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Integrative Structural Proteomics Analysis of the 20S Proteasome Complex

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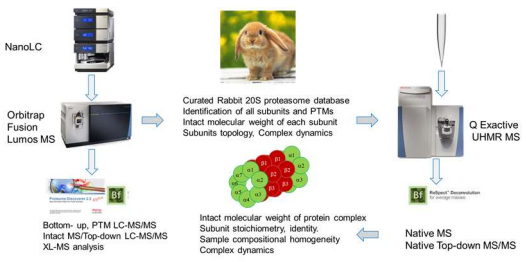
ABSTRACT

Introduction: The structural determination of protein complexes plays an important role in the fundamental understanding of their catalytic function. Multiple analytical methods, including hybrid approaches such as cryo-electron microscopy, crosslinking, and native mass spectrometry, are usually required for such type of analysis[1]. In this study we combined multiple mass spectrometry based structural proteomics techniques to characterize the rabbit 20S proteasome complex.
Methods: Rabbit 20S proteasome complex was obtained from Boston Biochem. LC-MS bottom up, crosslinking and intact/top down analysis were performed using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system and a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Mass Spectrometer. Native MS experiments were performed using a Thermo Scientific™ Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap ™ Mass Spectrometer. Data were analyzed with Thermo Scientific™ BioPharma Finder™ 3.0, Thermo Scientific™ Proteome Discoverer™ 2.3, and Thermo Scientific™ ProSightPC™ software.
Results: The 20S proteasome complex contains 28 subunits arranged into four stacked rings: seven alpha non catalytic subunits and seven beta-subunits[3]. In the first series of experiments we performed bottom-up, intact and top-down analysis using databases of proteasome proteins from rabbit, human, and all species in UniProt to create curated fasta database. We then used this curated rabbit 20S proteasome database for all other experiments. For structural characterization, we performed native MS intact and top-down experiments using a Q Exactive UHMR MS. The measured mass of the 28 subunits complex was consistent with expected value of 716 kDa and pseudo-MS3 experiments enabled unambiquous identification of alpha 6 subunit. Crosslinking experiments using DSSO are combined with restraints from native and top-down MS to validate the structure obtained through electron microscopy.

INTRODUCTION

The structural determination of protein complexes plays an important role in the fundamental understanding of biochemical pathway. Proteins interact both nonspecifically and specifically, with the specific interactions being important because of the downstream consequences. Multiple analytical methods are usually required for these kinds of analysis. These hybrid methods can consist of various structural techniques, such as cryo-electron microscopy (EM) and crosslinking or native mass spectrometry[1]. In this study we combined multiple mass spectrometry based structural proteomics techniques to characterize the rabbit 20S proteasome complex (Figure 1). The 20S proteasome is a multicatalytic enzyme complex expressed in the nucleus and cytoplasm of all eukaryotic cells and part of the 26S proteasome complex responsible for recycling of damaged, misfolded and short-lived regulatory proteins via ubiquitin-proteasome pathway[3]. The human and yeast 26S and 20S proteasome complexes are well studied and characterized[4], however there is no structure or even curated proteomics database available for rabbit proteasome complex.
Combining the results from different MS methods, including novel native top-down analysis using a pseudo-MS^3 approach[2], we were able to perform comprehensive analysis of this protein complex and develop a new workflow.

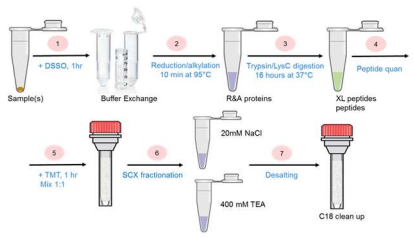
Figure 1. Overview of the study design



MATERIALS AND METHODS

Rabbit 20S proteasome complex was obtained from Boston Biochem (Boston, MA, USA). LC-MS bottom up, crosslinking and intact/top down analysis was performed using a UltiMate 3000 RSLCnano system and on the Orbitrap Fusion Lumos mass spectrometer. Peptides were separated using a 50cm Thermo Scientific™ EASY-Spray™ LC column and proteins were separated using a prototype MabPac column (150 um x 15 cm). For crosslinking, 20S proteasome complex in HEPES buffer (pH 8.0) was reacted with DSSO in a molar ratio of 1:100 (protein:cross-linker) for 1 hr at room temperature in presence or absence of 0.03% or 0.1%SDS to activate proteasomes. Reaction was quenched with 1M NH_4HCO_3. Complete sample prep workflow is outlined in Figure 2.

Figure 1. Crosslinking Sample preparation for Rabbit 20S proteasome. Buffer exchange was performed using an Amicon® centrifugal filter unit (10 kDa, EMD Millipore). Peptide concentrations were determined using the Pierce™ Quantitative Fluorometric Peptide Assay. Peptides were fractionated using polymer-based SCX spin columns with an increasing step gradient of 20mM NaCl followed by 400mM triethylammonium(TEA). SCX fractionated samples were desalted using a 70% ACN elution step on Pierce™ High pH Reversed-Phase Peptide spin columns before LC-MS/MS analysis.



