Analytical Biochemistry 446 (2014) 44–52

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Crude and purified proteasome activity assays are affected by type of microplate

Ziyou Cui [a], Jennifer E. Gilda [a], Aldrin V. Gomes [a,b,*]

[a] Department of Neurobiology, Physiology, and Behavior, University of California, Davis, CA 95616, USA
[b] Department of Physiology and Membrane Biology, University of California, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 20 August 2013
Received in revised form 4 October 2013
Accepted 10 October 2013
Available online 17 October 2013

Keywords:
Proteasome
Microplate
Proteolytic activity
Betulinic acid

ABSTRACT

Measurement of proteasome activity is fast becoming a commonly used assay in many laboratories. The most common method to measure proteasome activity involves measuring the release of fluorescent tags from peptide substrates in black microplates. Comparisons of black plates used for measuring fluorescence with different properties show that the microplate properties significantly affect the measured activities of the proteasome. The microplate that gave the highest reading of trypsin-like activity of the purified 20S proteasome gave the lowest reading of chymotrypsin-like activity of the 20S proteasome. Plates with medium binding surfaces from two different companies showed an approximately 2-fold difference in caspase-like activity for purified 20S proteasomes. Even standard curves generated using free 7-amino-4-methylcoumarin (AMC) were affected by the microplate used. As such, significantly different proteasome activities, as measured in nmol AMC released/mg/min, were obtained for purified 20S proteasomes as well as crude heart and liver samples when using different microplates. The naturally occurring molecule betulinic acid activated the chymotrypsin-like proteasome activity in three different plates but did not affect the proteasome activity in the nonbinding surface microplate. These findings suggest that the type of proteasome activity being measured and sample type are important when selecting a microplate.

The microplate is a critical component of many proteolytic assays as well as many high-throughput screens. Understanding the characteristics of different microplates is important for optimal performance when developing assays because different microplates have different inherent compositions. A comparison of four different black 96-well plates showed that proteasome assay performance is directly influenced by the properties of the microplate. Black microplates are used for fluorescence measurements due to their low autofluorescence, reduced well-to-well crosstalk, and ability to absorb scattered light, resulting in enhanced fluorescence signals.

A frequently used assay in our laboratory, and one that is becoming common in other laboratories, is the measurement of proteasome proteolytic activity [1–3]. The proteasome is a large complex composed of a 28-subunit 20S core that contains three different proteolytic activities (caspase-like activity, β1; trypsin-like activity, β2; and chymotrypsin-like activity, β5) [4,5]. The 20S core can exist on its own but is normally bound to one or two 19S regulatory complexes, forming the complex commonly referred to as the 26S proteasome. The 26S proteasome is the main proteolytic enzyme responsible for degradation of unwanted intracellular proteins in eukaryotic cells. Measurement of proteasome activity is important because proteasome dysfunction has been implicated in several diseases, including Alzheimer's disease, Parkinson's disease, cancer, and heart failure [6–10].

The most common method for determining the activity of the proteasome is by incubating the sample of interest with a fluorescently labeled proteasome substrate in the presence and absence of a specific proteasome inhibitor in a black 96-well microplate [11]. The most commonly used proteasome substrates are the peptides labeled with fluorescent 7-amino-4-methylcoumarin (AMC).[1] The excitation and emission spectra of free AMC are different from those of bound AMC, allowing AMC released by proteolytic cleavage (free AMC) to be easily measured with a fluorometer. Fluorescence measurements in black 96-well plates are common in applications where greater sensitivity than that achievable by simple absorbance measurements is required.

* Corresponding author at: Department of Neurobiology, Physiology, and Behavior, University of California, Davis, CA 95616, USA. Fax: +1 530 752 5582.
E-mail address: avgomes@ucdavis.edu (A.V. Gomes).

[1] *Abbreviations used:* AMC, 7-amino-4-methylcoumarin; BA, betulinic acid; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; NP-40, Nonidet P40; SDS, sodium dodecyl sulfate; DC, detergent-compatible; BSA, bovine serum albumin; GMBS, Greiner medium-binding surface; GHBS, Greiner high-binding surface; CMBS, Costar medium-binding surface; CNBS, Corning nonbinding surface.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.10.018

Several kits for measuring chymotrypsin-like proteasome activity only are commercially available, with some of them providing the microplate as well. As more labs use proteasome assays, an important variable to consider is the microplate used. Although the source of the substrate and inhibitor for the proteasome assays are usually stated in publications, the source of the microplate is often not given. There are many manufacturers of black 96-well microplates, and among them they offer a huge variety of plates. As such, many scientists use any available black microplate; however, there are many features that are important to take into consideration. The orientation of proteins in plate wells is unpredictable, and the binding between enzymes and substrates is a complex process affected by many parameters. Three main types of binding surfaces are available: non- or low-binding, medium-binding, and high-binding. Many microplates are composed of polystyrene, a polymer composed of a carbon chain with benzene rings placed at every other carbon, which provides a hydrophobic binding surface. The untreated polystyrene is referred to as medium-binding. Different treatments are performed to alter the structure of the polystyrene and, hence, the binding properties of the plate. The nonbinding surface has been treated to create a nonionic hydrophilic surface that is similar in structure to polyethylene oxide, which has a low affinity for proteins [12]. The high-binding surface has been treated so that some benzene rings have been modified to create hydrophilic groups, which generally increases binding of polar proteins [13]. To investigate whether the microplate chosen significantly affects proteasome activity measurements, four different sets of black 96-well flat-bottomed polystyrene plates were compared: one nonbinding microplate, two medium-binding microplates, and one high-binding microplate. The four different types of plates were used to determine proteasome activities in heart and liver cytosolic lysates and purified 20S proteasomes as well as the effect of different plates on standard curves using free AMC. The effect of betulinic acid (BA), a potential antitumor small molecule, on purified 20S proteasomes was also investigated and found to be affected by the plate used. Our results suggest that the type of microplate used affects proteasome activity measurements and demonstrate the problems associated with different microplates when comparing proteasome activity between different tissues.

