuge with fixed angle rotor capable of processing 30 mL sample at 30,000 x g such as an Avanti<sup>™</sup> J-25 with a JA-25.50 rotor (Beckman-Coulter).
10. Additional equipment: 100 and 500 mL beakers, clamp stands or other column supports, 1.5 mL microfuge tubes, glass rods, ice buckets and ice, liquid nitrogen, pestle and mortars, plastic spoons/spatulas, plastic funnels, silicone tubing, sterile pipettes, and stir plate and stir bars.

### **2.3 Affinity Proteasome Purification**

1. Protein extraction buffer (Buffer B): 50 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 10% (v/v) glycerol, 10 mM MgCl<sub>2</sub>, and 20 mM ATP (*see Notes 2 and 3*), supplemented just before use with 2 μM chymostatin, 2 mM phenylmethylsulphonyl fluoride, 5 mM dithiothreitol and (optionally) 0.6% (w/v) sodium metabisulphite (*see Notes 4 and 5*), with the final solution re-adjusted to pH 7.5 with KOH.
2. Cheesecloth and Miracloth.
3. 30 mL high-speed polycarbonate centrifuge tubes, such as Nalgene<sup>™</sup> Oak Ridge (Thermo Scientific)
4. Sigma anti-FLAG<sup>®</sup> M2 affinity gel (*see Note 6*).
5. 12 mL chromatography columns and end caps, such as PolyPrep<sup>®</sup> (Bio-Rad).
6. 10X FLAG peptide stock (DYKDDDDK; 5 μg/mL) in 10 mM Tris-HCl, pH 7.5 and 150 mM NaCl (*see Note 7*).
7. 1X FLAG elution buffer: 10X FLAG peptide diluted to a concentration of 1X (500 ng/mL) in Buffer B without inhibitors (*see Note 8*).
8. Preparative centrifuge with fixed angle rotor capable of processing 30 mL sample at 30,000 x g such as an Avanti<sup>™</sup> J-25 with a JA-25.50 rotor (Beckman-Coulter).
9. Additional equipment: 100 mL beakers, , clamp stands or other column supports, drip tray, 1.5 mL microfuge tubes, 15 mL and 50 mL screw top tubes, glass rods, ice buckets and ice, liquid nitrogen, microcentrifuge, pestle and mortars, plastic spoons/spatulas, plastic funnels, silicone tubing, sterile pipettes, stir plate and stir bars, and syringe.

### **2.4 Proteasome Activity Assays**



1. Assay buffer: 50 mM Tris-HCl, pH 7.0 and 2 mM MgCl<sub>2</sub>, supplemented just before use with 1 mM ATP and 2 mM β-mercaptoethanol, with the final solution re-adjusted to pH 7.0 with HCl. The succinyl-leucyl-leucyl-valyl-tyrosyl-7-amido-4-methylcoumarin (succinyl-LLVY-amc) substrate is then added to a final concentration of 100 μM.
2. Quenching buffer: 160 mM sodium acetate (pH 4.3).
3. 80 mM N-(benzyloxycarbonyl)leuciny-leuciny-leucinal (Selleckchem).
4. Fluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm, such as a TKO-100 (Hofer Scientific Instruments).
5. Additional equipment: 2 mL microfuge tubes, 37°C water bath, and sterile pipettes.

## **2.5 SDS-PAGE**

1. 40% (w/v) acrylamide/bis-acrylamide solution.
2. 4X SDS-PAGE resolving buffer: 1.5 M Tris-HCl, pH 8.8 and 0.5% (w/v) SDS.
3. 4X SDS-PAGE stacking buffer: 0.5 M Tris-HCl, pH 6.8 and 0.5% (w/v) SDS.
4. 7% (w/v) ammonium persulphate (APS).
5. N,N,N',N'-tetramethylethylenediamine (TEMED).
6. 100% 2-Propanol (isopropanol).
7. 5X SDS-PAGE sample buffer: 60 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromophenol blue.
8. 10X SDS-PAGE running buffer: 250 mM Tris-HCl, pH 8.3, 1.92 M glycine, and 1% (w/v) SDS.
9. Protein ladder, such as PageRuler™ pre-stained protein ladder (Thermo Scientific).
10. 25 μL gel loading syringe, such as a Gastight® syringe (Hamilton).
11. Additional equipment: gel clamps, gel combs, gel plates, gel running tank, power pack, spacers, and sterile pipettes.

## **2.6 Native Gel Electrophoresis**

1. 40% (w/v) acrylamide.
2. 2% (w/v) bis-acrylamide.
3. 50% (w/v) sucrose.
4. 5X TBE buffer: 445 mM Tris-HCl, pH 8.4, 445 mM boric acid, and 10 mM EDTA, obtained by a 1 in 2 dilution of a 10X stock.
5. Rhinohide™ Polyacrylamide Gel Strengthener (Thermo Scientific).
6. 1 M MgCl<sub>2</sub>.
7. 0.5 M ATP.
8. 7% (w/v) APS.
9. TEMED.

10. 100% 2-Propanol (isopropanol).
11. 0.005% (w/v) xylene cyanol.
12. 1X native-PAGE running buffer: 1X TBE buffer and 1 mM ATP.
13. 25  $\mu$ L gel loading syringe, such as a Gastight<sup>®</sup> syringe (Hamilton).
14. Additional equipment: gel clamps, gel combs, gel plates, gel running tank, power pack, spacers, and sterile pipettes.

## 2.7 Silver Staining

1. Fixing solution: 50% (v/v) ethanol, 12% (v/v) glacial acetic acid, and 0.05% (v/v) formaldehyde (*see Note 9*).
2. Washing solution: 50% (v/v) ethanol.
3. Sensitizing solution: 0.02% (w/v) sodium thiosulfate pentahydrate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ; *see Note 10*).
4. Staining solution: 0.2% (w/v) silver nitrate and 0.075% (v/v) formaldehyde (*see Note 9*).
5. Developing solution: 6% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde, and 0.0004% (w/v) sodium thiosulfate pentahydrate (*see Note 11*).
6. Stop solution: 50% (v/v) ethanol and 12% (v/v) glacial acetic acid.
7. Additional equipment: 500 mL beakers, nitrile gloves, protein-free glass container with a lid, razor blade, side-to-side shaker, sheet of white paper, sterile pipettes, and vacuum-powered aspirator.

## 3 Methods

### 3.1 Generation of Plant Material

To purify the 26S proteasome from *Arabidopsis* by either of the two methods described here, we typically grow seedlings in liquid culture under continuous light for 7 to 10 days after sowing. While the conventional purification can be performed simply using wild-type backgrounds, the affinity purification protocol requires the prior generation of *Arabidopsis* lines stably expressing an appropriate affinity-tagged proteasome subunit. We typically prefer to express the tagged subunit from its native promoter in a null mutant background, to ensure expression levels that are as close as possible to wild-type. However, it is likely that either constitutive expression from strong promoters such as cauliflower mosaic virus 35S, or tissue-specific expression from other appropriate promoters, can also be employed [94]. However, given that most, if not all, 26S proteasome subunits are essential, it is likely that tissue-specific promoters can only be used in conjunction with wild-type plants. Furthermore, when using genetic replacement, most tagged subunits must be first introduced by transformation of plants heterozygous for the corresponding subunit, followed by selfing of the double heterozygous transgenic plants to generate the double homozygous lines. We currently have

lines expressing *PAG1-FLAG* in the *pag1-1* background for CP-based purification [31], or expressing *FLAG-RPT4a* or *FLAG-RPT4b* in the *rpt4a-1* or *rpt4b-2* backgrounds, respectively, for purification via the RP base (D.C.G. and R.D.V., unpublished). We are now actively generating further lines based on other RP subunits to permit purification of the lid and various RP sub-complexes.

The long lead-time necessary to create these purification lines, followed by the bulking up of the double homozygous seeds, is a major limitation of the affinity approach compared to the conventional method. However, the advent of CRISPR genome editing technology [95, 96] may simplify creation of such lines. Fortunately, the ease with which tagged proteasomes can be isolated, combined with the greater reproducibility of the preparations, makes this approach worth the initial effort in generating the required germplasm. The affinity purification protocol requires only 5 to 10 g of fresh weight tissue, while the conventional purification protocol typically uses between 150 and 200 g of tissue, thus making the affinity approach also more appealing when tissue is limiting.