Native MS experiments were performed using a Q Exactive UHMR mass spectrometer in which the ability to perform pseudo-MS^3 scans for native top-down analysis and transmission of high m/z ions were improved by implementing several hardware and software modifications, the most important being pulsed trapping of ions in the injection flatapole region ('in-source trapping') and reduction of the frequency of RF voltages applied to injection flatapole, bent flatapole, quadrupole, transfer multipole, C-trap and HCD cell (Figure 4)[4]. The data were analyzed with the BioPharma Finder 3.0, ProSightPC 4.1 and Proteome Discoverer™ 2.3 (utilizing the ProSightPD and XlinkX 2.0 node) software packages.

RESULTS

As only homology based amino acid sequences for the rabbit proteasome subunits are available, we started from bottom-up analysis using rabbit TreEMBL database. We identified 224 proteins and 17 different types of proteasome subunits with sequence coverage from as low as 25% up to as high as 90%. In the next series of experiments we performed LC-MS intact/top-down analysis using CID or EThcD on the Orbitrap Fusion Lumos MS, and identified eleven of the main subunits using rabbit fasta file. To characterize the last 3 subunits, alpha 1, 3 and 7, we created a focused UniProt flatfile database using the sequences across all species for these 3 proteins. The search results from ProSightPD were compared with the bottom-up and intact analysis data. Figure 3 and Table 1 show summary of these experiments. All alpha subunits and 2 beta subunits were N-acetylated and the alpha 3 subunit was found to be phosphorylated. Nine subunits were truncated at N-terminus and several had sequence variants. The results were combined to create curated rabbit 20S proteasome database used in subsequent studies.

Figure 3. Denaturing intact/top-down analysis of 20Ss proteasome. A. Monoisotopic deconvoluted masses. B. Example of sequence validation. Modified residues are in blue, confirmed sequence is in red and sequence from bottom-up is in bold. C. Proteoforms of alpha 3 subunits.

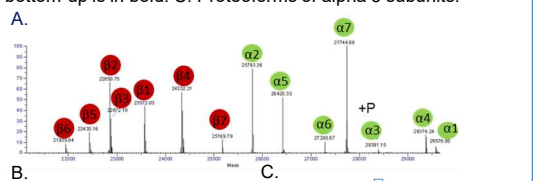


Table 1. Rabbit 20S proteasome subunits characterized by bottom-up and top-down LC-MS/MS.

Uniprot Accession	Subunits	Theo. Monoisotopic Mass (Da)	Theo. Average Mass (Da)	Modifications
G1SU71	Beta 1	23571.93	23586.97	29-241
G1T4X8	Beta 2	22850.66	22865.28	N-acetyl
G1SHV9	Beta 3	22874.43	22889.76	N-acetyl, 2-205
G1T918	Beta 4	24332.09	24347.72	46-264
G1T4Q9	Beta 5	22430.10	22443.34	63-266
G1T235	Beta 6	21939.84	21952.15	35-239
G1SWK8*	Beta 7	25169.79	25184.80	68-302
G1SDA8*	Alpha 1	29577.61	29595.61	N-acetyl
G1T2L1	Alpha 2	25793.24	25809.43	N-acetyl, 2-234
G1SZ14*	Alpha 3	28391.04	28408.83	N-acetyl, P
G1T519	Alpha 4	29374.24	29392.64	N-acetyl, 2-260
G1T670*	Alpha 5	26420.22	26437.07	N-acetyl
G1T9V4	Alpha 6	27290.76	27308.28	N-acetyl, 2-246
G1SWI7*	Alpha 7	27744.68	27761.66	N-acetyl

*Uniprot accession numbers provided only as reference as actual sequences were significantly different.

To evaluate the structure of the 20S proteasome complex we conducted native MS intact and top-down experiments using a Q Exactive UHMR MS (Figure 4). Using unique capabilities of this instrument we were able to determine the molecular weight of the intact complex with high accuracy (Figure 5, A), its composing subunits after dissociation in the injection flatapole region (Figure 5, B), and finally identify the alpha 6 subunit via quadrupole selection and fragmentation in the HCD cell (Figure 5, C), enabling sequence analysis through the MS^3 spectrum (Figure 5, D). As expected during MS^2 analysis, only the outer ring alpha subunits were ejected from the complex, all of them were identified based on accurate intact mass. Alpha 6 as well as the alpha 2 and 5 subunits were also identified by top-down using pseudo MS^3. Figure 5D shows comparison of sequence coverage obtained by native HCD top-down vs. denaturing EThcD. Results reflects difference in fragmentation techniques as well as protein conformation during analysis but in both cases alpha 6 was identified with high confidence.
The rabbit 20S proteasome complex structure is unknown. Although it is homologous to human complex, our native MS experiments demonstrate that it is not identical. We thus performed crosslinking experiments to confirm their level of similarity.
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Figure 4. Schematic of the Q Exactive UHMR MS.

