HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2019 March 21.

Published in final edited form as:

Methods Mol Biol. 2018 ; 1844: 277–288. doi:10.1007/978-1-4939-8706-1_18.

Methods to Rapidly Prepare Mammalian 26S Proteasomes for Biochemical Analysis

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Abstract

Rapid, gentle, isolation of 26S proteasomes from cells or tissues is an essential step for studies of the changes in proteasome activity and composition that can occur under different physiological or pathological conditions and in response to pharmacological agents. We present here three different approaches to affinity purify or to prepare proteasome-rich cell fractions. 1) The first method uses affinity tags fused to proteasome subunits and has been useful in several cell lines (see Table 1) for studies of proteasome structure by cryo-electron microscopy and composition by mass spectrometry. 2) A second method uses the proteasome's affinity for a ubiquitin-like (UBL) domain and can be used to purify these particles from any cell or tissue. This method does not require expression of a tagged subunit and has proven very useful to investigate how proteasomes activity changes in different physiological states (e.g. fasting or aging), with neurodegenerative diseases, and with drugs or hormones that cause subunit phosphorylation. 3) A third, simple method that is based on the 26S proteasome's high molecular weight (about 2.5 MDa), concentrates these particles greatly by differential centrifugation. This method maintains the association of proteasomes with ubiquitin (Ub) conjugates and many other loosely-associated regulatory proteins and is useful to study changes in proteasome composition under different conditions (see Table 2).

Introduction

Rigorous studies of proteasome composition, regulation and its detailed mechanisms require proteasome purification or at least isolation away from other cytosolic enzymes. The ATP-dependent degradation of ubiquitinated proteins proceeds through multiple steps (1), which can not be analyzed without purification of the 26S complex. Also, analysis of its regulation, the effects of pharmacological and physiological stimuli and disease processes requires rapid and gentle purification of 26S proteasomes, without which superficial or even misleading conclusions about proteasome activity may be obtained (e.g. through reliance on peptidase activities in crude extracts). Purification is essential to analyze proteasomal activities free of cytosolic, deubiquitinating enzymes (DUBs), Ub ligases, proteases, and ATPases that can mask or alter 26S activity. Moreover, it is increasingly clear that 26S proteasomes within cells are a heterogeneous mixture of active and inactive forms (2) and may contain one or

[7]·To study how proteasome composition changes during in vitro incubations and the effects on Ub conjugate binding, protein-bound affinity gels were washed 3 times with 150 μL wash buffer. Spin at 300 × g for 1 min. The bound FLAG-tagged proteasomes were incubated at either 4° or 37 °C for 20 min [Note 8] before the final wash and elution.

two 19S regulatory particles per 20S core, alternative activating complexes (e.g. PA28α or PA200), or sub-stoichiometric amounts of different associated proteins that can alter degradation rates (e.g. shuttling factors, Ub ligases, and DUBs). Another important source of heterogeneity is the presence of ubiquitinated substrates on some cell proteasomes (2) since Ub conjugates activate the proteasomes (3) and alter the levels of other associated proteins (4). Finally, several post-synthetic modifications of proteasome subunits (e.g. phosphorylation (5–7) and ubiquitination (8)) can change the proteasomes' activity (See Chapter of VerPlank and Goldberg). Thus, with the proteasomes (as with other biochemical preparations), clean results and conclusions require clean preparations.

Until the past decade, biochemical and structural studies of 26S proteasomes utilized various classical multi-step purification methods (i.e. ion exchange and size exclusion chromatography). Aside from being lengthy and time-intensive methods, these efforts strip away 26S-associated proteins, decrease the ability to degrade Ub conjugates, and lose certain regulatory properties. Therefore, we have developed several methods to rapidly and gently isolate 26S particles that maintain proteasome activity and regulatory features better than conventional purification methods. These approaches have proven very useful for various studies of proteasome mechanis`ms and regulation (see chapter by Kim et al and VerPlank and Goldberg).