1. Where desired, generate transgenic *Arabidopsis* lines expressing an affinity epitope-tagged proteasome subunit by cloning the appropriate gene and introducing it into *Arabidopsis* by the *Agrobacterium tumefaciens*-mediated floral dip method [97]. Generation of the *PAG1-FLAG* line was previously described in detail [31].
2. Once plants of the required genotype are obtained, seeds should be bulked by growing at least one tray of 36 individual plants under standard growth conditions (*see Note 12*) and harvesting (*see Note 13*).
3. To sterilize *Arabidopsis* seeds, first allow them to hydrate for 30 minutes in at least 10 volumes of sterile distilled H<sub>2</sub>O, with gentle shaking (*see Note 14*).
4. Collect the seeds by centrifugation (60 x g for 1 minute), pour off H<sub>2</sub>O, and incubate seeds for 5 minutes in at least 10 volumes of bleach solution with shaking (*see Note 15*).
5. Collect the seeds by centrifugation (60 x g for 1 minute), pour off bleach solution, and incubate seeds for 5 minutes in at least 10 volumes of ethanol solution with shaking (*see Note 15*).
6. Collect the seeds by centrifugation (60 x g for 1 minute), pour off ethanol solution, and wash seeds at least 8 times in 10 volumes of sterile distilled H<sub>2</sub>O.
7. Incubate seeds in sterile distilled H<sub>2</sub>O (in a volume equal to 1 mL per liquid culture to facilitate even seed transfer into Erlenmeyer flasks) in the dark at 4° C for 4 to 5 days to stratify (*see Note 16*).
8. Autoclave 50 mL of liquid GM medium in a 250 mL Erlenmeyer flask capped with two layers of aluminum foil (*see Note 17*), and allow to cool to room temperature in a laminar flow hood.

9. Aliquot seeds (1 mL per liquid culture from subheading 3.1.7) by gently lifting the foil cover from the flask and transferring seeds using a sterile 1 mL pipette tip (*see Note 18*). Close the foil cover.
10. Grow the liquid cultures at 21-23° C under continuous light (75-100  $\mu\text{mol}/\text{m}^2/\text{sec}$ ) for 10 days on a suitable platform with gentle shaking (90 rpm; *see Note 19*).
11. Where desired, treat seedlings with appropriate chemical compounds or growth conditions immediately prior to harvesting, to assess possible effects on 26S proteasome composition (*see Note 20*).
12. After 10 days of growth, harvest tissue by removing seedlings from the flask and gently patting dry with paper towels to remove excess liquid (*see Note 21*). Weigh the tissue, wrap in aluminum foil, and immediately freeze in liquid nitrogen. Tissue can be stored indefinitely at -80° C.

### **3.2 Conventional Purification of the 26S Proteasome from Arabidopsis Seedlings.**

While the affinity purification protocol described in the next section works well for purifying intact 26S proteasomes or individual CP and RP sub-complexes, its reliance on the availability of transgenic lines expressing epitope-tagged proteasome subunits can limit its use in cases where the required lines are unavailable, or in plant species for which the generation of such lines is challenging. In such cases, the conventional proteasome purification protocol may be useful, as it can be accomplished with wild-type plants. Although this protocol requires much greater quantities of plant tissue (typically 150 to 200 g), one advantage is the large amounts of proteasomes that can be obtained (about 200  $\mu\text{g}$ ), even if the purification efficiency is lower ( $\sim 1 \mu\text{g}/\text{g}$  [92]). Compared to the affinity protocol, the conventional protocol requires considerably more time, is less efficient, and is not as amenable to processing multiple samples at once. We therefore recommend using the affinity purification method where possible. A flow chart outlining the various steps in this procedure is shown in Figure 3A, while SDS-PAGE analysis of fractions collected from the final size exclusion step of this protocol is shown in Figure 4B. The identity of each subunit as determined by mass spectrometric (MS) analysis of the polypeptides separated by SDS-PAGE is provided for reference in Figure 4C.

1. Prepare 500 mL fresh Buffer A as described above, omitting PMSF until just before use.
2. Pre-chill a pestle and mortar with liquid nitrogen, add the frozen tissue sample, and grind to a very fine powder (*see Note 22*), adding more liquid nitrogen as needed to prevent thawing (*see Note 23*).
3. Transfer ground tissue to a 500 mL beaker on ice using a plastic spoon pre-chilled in liquid nitrogen (*see Note 24*).

4. Add PMSF to Buffer A, mix well, and add the required volume to the frozen tissue (1.25 mL per gram of fresh weight). Stir gently with a glass rod to ensure even distribution of buffer throughout the tissue, and leave to thaw on ice. Once the mixture has thawed completely, enable protein extraction by incubating on ice for an additional 10 minutes with occasional stirring.
5. Filter the protein extract through four layers of cheesecloth and two layers of Miracloth into pre-cooled 30 mL high-speed polycarbonate centrifuge tubes on ice. Squeeze out any excess liquid into the centrifuge tubes, until the particulate material left behind is mostly dry (*see Note 25*).
6. Clarify the protein extract by centrifugation at 30000 x g for 15 minutes at 4° C in a pre-cooled centrifuge and rotor.
7. Transfer the supernatant to a new 500 mL beaker. Add PEG 8000 to a final concentration of 2% (w/v), from a 40% stock containing 200 mM ATP. Stir the mixture for 30 minutes at 4° C, and re-clarify by centrifugation at 30000 x g for 45 minutes at 4°C (*see Note 26*).
8. Transfer the supernatant to a new 500 mL beaker. Add PEG 8000 to a final concentration of 10% (w/v), from a 40% stock containing 40 mM ATP. Stir the mixture for 30 minutes at 4° C, and collect the precipitate by centrifugation at 12000 x g for 15 minutes at 4° C (*see Note 26*).
9. Re-suspend the pellet in 1 mL Buffer A and clarify by centrifugation at 30000 x g for 15 minutes at 4° C.
10. Apply supernatant to a 6 mL anion exchange chromatography column, pre-equilibrated in Buffer A according to the manufacturer's instructions (*see Note 27*) and connected to an appropriate FPLC system.
11. Elute using a 240 mL gradient of 0 to 1 M KCl in Buffer A at a flow rate of 2 mL/min, collecting fractions of 3 mL. The 26S proteasome will typically elute between 260 and 280 mM KCl. Fractions within this range can be tested for proteasome activity to confirm elution and identify the peak fractions (*see Subheading 3.4.1*).
12. Pool all fractions containing reasonable 26S proteasome activity into a 100 mL beaker. Add PEG 8000 to a final concentration of 10% (w/v), from a 40% stock containing 40 mM ATP. Stir the mixture for 30 minutes at 4° C, and collect the precipitate by centrifugation at 12000 x g for 15 minutes at 4° C.
13. Re-suspend the pellet in 500 µL Buffer A adjusted to 20% (v/v) glycerol and clarify by centrifugation at 30000 x g for 15 minutes at 4° C.
14. Apply supernatant to a size exclusion chromatography column, pre-equilibrated in Buffer A according to the manufacturer's instructions (*see Note 28*) and connected to an appropriate FPLC system.
15. Elute in Buffer A at a flow rate of 0.1 mL/min, collecting fractions of 500 µL. The 26S proteasome will typically elute between fractions 16 and 22. Fractions within this range can

be tested for proteasome activity (*see Subheading 3.4.1*) to confirm elution. Fractions containing reasonable 26S proteasome activity should be pooled and immediately frozen in liquid nitrogen. They can then be stored indefinitely at -80° C.

16. Assess the different fractions for purity by SDS and/or native-PAGE (*see Subheadings 3.4.2 and 3.4.3, respectively*) combined with silver staining for total protein (*see Subheading 3.4.4*), or by immunoblot analysis with various 26S proteasome subunit antibodies. Fortunately, antibodies against several *Arabidopsis* subunits are now commercially available, and these can likely also be used with other related plant species.

### **3.3 Affinity Purification of the 26S proteasome from *Arabidopsis* Seedlings.**

The *Arabidopsis* 26S proteasome exists *in planta* as a diverse array of complexes containing multiple subunit isoforms and interacting proteins [31, 92, 98]. To facilitate biochemical analysis of the plant particle, we developed a rapid and robust affinity purification protocol that enables isolation of intact 26S proteasomes, and the individual CP and RP sub-complexes, by genetically replacing individual subunits with FLAG-tagged versions [31]. Such a strategy was based on a similar approach used successfully with yeast, where the proteasome subunits Pre1, Rpt1 and Rpn11 were appended with either FLAG or Protein A tags to permit effective affinity enrichment [93]. Using the recently described structures of the 26S proteasome [6, 22, 23], we identified subunits in the CP (PAG1) and RP (RPT4) which had solvent exposed N- or C-termini that were potentially appropriate for appending the epitope tag (Figures 1B and 1C).

