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Comparison of label-free quantification methods for the determination of protein complexes subunits stoichiometry



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ABSTRACT

Protein complexes are the main molecular machines that support all major cellular pathways and their in-depth characterization are essential to understand their functions. Determining the stoichiometry of the different subunits of a protein complex still remains challenging. Recently, many label-free quantitative proteomic approaches have been developed to study the composition of protein complexes. It is therefore of great interest to evaluate these different methods in a stoichiometry oriented objective. Here we compare the ability of four absolute quantitative label-free methods currently used in proteomic studies to determine the stoichiometry of a well-characterized protein complex, the 26S proteasome.

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As protein complexes regulate most cellular pathways, determining their composition, structural organization and dynamics are important challenges of modern biology. Affinity purification coupled to mass spectrometry (AP-MS) is a well-suited tool to access the protein composition of cellular protein complexes [1–3]. Most major cellular protein complexes have now been well characterized using this approach. Several large scale studies based on AP-MS have also been performed and have revealed that at least 500 protein

complexes are present in *Saccharomyces cerevisiae* [4] or *Drosophila melanogaster* [5] cells. Chemical cross-linking methods have also been successfully used in association with AP-MS strategies. *In vitro* crosslinking, performed after the purification step, enables to map protein–protein interactions within protein complexes [6], whereas *in vivo* crosslinking helps to preserve the integrity of the complex during the biochemical steps [7]. However, quantitative data about the stoichiometries of the proteins involved in these complexes

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and their dynamics upon cell stress conditions are still missing to understand their function.

During the last decade, several targeted proteomic approaches relying on the isotope dilution method have been developed to measure the absolute abundance of proteins [8]. In these strategies, the sample is spiked with defined amounts of an isotope-labeled analog, either a proteotypic peptide or a protein. Although these strategies are the most precise to determine protein concentrations in biological samples, they require time-consuming optimizations at different levels and specific mass spectrometry acquisition methods. Therefore, quantification, because it is targeted, is performed in a separate experiment from the one used to identify the protein complexes subunits. Alternatively, several label free MS-based relative quantification methodologies are widely used to define protein complexes composition or compare multiple proteomes. They have more recently been applied to approximate the absolute quantities of proteins [9]. These strategies have been used to evaluate the highly dynamic intracellular protein concentrations and their correlation with mRNA levels [10], but also to estimate protein complexes subunits stoichiometry [11–15]. Recent reports have compared the accuracy of different label-free quantification methods using the UPS (Universal Proteomics Standard, Sigma–Aldrich) protein standards spiked in *Escherichia coli* lysate as background [16,17]. However, the protein concentration dynamic range in an *E. coli* lysate and in an affinity purified protein complex sample are very different. This variation in background protein dynamic range can affect the behavior and the precision of the different label-free quantification methods applied to the determination of the stoichiometry of protein complexes subunits. In this report, we evaluated the precision of four label-free quantification approaches on protein complexes in which subunits stoichiometry has been clearly established.

In order to compare label-free quantitative methods, we chose a structurally well characterized protein complex as model, the 26S proteasome. The proteasome is a macromolecular complex of 2.4 MDa responsible of the degradation of most intracellular proteins [18]. The 26S proteasome is composed of two sub-complexes, a 20S core particle bearing the catalytic subunits responsible for the proteolytic activities of the proteasome, and a 19S regulatory particle which function is to recognize, unfold and translocate the substrate into the core particle [19]. The subunits stoichiometries within these complexes are very well characterized, in particular for the 20S core particle which crystal structure is available [20,21]. It is composed of constitutive ($\alpha 1$ – $\alpha 7$ and $\beta 3$, $\beta 4$, $\beta 6$ and $\beta 7$) and catalytic ($\beta 1$, $\beta 2$, $\beta 5$, called “standard”, and their respective “immuno” counterparts $\beta 1i$, $\beta 2i$, $\beta 5i$) subunits (Fig. 1A) [18]. All constitutive subunits are incorporated at a stoichiometry of 2 proteins per 20S core particle (Fig. 1A) whereas the integration of catalytic subunits into the 20S proteasome is more complex and leads to several 20S subtypes possibly bearing a mixed assortment of standard and immunosubunits. Therefore, the stoichiometry of each catalytic subunit is expected to be variable across tissues or cell types [22]. Recent reports have largely contributed to major breakthrough in the knowledge of 26S proteasome structure using cryo-electron microscopy combined with other techniques [23,24].

