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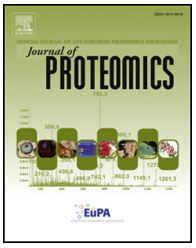

Protein identification and quantification by data-independent acquisition and multi-parallel collision-induced dissociation mass spectrometry (MS^E) in the chloroplast stroma proteome

Stefan Helm, Dirk Dobritzsch, Anja Rödiger, Birgit Agne, Sacha Baginsky

Institute of Biochemistry and Biotechnology, Martin Luther University Halle-Wittenberg, Weinbergweg 22, 06120 Halle (Saale), Germany

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ABSTRACT

We report here a systematic evaluation of a multiplex mass spectrometry method coupled with ion mobility separation (HD-MS^E) for the identification and quantification of proteins in the chloroplast stroma. We show that this method allows the robust quantification of reference proteins in mixtures, and it detects concentration differences with high sensitivity when three replicas are performed. Applied to the analysis of the chloroplast stroma proteome, HD-MS^E identified and quantified many chloroplast proteins that were not previously identified in large-scale proteome analyses, suggesting HD-MS^E as a suitable complementary tool for discovery proteomics. We find that HD-MS^E tends to underestimate protein abundances at concentrations above 25 fmol, which is likely due to ion transmission loss and detector saturation. This limitation can be circumvented by omitting the ion mobility separation step in the HD-MS^E workflow. The robustness of protein quantification is influenced by the selection of peptides and their intensity distribution, therefore critical scrutiny of quantification results is required. Based on the HD-MS^E quantification of chloroplast stroma proteins we performed a meta-analysis and compared published quantitative data with our results, using a parts per million normalization scheme. Important pathways in the chloroplast stroma show quantitative stability against different experimental conditions and quantification strategies.

Biological significance

Our analysis establishes MS^E-based *Hi3* quantification as a tool for the absolute quantification of proteins in the chloroplast stroma. The meta-analysis performed with a parts per million normalization scheme shows that quantitative proteomics data acquired in different labs and with different quantification strategies yield comparable results for some metabolic pathways, while others show a higher variability. Our data therefore indicate that such meta-analyses allow distinguishing robust from fine-controlled metabolic pathways.

Introduction

Large-scale protein quantification by data independent multi-parallel collision induced dissociation (MS^E) mass spectrometry

Quantitative proteomics comes in various flavors and many methods and approaches were developed that enable

E-mail address: sacha.baginsky@biochemtech.uni-halle.de (S. Baginsky).

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experimentalists to adapt protein quantification to their particular purpose [1–10]. In recent years, new mass spectrometric acquisition techniques were introduced that sample in a data-independent fashion to circumvent problems with the stochastic nature of peptide sampling in data-dependent acquisition. One of these methods is referred to as MS^E. In MS^E, the mass spectrometer switches between low and high collision energy with quadrupole settings that allow all precursor ions within a chosen molecular mass window to pass through. Thus, all peptides within the mass window will be detected and fragmented. The downside of this approach is the lost connection between precursor and fragment ions, and algorithms are required to re-establish this connection by aligning elution profiles of precursor and product ions in the chromatography. On the other hand, the rapid cycling between low and high-collision energy and the fact that all peptides are fragmented preserves more accurate quantitative information compared with the standard data-dependent acquisition modes. Thus MS^E is perfectly suitable for label-free quantification based on peptide XIC [11]. High-definition (HD)-MS^E is based on the same principle as MS^E but additionally uses ion mobility (IMS) as a further peptide separation step. Because precursor ions are fragmented after the IMS cell, ion mobility is an additional characteristic feature for the alignment of precursor and product ions. This increases its sensitivity compared to MS^E alone because more peptides can be assigned with higher confidence [12].