As mentioned above, the affinity method has considerable advantages compared to previous conventional chromatographic approaches [92] as it is both faster and more reliable, produces higher yields per gram of tissue (~6 µg/g), and allows purification of the CP and RP separately by omitting ATP from the buffers and/or performing a high salt wash step prior to elution. Additionally, it also avoids the harsh buffer conditions necessary for conventional purification, which has allowed the identification of less tightly bound core and accessory components, such as various CP and RP assembly chaperones, the ubiquitin receptors RPN13 and DSS1/SEM1, and the alternate capping particle PA200 [31, 32]. This milder more rapid technique also prevents breakdown of some subunits, in particular RPN10, which is sensitive to post-homogenization proteolysis [92]. One caveat is that the epitope tag, given its exposed position and flexible structure, might be sensitive to proteolytic cleavage following tissue homogenization. For the PAG1-FLAG protocol, chymostatin was found to effectively block the interfering protease in *Arabidopsis* [31]. Whether this inhibitor is effective in other plants remains to be tested. A flow chart outlining the various steps in this affinity procedure is shown in Figure 3B, while an example of such preparations analyzed by SDS-PAGE followed by immunoblotting with antibodies against several proteasome subunits, are shown in Figures 5A and

5B, respectively. Keep in mind that ATP should be included in all purification steps if the final desired outcome is the fully assembled 26S particle.

1. Prepare 100 mL fresh Buffer B as described above, omitting PMSF until just before use (*see Note 29*).
2. Pre-chill a pestle and mortar with liquid nitrogen, add the frozen tissue sample, and grind to a very fine powder (*see Note 22*), adding more liquid nitrogen as needed to prevent the sample from thawing (*see Note 23*). If multiple samples are to be processed, tissue should be kept frozen at this stage until the other samples are completed.
3. Transfer ground tissue to a 100 mL beaker on ice using a plastic spoon pre-chilled in liquid nitrogen (*see Note 24*).
4. Add PMSF to Buffer B, mix well, and add the required volume to the frozen tissue (1.25 mL per gram of fresh weight). Stir gently with a glass rod to ensure even distribution of buffer throughout the tissue, and leave to thaw on ice. Once the mixture has thawed completely, enable protein extraction by incubating on ice for an additional 10 minutes with occasional stirring.
5. Filter the protein extract through two layers of Miracloth into a pre-cooled 30 mL high-speed polycarbonate centrifuge tube on ice. Squeeze out any excess liquid into the centrifuge tube, until the particulate material left behind is mostly dry (*see Note 25*).
6. Clarify the protein extract by centrifugation at 30000 x g for 30 minutes at 4° C in a pre-cooled centrifuge and rotor. Immediately pour the supernatant into a pre-cooled 15 mL screw top tube on ice, being careful not to disturb the pellet. Take a 50 µL sample (diluted 1 in 10 in Buffer B) for subsequent PAGE analysis if needed. Keep the clarified protein extract on ice, and proceed immediately to the affinity purification step.
7. Transfer 100 µL of the Sigma anti-FLAG<sup>®</sup> M2 resin (50% slurry) into a 1.5 mL microfuge tube (giving a final bead volume of 50 µL). If more than one sample is being processed, this volume should be scaled up accordingly.
8. Collect the beads at 8000 x g for 1 minute at 4° C in a micro-centrifuge, remove the excess liquid, and wash the beads by re-suspending them in 1 mL of Buffer B (*see Note 30*). Repeat this washing three more times, and then re-suspend the beads in approximately 250 µL of Buffer B, again scaling up this volume if required for additional samples (*see Note 31*).
9. Prepare 1X FLAG elution buffer by diluting the 10X stock with Buffer B, making enough for 250 µL per purification (*see Notes 8 and 31*).
10. Setup the 12 mL chromatography column in an appropriate stand at 4° C, and pipette 250 µL of pre-washed anti-FLAG<sup>®</sup> M2 resin into the column. Allow the buffer to drain into a drip tray.

11. Apply the clarified protein extract onto the column and collect the flow through in the same 15 mL screw top tube (*see Note 32*). Once all the extract has flowed through, apply it again to the column two more times. After the third flow-through, take a 50  $\mu$ L sample (diluted 1 in 10 in Buffer B) for subsequent PAGE analysis if needed.
12. Wash the column three times with 40 volumes (i.e. 2 mL) of Buffer B. After the third wash, perform a final wash with 400  $\mu$ L of Buffer B (*see Note 33*), and save this for subsequent PAGE analysis if needed. Allow the column to drain completely, and cap the end.
13. *Optional step*: add 250  $\mu$ L of 800 mM NaCl without ATP to the column and mix by gentle pipetting with a cut P1000 tip. This will encourage dissociation of the RP from the CP, and will likely wash off many associated accessory proteins, thus permitting subsequent elution of the CP (for a PAG1-based purification) or RP (for an RPT4-based purification) by themselves. Incubate at 4° C for 30 minutes, remove the column cap, and collect the eluent.
14. Add 250  $\mu$ L of FLAG elution buffer to the column and mix by gentle pipetting with a cut P1000 tip. Incubate at 4° C for 30 minutes, remove the column cap, and collect the eluent containing purified 26S proteasomes, or CP and RP sub-complexes (*see Note 34*). Freeze samples immediately in liquid nitrogen. They can then be stored indefinitely at -80° C. The FLAG resin can be reused at least once more (*see Note 35*)
15. Analyze the different fractions and assess the elution for purity by SDS and/or native-PAGE (*see Subheadings 3.4.2 and 3.4.3*) combined with silver staining for total protein (*see Subheading 3.4.4*), or by immunoblot analysis with various 26S proteasome subunit antibodies.

### **3.4 Analysis of Purified Arabidopsis 26S Proteasomes.**

Following the above protocols should yield pure 26S proteasome preparations with minimal contaminants. Indeed, the only major contaminating proteins we have routinely encountered from these protocols are tripeptidyl peptidase II and fatty acid  $\alpha$ -dioxygenase from the conventional method [92], and nitrilase, ribulose biphosphate carboxylase/oxygenase (Rubisco), and HSC70 from the affinity purification method [31]. However, in order to assess the peptidase activity of the preparations and confirm their protein composition and assembly status, we usually perform fluorescence-based activity assays and analyze the preparations by both SDS- and native-PAGE. The preparations can also be analyzed by immunoblot with antibodies against specific proteasome subunits, or by mass spectrometry [31], but these will not be described in this chapter. We encourage analyzing samples from all steps in the protocols to help with trouble shooting should problems arise.

#### **3.4.1 Proteasome Activity Assays**



The activity of purified *Arabidopsis* proteasomes can be assayed by monitoring the cleavage of a fluorogenic peptide substrate [99]. The most commonly used is succinyl-LLVY-amc, which is cleaved by the chymotrypsin-like activity of the  $\beta 1$  subunit (PBA1 in *Arabidopsis*) to release free AMC, which can be monitored by fluorescence-based assays. Alternative substrates are available that can be cleaved by the trypsin-like and caspase-like activities of the  $\beta 2$  (PBB1) and  $\beta 5$  (PBE1) subunits, including Z-ARR-amc and Z-LLE-amc, respectively [99]. Because these substrates are based on short tri- or tetra-peptide sequences, they easily diffuse into the central chamber of the CP, regardless of the presence or absence of the RP. To enable assaying of the 26S proteasome more selectively, larger fluorogenic substrates have been developed that require specific import into the CP chamber [35], although to our knowledge these have not yet been tested on plant proteasomes. While proteasome activity can be assayed from crude plant extracts with these peptides, it should be mentioned that plants have other proteolytic activities capable of degrading succinyl-LLVY-amc. Consequently, control assays in the presence of proteasome inhibitors such as MG132 or epoxomicin should also be performed to confirm that hydrolysis is due to proteasomes and not other contaminating proteases. Typical results from this protocol are shown for different fractions of the conventional purification following size-exclusion chromatography in Figure 4A.