It is now established that mot 19S subunits are incorporated at a stoichiometry of 1 into the 19S regulator (Fig. 1B), except for the ubiquitin receptor Rpn13 which has been described to be dynamic and probably sub-stoichiometric [25,26]. Considering the stoichiometries of the 19S and the constitutive 20S subunits, the 26S proteasome therefore provides a valuable tool to compare label-free quantitative methods for the determination of the relative quantities of subunits within a protein complex. Proteasome complexes were purified from different *in vivo* crosslinked cells using the MCP21 antibody which allows to immunoprecipitate the whole 26S proteasome complex in a single step, as previously described [27]. A total of 24 biological replicates of 26S proteasome purified from nine different human cell lines were used in this study. Samples were prepared for mass spectrometry, as previously described [27]. The peptides mixtures were analyzed using nano-LC–MS/MS using a LTQ-Orbitrap XL mass spectrometer. Data were searched using Mascot server and validated and quantified using the MFPaQ software [28], as previously described [27]. The stoichiometries of the different proteasome subunits were determined by label-free quantitative proteomics. Four quantitative approaches were compared: the TOP 3 [29], the iBAQ [10], the sum of the MS intensities normalized by the molecular weight [30] (MS1 based quantification approaches – called “MS1 over MW”) and the spectral counting normalized by the molecular weight [31] (MS2 based quantification approach – called “MS2 over MW”). The TOP3 is calculated as the mean of the three highest peptides areas measured for each protein. The iBAQ corresponds to the sum of all the peptides intensities divided by the number of observable peptides of a protein. The MS1 over MW and MS2 over MW were obtained by dividing respectively, the sum of peptides intensities or the sum of MS/MS events for the peptides of a protein by its molecular weight. Each of these methods has been described as a valuable tool to estimate protein abundance and to compare the relative quantities of proteins [32]. For each quantitative approach, we calculated the ratio of the subunit abundance index (SAI) (abundance value obtained for each 26S proteasome subunit using the four label-free quantitative methods) over the mean of the abundance indexes (MAI) (mean of the SAI of the 20S or the 19S complexes) for all the subunits of the complex. This ratio, also called hereafter “observation”, gives the deviation of each quantitative method from the expected value of 1. The distribution of all the ratios of the 20S constitutive (264 observations) and 19S (except rpn13) (384 observations) subunits were computed and box-plots were obtained for the different quantitative approaches (Fig. 1C and D). As 11 constitutive subunits of the 20S proteasome ($\alpha 1$ – $\alpha 7$, $\beta 3$, 4, 6 and 7) and 16 subunits of the 19S regulator (Rpt1, 3–6, Rpn1–3 and Rpn5–12) were quantified in 24 biological replicates of purified proteasome complexes, we obtained 264 observations for the 20S and 384 ones for the 19S complexes. The mean standard deviation, defined as the deviation from the expected value of 1, was calculated for the TOP3, the iBAQ, the MS1 over MW and the MS2 over MW methods and was found to be equal to 28.1, 31.8, 33.6 and 26.6%, respectively, for the 20S (Fig. 1C) and 20.9, 29.3, 31.7 and 23.3%, respectively, for the 19S (Fig. 1D). The four label-free quantification methods therefore show low standard deviations, below 30%

