Chapter 12

Determination of Proteasomal Activities

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Abstract

The proteasome is an important component of the intracellular system for the turnover of proteins. Themammalian proteasome is engaged to degrade a bulky fraction of soluble intracellular proteins both in an ubiquitin-dependent and independent manner. The proteasome is composed by a central catalytic core – the 20S proteasome – where three different proteases are located, whose activities can be measured. A detailed protocol for measuring accurately the three activities of the 20S proteasome in cell and tissue homogenates, using specific fluorogenic substrates and a microplate reader fluorometer, are described. Successful applications of this method include determining changes in the proteasomal activities during aging, anti-aging interventions, cell cycle analysis, and in various disease states including neurodegenerative diseases and cancers.

Key words: 20S Proteasome, Turnover of proteins, Chymotrypsin-like activity, Trypsin-like activity, Caspase-like activity, Microplate reader fluorometer

1. Introduction

In order to maintain cellular homeodynamics, macromolecules within a cell are submitted to constant repair and turnover. In the case of proteins, cells have several mechanisms of turnover, which include the ubiquitin and non-ubiquitin proteasome-mediated pathways, calpains or calcium-dependent proteases, and lysosome-mediated pathways, such as macroautophagy, microautophagy, and chaperone-mediated autophagy. The proteasome system represents about 1% of the total cellular protein, is present both in the cytosol and the nucleus of mammalian cells, and is responsible for the degradation of a large portion of soluble intracellular proteins (1, 2). It is a multicomponent enzymatic system incorporating different regulators and the catalytic core – the 20S proteasome.

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Mammalian 20S proteasome is composed of two alpha and beta rings in the order αββα, arranged in a barrel-shaped structure of about 700 kDa. Each ring contains seven different subunits (α1-α7 or β1-β7) totalizing two each of different 14 types of subunits (3). The protease active site of β subunits in the inner rings is located inward to form the proteolytic chamber (4). The polypeptide substrate enters the opening formed by the outer rings, and it is degraded by the proteolytic chamber in small peptides (2–30 amino acids), which are further degraded by intracellular peptidases (3, 5). The 20S proteasome core alone is able to recognize and degrade oxidized and unfolded proteins in an ATP-independent and ubiquitin-independent manner, being important for the rapid removal of damaged proteins following oxidative stress (2, 3, 6, 7). It has been suggested that the exposure of hydrophobic amino acids at the surface of the proteins after being damaged may represent the recognition signal for the degradation by the 20S proteasome (5, 6).

The activity of the proteasome needs to be controlled in view of its abundance in the cytosol, and therefore, the 20S proteasome is strictly regulated. Different regulators influence the activity of the 20S proteasome, of which the 19S and the 11S regulators are the most understood (3). The 19S regulator is able to bind the 20S core proteasome on each side forming the 26S proteasome complex with a molecular weight of 2,000 kDa (4). The 26S proteasome is mainly responsible for the recognition and ATP-dependent degradation of poly-ubiquitinated proteins, although it also degrades non-ubiquitinated proteins. The 19S regulator is composed of a lid and a base-ring responsible for the recognition and ubiquitin recycling by poly-ubiquitin-cleaving hydrolases and the ATP-dependent unfolding of polyubiquitinated proteins, respectively, which are then degraded by the 20S core of the 26S proteasome (5, 7). Besides the removal of abnormal proteins, the ubiquitin system is believed to be responsible for the rapid degradation of normal short-lived proteins, such as cyclins, cyclin-dependent kinases, transcription factors, cell surface receptors, and structural proteins, important for the synchronized continuation of many cellular processes, including cell cycle progression, stress response, and cell differentiation (1, 7).