1. Add 10  $\mu\text{L}$  of purified proteasome to 1 mL of assay buffer containing 100  $\mu\text{M}$  fluorogenic substrate (e.g. succinyl-LLVY-amc). If the CP has been specifically purified by itself, then the assay buffer should also contain 0.02% SDS (*see Note 36*).
2. Set up parallel reactions containing 80  $\mu\text{M}$  MG132, which should inhibit most substrate cleavage by the CP or the 26S particle. This will confirm the amount of fluorescence signal resulting from proteasomal cleavage of the substrate, compared to non-specific background hydrolysis (*see Note 37*).
3. Mix and incubate the reactions for exactly 20 minutes at 37°C (*see Note 38*).
4. Stop the reaction by adding 500  $\mu\text{L}$  of quenching buffer and mixing thoroughly.
5. Measure the fluorescence of each sample at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.
6. Data for each technical and/or biological replicate should be averaged and then normalized to the control (if required).

#### 3.4.2 SDS-PAGE of 26S Proteasomes Purified from *Arabidopsis*

To determine the protein composition of both the affinity and conventional proteasome purifications, the elution fractions can be analyzed by SDS-PAGE followed by silver staining (*see Subheading 3.4.4*). We typically analyze our purifications on 11% (w/v) polyacrylamide gels, although in some cases, the use of gradient gels might provide better resolution of individual proteasome subunits. While we use 12 x 14 cm resolving gels with 0.75 mm spacers, it is likely that any gel system will

provide adequate results, and the gel recipes can be adapted accordingly. SDS-PAGE followed by silver staining of the conventional and affinity purifications can be seen in Figures 4C and 5A, respectively. Following SDS-PAGE individual subunits can be detected by immunoblot using appropriate antibodies. This is shown in Figure 5B. If warranted, native-PAGE followed by SDS-PAGE in the second dimension can be employed to visualize the subunit composition of the various sub-complexes

1. Thoroughly clean the electrophoresis plates and other gel equipment by soaking for 30 minutes in a 1% (v/v) detergent solution, rinse with sterile, distilled H<sub>2</sub>O, and then rinse with 95% ethanol (*see Note 39*).
2. Assemble the gel apparatus and prepare the resolving gel according to the following recipe: 6.91 mL sterile distilled H<sub>2</sub>O, 3.75 mL 4X SDS-PAGE resolving buffer, 4.13 mL 40% (w/v) acrylamide/bis-acrylamide (29:1), 200 μL 7% (w/v) APS, 10 μL TEMED. This recipe is sufficient for a single 12 cm x 14 cm x 0.75 mm gel, and can be scaled up if multiple gels are needed.
3. Upon addition of APS and TEMED, immediately pour the solution between the gel plates, overlay with 1 mL isopropanol (*see Note 40*), and leave to polymerize for 20 to 30 minutes.
4. After the resolving gel has set, pour off the isopropanol and absorb any excess liquid with a paper towel. Prepare the stacking gel according to the following recipe: 2.22 mL sterile distilled H<sub>2</sub>O, 0.88 mL 4X SDS-PAGE stacking buffer, 0.35 mL 40% (w/v) acrylamide, 50 μL 7% (w/v) APS, 5 μL TEMED. This recipe is sufficient for a single 12 cm x 14 cm x 0.75 mm gel, and can again be scaled up if multiple gels are needed.
5. Upon addition of APS and TEMED, immediately pour the solution between the gel plates. Insert the gel comb, being careful to avoid trapping bubbles, and leave to polymerize for 15 to 20 minutes.
6. After the stacking gel has set, carefully remove the comb and rinse out the wells with 1X SDS-PAGE running buffer. Remove sufficient running buffer from the wells to accommodate the sample volume (*see Note 41*).
7. Add 5X SDS-PAGE sample buffer to each sample and heat for 5 minutes at 95° C. We typically load 32 μL of elution from the affinity purification mixed with 8 μL of 5X sample buffer, which corresponds to around 0.5 μg of purified proteasome.
8. Carefully load the gel, including molecular weight markers, then place into the electrophoresis tank with 1X SDS-PAGE running buffer, and run at 10 to 20 mA per gel until the bromophenol blue dye front has migrated almost to the bottom of the gel (in around 5 to 6 hours).
9. Remove the gel from between the electrophoresis plates and proceed to downstream analyses.

### 3.4.3 Native-PAGE of 26S Proteasomes Purified from *Arabidopsis*

The sub-complex organization of either conventionally or affinity purified proteasomes can be determined by native PAGE followed by a variety of visualization techniques. While SDS-PAGE allows separation of the individual components of the 26S proteasome, native-PAGE permits visualization of the entire particle and its sub-particles. By silver staining these native gels (see section 3.4.4), it is possible to visualize the free CP, free RP, CP singly capped by RP (CP-RP<sub>1</sub>), CP doubly capped by RP (CP-RP<sub>2</sub>), or alternatively capped CP (e.g. CP-PA200). An example of native-PAGE analysis of affinity purified 26S proteasomes by followed by silver staining is shown in Figure 5C. In addition, two-dimensional separation by native- and SDS-PAGE can further resolve the individual proteins within these sub-complexes.

1. Thoroughly clean the electrophoresis plates and other gel equipment by soaking for 30 minutes in a 1% (v/v) detergent solution, rinse with sterile, distilled H<sub>2</sub>O and then rinse with 95% ethanol (*see Note 39*).
2. Assemble the gel apparatus and prepare the resolving gel according to following recipe: 8.06 mL sterile distilled H<sub>2</sub>O, 1.68 mL 40% (w/v) acrylamide, 0.87 mL 2% (w/v) bis acrylamide, 0.7 mL 50% (w/v) sucrose, 3 mL 5X TBE buffer, 75 μL 1 M MgCl<sub>2</sub>, 30 μL 0.5 M ATP, 370 μL Rhinohide™, 200 μL 7% (w/v) APS, 15 μL TEMED (*see Note 42*). This recipe is sufficient for a single 12 cm x 14 cm x 0.75 mm gel, and can be scaled up if multiple gels are needed.
3. Upon addition of APS and TEMED, immediately pour the solution between the gel plates, overlay with 1 mL isopropanol (*see Note 40*), and leave to polymerize for 20 to 30 minutes.
4. After the resolving gel has set, pour off the isopropanol and remove any excess liquid with a paper towel. Prepare the stacking gel according to the following recipe: 1.16 mL sterile distilled H<sub>2</sub>O, 220 μL 40% (w/v) acrylamide, 1.09 mL 2% (w/v) bis acrylamide, 161 μL 50% (w/v) sucrose, 0.7 mL 5X TBE buffer, 17.5 μL 1 M MgCl<sub>2</sub>, 7 μL 0.5 M ATP, 87 μL Rhinohide™, 52.5 μL 7% (w/v) APS, 5 μL TEMED. This recipe is sufficient for a single 12 cm x 14 cm x 0.75 mm gel, and can again be scaled up if multiple gels are needed.
5. Upon addition of APS and TEMED, immediately pour the solution between the gel plates. Insert the gel comb, being careful to avoid trapping bubbles, and leave to polymerize for 15 to 20 minutes.
6. After the stacking gel has set, carefully remove the comb and rinse the wells with 1X native-PAGE running buffer. Remove sufficient running buffer from the wells to accommodate the sample volume (*see Note 41*).
7. Add xylene cyanol to each sample to a final concentration of 0.0005% (w/v) (*see Note 43*). We typically load 32 μL of elution from the affinity purification, which corresponds to around 0.5 μg of purified proteasome.

- Carefully load the gel, then place into the electrophoresis tank with 1X native-PAGE running buffer, and run at 50 V at 4°C until the xylene cyanol dye front has migrated almost to the bottom of the gel (around 16 to 18 hours).
- Remove the gel from between the electrophoresis plates (*see Note 45*) and proceed to downstream analyses.

#### 3.4.4 Silver Staining

Silver staining is a highly sensitive method for detecting proteins after separation by native- or SDS-PAGE. Silver cations bind to proteins within the gel, and become visible to the naked eye upon reduction to solid silver. This technique allows direct visualization of the many proteins that make up the 26S proteasome complex, and is therefore a useful tool to validate the purity and subunit composition of the preparations. Examples are shown in Figures 4B, 4C and 5A, which show silver stained gels of the conventional and affinity purified 26S proteasomes. It is very easy to see the high level of purity obtained from the affinity protocol, as there are very few proteins remaining in the wash fraction.

