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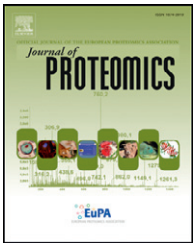

Mass spectrometry-based label free quantification of gel separated proteins

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ABSTRACT

Co-migrating proteins in 2D PAGE spots cause difficulties in the assignment of quantitative data obtained from the staining density of a gel spot. We present an application of a label free LC-MS quantitative method that can overcome problems like this. Protein mixtures were prepared with varying compositions, and were run on either 1D or 2D PAGE. Relative and absolute quantitative evaluation was carried out using a simple but reliable method based on integrated MS signals of the three most intensive peptides of each protein. The efficacy of digestion and peptide extraction is, however, influenced by different factors in gel from those in solution, hence the method had to be validated via a quantitative assessment of proteins from 1D or 2D gels. Our findings suggest that a reliable relative quantification is viable using peptide ion intensities when protein levels in two gels have to be compared. In the case of 2D gels, label free MS quantification provides more precise results on changes of protein expression levels than gel spot intensity-based measurements, especially in the case of overlapping proteins. Absolute amounts of different proteins in 1D or 2D gels can be evaluated to a reasonable precision.

1. Introduction

Tools of proteomics are widely used in the identification of key proteins and the corresponding biological processes via measurements of different protein expression levels in various biological samples. In the traditional and still the most commonly applied workflow, 2D gel electrophoresis is used for the separation and relative quantification of proteins using stained spot intensities. In the next step of this protocol, selected spots are excised and subjected to enzymatic cleavage with an enzyme of known cleavage specificity. Peptides produced in the digestion procedure are extracted from the gel matrix and transferred for protein identification using mass spectrometry. Using this approach quantification is achieved at the protein level, compared to gel-free methodologies, where both quantification and identification are carried out on digested peptides [1,2].

2DGE has a high resolving power, and a few thousand protein spots can be separated and visualised on a single gel. But this number is still far from the complexity of most biological samples [3]. A frequently occurring issue, related to 2DE spots containing multiple proteins, is the difficulty in assigning gel-based quantitative data (one data per spot) to a single protein (several proteins per spot) [4–7]. In such cases the costly and laborious biological work [3] may be of little value or some additional expensive protein-specific (e.g., immunochemical) validation should be performed for each single overlapping protein.

A mass spectrometry-based quantitative analysis of proteins separated by gel electrophoresis was carried out by several laboratories using different techniques. Havlis and Shevchenko [8] measured absolute quantities of reference proteins recovered from 1D polyacrylamide gels by isotope labelled peptides. They found differences in the recoveries of

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proteins from gels and from solution, but unfortunately their analytical procedure can be applied only to a limited number of peptides of preselected proteins, and cannot be incorporated into the daily routine identification workflows.

In-gel ^{18}O labelling was also evaluated for the MS-based quantification of proteins separated by gel electrophoresis [7]. The disadvantage of this approach is the high cost and additional work of labelling. In addition, the general benefit of labelling (e.g. the simultaneous measurement of labelled and unlabelled peptides) can be lost because of variability introduced by incomplete and sometimes poorly reproducible labelling in gels [9].

A label-free quantitative analysis of proteins from 1D PAGE was carried out by Gao et al. [10] via a combination of spectral counting, ion intensity measurement and integrated peak area measurement. Their work focused on the application of 1D PAGE as a prefractionation technique for an LC-MS based quantitative analysis. They observed a good correlation among the three quantities used in their algorithm, but the absolute quantitative assessment of proteins was not addressed and only 1D gels were analysed.

Yang et al. [11] compared proteins identified by MALDI and LC-MS in 2D gels, and found that LC-MS identified multiple proteins in 75% of spots, while MALDI detected at least two proteins only in 19% of spots. The exponentially modified protein abundance index (emPAI) was used in LC-MS to evaluate the approximate contribution of each protein to the spot volume. The accuracy of the results is limited by the spectral counting method, and the absolute quantities of proteins were not estimated.