The 11S regulator is a hexa-or hepta-meric ring structure that also binds to either end (α-ring) of the 20S core proteasome forming an 11S-20S-11S structure. This complex is inducible by the interferon-γ and is specially expressed in tissues involved in immune response, and is therefore also known as the immunoproteasome (3, 8). Interferon-γ also induces three alternative subunits of the inner ring of the 20S proteasome core – βi subunits, which exchange constitutive catalytic active units in the β-rings, modifying and enhancing the catalytic activity of the proteasome. The immunoproteasome containing all three βi subunits

produces peptides with about 8–10 amino acids that are suitable to be presented as antigen by the class I major histocompatibility complex (MHC-I) (3). The immunoproteasome is mainly located in the cytosol, but there is a special nuclear 11S regulator that is not induced by interferon-γ, which is particularly abundant in the brain (5, 8).

It was shown that the different proteasome forms might be distributed in the cells, and also a hybrid proteasome (11S-20S-19S) was identified (9). These studies have also shown that the free 20S proteasome levels exceed all other forms by two- to threefold. All forms with a regulator attached to the α-rings, where the 20S proteasome core and the regulators are superimposed opening a channel into the proteolytic chamber, result in increased activity (3).

In the eukaryotic 20S proteasome, only three β-subunits in each of the inner rings possess catalytic activities: β1 – a peptidyl-glutamyl-peptide-hydrolase or caspase-like activity that cleaves after acid residues such as glutamic acid; β2 – a trypsin-like activity that cleaves after basic residues like arginine or lysine; and β5 – a chymotrypsin-like activity that cleaves after large hydrophobic residues such as tyrosine or phenylalanine (4, 5, 10). The active center of each of these subunits is a threonine residue at the *N*-terminus of their protein chain, where the hydroxyl group acts as nucleophile defining a new type of protease as compared with serine and cystein proteases (10).

As stated above, the composition of β-subunits in the inner ring can be modified to form the immunoproteasome by incorporating inducible forms by de novo synthesis replacing their constitutive counterparts. These modify proteasome peptidase activities by increasing chymotrypsin-like and trypsin-like activities and decreasing caspase-like activity, which would be important to produce peptides with higher affinity for MHC class I complex (5).

Several specific proteasome inhibitors have been developed. Some widely used inhibitors are natural compounds such as epoxomycin and lactacystin, which act as irreversible inhibitors (5). Also, peptide aldehydes have been synthesized, such as Cbz-Leu-Leu-Leucinal (MG132), which are competitive inhibitors, and are able to enter cells. It was shown that MG132 is a more potent inhibitor against the activated 20S core than against the 26S proteasome. Nevertheless, it reduces the degradation of ubiquitin-conjugated proteins by the 26S complex without affecting its ATPase or isopeptidase activities (4). However, MG132 has also some affinity to inhibit lysosomal and calcium-activated proteases (4, 11).

1.1. Method-Background for Estimating Proteasome Activity

The three peptidase activities of the proteasome can be assayed using fluorogenic synthetic peptide substrates, which provide a convenient and sensitive way to monitor the proteasome activity in crude homogenates and purified fractions. These substrates

consist of a bulky blocking group on the *N*-terminus followed by a three to four amino acid residue peptides with a fluorophore, usually amido-4-methylcoumarin (AMC), at the *C*-terminus. The proteasome cleaves an amido bond between an amino acid and the fluorophore, resulting in the release of a highly fluorescent product that can be followed by fluorometry (12). Depending on the amino acid sequence of the synthetic peptide, the three proteasome activities can be distinguished. Some of the most commonly used substrates are succinyl-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-amc) for chymotrypsin-like activity, *N*-*t*-butyloxycarbonyl-Leu-Ser-Thr-Arg-AMC (Boc-LSTR-amc) for trypsin-like activity, and *N*-benzyloxycarbonyl-Leu-Leu-Glu-AMC (Z-LLE-amc) for caspase-like activity, respectively (10).