- Nitrile gloves should be worn throughout this protocol to avoid fingerprints (*see Note 46*). Disassemble the gel apparatus (*see Subheadings 3.4.2 and 3.4.3*) and remove the stacking gel with a razor blade.
- Transfer the gel into an appropriate glass container with lid (*see Notes 45 and 47*). Add 200 mL of fixing solution and allow gel to fix for 1 to 16 hours (if performing subsequent MS analysis *see Notes 9 and 48*). This and all subsequent steps should be performed with gentle sideways shaking unless otherwise stated (*see Note 49*).
- Remove the fixing solution (*see Note 50*) and wash gel for three periods of 10 minutes with 150 mL of washing solution.
- Remove the final ethanol wash and add 200 mL sensitizing solution (*see Notes 51 and 52*). Incubate for exactly 1 minute while shaking the glass container relatively vigorously to guarantee even uptake of the solution.
- Remove the sensitizing solution and wash gel three times for 20 seconds each with 150 mL sterile, distilled H<sub>2</sub>O.
- Add 200 mL staining solution and allow gel to stain for 1 to 16 hours (if performing subsequent MS analysis *see Notes 9 and 48*).
- Remove the staining solution and wash gel three times for 20 seconds each with 150 mL sterile, distilled H<sub>2</sub>O. Change gloves so as not to contaminate the developing gel with concentrated silver staining solution.
- Prepare developing solution within 1 hour of use (if performing subsequent mass spectrometric analysis *see Notes 11 and 51*). Add 250 mL developing solution to the gel and

allow it to develop by eye. Typically, we shake the gel by hand, and place a sheet of white paper underneath the gel container to help visualize the emerging bands.

9. A few seconds before the desired exposure is reached, remove the developing solution and wash gel twice for 5 seconds with 150 mL sterile, distilled H<sub>2</sub>O.
10. Add 200 mL stop solution to the gel and incubate for 10 minutes.
11. Remove the stop solution and wash gel for 20 minutes with 150 mL of washing solution.
12. Image the gel on an appropriate imaging platform.

## 4 Notes

1. Wide-mouth Erlenmeyer flasks allow for better aeration of the culture, promoting healthier seedling growth. However, standard sized flasks can be used with only a minor impact on fresh weight yield.
2. We have found that using an ATP concentration of 20 mM, instead of the previously published 10 mM, gives a more robust association between the CP and the RP, allowing for better purification of the intact 26S particle.
3. We switched from the previously published Tris-based extraction buffer to the HEPES-based buffer because HEPES has a more appropriate *pKa* for buffering the acidic extracts that result from vacuolar lysis during tissue homogenization.
4. We tested many different protease inhibitors, including aprotinin, antipain, benzamide, E64d, leupeptin, pepstatin and PMSF, and found that chymostatin was the best at preventing cleavage of the FLAG tag from PAG1 [31].
5. Sodium metabisulfite acts as a protective agent against polyphenol modification, and we have found that its addition results in a slight increase in band sharpness when proteasomes are analyzed by SDS-PAGE.
6. It is important to use the anti-FLAG<sup>®</sup> M2 affinity gel rather than the anti-FLAG<sup>®</sup> M1 affinity gel, because the latter does not bind C-terminal FLAG-tags.
7. We obtain our FLAG peptide from the University of Wisconsin Biotechnology Center Peptide Synthesis Facility, but any other commercial source is likely to be suitable. We normally make aliquots of 100  $\mu$ L to limit the number of freeze-thaw cycles, and the peptide is stable at -20° C for up to one year.
8. The 1X FLAG elution buffer is generated by dilution from a 10X stock with Buffer B lacking the protease inhibitors chymostatin and PMSF, which may have an adverse effect on subsequent activity assays.
9. For mass spectrometry safe silver staining, formaldehyde should be omitted from these steps, as it permanently crosslinks proteins to the gel, reducing the recovery of tryptic peptides.
10. Commercially available sodium thiosulfate typically comes in a large crystalline form that is impractical for weighing the small amount necessary for silver staining. We therefore grind the sodium thiosulfate into a fine powder using a mortar and pestle, and keep this powder in 1.5 mL microfuge tubes stored in an airtight bag containing a desiccant for up to 6 months.
11. Formaldehyde must be included at this step, even for mass spectrometry safe silver staining, as it catalyzes the reduction of silver ions into solid silver for the visible staining of proteins. Because of the short development time, and thus the short exposure to formaldehyde, suitable tryptic peptides are still recoverable for mass spectrometry [100].

12. For standard plant growth conditions, we sow seeds on solid GM medium containing 0.7% (w/v) agar and grow for 2 to 3 weeks at 21-23° C with a light intensity of 75-100  $\mu\text{mol}/\text{m}^2/\text{sec}$  under a long day photoperiod (16 hours light/8 hours darkness). Seedlings are then transferred to soil (mixed in a 1:1 ratio with organic Coco Coir planting mixture supplemented before use with 2 g/L Peters 20-20-20 fertilizer, 80 mg/L  $\text{Ca}(\text{NO}_3)_2$ , and 80 mg/L  $\text{MgSO}_4$ ), and grown under the same conditions as above for a further 6 to 8 weeks, until the plants are fully dry.
13. Care should be taken when harvesting seeds to be used for liquid culture, as they are particularly susceptible to contamination. All additional plant material should be removed from the seeds prior to sterilization.
14. We typically sterilize approximately 80 to 100 mg of seeds per 50 mL liquid culture, which should generate at least 5 to 8 g fresh weight of tissue after 10 days of growth.
15. We have found that using liquid-phase rather than vapor-phase seed sterilization considerably reduces liquid culture contamination. However, it is necessary to avoid extended incubation times in the bleach or ethanol sterilization solutions, as this will reduce seed viability.
16. Extending the stratification period to 4 or 5 days seems to increase the consistency of seed germination.
17. Because of the problems with contamination, we keep our liquid culture flasks scrupulously clean, soaking them in 10% bleach for at least 2 days after each use, and rinsing thoroughly with sterile, distilled  $\text{H}_2\text{O}$  prior to addition of the medium and autoclaving.
18. It may be necessary to cut off the end of the pipette tip with a sterile razor blade to facilitate easier transfer of the seeds.
19. We find that seedlings grown for between 7 and 10 days provide the best source of proteasomes, as they have a high peptidase activity but contain less of the starch and polyphenols present in older tissue that can interfere with the purification.
20. Examples could include treatment with proteasome inhibitors such as MG132, *clasto*-lactacystin  $\beta$ -lactone, epoxomicin or bortezomib, treatment with various plant hormones such as abscisic acid, nutrient starvation to induce autophagy, or heat shock.
21. Press tissue gently 4 or 5 times between 5 to 10 paper towels to remove excess liquid, which helps in obtaining an accurate fresh weight of the tissue.
22. Grinding is typically performed for 1 minute per gram of tissue. It is not uncommon to process multiple tissue samples at once therefore, once grinding is complete, add additional liquid nitrogen while processing the other samples to prevent thawing.
23. Care should be taken when re-applying liquid nitrogen, as the fine powder can often become dispersed, risking tissue loss and contamination of adjacent samples.

24. Pre-chilling the plastic spoon is essential to stop thawing of the tissue occurring during transfer from the mortar to the glass beaker.
25. This step removes most large particles of plant material. Care should be taken when squeezing out the remaining liquid that the Miracloth does not burst and spray insoluble material into the centrifuge tube.
26. These initial PEG precipitations do little to enrich the 26S proteasome; however, these steps help remove peptidases that interfere with 26S proteasome activity assays. When purifying an enzymatic complex based on activity, removing interfering components becomes a crucial step.
27. We store our UNO<sup>®</sup> Q6 anion exchange chromatography column in 20% (v/v) ethanol containing 0.1 M NaCl. To prepare the column for use we wash with 5 column volumes of sterile, distilled H<sub>2</sub>O, 5 column volumes of 20 mM Tris-HCl, pH 6.0, 5 column volumes of Buffer A containing 1 M NaCl, 5 column volumes of 20 mM Tris-HCl, pH 6.0, and 5 column volumes of Buffer A, with a flow rate of 1 mL/min. All buffers are filtered and de-gassed before use.
28. We store our size exclusion chromatography column in 20% (v/v) ethanol. To prepare the column for use we wash with 5 column volumes of sterile, distilled H<sub>2</sub>O followed by 5 column volumes of Buffer A, with a flow rate of 0.1 mL/min. All buffers are filtered and de-gassed before use.
29. Buffer B can be prepared up to a week in advance provided that the ATP, DTT and protease inhibitors are omitted, and the buffer is filter sterilized with a 0.22 μm filter. However, it is important to remember to re-adjust the pH after adding ATP.
30. Beads should be washed by gentle re-suspension in 1 mL Buffer B using a cut P1000 pipette tip, and then further mixed by inverting the tube 3 or 4 times.
31. We have found that it helps to prepare slightly more of the beads and elution buffer than we might expect to be necessary, to avoid having insufficient quantities, especially if we plan on performing more than one purification simultaneously.
32. It is important to collect the flow-through by allowing it to run down the wall of the screw top tube, rather than dripping to the bottom. This more gentle approach helps to maintain the integrity of the complex, preventing unwanted dissociation into sub-complexes, and loss of additional interacting proteins.
33. We have found that this additional wash step greatly reduced the number of contaminating proteins ending up in the elution fraction. Add the final 400 μL wash in the chromatography column without touching the sides, as this provides a clean surface for the final 250 μL elution.
34. To ensure maximum elution, once the majority of the buffer has dripped through we put a cap on the column which is connected to a syringe by a short length of silicone tubing, and



apply a small amount of pressure by pushing down on the syringe. This expels the final few drops of elution buffer, containing purified proteasomes, from the column.