1) Affinity Purification of Proteasomes Bearing a Tagged Subunit

Several laboratories have fused affinity tags to various proteasome subunits, including β4 of the 20S core (9), and Rpn11 (10–12), and the sub-stoichiometric subunit Dss1 (13) of the 19S particle and used them to purify active proteasomes (Table 1). Since the intracellular pools of free 26S subunits are very small, affinity-tag methods primarily yield intact proteasome particles. However, depending on the tagged subunits and the cell source, the resulting 26S proteasomes may be co-purified with free 20S proteasomes or 19S regulatory particles. For example, if tagged-Rpn11 is used, one may purify a mixture of singly and doubly capped 26S particles and some free 19S, especially in certain cells types or experimental conditions (e.g. ATP-deficient cells) where there are abundant free 19S complexes. Using tagged-β4 purifies normally a mixture of 26S and 20S proteasomes, but 20S particles can be specifically purified using tagged-β4 if ATP is omitted from the buffers to promote dissociation of 26S into 19S and 20S components.

In yeast, the affinity tagged subunits are integrated into the endogenous genomic loci, and thus all cellular proteasomes are tagged and can be purified by this approach. The mammalian cell lines presently in use stably over-express the tagged subunit off a multi-copy plasmid and therefore still have an endogenous untagged copy of the subunit gene. Because only the proteasomes that incorporated the tagged-subunits can be isolated by this approach, many cytosolic proteasomes are not purified. It is very likely that the increased use of CRISPR-Cas9 genome editing methods should change this situation and introduce tags on both genomic copies of a given subunit, and thereby improve the purification yields. These affinity tag approaches co-purify more of the proteasome-associated proteins, even the weakly bound ones and Ub conjugates than the UBL method (discussed below). Consequently, these tagged particles are particularly useful for studies of the changes in 26S

composition under different conditions, as many 26S-associated proteins cycle rapidly on and off the particles at 37°C (4).

2) The UBL Affinity Method

The proteasome's strong affinity for proteins containing UBL domains is the basis for another very useful affinity purification method. The UBL method uses a GST-tag fused to the UBL domain of the shuttle factor Rad23b. 26S proteasomes, bind to GST-UBL columns without the need for tagging proteasome subunits and then can be eluted from the GST-UBL resin by addition of high concentrations of the ubiquitin-interacting motif (UIM) derived from the Ub-binding subunit Rpn10/S5a/PSMD4. The chief advantage of the UBL affinity method is that it can gently and rapidly purify proteasomes or immunoproteasomes from a wide diversity of cells and tissues without any genetic manipulations. Therefore, it has been successfully used to characterize proteasomes from various cultured cells and yeast, as well as rabbit and mouse muscles (14), spleen (15), peripheral neurons, and brains (16–18), and also to identify proteasome adaptations during different physiological states (e.g. fasting or after hormone treatments) (16,18).

It is not clear which of several potential UBL binding sites on the proteasome is targeted by this method. In yeast, the Rad23 UBL domain binds to the T1 site of Rpn1 (19). (The other potential site on Rpn1, T2, binds the DUB Ubp6 (19)). In mammalian cells, both the Pru domain of Rpn13/ADRM1 and the two UIM regions of Rpn10 have been reported to be important for the binding of Rad23b to the proteasome (20). All these potential UBL binding sites also can bind Ub conjugates and some other UBL-containing proteins. Consequently, this method dislodges several proteins from the proteasomes, including most ubiquitinated substrates and proteins that depend on the Ub conjugates to associate with proteasomes (e.g. Ube3c/Hul5). However, this method co-purifies other important 26S-associated proteins (e.g. the DUB Usp14) and has been very valuable for mechanistic studies of the multiple steps during Ub conjugate degradation (e.g. ATP hydrolysis, activation by substrates and proteasome regulation).

3) Isolation of Proteasomes by Differential Centrifugation

Because of their high molecular weights, proteasomes can be quickly isolated from cells or tissue extracts by differential centrifugation. This method can capture more than 99% of the proteasomes in the enriched fraction (see Table 2) and is thus advantageous for large-scale preparations. This rapid preparation of proteasome-rich fractions without any prior genetic manipulations also isolates most Ub conjugates and many other proteins that associate with the proteasomes. This approach however inevitably co-isolates in the proteasome-rich fraction other high molecular weight complexes (e.g. p97/VCP) and 26S and 20S particles cannot be reliably separated by this method. Thus, it is an enrichment method rather than a means of purification. However, differential centrifugation if used prior to an affinity purification method may improve the purity of the proteasomes obtained.

