



1 Overview

The aim of the following studies was the development of a method suitable for comparative analysis of two highly complex protein mixtures. This includes differential quantitative determination of protein expression, post-translational modifications and isoforms. In order to obtain this information, the whole sequence of every differential expressed protein has to be analyzed.

This challenge was achieved by isotope labelling of the high abundant free amino groups in proteins, which allows the usage of any separation method for proteins and peptides. Quantitation and identification of differential expressed proteins is then performed using high throughput mass spectrometry.

2 Introduction

Quantitative proteome analyses usually are accomplished by 2D-electrophoresis (2DE) followed by mass spectrometric protein identification. Although this method is well established, quantitative determination is not accurate and the reproducibility of the 2D-gels is very poor. Recent developments, like the ICAT reagent [1] or GIST [2] methodology have shown to be powerful alternatives to comparative 2D gel imaging analysis. Nevertheless, these methods also have their limitations. Here we describe a new method termed Isotope Coded Protein Label (ICPL) which is based on isotopic labelling of all free amino groups in proteins.

Compared to the ICAT reagent, that modifies the low abundant amino acid cysteine in proteins, higher sequence coverage and thus more information about post-translational modifications and isoforms are obtained with ICPL.

With the GIST approaches isotope labelling of peptides is performed after enzymatic cleavage of the proteins. Although almost every peptide is modified using this strategy, the highly demanded quantitatively controlled separation dimension on the protein level is lost.

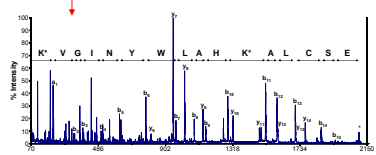
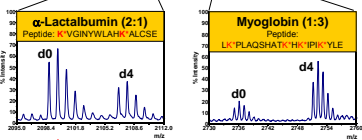
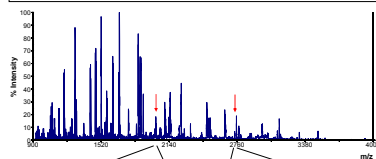
The efficiency of the ICPL method is demonstrated by comparative analysis of two *E. coli* - proteomes spiked with different amounts of five standard proteins.

6 Labelling of standard proteins

To illustrate the ICPL workflow, 2 mixtures containing the same 6 standard proteins in various amounts were treated as described under 3. Isotopic labelling of all free amino groups was performed using activated nicotinic acid. As nicotinic acid contains 4 hydrogens in the light version which are replaced by 4 deuterium atoms in the heavy version, the mass difference per modified amino group is 4.

After derivatization, the combined protein mixtures were enzymatically cleaved using endopeptidase Glu-C. The obtained peptide mixture was then applied to a MALDI target without further separation and analyzed. The extended views are each showing one peptide pair origin from myoglobin and α -lactalbumin. Identification of the peptide/protein is finally carried out by MS-sequencing followed by database search.

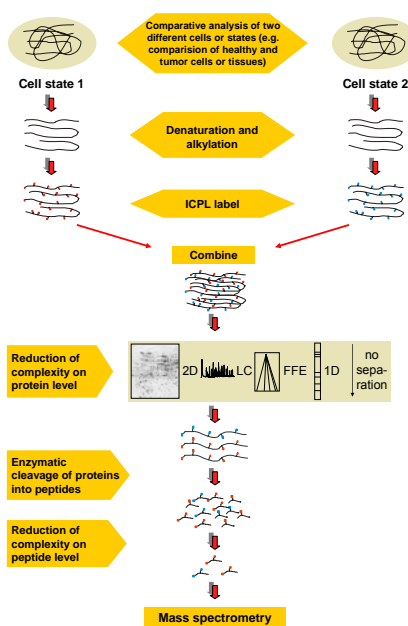
MALDI-TOF-spectra of labeled peptides



Results

- The ratio of the 6 proteins could be determined very precisely, having a standard deviation of less than 6%
- The sequence coverage of the proteins ranged from 32.5 to 82.3% (labelled peptides only)
- All analyzed peptides containing a free amino group have been modified
- No side reactions, that would result in additional isotopic peptide pairs including no free amino groups, could be identified

3 Workflow



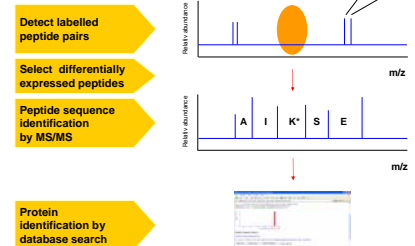
4 Mass spectrometry

1) Quantitation

The ratio of an isotope labelled peptide pair is calculated by comparing their intensities. This provides relative quantitative information about the differential protein expression of the 2 cell states.