35. The resin can be re-used one additional time with only slightly decreased binding activity. To regenerate, wash the column with 10 mL Buffer B and re-suspend the beads in 50% (v/v) glycerol in Tris-buffered saline. Store the resin for up to 1 month at -20° C until further use.
36. Purified CP alone has low activity against fluorogenic peptide substrates because the axial channels that allow substrate entry into the CP are closed [28]. The addition of 0.02% (w/v) SDS to the assay allows activation of the CP by selectively denaturing this CP gate, thereby allowing substrate entry. Assay of the 26S proteasome holoenzyme does not require the addition of SDS, because the association of the RP with the CP opens the channels.
37. We typically perform three technical replicates of each reaction (plus and minus MG132), and three independent biological replicates if we are comparing activity levels between different samples.
38. To ensure a precise incubation time for each sample, we typically stagger the start of each assay by 30 seconds.
39. Because silver staining is extremely sensitive, we use dedicated gel buffers, gel tanks, sets of gel plates and other gel equipment for performing this technique. The buffers are made with sterile, distilled H<sub>2</sub>O and are autoclaved before use, whereas the other equipment is maintained in a scrupulously clean condition by regular washing with detergent and sterile, distilled H<sub>2</sub>O.
40. H<sub>2</sub>O-saturated butanol can be used instead. Alternatively, 10% glycerol can be included in the resolving gel, and the stacking gel (lacking glycerol) can immediately be poured directly on top of the resolving gel.
41. Filling the wells only half-way helps prevent spill-over of the sample into neighboring lanes, which can often be detected by the sensitive silver staining technique and may cause problems in interpretation of results.
42. We add Rhinohide™ to our native gels to strengthen them given their very low percentage of acrylamide. Despite this, the gels remain extremely fragile.
- 43.
44. The elution fractions should already contain 10% (v/v) glycerol. If not, sterile glycerol should be added to the samples prior to loading.
45. The native gels must be handled with great care at this stage. We have found that while wearing gloves, the fingertips should be soaked in running buffer before handling the gel, which helps prevent them from sticking to the gel and causing breakage.
46. Nitrile gloves should be used in preference to powdered latex gloves, as the powder can leave a residue on the gel that will cause visual artefacts to appear upon staining.

47. The glass container should be kept scrupulously clean. Typically, we wash before use by incubating with a 1% (v/v) detergent solution for at least 30 minutes, and then rinsing once with sterile, distilled H<sub>2</sub>O and once with 95% (v/v) ethanol.
48. To perform mass spectrometric safe silver staining, formaldehyde should be omitted during the fixing and staining steps. As a result, prolonged fixing and/or staining will decrease band sharpness, and so this step should be limited to a maximum of 1 to 2 hours.
49. We typically use a platform with a sideways shaking mechanism, as we have found that this type of motion leads to reduced gel breakage, particularly for the fragile native gels. It also prevents the gel sticking to the sides of the glass container and not remaining uniformly submerged in solution, which can cause differential staining across the gel.
50. For the high percentage SDS gels (10-14% (w/v) acrylamide), we typically pour off all solutions while gently holding the outer edges of the gel with our gloved fingers. However, as the native gels are much more fragile, we instead remove solutions using a vacuum aspiration system, which avoids unnecessary maneuvering of the gel.
51. We have found that the gels have a tendency to curl in upon themselves following addition of this solution, so we hold the gel corners down with our gloved fingers to ensure uniform solution uptake.
52. To save time and effort, we retain 5 mL of the sensitizing solution for subsequent use in the developing solution, which results in the required 0.0004% sodium thiosulfate concentration when using 250 mL of developer.

## Acknowledgements

The authors wish to thank Peizhen Yang, Adam J. Book, and Joseph M. Walker for their extensive work on the initial development of these protocols. We also wish to thank Erin Gemperline for critical reading of the manuscript. This work, R.S.M. and D.C.G. were supported by the U.S. Department of Energy Office of Science; Office of Basic Energy Sciences; Chemical Sciences, Geosciences and Biosciences Division (grant number DE-FG02-88ER13968).