The different peptidase activities of the proteasome are optimal at neutral to weakly alkaline pH values depending on the substrates, but buffers at pH 7.5–8.0 work well for assaying proteasome activities (13). When the proteasome activity is determined in crude homogenates, and since the substrates are not 100% specific for the proteasome, other intracellular proteases may cleave the peptides giving overestimated results. Therefore, the non-specific proteolysis is commonly determined in the presence of a proteasome inhibitor, such as lactacystin or MG132, although care has to be taken in the interpretation of the results, since other proteases might be also inhibited by these reagents (11, 13).

To measure the ATP-dependent proteasome activity (26S proteasome), crude homogenates and proteasome activity have to be performed in the presence of ATP and 10–20% glycerol or 0.25 mM sucrose to isolate integral 26S proteasome and to stabilize the complex, respectively (12, 14). In addition, KCl is also needed to further decrease the contribution of the 20S proteasome to peptide cleavage by suppressing its spontaneous activation (14). It was demonstrated that different concentrations of ATP in the assay method affect differentially the proteasome activity, and, therefore, for each case, the concentration of ATP for optimal determination of 26S proteasome activity should be calculated (14). A detailed method for measuring 26S proteasome in purified proteasome fractions and in crude homogenates can be found in (12).

A detailed protocol for measuring different activities of the 20S proteasome in a microplate reader fluorometer using fluorogenic substrates is presented. This protocol is modified from combining protocols from other papers, and from our own experience (15–17).

2. Materials

2.1. Buffers and Stock Solutions

1. Proteasome Assay Buffer (PAB): 10 mM HEPES-NaOH, pH 8.0, 50 mM NaCl, 1 mM Na_2EDTA, 250 mM sucrose. Store solution at 4°C.
2. Proteasome Lysis Buffer (PLB): PAB with 0.2% Triton X-100 added fresh. Keep at 4°C.
3. Stock solutions of fluorogenic substrates: Commercially available fluorogenic substrates (e.g., Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO) at 10 mM: Suc-LLVY-amc, Boc-LSTR-amc and Z-LLE-amc. Store in aliquots at −20°C and protect from light.
4. Stock solution of MG132: dissolved in DMSO at 20 mM. Store in aliquots at −20°C.

2.2. Assay Solutions

Make the following assay solutions just before use in the 20S proteasome activity assays (see Subheading 3.2):

1. Assay Buffer (AB): Add DMSO to PAB to a final concentration of 2% (20 µl of DMSO to each 1 ml of PAB).
2. Inhibitor Buffer (IB): For each 1 ml of PAB, add 20 µl of stock solution of MG132 (20 mM in DMSO). Final concentration of the inhibitor in the assay will be ~35 µM.
3. Substrate Solutions (protect from light):
    1. *Chymotrypsin-like activity*: Add 20 µl of Suc-LLVY-amc stock for each 1 ml of PAB. Final concentration of the substrate in the assay will be ~21 µM.
    2. *Trypsin-like activity*: Add 32 µl of Boc-LSTR-amc stock for each 1 ml of PB. Final concentration of the substrate in the assay will be ~34 µM.
    3. *Caspase-like activity*: Add 100 µl of Z-LLE-amc stock for each 1 ml of PB. Final concentration of the substrate in the assay will be ~105 µM.

3. Methods

3.1. Sample Preparation

For measuring the three proteasomal activities in triplicate and for protein estimation, about 100–200 mg of tissue or $0.5–3 \times 10^6$ cells (depending on cell size) will be sufficient, following the procedures outlined below.

3.1.1. Tissues/Organs

1. Immediately after removing the organ/tissue rinse it with ice-cold PBS (phosphate buffer saline), mince with scissors (~2 mm^3 portions), and then immerse and rinse in ice-cold PBS.
2. Homogenize tissue with ice-cold PLB in a ratio 1:5 (5 ml PLB/g of tissue) using a Polytron homogeneizer (~10 passes).
3. Centrifuge the homogenate for 5 min at 500 × *g* at 4°C and take the supernatant to a tube. Rehomogenize the pellet and centrifuge as above.
4. Combine both supernatants and centrifuge for 10 min at 10,000 × *g* at 4°C and take the supernatant to a clean tube. Maintain the homogenate in ice for protein quantification.
5. After protein quantification measure proteasome activities (see Subheading 3.2 and Note 1).

