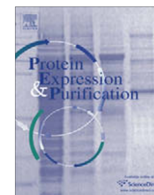
Protein Expression and Purification 67 (2009) 156–163

Contents lists available at ScienceDirect

Protein Expression and Purification

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



Rapid purification method for the 26S proteasome from the filamentous fungus *Trichoderma reesei*

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A R T I C L E I N F O

Article history:
Received 4 March 2009
and in revised form 22 April 2009
Available online 19 May 2009

Keywords:
26S proteasome
Trichoderma reesei
Filamentous fungus
Anion exchange chromatography
Purification

A B S T R A C T

We have developed a fast and simple two column chromatographic method for the purification of the 26S proteasome from the filamentous fungus *Trichoderma reesei* that simplifies the overall procedure and reduces the purification time from 5 to 2.5 days. The combination of only the anionic exchange POROS® HQ column (Applied Biosystems) together with a size exclusion column has not been used previously for proteasome purification. The purified complex was analysed further by two-dimensional electrophoresis (2DE) and examined by transmission electron microscopy (TEM). A total of 102 spots separated by 2DE were identified by mass spectrometry using cross-species identification (CSI) or an in-house custom-made protein database derived from the *T. reesei* sequencing project. Fifty-one spots out of 102 represented unique proteins. Among them, 30 were from the 20S particle and eight were from the 19S particle. In addition, seven proteasome-interacting proteins as well as several non-proteasome related proteins were identified. Co-purification of the 19S regulatory particle was confirmed by TEM and Western blotting. The rapidity of the purification procedure and largely intact nature of the complex suggest that similar procedure may be applicable to the isolation and purification of the other protein complexes.

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Introduction

The 26S proteasome is a 2.5 MDa proteinase complex found in the cytoplasm and nuclei of all animal, plant and fungal cells [1]. It is involved in a multitude of cellular processes including cell differentiation, signal transduction, cell-cycle control, regulation of metabolic activity and stress response. This organelle functions to remove and recycle misfolded proteins as well as intracellular regulatory proteins rapidly and selectively [2,3]. Eventually, 80–90% of total cellular proteins are degraded by the 26S proteasome [4]. Depending on protein breakdown rates, the proteasome can constitute up to 1% of the cellular protein content [5].

The 26S proteasome is composed of at least 32 different subunits arranged in two sub-complexes: the 20S proteolytic complex, called the core particle (CP)[1] and the 19S regulatory particle (RP). The cylindrical structure of the 20S proteasome is composed of four heptameric rings of α and β-subunits. The two outer rings consist of seven α-subunits while the two central rings are composed of seven β-subunits. The centre of the multicatalytic protease chamber contains three subunits which perform the proteolytic function. Subunit β1 has peptidyl-glutamyl peptide-hydrolysing activity, subunit β2 has trypsin-like activity and subunit β5 has chymotrypsin-like activity [6,7]. The exact number of 19S complex subunits in different organisms is unknown. In yeast, as well as mammals, 19 different subunits have been identified [8,9].

The 19S RP associates with the 20S proteasome via an ATP-dependent reaction to form an active 26S complex. The RP of the 26S proteasome contains six ATPases of the "AAA" family (RPT; for Regulatory Particle Triple-A ATPase subunit) and four non-ATP-

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[1] *Abbreviations used:* CP, core particle; RP, regulatory particle; WB, washing buffer; IEF, isoelectric focusing; IPG, immobilized pH gradient; TEM, transmission electron microscopy; UPR, unfolded protein response; PiPs, proteasome-interacting proteins; CDC, cell division control.

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doi:10.1016/j.pep.2009.05.007

ase subunits (RPN), which form the base complex at the interface between the 19S RP and 20S CP [10]. The ATPases play a role in substrate binding and unfolding and assist in the translocation of the unfolded substrate into the 20S proteasome cavity [11]. The lid of the 19S RP is composed of nine non-ATPase subunits and its major activity is proposed to be deubiquitination of proteins. Therefore, the lid is required for the recognition and degradation of ubiquitinated target proteins [10].

The unicellular yeast *Saccharomyces cerevisiae* is an acknowledged model of a eukaryotic cell and the majority of published purification methods for the 26S proteasome come from yeast studies [8,11,12]. 26S proteasomes have been isolated from higher plants (e.g. [13,14]), mammalian cells (e.g. [15,16]) and from yeast [8,17]. Isolation of the 20S proteasome particle from a filamentous fungus also has been reported [18]. While the earlier proteasome purification methods involved the application of glycerol gradients, PEG precipitation followed by multistep chromatography or His-tags combined to immunochromatography, more sophisticated high affinity chromatography systems can be applied to simplify this process. We present here a simple and rapid isolation and purification method for the entire and enzymatically active 26S proteasome from the industrially exploited filamentous fungus *Trichoderma reesei*. This method can be applied to the purification of other large protein complexes of interest.

Materials and methods

Strain and cultivation

T. reesei strain RutC-30 [19] was grown in five 2 L conical flasks (500 mL culture volume) in minimal medium [20] supplemented with 2% glycerol (v/v) at 28 °C for 48 h, with shaking at 250 rpm.