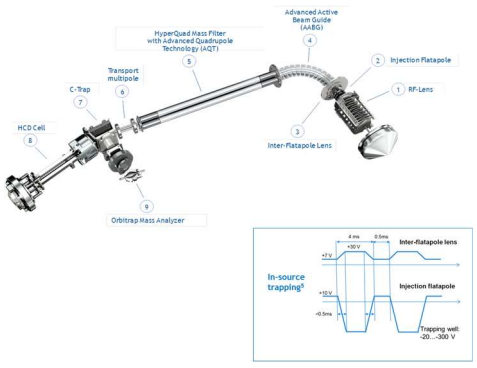


Figure 5. Native MS and MS/MS analysis of the 20S proteasome complex. A. MS^1 analysis. B. MS^2 analysis using in-source trapping. C. Alpha 6 subunit MS^3 analysis using quadrupole isolation and HCD fragmentation. D. Top-down analysis of alpha 6 subunit by HCD (native) and EThcD (denaturing).

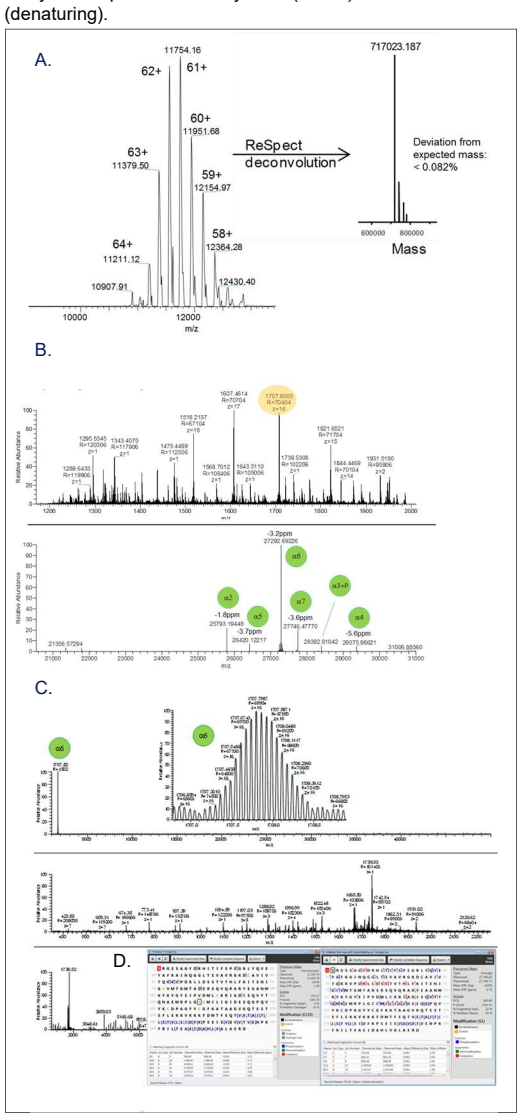
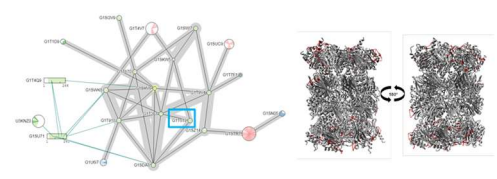
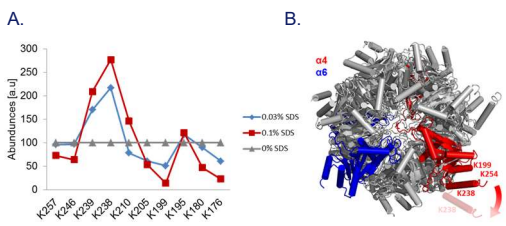


Figure 6. DSSO crosslink mapping of the Rabbit 20S proteasome. Crosslinking map was generated using *xiNET*(A) and visualization was performed using Yasara viewer (B). Lys-Lys contacts identified by DSSO crosslinking experiments mapped onto human 20S proteasome homology structure with distances (Cα-Cα) less than 30Å.



Using the DSSO crosslinker (Figure 2) we identified 150 unique crosslinked peptides, including 94 inter-protein (Figure 6, A). However we could only map 58 crosslinked sites to the human structure (Figure 6, B). Few crosslinked peptides were localized for beta or alpha 1, 3, 7 subunits as those sequences were most unique for the rabbit 20S proteasome complex. The 20S proteasome *in vitro* can efficiently degrade peptides or proteins if it is activated by addition of low concentrations of SDS[4] . Using the QMIX[5] method, we were able to confirm that SDS indeed causes some conformational changes at least in the outer alpha subunits ring for the alpha 4 subunit as shown in Figure 7. For example, we monitored changes in amount of crosslinked peptides between Lys 254 at C-terminus vs. other Lys and observed increased distance between Lys 254 and Lys 199 with increasing SDS concentration.

Figure 7. Quantitative profiling of proteasome subunit alpha 4(G1T519) using QMIX workflow upon SDS activation. Relative abundances of K254 containing DSSO crosslinked peptides (A), visualization of mapped Lys (B)



CONCLUSIONS

Combining results from different MS methods, including novel native top-down-pseudo MS^{3}approach, we were able to perform comprehensive analysis of the rabbit 20S proteasome.

The QMIX workflow for comparative structural analysis of proteins and protein complexes was successfully applied to study dynamics of rabbit 20S proteasome upon SDS activation

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TRADEMARKS/LICENSING

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