Our current study mainly focuses on finding a simple, but reliable method to estimate the relative contribution of co-migrating proteins to a 2D GE spot staining intensity. Usually this is carried out by ranking proteins based on the number of unique peptides assigned to each one. This approach is normally used because in most cases no other quantitative information is available from mass spectrometric measurements. Spectral counting approaches are generally considered to be semiquantitative methods for protein analysis, but provide acceptable results only in the case of relatively large quantitative differences, and only with proteins for which the number of identified peptides exceeds a certain threshold. Ion intensities are not reliable measures of the analyte concentration on mass spectrometers which work with a cycle time in the range of one second, because the generally applied data dependent ion selection methods truncate the chromatographic peaks when switching to MS/MS data collection. In the case of protein digests, an absolute quantification based on MS signal intensities is even more questionable because several signals have to be combined, which are produced by different peptides that have highly variable ionisation properties. Moreover, these peptides are produced in enzymatic digestion processes that are not yet well understood quantitatively, and depend on the protein sequence and digestion protocol as well. The method of Silva et al. [12] eliminates both peak shape and peptide selection-related problems. A data independent MS/MS data collection is applied to allow a simultaneous identification and quantification even on relatively slower mass spectrometers and the absolute quantitative evaluation of proteins is based on the intensity sum of the three most intensive tryptic peptides (Top3 peptides) of each protein. Nowadays the Top3 method for the absolute evaluation of proteins in solutions is commonly used. It also has been adapted for use with different instruments [13,14] and has been incorporated into several commercial proteomics database search engines and software platforms (e.g. Mascot (Matrix Science Inc., Boston, MA) and Scaffold (Proteome Software Inc., Portland, OR)). However, a validation of the correlation of the Top3 intensity to protein quantity is still an open question for proteins digested in gel, as different physicochemical processes and properties may play a big role in individual peptide ion intensities and intensity ratios.

Getie-Kebtie et al. [15] analysed protein mixtures from 1D gels using the Top3 label free quantitative methodology, however their results are ambiguous regarding absolute quantification of co-eluting proteins, which may be attributed to several methodological differences compared to the previous publications. Getie-Kebtie and his coworkers in their work applied MALDI ionisation which may have different preferences for relative ionisation efficiency of different peptides than in ESI-LC-MS. They applied their new rapid pressure assisted in-gel digestion protocol in the quantitative measurements, but no quantitative comparisons were carried out to traditional in-gel or in-solution digestion protocols nor at protein neither at peptide level. Therefore it is hard to decide that the highly protein dependent response factors they have calculated, are caused by protein/peptide structure/size, digestion efficiency or by the ionisation method. They obtained however, good linear correlation between Top3 intensity and protein amount from sequential dilution series of protein mixtures, so relative quantification was found to be feasible from 1D gels.

In order to validate the reliability (accuracy and reproducibility) of the Top3 label free quantitative MS method, standard protein mixtures were prepared, electrophorised into 1D PAGE gels or spiked with *E. coli* lysate, and separated on 2D GE. The composition of each of the four different protein mixtures containing five proteins is shown in Table 1. The amount of Bovine albumin (ALBU_BOVINE) was kept constant in the mixtures, this protein being included as an internal standard for the normalisation of quantitative data. The results obtained by normalisation on this in-gel digested protein, and results with a commercial Rabbit Phosphosrylase B (PYGM_RABIT) digest added to samples just before LC-MS analysis were compared. The quantity of four other proteins was varied between 0.1 and 1 μg/sample (2.1 μg total protein in each case). The relatively narrow (one order of magnitude) dynamic range was selected because the most problematic part is that of resolving small quantitative changes of proteins present in a single 2DGE spot at similar concentrations.

For the validation in 1D gels, the samples were run on 1D gel electrophoresis, in-gel digested, and the samples were simultaneously digested in solution too. In both cases each sample was processed in triplicate; consequently, each protein mixture was represented by three replicate 1D gel samples and three solution samples. All samples were analysed by LC-MS in triplicate, thus 9 parallel measurements were performed using each digestion protocol for the protein mixtures.

In order to achieve our main goal, namely the quantification of comigrating proteins in 2D gel, the previously described