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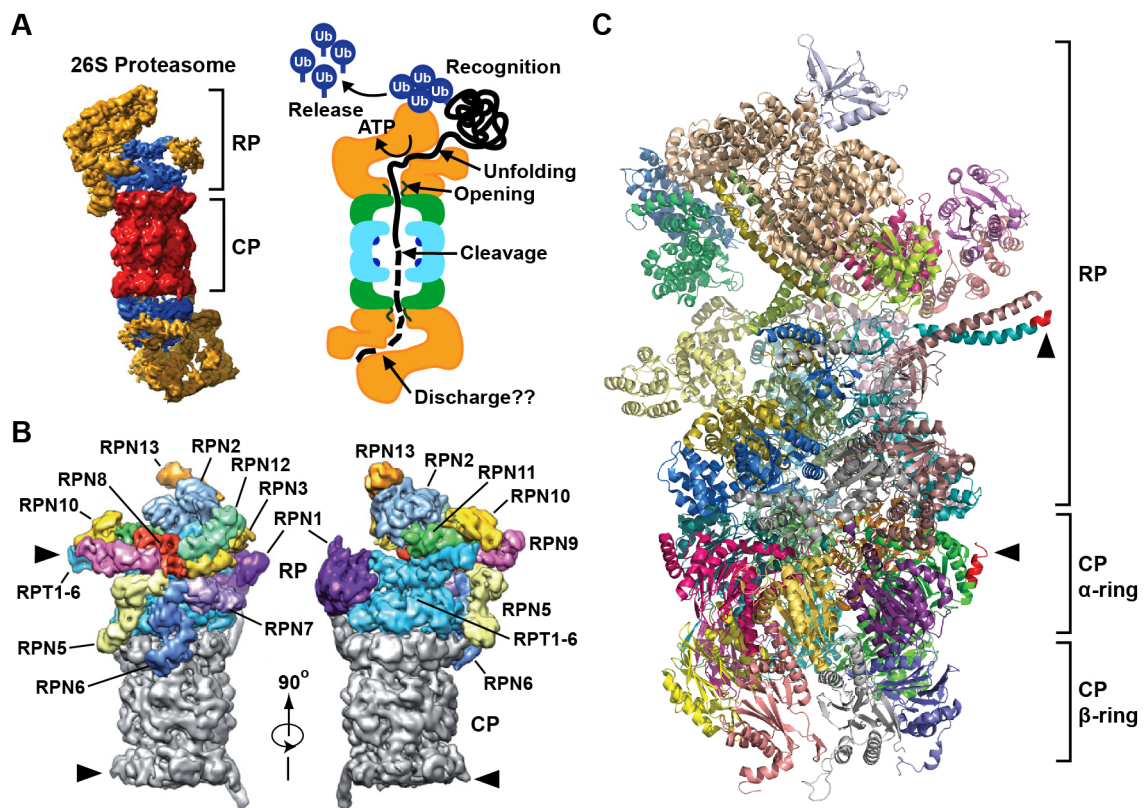
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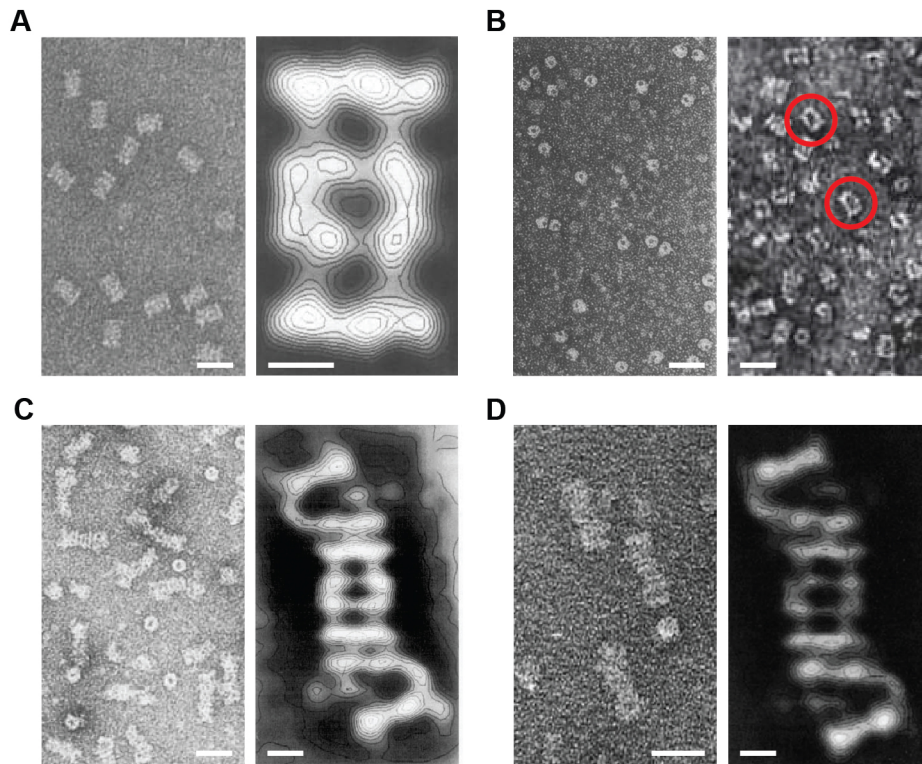
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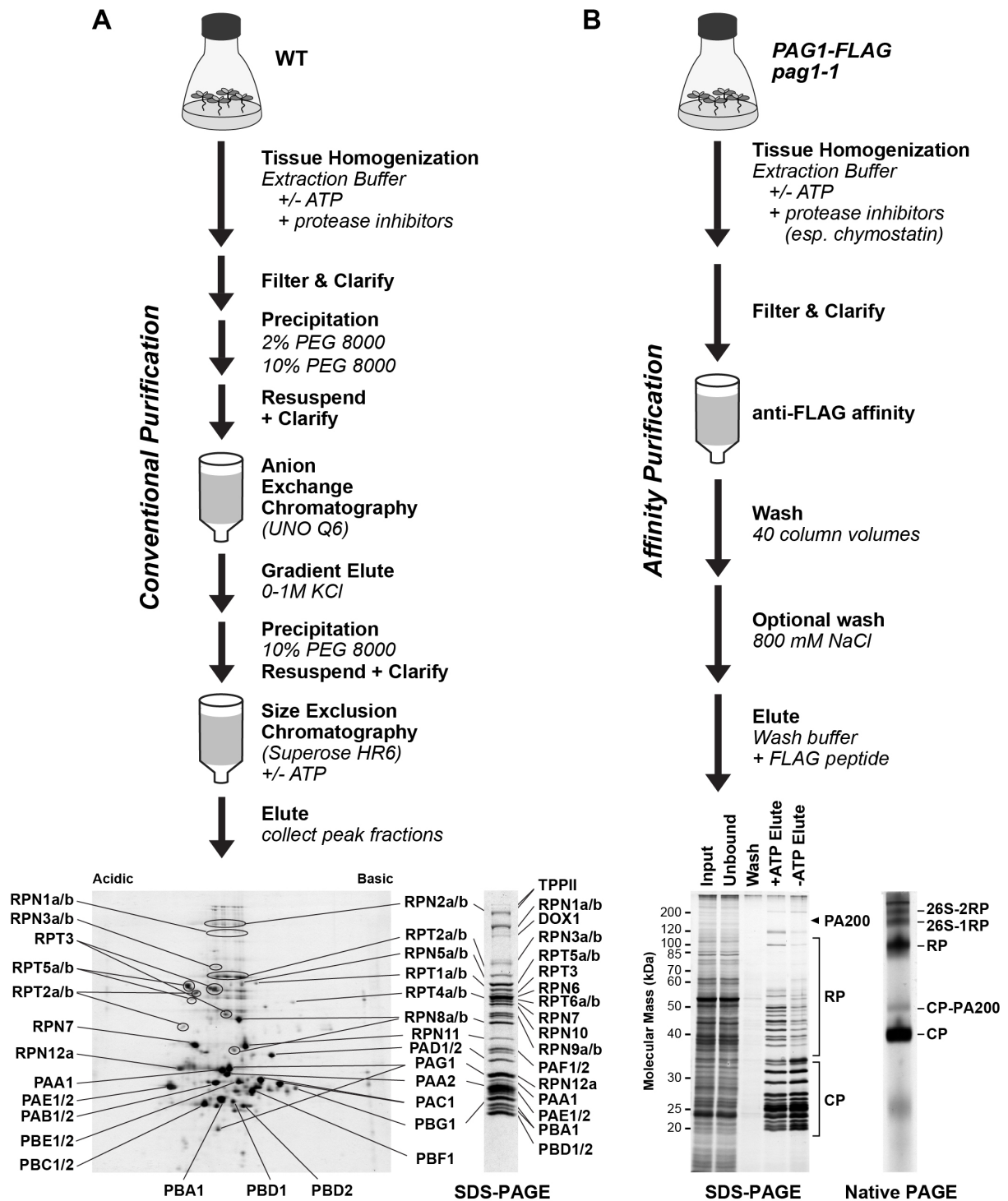
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**Figure 1.** Structure of the 26S proteasome. (A) Schematic representation of the 26S proteasome, with a 3-D structure as determined by electron microscopy (EM) shown on the left, and a cartoon representation of the holoprotease shown on the right. Within the EM structure, the CP is shown in red, the RP base is shown in blue, and the RP lid is shown in yellow. Specific functions within the CP and RP are shown on the right. The EM structure is modified from reference [23]. (B) A detailed view of the subunit architecture of the 26S proteasome RP. The CP is shown in grey, the RPT ring is shown in blue, and all additional RPN subunits are shown in different colors, with their identity labeled. The positions of the FLAG tags on PAG1 and RPT4 are indicated by black arrowheads. These structures are modified from reference [22]. (C) A structural model of the 26S proteasome from yeast at sub-atomic resolution modified from PDB ID 4CR2 [37]. The RP subunits, as well as the CP  $\alpha$  and  $\beta$  rings are shown. Highlighted in red, and indicated by black arrowheads, are the positions where FLAG affinity tags have been successfully used to enrich for *Arabidopsis* 26S proteasomes.