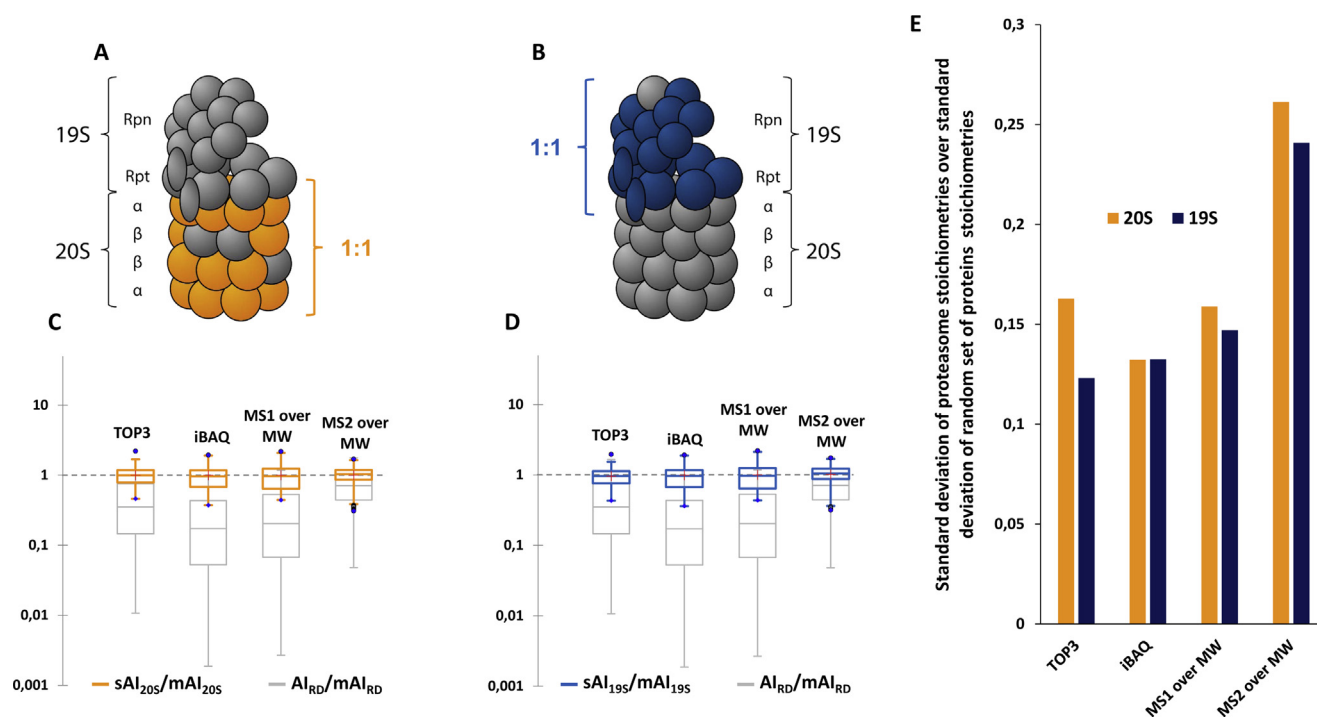


Fig. 1 – Comparison of four label-free quantification methods for the determination of the stoichiometry of the 26S proteasome subunits. (A and B) Composition and subunits stoichiometry of the 20S (A) and the 19S (B) proteasome. Distribution of the ratio of abundance index of each subunit (sAI) over the mean abundance indexes of all the subunits (mAI) of the 20S (C) and the 19S (D) proteasome, as determined by Top3, iBAQ, “MS1 over MW”, and “MS2 over MW” label-free quantification methods. The 20S (C) and 19S (D) ratio distribution are represented with blue and orange box-plots, respectively. Gray box-plots represent the distribution of the ratios of the abundance index of each protein of the random proteins set (AI_{RD}) over the mean abundance indexes of all the proteins in the same random protein set (mAI_{RD}) (C and D), as determined by four different label-free quantification methods. Red crosses and blue dots represent the mean and extreme values, respectively. (E) Ratio of the standard deviations of the stoichiometries of the proteasome subunits over the standard deviations of the stoichiometries of the randomly chosen proteins.

in most cases, when applied to the determination of the proteasome subunits stoichiometry. This relatively good ability to determine subunit stoichiometry within the complex might be explained by the low dynamic range observed in such biological sample. Greater deviations would probably be expected for higher protein dynamic ranges.

To study further the behavior of the quantitative methods in their ability to discriminate differences in the stoichiometry of several protein complex subunits, we then used a set of 16 randomly selected proteins identified in the proteasome affinity-purification samples as background proteins [7], which served as a negative control. The ratio distribution (384 observations) of the abundance index of the protein in the random set (AI_{RD}) over the mean of all the abundance indexes of all the proteins in the same random set (mAI_{RD}) was compared for each quantitative approach, as already performed for the 20S and 19S subunits.

The distributions of proteasome subunits ratios (19S and 20S) show median values close to the mean value of 1 (median values of 1.041, 0.886, 0.961, and 0.915 for the 20S calculated with the TOP 3, iBAQ, “MS1 over MW”, and “MS2 over MW” quantitative methods, respectively, and median values of 0.999, 0.964, 0.969, and 1.032 for the 19S, respectively) (Fig. 1C and D), which is representative of a symmetric distribution like

the normal distribution. This can be explained by the fact that all the ratios are representative of an expected stoichiometry of 1 between the different subunits of the proteasome complexes, and can thus be interpreted as multiple observations of the same relative quantity of proteins. Conversely, in the case of the proteins chosen randomly, the median ratio values are highly different from the mean ratio of 1 (median values of 0.351, 0.172, 0.204, and 0.71 calculated with the same quantitative methods) (Fig. 1C and D). This therefore confirms the selection of a set of proteins which are uncorrelated regarding their stoichiometry.

Strikingly, the random set ratio values distribution observed for the MS1-based quantitative methods (TOP 3, iBAQ, and “MS1 over MW”) were also found to be more scattered (Fig. 1C and D) than the ones obtained for the MS2 based quantification approach, “MS2 over MW” (Fig. 1C and D). When we then compared the standard deviations of the stoichiometries of the proteasome subunits with the ones of the randomly chosen proteins, we observed that the ratios of these standard deviations were higher for the “MS2 over MW” method (0.24 and 0.26, for 20S and 19S complexes, respectively) than for the MS1-based quantification methods (Fig. 1E). This result shows that the MS2-based quantification method is less discriminant than the methods based on the integration

of the MS1 signal in the objective to highlight a difference (or an absence of difference) in the stoichiometry of protein complex subunits. This could be probably explained by the fact that the MS2-based quantification method has a lower dynamic range compared to MS1 based approaches [32]. This result suggests that the “MS2 over MW” method tends to flatten the stoichiometry values between the subunits of a protein complex.

To conclude this study, we have compared four label-free quantitative methods for the determination of the 26S proteasome complex subunits stoichiometry. We have found that all the quantification methods have low standard deviations (less than 34%) and represent straightforward and reasonably precise tools to determine the stoichiometry of the different proteins involved in a complex. Importantly, this quantification can be performed using the same experimental data as the one used to identify the protein complexes subunits and do not require any specific optimization steps, as needed for isotopic dilution methods. However, the spectral counting approach flattens the measured ratios, as determined using the random set of proteins, which can bias the determination of the relative stoichiometries of the different subunits composing a protein complex. In the goal to determine the relative abundances of different subunits of a protein complex, label free MS1-based quantification methods might represent alternative and straightforward strategies to isotopic dilution MS-based approaches.

Conflict of interest

The authors declare no conflict of interest.

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