Materials and methods

Reagents

BA (high purity) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Purified 20S murine proteasome was obtained from Boston Biochem (Cambridge, MA, USA). Fluorotrac 200 (medium-binding, cat. no. 655076) and Fluorotrac 600 (high-binding, cat. no. 655077) were obtained from Greiner Bio-One (Germany). Costar nontreated surface (medium-binding, cat. no. 3915) and Corning nonbinding surface (cat. no. 3991) were purchased from Corning (Corning, NY, USA).

Heart and liver lysate preparation

Livers and hearts from 3-month-old rats were obtained from Pel-Freez (Rogers, AR, USA). Tissues were minced with a razor blade and then immediately homogenized using a dounce homogenizer with 2 ml buffer/0.1 g tissue of 26S buffer (50 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA], 150 mM NaCl, and 5 mM $MgCl_2$, pH 7.5) and freshly added dithiothreitol (DTT, 0.5 mM) and subsequently centrifuged at 12,000*g* for 30 min at 4 °C. The supernatant (cytosolic lysate) was quantified using a Nanodrop 2000C (Thermo Scientific), diluted to 2 μg/μl in 26S buffer, and stored at −80 °C in small aliquots. Each aliquot was used for one set of assays only (no freeze–thaw cycles).

Lysate 26S proteasome activity assay

The 26S proteasome assay was carried out in a total volume of 100 μl in 96-well plates with 100 μM adenosine triphosphate (ATP) in 26S buffer using 20 μg of protein supernatants. Assays were initiated by the addition of fluorescently labeled substrate: succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC), Boc-Leu-Ser-Thr-Arg-AMC (Boc-LSTR-AMC, Bachem), and Z-Leu-Leu-Glu-AMC (Z-LLE-AMC) for chymotrypsin-like (β5), trypsin-like (β2), and caspase-like (β1) activity measurements, respectively. The final concentration of substrate in each assay was 100 μM, as described previously [1,3,14].

These substrates are cleaved by the proteasome, releasing free AMC, which was then measured spectrofluorometrically using a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) at an excitation wavelength of 390 nm and an emission wavelength of 460 nm. Fluorescence was measured at 15-min intervals for 2 h. All assays were linear in this range. Each assay was conducted in the absence and presence of the specific proteasomal inhibitor bortezomib (LC Laboratories, Woburn, MA, USA): 20 μM for chymotrypsin-like (β5) activity and 100 μM for trypsin-like (β2) and caspase-like (β1) activities.

To properly compare different plates, all of the samples and reagents used were aliquoted into small aliquots, kept at −20 °C (β1, β2, and β5 substrates and AMC standard) or −80 °C (heart and liver lysates, 20S proteasomes, bortezomib, and ATP), and thawed 30 min before use.

Lysate 20S proteasome activity assay

The 20S proteasomal activities in heart and liver lysates were determined in a similar fashion to the 26S assays, using the same substrates and inhibitors but a different buffer. Assays were carried out in buffer containing 25 mM Hepes, 0.5 mM EDTA, 0.05% (v/v) Nonidet P40 (NP-40), and 0.001% (w/v) sodium dodecyl sulfate (SDS).

Purified 20S proteasome activity

Measurement of purified murine 20S proteasome activities were carried out in a total volume of 100 μl in 96-well plates with 20 mM Tris buffer (pH 7.5). Assays were initiated by the addition of fluorogenic substrates as described for tissue lysates. No proteasome inhibitors were used for these assays. All other conditions were similar to the 26S proteasome measurements. For measurements in the presence of BA, BA (10 μg/ml final concentration) was added to the proteasome and incubated for 20 min before the addition of the proteasome substrate.

AMC standard curve

AMC standard curves were obtained by making dilutions ranging from 0 to 500 nM and from 0 to 8 μM. Measurement of free AMC fluorescence values was carried out in a total volume of 100 μl in 96-well plates using 20 mM Tris buffer (pH 7.5).

Protein concentration determination

Protein concentrations were determined using four independent methods: (i) the Bradford assay (Bio-Rad Bradford reagent), (ii) the detergent-compatible (DC) assay (Bio-Rad DC kit), (iii) absorbance at 280 nm using a bovine serum albumin (BSA)