2) Identification

Only peptides from differential expressed proteins (ratio not 1:1) are then selected for identification by sequencing (MS/MS).



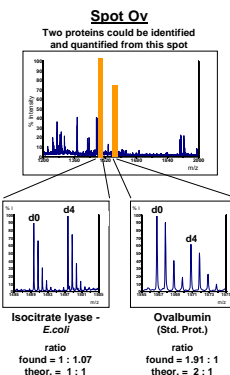
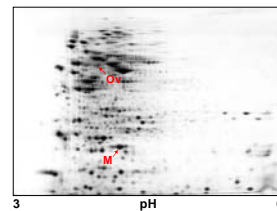
5 MS-parameters

Sample preparation: The modified proteins were digested, diluted (except in-gel digests) and mixed 1:1 with a α -cyano-4-hydroxynicotinic acid (CHCA) solution. 0.4 μ l of the mixture containing 500 fmol of peptides were then applied to a stainless steel MALDI target.

Instrument: Proteomics analyzer 4700 (MALDI-TOF/TOF)

Database search: GPS-Explorer / Mascot

7 Analysis of a spiked *E. coli* - proteome



Comparative analysis of two *E. coli* - proteomes, spiked with different amounts of 5 standard proteins. Separation was performed using 2 dimensional gel-electrophoresis.

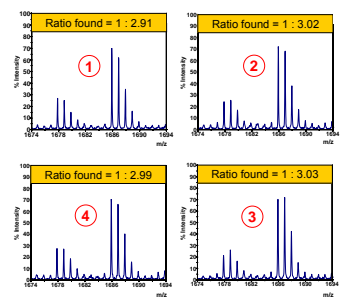
Because the same amount of *E. coli* proteins was labelled in both samples, their ratios were 1:1. For simulating different protein expression, the ratios of the added standard proteins were not 1:1, but varying from 4:1 to 1:4. From the MS-spectrum on the left (Spot Ov) one protein from *E. coli* and one added standard protein could be identified and, more importantly, correctly quantified. Accurate quantification of co-migrating proteins during 2DE is not feasible by differential image analysis of 2D-gels.

Results

- All added standard proteins could be identified from this 2D-gel with their correct quantities having mean differences between 0.1 and 7%
- The ratios of the analyzed *E. coli* proteins were all 1.1 ± 0.09
- The experiment demonstrates that the precise quantitation and high sequence coverage of proteins are maintained, even when analyzing highly complex protein mixtures
- Using ICPL, multiple proteins having the same position on a 2D-gel can still be identified and, more importantly, correctly quantified

8 Co-migration of light and heavy labelled proteins during 2DE

To check if proteins labelled with the heavy or the light reagent behave similar during 2DE, Spot M from the 2D-gel (see 7) was divided into 4 quadrants as illustrated on the left. Each part was individually excised, digested and analyzed by MALDI-MS.



Result

The standard deviation of the ratios determined was less than 2%, clearly demonstrating co-migration of proteins labelled with either the heavy or light version of the tag

9 Conclusion

In summary, the new ICPL approach presented here provides accurate quantitative determination and high sequence coverage of differential expressed proteins in highly complex protein mixtures. It offers easier access to basic proteins during 2DE and is feasible to quantify multiple proteins that migrate to the same position on a 2D-gel. We have currently evaluated new reagents, that allow multiplexed experiments as well as LC/LC-MS/MS analysis. In the latter case, the deuterium isotopes of the heavy nicotinic acid label were replaced by ^{13}C -isotopes. This ensures co-elution of derivatized peptides during RP-LC which is required for correct quantification. With the opportunity of using any separation method and protein sample, this new strategy is suitable to challenge comprehensive quantitative proteome analysis.

10 References

1. Gygi, S. P. et al; Nat. Biotechnol. 1999, 17, 994-999
2. Chakraborty A. et al; J Chromatogr A 2002, 949, 173-184