Isolation of the 26S proteasome

T. reesei mycelia were harvested by centrifugation at 4000*g* for 20 min, washed three times with distilled water and placed in two times the sample volume of buffer A (50 mM Tris, pH 7.5, containing 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 10% (v/v) glycerol, 0.1% (v/v), and fungal and yeast protease inhibitor cocktail [Sigma, P8215]). Complete cell lysis was achieved by twice passing the fungal mycelia through a French Press (French® Press Cell Press, Thermo Scientific, USA) at 15,000 psi. The extract was centrifuged at 4000*g* for 30 min and the supernatant spun at 100,000*g* for 1 h at 4 °C. The final supernatant was filtered through Miracloth (Calbiochem®) and a membrane filter (pore size 0.2 μm) to remove lipid material. The extract was concentrated to 6 mL using an Ultra free concentrator with molecular weight cut-off of 5 kDa (Amicon® Ultra-15, Millipore, USA). The protein concentration of the fungal extract was determined using a modified Bradford method [21] with bovine serum albumin as a standard. The fungal extract was stored at −80 °C until used.

The extract was applied onto a 1 mL POROS® HQ column (anion exchange, Applied Biosystems) at 5 mL min^{-1}, using an ÄKTA Explorer 10S FPLC system (GE Healthcare), washed with 25 mL buffer B (10 mM Tris–10 mM NaH_2PO_4, pH 7.5–1 mM ATP) at 5 mL min^{-1} before eluting using a linear salt gradient of 0–1 M NaCl in buffer B at 5 mL min^{-1}. One milliliter fractions were collected and assayed for chymotrypsin activity using a fluorometric assay (20S Proteasome Assay Kit AK-740, Biomol, USA) following the manufacturers' instructions. Fractions positive for chymotrypsin-like activity were pooled and concentrated as mentioned earlier. The concentrated sample was separated on a 150 mL size exclusion column containing Sephacryl S-500 HR (GE Healthcare Life Sciences, Uppsala, Sweden) at 0.5 mL min^{-1} with buffer C (1 M NaCl in buffer B). Fractions of 1 mL were collected and assayed for chymotrypsin-like activity as described above. Fractions containing high chymotrypsin-like activity (eluting approximately at 230–280 mM NaCl) were pooled and concentrated to 1 mL and stored at −80 °C until used. Protein concentration was determined as previously described.

1D SDS–PAGE and Western blotting

The purified 26S proteasome was separated on one-dimensional 12–20% (w/v) SDS–PAGE gel and protein bands were visualised with Coomassie Colloidal Blue 250G (CCB; 17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250). For Western blotting, proteins were separated by SDS–PAGE as above and blotted onto PVDF (Immobilon-P, Millipore, USA) membranes at 30 V for 1 h. Following blocking with 3% (w/v) skim milk in the washing buffer (WB; PBS–0.5% Tween 20) for 1 h, the membranes were incubated for 1 h with primary antibody (rabbit polyclonal anti-20S and five anti-19S proteasome subunits Ig, Abcam Inc., USA; 1:2000 dilution). After washing the blot with WB three times, it was incubated for 1 h with secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA; 1:500 dilution). After three washes with WB, the secondary antibody was detected with alkaline phosphatase substrate (*FAST*™ BCIP/NBT; 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma).

Isoelectric focusing and 2D SDS–PAGE

A buffer exchange was performed using a 5 kDa centrifugal concentrator to remove residual salt from chromatograph fractions prior to isoelectric focusing (IEF) and 2D electrophoresis. For each 2DE gel, a 300 μg portion of concentrated sample was purified using a 2D Clean up Kit following the manufacturer's instructions (GE Healthcare, USA). Precipitated proteins were resuspended in 200 μL of sample solution (7 M urea, 2 M thiourea, 1% (w/v) C7Bz0, 40 mM Tris, 5 mM tributylphosphine, 10 mM acrylamide, 1 mM PMSF, and 0.1% (v/v) protease inhibitor cocktail). The samples were used directly to passively rehydrate 4–7, 11 cm immobilized pH gradient (IPG) strips (Bio-Rad, CA, USA) by applying 200 μL of each sample. IPGs were focused to a total of 80,000 Volt hours (Vh) using a three-step focusing program. The focusing program included a rapid ramp to 300 V for 4 h, a linear ramp to 10,000 over 8 h, and a 10,000 V step until 80,000 Vh were reached. IPGs were equilibrated for 20 min in 6 M urea, 2% (w/v) SDS, 50 mM Tris–HCl buffer, pH 8.8, 0.1% (w/v) bromophenol blue for 20 min. The IPGs were then placed on top of Bio-Rad Criterion™ XT Precast Gel, 4–12% (Bio-Rad, CA, USA), and run at 30 mA constant until the bromophenol blue dye reached the bottom of the gel. Gels were fixed in 10% (v/v) methanol, 7% (v/v) acetic acid solution for 30 min, then stained with Coomassie colloidal blue G250 (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250).

Mass spectrometry and protein identifications

Protein spots were excised manually and gel pieces were destained and dried. Each gel spot was subjected to in-gel digestion with trypsin as described previously [18]. Mass fingerprints of tryptic peptides were generated by matrix assisted laser desorption/ionization-time of flight–mass spectrometry (MALDI-TOF–MS) using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750–3500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were fragmented by collision-induced

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