A new strategy for quantitative proteomics using isotope-coded protein labels

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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

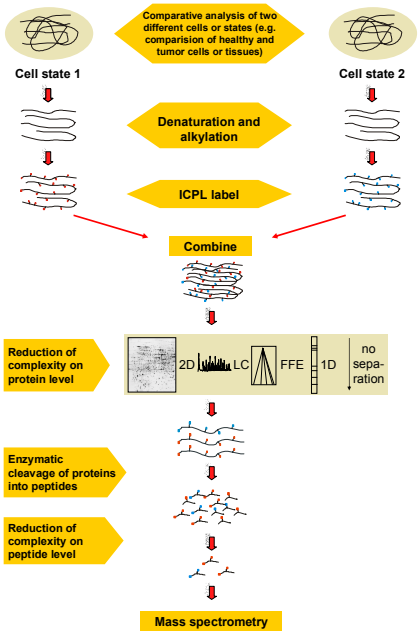
Quantitative proteome analyses usually are accomplished by 2D-electrophoresis (2DE) followed by mass spectrometric protein identification. Although this method is well established, quantitative determination is not accurate and the reproducibility of the 2D-gels is very poor. Recent developments, like 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.

3 Workflow



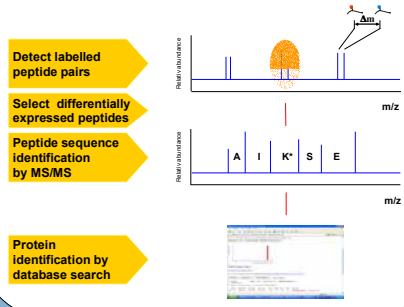
4 Mass spectrometry

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).

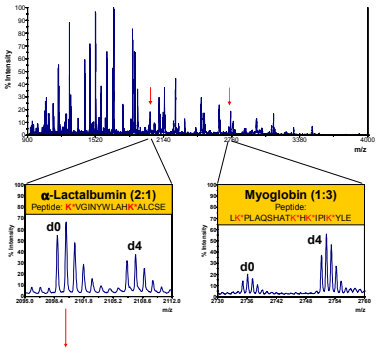


5 MS-parameters

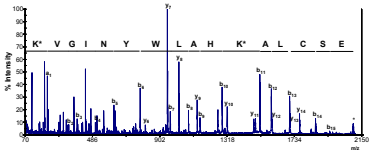
Sample preparation:	The modified proteins were digested, diluted (except in-gel digests) and mixed 1:1 with a α-cyano-4-hydroxycinnamic acid (CHCA) solution. 0.4 µl of the mixture containing 500 fmol of peptides were then applied to a stainless steel MALDI target.
Instrument:	Proteomics analyzer 4700 (MALDI-TOF/TOF)
Database search:	GPS-Explorer / Mascot

6 Labelling of standard proteins

1. Quantification
MS-spectra of labeled peptides



2. Identification
MS/MS-spectra of selected peptide



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.

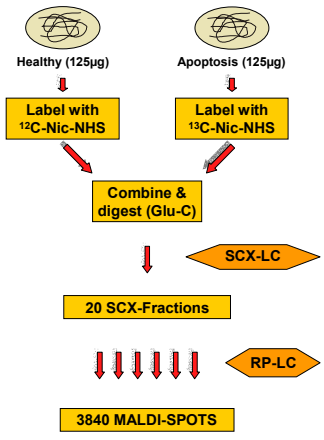
After derivatization, the combined protein mixtures were enzymatically cleaved using endopeptidase Glu-C. The obtained peptide mixture was then applied to a MALDI target without further separation and analyzed. The extended views are each showing one peptide pair origin from myoglobin and α-lactalbumin.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

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

7 Comparative analysis of healthy and apoptotic human HEP-G2 cells

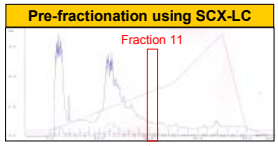
Workflow



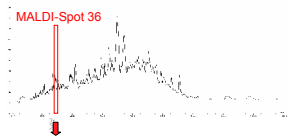
The aim of this study was the discovery of new proteins that change during apoptosis and are specific for apoptotic cells (marker proteins). (For details refer to poster "MONACO – Proteomic ..." presented by Cornelia Ciosto).

For this experiment, the heavy 4-^{2}H-nicotinic acid was replaced by the 6-^{13}C version. This ensures co-elution of derivatized peptides during reversed phase liquid chromatography (RP-LC) analysis which is required for accurate protein quantification.

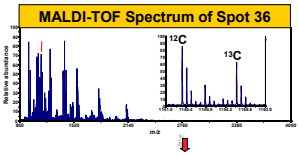
Pre-fractionation using SCX-LC



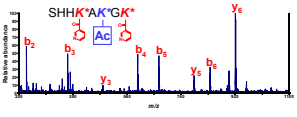
RP-LC of SCX-Fraction 11



MALDI-TOF Spectrum of Spot 36



MALDI-MS/MS Spectrum of the indicated Peptide Pair identified as Histone H2AA (Ac at K127)



Results

- In total **3840** MS-spectra (13.3h) and **13462** pairs for MS/MS-analysis (37.4h) were acquired
- Only **952** of **13462** pairs (7.07%) could be correctly identified
- This corresponds to **416** unique proteins (average = 2.3 peptides/protein)
- **31** proteins were found to be up- and **33** down-regulated during apoptosis
- **25** acetylation, **2** phosphorylation and **2** methylation sites could be identified

8 Conclusion

In summary, the new ICPL approach presented here provides accurate quantitative determination and high sequence coverage of differential expressed proteins even when analyzing highly complex protein mixtures. With the opportunity of using any separation method and protein sample, this new strategy is suitable to challenge comprehensive quantitative proteome analysis.