3.1.2. Culture Cell Monolayers

1. Treat cells in culture as desired.
2. Wash cell monolayer twice with PBS or Hanks solution.
3. Add ice-cold PLB, spread it through the cell layer, and keep in ice for 5 min (see Note 2).
4. Scrap the cell layer with the PLB to a microtube and keep on ice.
5. Sonicate the cell homogenate twice for 6sec at lower amplitude with an interval of 2 min on ice between sonications.
6. Centrifuge the cell homogenate for 10 min at 10,000 × *g* at 4°C and take the supernatant to a clean microtube. Maintain the homogenate in ice for protein quantification and then perform the proteasome activity assays (see Subheading 3.2 and Note 1).

3.1.3. Cell Pellets

1. Cell monolayers described above (see Subheading 3.1.2) can be scrapped in PBS or Hanks solution instead of PLB. In that case, centrifuge scrapped cells at 1,000 × *g* at 4°C and keep the cell pellet. Cells that grow in suspension can also be washed with two cycles of centrifugation and resuspension using PBS or Hanks solution at 4°C to obtain a clean cell pellet (see Note 3).
2. Add ice-cold PLB to cell pellet and resuspend it. Maintain in ice for 5 min (see Note 4).
3. Continue the protocol as described above after Step 5 in Subheading 3.1.2.

3.2. 20S Proteasome Activity Assays

1. Set the microplate reader fluorometer to 37°C (see Note 5 and 6).
2. Pipette in triplicate for a flat-bottom black 96-well plate 10 µg of protein for each sample (see Note 7 and 8).

3. For 2 wells of each sample, pipette 200 µl of AB using a repeating pipette. To the other well for the same sample, pipette 200 µl of IB.
4. Incubate at 37°C for 10 min in the fluorometer (see Note 9).
5. Meanwhile, prepare the substrate solution to the proteasome activity that will be measured (see substrate solutions in Subheading 2.2).
6. After pre-incubation, take the plate from the fluorometer and using a repeating pipette, add 25 µl of the chosen substrate solution (e.g., Suc-LLVY-amc substrate solution for measuring the chymotrypsin-like activity).
7. Mix the plate for 5–10 s using the built-in automixing feature of the microplate reader fluorometer and read the fluorescence intensity along the time using ex_{380nm}/em_{460nm} (AMC) in the fluorometer for around 20 min in 1 min intervals at 37°C.
8. Take the slope (reaction kinetic) obtained from each sample with and without the proteasome inhibitor.

3.3. Calculation of the Proteasome Activity

To express any peptidase activity of the proteasome, the slopes of the linear kinetics of the reaction are taken by using the instrument software. The slope obtained in the replicate measured in the presence of MG132 is subtracted to the average of the other two replicates to eliminate the proteasome non-specific activity. This value can then be expressed as percentage of the sample considered as the control of the experiment.

Instead of the relative activity between the samples, the results can be expressed as specific activity of the enzyme by using the fluorescence intensity (FI) of one AMC standard obtained in the same final volume of the assay and the following equation:

Specific activity [nmol AMC/min/mg] = [slope (FI/min) × AMC standard concentration (µM) × volume of assay (ml)]/[fluorescence of AMC standard (FI) × quantity of protein (mg)].