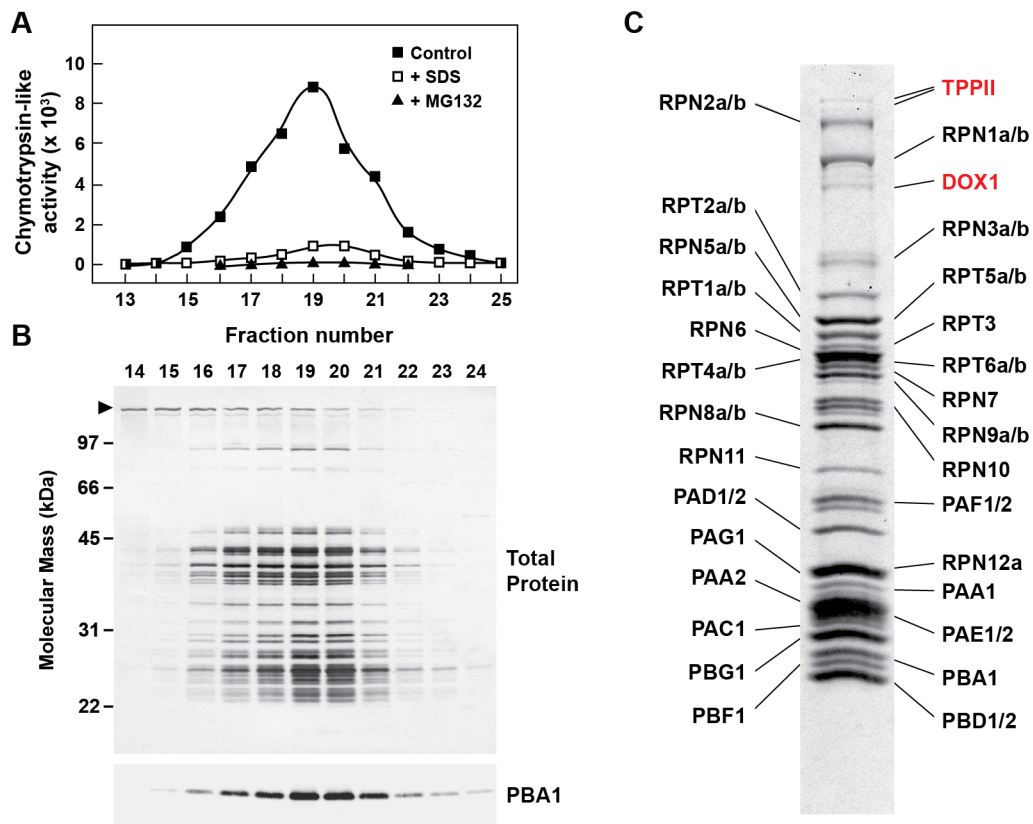
**Figure 2.** Electron microscopy images of 20S and 26S proteasomes from mammals and plants. (A) Images of 20S proteasomes purified from rat skeletal muscle. On the left is an electron micrograph of the 20S particles negatively stained with sodium phosphotungstate, while on the right is a close-up image with overlaid contour plots generated by correlation averaging of approximately 300 individual images negatively stained with ammonium molybdate. (B) Images of the first 20S proteasomes purified from different plant species. On the left are proteasomes isolated from tobacco leaves, while on the right are proteasomes from potato tubers, both negatively stained with uranyl acetate. The typical barrel-shaped structures are indicated with red circles. (C) Images of 26S proteasomes purified from rat liver. On the left is an electron micrograph of the 26S particles negatively stained with uranyl acetate, while on the right is a close-up image with overlaid contour plots generated by correlation averaging of 215 individual images. (D) Images of 26S proteasomes purified from spinach leaves. On the left is an electron micrograph of the 26S particles negatively stained with uranyl acetate, while on the right is a close-up image with overlaid contour plots generated by correlation averaging of 450 individual images. In all cases, scale bars represent 25 nm for the electron micrograph images and 5 nm for close-up images generated by averaging. The images were modified from references [71, 72, 74, 85, 88].



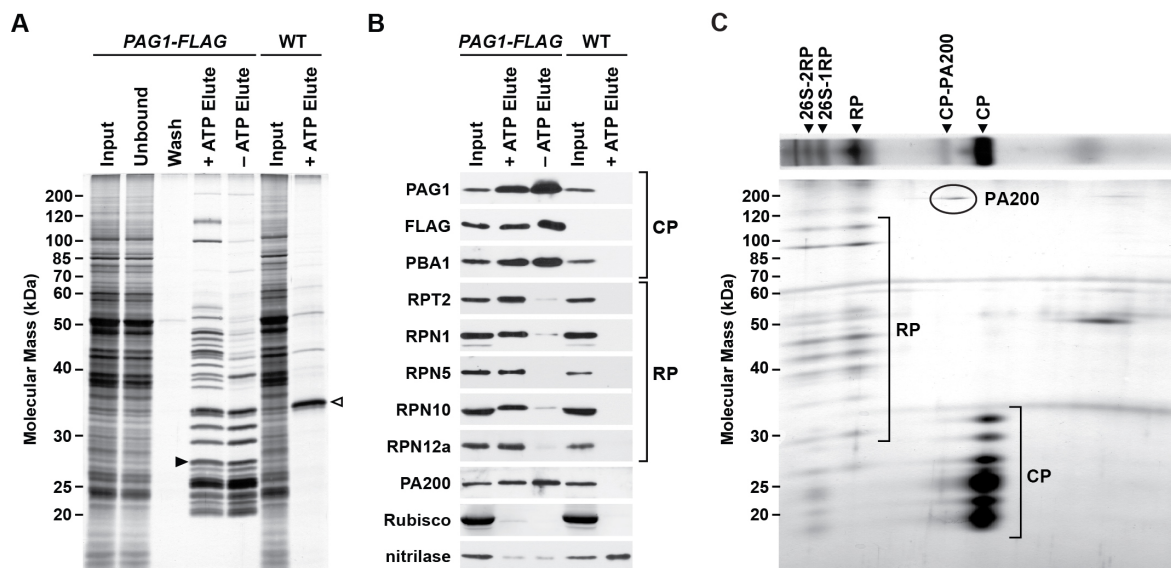
**Figure 3.** Flow chart of the two protocols for purification of the 26S proteasome from Arabidopsis. (A) The conventional purification protocol begins with homogenization of plant tissue followed by protein extraction, clarification, and two consecutive precipitation steps with 2% (w/v) and 10% (w/v) PEG 8000. The resulting protein pellet is resuspended, clarified, and subjected to sequential anion exchange and size exclusion chromatography. The presence of proteasomes in the final elution fractions is determined by activity assay and native- or SDS-PAGE followed by silver stain. Representative preparations analyzed by isoelectric focusing and/or SDS-PAGE are shown (modified

from reference [92]). (B) The affinity purification protocol begins with homogenization of appropriate transgenic tissue expressing a FLAG-tagged proteasome subunit, followed by protein extraction and clarification. The extract is then loaded onto an anti-FLAG affinity column, the column is extensively washed, and bound proteasomes are competitively eluted with FLAG peptide. A representative preparation, together with samples from the various purification steps, analyzed by SDS-PAGE is shown (modified from reference [31]).





**Figure 4.** Conventional purification of 26S proteasomes from wild-type *Arabidopsis* plants. Fractions enriched for 26S proteasome activity following anion exchange were subjected to size exclusion chromatography with a Superose™ HR6 column. (A) An elution profile of 26S proteasome peptidase activity as assayed with the fluorogenic substrate succinyl-LLVY-amc in the absence (black square) or presence (black triangle) of 80  $\mu$ M MG132 or 0.02% (w/v) SDS (white square). Peak 26S proteasome activity is observed in fractions 16 to 21. (B) An elution profile of the 26S proteasome as analyzed by SDS-PAGE followed by protein staining with Coomassie Blue (upper panel), or by immunoblot analysis with antibodies against the CP subunit PBA1 (lower panel). The arrowhead indicates the migration position of the contaminating TPPII polypeptides. (C) Identification of 26S proteasome components as identified by mass spectrometry (MS). Purified 26S proteasomes were resolved by SDS-PAGE, and the gels were stained for total protein with silver. Bands were excised, digested with trypsin, and identified by MALDI-TOF MS. The identities of the various bands are indicated, with only those bands that allowed unambiguous identification of the protein being labelled. The two principle contaminating proteins, TPPII and DOX1, are highlighted in red. This figure was modified from reference [92].



**Figure 5.** Affinity purification of 26S proteasomes from *pagl-1* *PAG1-FLAG* plants. (A) SDS-PAGE analysis of the affinity purification steps. Total protein extracts from 10 day old wild-type (WT) and *pagl-1* *PAG1-FLAG* plants were incubated with anti-FLAG affinity resin, washed, and competitively eluted with the FLAG peptide. The procedure was performed in the presence or absence of ATP, and the input, unbound, washed and eluted fractions were subjected to SDS-PAGE and the gel stained for protein with silver. The black arrowhead indicates the PAG1-FLAG protein, while the open arrowhead identifies nitrilase, which is non-specifically enriched during the purification. (B) Immunoblot detection of various 26S proteasome subunits in the affinity-purified preparations shown in A. Subunits tested include the CP subunits PAG1 and PBA1, the RP subunits RPT2, RPN1, RPN5, RPN10 and RPN12a, and the alternate capping particle PA200. Other proteins tested include the Rubisco small subunit and nitrilase. (C) Separation of the various proteasome complexes by native PAGE. 26S proteasomes affinity-purified from *pagl-1* *PAG1-FLAG* plants as in A were fractionated by native PAGE in the presence of ATP, and the gel was stained for total protein with silver. The migration of the CP alone, the CP-PA200 complex, the RP alone, and 26S proteasomes singly or double capped with RP (26S-1RP and 26S-2RP) are indicated. Additionally, proteasomes can be further subjected to denaturing SDS-PAGE in the second dimension, further confirming the identity of the different species observed by native PAGE. This figure was modified from reference [31].