1. Purification of FLAG-Dss1 tagged proteasomes using anti-FLAG affinity gels

Materials

1. HEK293 cells stably expressing FLAG-Dss1 (13).
2. Cell lysis buffer: 25 mM HEPES (pH 7.4), 1X protease inhibitors cocktail (Roche), 10% glycerol, 1 mM ATP-Na (pH7.4), 5 mM $MgCl_2$ [Note 1].
3. Wash buffer: same as the cell lysis buffer but without protease inhibitors
4. Anti-FLAG M2 affinity gel (Sigma)
5. 0.2 μm nylon membrane centrifugal filters (VWR)

Methods: Perform all steps on ice or at 4°C.

Preparation of Crude Extract

1. HEK293 cells stably expressing FLAG-Dss1 were grown to 90% confluency in one 100 mm culture dish [Note 2, 3], washed with 10 mL PBS, and then harvested by scraping into 4 mL ice cold PBS.
2. Cell pellets were collected by centrifugation at 350 × g for 5 min and then resuspended in 0.4 mL cell lysis buffer.
3. Cells were lysed by sonication with 4 rounds of 10 sec bursts (12 micron amplitude) and 50 sec rests on ice.
4. Cell debris was cleared by centrifugation at 10,000 × g for 10 min, and the resulting supernatants were further centrifuged at 100,000 × g for 30 min [Note 4]. The supernatants (S100 cell extracts) were then utilized in further purification steps.

Affinity Purification

1. Prepare the FLAG-affinity gel as advised by the manufacturer. An affinity gel volume that binds > 4 μg FLAG fusion protein was used for cell extracts from one 100 mm dish.
5. Incubate the cell extracts with the affinity gel at 4 °C for 1 h.
6. Pellet the protein-bound affinity gel by centrifugation at 300 × g for 1 min in the cold. Collect the supernatants to monitor the amount of unbound proteins.

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1. DTT (1 mM) was omitted in the cell lysis and wash buffers because reducing agents disrupt the disulfide bridges holding the antibody chains of the FLAG-M2 affinity gel (Sigma) together.
2. These purification procedures are used for small-scale analytical preparations, and thus protein concentrations are too low to be reliably measured by standard assays, such as the Bradford assay. To quantify the concentration of purified proteasomes, we must rely on with known concentrations of reference proteins. For this purpose, the FLAG peptides or a 19S or 20S proteasome subunit can be expressed in *E. coli* and purified. Known concentrations are then used to create a standard curve to determine the concentrations of the purified proteasomes by western blot (4).
3. To scale up the preparations, one should determine the optimal affinity gel and cell extract ratio.
4. Longer spins of the cell extracts may precipitate proteasome particles, leading to a loss of proteasomes from the cell extracts.

7. The protein-bound affinity gels were washed four times with 150 μL of the wash buffer. Spin at 300 × g, for 1 min.
8. The bound FLAG-tagged proteasomes were eluted with 45 μL of elution buffer (200 ng/mL 3XFLAG) for 30 min twice [Note 5].
9. Combine the eluted proteasomes and apply them to 0.2 μm nylon membrane centrifugal filters to remove the trace amount of affinity gel. Spin at 1,500 × g for 1 min. Purified proteasomes are collected at the bottom of the 1.5 mL tube.

Assay and Storage

1. Measure proteasome peptidase activity usually using Suc-LLVY-amc as the substrate (see chapter by Kim et al.) [Note 6].
2. Store purified proteasomes at −20 °C for short-term storage or −80 °C for prolonged storage.

2. Proteasome purification by the UBL method

Materials

1. His_{10}-UIM (14)
2. GST-UBL (14)
3. 150 mL affinity purification buffer (APB): 25 mM HEPES-KOH (pH 7.4), 10% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT. Always add ATP and DTT immediately before the purification [Note 1].
4. Glutathione-sepharose (GE Healthcare). Equilibrate 500 μL 50% slurry of glutathione-sepharose by washing three times in 2.5 mL APB. Collect the resin at 100 × g for 5 minutes; discarding the supernatant.
5. Ni-NTA agarose (Qiagen). Equilibrate 5 × 60 μL 50% slurries of Ni-NTA agarose by washing three times in 300 μL APB. The agarose sediments quickly by gravity. Carefully aspirate off the supernatant.
6. 20-mL empty column pack (Bio-Rad)

Methods—Preparation of Crude Extract

Perform all steps on ice or at 4°C.