An example is given for understanding how proteasome specific activity is calculated in an assay performed with 10 µg of protein and in a final volume of 230 µl:

1. Calculate proteasome activity:
   1.1. Slope of activity measured without MG132: 22 FI/min
   1.2. Slope of activity measured with MG132: 2 FI/min
   1.3. Proteasome activity will be: 22-2 = 20 FI/min
2. Measure fluorescence of an AMC standard:
   2.1. Fluorescence of 5 µM AMC standard: 330 FI
   2.2. Fluorescence of blank: 30 FI
   2.3. AMC standard fluorescence will be: 330-30 = 300 FI

3. Calculate proteasome specific activity using previous equation:

$$\text{Proteasome specific activity} = (20 \times 5 \times 0.23)/(300 \times 0.01) = 7.67 \text{ nmol AMC/min/mg}$$

4. Conclusions

With the present protocol, the three different activities of the proteasome can be estimated in cell lysates using fluorogenic substrates. By measuring the 20S proteasomal activity in cell homogenates, many sources of interferences may occur, such as due to the use of the general proteasome inhibitor MG132 that may also inhibit other proteases, or due to the lack of 100% specificity of the proteasome substrates. However, if the protocol is performed as described above and by using the correct experiment controls run in parallel with the treatment conditions, it will give effective estimation of any changes in proteasomal activity. In addition, in opposition to the end-point measurements, estimation of proteasomal activities in a microplate reader fluorometer by following the kinetics of the reaction along the time will increase the accuracy of the assay, if care is taken to pipette precise volumes of the different solutions and protein.

Finally, it may be noted that the above protocols for the estimation of proteasomal activities have been successfully applied for determining changes during aging, anti-aging interventions, cell cycle analysis, and in various disease states, including neurodegenerative diseases and cancers (15–18).

5. Notes

1. Clean supernatant can be stored in aliquots at −80°C (or −20°C for shorter time) before proteasome activity assays, but it will decrease the activity by about 10% per each thawing. Avoid repeated freeze/thaw cycles of the sample.
2. As a guide, use 300 µl of PLB for each T75 flask or 60 µl per well of a 6-well plate. The final concentration of protein of the homogenate should be higher than 1 mg/ml, otherwise decrease the added PLB to the samples accordingly.
3. Cell pellets can be then either immediately used for preparation of cell homogenates, or stored at −80°C for later homogenization and proteasome activities assays.
4. As a guide, use 250 µl of PLB for a cell pellet taken from a T75 flask. The final concentration of protein of the homogenate

should be higher than 1 mg/ml, otherwise decrease the added PLB to the samples accordingly.

5. If a microplate reader fluorometer is not available, a kinetic assay can be replaced by end point measurements. For that, make the assay as mentioned in the Subheading 3.2 in microtubes at 37°C (protecting from light) and stop the reaction with 300 μl of an acidic solution (30 mM sodium acetate, 70 mM acetic acid, 100 mM sodium chloroacetate, pH 4.3). After addition of 2 ml of distilled water, read the fluorescence in a fluorometer with the proper excitation and emission filters. The time of incubation has to be previously tested and has to be within the linear response.
6. To calibrate the plate reader fluorometer, a calibration curve with a range of AMC concentrations (0.1–v10 μM) has to give a linear response.
7. The quantity of protein necessary for accurate results for each peptidase activity should be determined previously for each cell type or organ/tissue with preliminary assays. The correct protein load should give a linear response along the time (at least in the first 30 min) and not exceed during the assay 1.5× the signal generated by 5 μM of AMC standard. In addition, to increase accuracy, the chosen protein quantity should be pipetted in a similar volume between samples, that is, different samples should have similar protein concentration, preferably higher than 1 mg/ml. If the pippetted volume of sample differs much between samples, since the fluorescence intensity also depends on the height of the liquid in a well, the comparison of the results between different samples may be affected.
8. Pipet also a standard curve, or at least one standard within the linear range (e.g., 5 μM AMC), if the results are going to be given in nmol of AMC produced/min/mg protein (see Subheading 3.3). For the standards, one should take the fluorescent intensity in an endpoint measurement, and not the slope in a kinetic assay as in the samples.
9. This pre-incubation procedure will allow AB and IB to stabilize at 37°C and also to give time to the MG132 to completely inhibit proteasome.

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