MS^E allows absolute protein quantification by comparing the intensity read-out of proteotypic sample peptides with those of an internal standard. The correlation between signal intensity of up to three most intense ions and protein concentration is used to infer the abundance of proteins in the sample. Silva and colleagues have shown that the count of measured signal intensity per amount of protein (so called *response factor*) is constant for all proteins tested [11] provided that the three peptides with the highest XIC read-out can be used for response factor calculation. Recent analyses showed that this quantification strategy is not restricted to MS^E, but also works in the data-dependent acquisition mode [13]. The advantage of this approach lies in its applicability to samples of low complexity. Furthermore it enables the quantification of proteins from non-model crop plant species, because only three proteotypic peptides must be identified for quantification thus increasing the chances for homology-based peptide detection [11,14].

Quantification of chloroplast stroma proteins

The chloroplast proteome has been studied intensely and high-quality plastid proteome maps are now available [15,16]. However, quantitative information on the identified proteins is not easily accessible from the published data. Many quantitative chloroplast proteome analyses used either 2-dimensional gel electrophoresis or spectral counting. For example, Bischof and colleagues used normalized spectral counting (nSpC) to compare the proteomes of wildtype plastids with non-photosynthetic plastids of a plastid protein import mutant [17]. Motohashi and colleagues used nSpC to identify protein *regulons* in different albino/pale-green mutants [18]. Kim and colleagues used normalized spectrum abundance factor (NSAF) quantification to compare the wildtype chloroplast proteome with that of a *clp* mutant [19]. All analyses were carried out at the level of the entire cell, and proteins were allocated to the chloroplast based on proteome reference tables a posteriori. Although the purposes of the aforementioned studies were confined by a specific hypothesis, quantification of proteins in wildtype chloroplasts was done in all studies. So far, this quantitative information for enzymes of the chloroplast metabolism was not used for metabolic modeling or other predictive analyses of organellar metabolism. This is owed to the fact that protein quantification was performed in different labs and under different conditions, and that different quantification and normalization schemes were used.

We report here a systematic assessment HD-MS^E based protein quantification in the chloroplast stroma and compare our data with published information on chloroplast protein quantities. To make the different data sets comparable, we normalized protein abundance with the parts per million normalization scheme that was originally developed for a quantitative metaproteome analysis between *Arabidopsis*, *Drosophila* and *Caenorhabditis elegans* [20,21]. Our analysis shows that the quantification of proteins by HD-MS^E yields robust results, despite the wide dynamic range of protein concentrations in the chloroplast stroma. In individual cases, careful examination of peptide quantification characteristics may be necessary. This concerns the question which peptides were selected for XIC measurements as well as the deviation of XIC among the three most intense proteotypic peptide ions. Both parameters constitute a potential error source for quantification accuracy. The HD-MS^E-based quantification showed that the quantitative distribution of proteins in different chloroplast functions is surprisingly robust between different published studies. This also entails the distribution of enzymes within one pathway, suggesting that quantitative meta-analyses as reported here are suitable to reveal basic robustness principles in the cellular metabolism.

Materials and methods

Materials

LC-MS grade solvents, including water with 0.01% (w/v) formic acid, water with 0.1% (w/v) trifluoroacetic acid and acetonitrile with 0.1% (w/v) formic acid were obtained from Carl Roth (Karlsruhe, Germany). Porcine sequencing grade modified trypsin was obtained from Promega (Mannheim, Germany).

Plant material

Arabidopsis thaliana plants (Columbia-0) were used for the preparation of stroma extracts. These were either grown on plates at 22 °C, 8 h light, 16 h darkness, 150 μmol m^{-2} s^{-1} on ½ MS media (replicate 1), containing 0.8% sucrose, or on soil in the greenhouse (replicates 2 and 3). All plants were harvested on day 21–28. Cell walls were either enzymatically digested cellulase 0.015 g/mL, macerozym 0.0375 g/mL [17] or disintegrated in a blender as described earlier [22]. Chloroplasts were isolated on Percoll (GE Healthcare, Solingen, Germany) gradients. Purified intact chloroplasts were then lysed in hypotonic medium. The

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