1. Collect cells from about ten - thirty 150 mm dishes [Note 2] by scraping in 1X PBS and pooling the cells from three 150 mm dishes in a 15 mL conical tube.

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5. Compared to a single elution, multiple elutions using smaller buffer volumes each time can result in a more complete elution.
6. Perform the proteasome activity assays on the same day because repeated freeze-thaw cycles decrease proteasome activity.
1. The purification buffer can be prepared with 150 mM NaCl. The GST-UBL also isolates a complex containing the hexameric ATPase p97/VCP and associated factors, but the use of NaCl (150 mM) prevents the p97 complex from binding to the GST-UBL (21). However, this salt concentration also reduces the amount of proteasomes and certain proteasome-associated proteins (21). We do not recommend adding NaCl when purifying from liver or spleen. For reasons that are unclear, 150 mM NaCl increases the amount of free 19S in the purified proteasomes from liver extract, although adding NaCl to purified 26S particles from liver does not cause their disassembly. Yeast proteasomes seem to have a lower affinity for the UBL domain of mammalian Rad23b and do not bind in the presence of salt.

The cells are spun at 100 × g for 5 minutes, and the supernatants removed by aspiration.

2. Add 2.5 mL APB to each of the tubes containing the cell pellet. Vortex to resuspend the cells.

3. Lyse the cells by sonication [Note 3]. We use six 10 sec bursts (12 micron amplitude) and at least 10 sec recovery periods on ice. Pool the lysates.

4. Clear the lysates by centrifugation at 100,000 × g for 30 min [Note 4].

5. Aliquot a small sample of clarified lysate (S100) to measure the protein concentration by a Bradford assay.

Affinity Purification

1. Combine 80–100 mg protein from the S100 lysate in 10 – 20 mL with 1 mg GST-UBL and 250 μL bed volume of glutathione-sepharose.

2. Gently rotate the S100, GST-UBL, and glutathione-sepharose mixture for 2 h.

3. Pour the S100, GST-UBL, and glutathione-sepharose mixture into an empty 20-ml column. Set aside the flow-through for later analysis of unbound proteins.

4. Wash the resin twice with 10 mL (40X bed volume) APB. Then, close the column with a plastic cap.

5. Cut the tip of a P1000 pipet and add 250 μL of HIS_{10}-UIM (0.5 mg/mL) [Note 5] to the 250 μL GSH-resin in the column body. Gently pipet the resin up and down to mix. Incubate for 15 min.

6. Open the column and collect the HIS_{10}-UIM eluate into a 1.5-ml tube containing 30 μL bed volume of washed Ni-NTA agarose [Note 6].

7. Repeat the elution with another 250 μL of HIS_{10}-UIM and collect into another tube with 30 μL bed volume of washed Ni-NTA agarose [Note 7]. Perform a total of five elutions.

8. To remove the UIM, incubate the 500 μL HIS_{10}-UIM eluate with the Ni-NTA for 20 min on a rotating wheel. Spin for 1 min at 500 × g to sediment Ni-NTA.

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2. The necessary amount of cells varies with the cell type and should be optimized for each line. The goal should be to harvest 10 – 20 mL of lysate containing 80–100 mg total protein, assuming that proteasomes represent 1–2% of the cell protein content, which is an approximation not necessarily true for all cell types or tissues. This procedure can be scaled down to a smaller size, if necessary for particularly valuable tissue sources, although such a modification tends to decrease the concentration of proteasomes obtained and consequently yields a less stable preparation.

3. The lysis of tissues is discussed in a previous description of this method (14).

4. We have found that 30 minutes is sufficient to separate the proteasomes from larger microsomal components. Longer spins begin to pellet most proteasomes.

5. Add ATP to 1 mM in the HIS_{10}-UIM solution immediately prior to elution.

6. We have found that Qiagen Ni-NTA provides consistently more active proteasome preparations than several other sources of this ligand.

7. Immediately after eluted proteins are added to the Ni-NTA agarose, begin to rotate for 20 min (step 8). Prolonged incubation with Ni-NTA agarose seems to decrease proteasome activity. Conversely, a shorter incubation causes HIS_{10}-UIM to be insufficiently cleared, and the presence of HIS_{10}-UIM in the 26S preparation can also decrease proteasome activity. Therefore, it is important to incubate specifically for 20 min.

9. Transfer the supernatant into a 500-μL filter unit (0.45 μm) and spin for another 5 min at 10,000 × g to remove the Ni-NTA. The flow-through contains the purified 26S proteasomes (but not the eluting peptide His_{10}-UIM).

10. Determine the protein concentration of the 26S preparation. Typically, the first elution has very little 26S. The second and third elution fractions contain the bulk of the proteasomes and can be combined. Aliquot and store at −80°C. To maintain the structural integrity of the 26S as well as possible, avoid freeze–thaw cycles.

3) Isolation of proteasomes by differential centrifugation

Materials

1. Cells or tissues of interest
2. Cell lysis buffer: 25 mM HEPES (pH 7.4), 1X protease inhibitors cocktail (Roche), 10% glycerol, 1 mM ATP-Na (pH7.4), 5 mM $MgCl_2$
3. Wash buffer: Same as the cell lysis buffer without protease inhibitors
4. Thick-wall, polycarbonate centrifuge tubes (11X 34 mm) (Beckman Coulter)

Methods

Perform all steps on ice or at 4°C.

1. Cells grown to 90% confluency in one 100 mm cell culture dish were washed with 10 mL PBS and then collected by scraping into 4 mL ice cold PBS.
2. Cell pellets were collected by centrifugation at 350 × g for 5 min and then resuspended in 0.4 mL cell lysis buffer.
3. Cells were lysed by sonication with 4 rounds of 10 sec (12 micron amplitude) bursts and 50 sec rests on ice. Cell debris was cleared by centrifugation at 10,000 × g for 10 min, and the cell extracts (S100) were prepared by further centrifugation of the resulting supernatants at 100,000 × g for 30 min.
4. Pellet proteasomes by centrifuging 200 μL S100 at 150,000 × g for 1 h [Note 1]. Collect supernatant (S150).
5. Rinse proteasome pellets with 200 μL wash buffer once and resuspend the proteasome pellet in 200 μL wash buffer by sonication with 4 rounds of 10 sec (12 micron amplitude) bursts and 50 sec rests on ice [Note 2].

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1. The centrifugal force was determined to pellet more than 99% of proteasomes while minimizing the co-isolation of other proteins. The optimal centrifugal force may vary with preparations of different sources.
2. To reduce contaminating proteins in the proteasome-rich fraction, resuspend and pellet proteasomes again by further centrifugation at 150,000 × g for 1 h and repeat step 5.

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Table 1.

Cell lines expressing tagged-proteasome subunits

Tag	Tagged-Subunit	Strain	Source	Reference
3XFLAG	Dss1	HEK293F	Human	(13)
3XFLAG	β4	Myoblast	Human	
HTBH *	Rpn11	HEK293	Human	(12)
3xFLAG	Rpn11	YYS40	S. cerevisiae	(10)
Protein A	Rpn11	SDL66	S. cerevisiae	(11)
Protein A	β4	Y-SDL135	S. cerevisiae	(9)

* The HTBH tag consists of two hexahistidine tags, a TEV cleavage site, and a bacterially derived peptide that induces biotinylation in vivo (12).

Table 2.

Nearly all the proteasomes can be isolated by differential centrifugation at 150,000 × g for 1 h. Ub conjugates and shuttling factors (e.g. Rad23b and Ddi2) are co-isolated from normal cells. However, in the absence of Ub conjugates (after E1 Ub-activating enzyme inhibitor MLN7243 treatment), the levels of Rad23b and Ddi2 in the proteasome-rich fraction decreased markedly. HEK293 cells were treated with 5 μM of the E1 inhibitor (MLN7243) for 1 h. Proteasome-rich fractions were prepared by the differential centrifugation method. Levels of proteins in total cell extracts and proteasome-rich fractions were analyzed by infrared Western blot. These data represent proteins recovered in the proteasome-rich fraction as a percentage of proteins from the cell extract.

Subunits and proteasome associated proteins	Untreated Control	After depletion of Ub conjugates
Ub conjugates	81 +− 4%	Not detectable
Rpt6 (19S)	96%	98%
β5 (20S)	100 +− 0%	100 +− 0%
Rad23b	33 +− 4%	9 +− 2%
Ddi2	23 +− 4%	11%
p97 (VCP)	95 +− 5%	98%