Bruker Daltonics

Application Note # MT-86

Quantitative Proteome Analysis by Labeling of Arginine and Lysine with SILAC, SDS-PAGE, and nano-LC-MALDI-TOF/TOF Mass Spectrometry

Quantitative proteome analysis of cisplatin-induced apoptosis in total Jurkat T cell lysates was performed in order to identify modified proteins. Proteins were labeled in cell culture with stable isotopes of arginine and lysine, fractionated by SDS-PAGE and analyzed by offline nano-liquid chromatography coupled to MALDI-TOF/TOF-MS. Data analysis was performed using the new WARP-LC 1.1 quantification software. Metabolic labeling of proteins with different isotopes ($^{12}C_6$ or $^{13}C_6$, respectively) of arginine and lysine residues with subsequent tryptic digestion resulted in detection of all peptides as pairs in the mixture. Combined proteins derived from non-apoptotic and apoptotic cells revealed predominantly a heavy-to-light (H/L) ratio of about 1, indicating that most of the proteins were unchanged during apoptosis. Therefore, only about 2000 MS/MS spectra were acquired in total from the 60 gel slices due to the requirements to select peptides (H/L > 1.5 or L/H > 1.5) for MS/MS analysis although up to 1000 chromatographic compounds were detected per gel slice. As a result, several modified proteins with at least two peptide pairs were found by comparison of control and apoptotic Jurkat T cells.

Introduction

Online LC-ESI-MS is typically preferred to offline LC-MALDI-MS for quantitative proteome analysis, because it is faster and more MS/MS spectra can be generated. However, more time is still required on data analysis and validation than on performing LC runs. Moreover, quantitative protein data generated by LC-MALDI-MS were shown to be particularly accurate and are easier to evaluate as only single charged peptide ions are generated. Furthermore, the sample on MALDI targets is not fully used and can be re-analyzed.

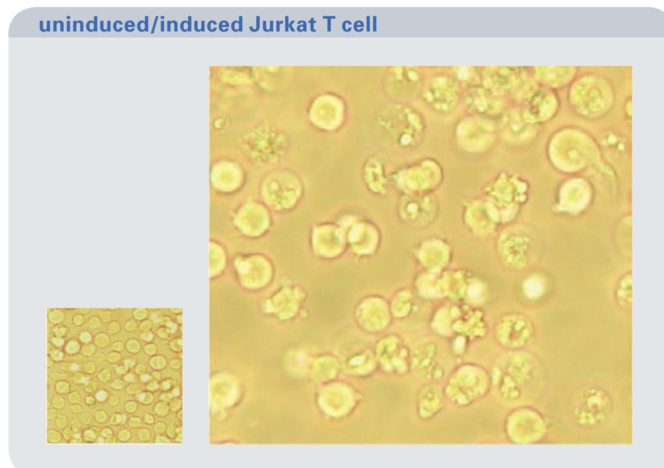


Fig. 1. The Jurkat T cell line E6. Healthy (left) and after induction of apoptosis (right).

Materials and Methods

Cell Culture, SILAC and Induction of Apoptosis

The Jurkat T cell line E6 was maintained in RPMI tissue culture medium without arginine and lysine supplemented with 10 % dialyzed fetal calf serum, penicillin (100 U/ml)/ streptomycin (100 µg/ml) (all Invitrogen) at 37°C in 5.0 % CO_2 and 100 mg/l of L-arginine-$^{12}C_6$ monohydrochloride and 100 mg/l L-lysine-$^{12}C_6$ monohydrochloride (light) or 100 mg/l L-arginine-$^{13}C_6$ monohydrochloride and 100 mg/l L-lysine-$^{13}C_6$ monohydrochloride (heavy) (Sigma), respectively. Cell population doublings were performed four times to verify full incorporation of arginine and lysine by SDS-PAGE and peptide mass fingerprinting of five gel bands. Apoptosis was induced after five cell population doublings by adding 60 µg/ml cisplatin for 16 h at 37°C in 5.0 % CO_2 to 2x106 Jurkat T cells grown in arginine-$^{13}C_6$ and lysine-$^{13}C_6$ containing medium (Fig. 1).

One-dimensional Gel Electrophoresis

Total lysates of control and apoptotic cells containing equal amounts of protein were pooled. SDS-PAGE was performed with 15 % w/v acrylamide and 0.2 % bisacrylamide for protein separation and was stained with Coomassie Brilliant Blue G-250 (Serva).

In-gel Digest and Mass Spectrometry

The Coomassie Blue G-250 stained single gel lanes were excised for in-gel digestion with 0.1 µg of trypsin (Promega) in 20 µl 25 mM ammonium bicarbonate, pH 7.8 at 37°C for 16 h. The purified and dried peptides were dissolved in 6 µl 0.1% trifluoroacetic acid (TFA) in water. An UltiMate™ 3000 nanoLC system (LC-Packings) equipped with a FLM 3X00 (integrated flow manager) and micro-vacuum degasser was used for peptide separation. The peptides were loaded onto a C18 PepMap 100 column (3.0 µm, 150 mm x 75 µm) for gradient elution (eluent A, 0.05 % TFA in water and 5 % acetonitrile; eluent B: 0.05 % TFA in 95% acetonitrile) from 0 to 50% eluent B within 40 min and for further 5 min from 50% to 90% eluent B. After 15 min, the eluted sample fractions were continuously diluted with 0.5 µl/min -cyano-4-hydroxycinnamic acid (CHCA) and spotted onto a MALDI target plate (MTP 384 ground steel) using a Probot (LC-Packings) with an interval of 30 sec resulting in 60 fractions for each gel slice. MALDI-TOF-MS spectra were acquired using an ultraflex II MALDI-TOF/TOF controlled by FlexControl 2.4 after external calibration. Peak lists were created from the raw data by FlexAnalysis 2.4 (all Bruker Daltonics) using mass-range 900-4000 Da (MS), and minimal signal-to-noise ratio 6 as peak filter settings. Only MS/MS spectra of pre-selected peaks (peaks pairs with a mass

Table 1 Compilation of software definitions for the reported SILAC experiments

Software	Specification	Definition
Mascot	mod-file	SILAC_light (KR)
Mascot	mod-file	SILAC_C13_heavy (KR)*
WARP-LC	SILE method tab	SILAC_light
WARP-LC	SILE method tab	SILAC_C13_heavy
BioTools Method	Mascot Search Parameters	Variable Modifications: SILAC_light (KR)
BioTools Method	Mascot Search Parameters	Variable Modifications: SILAC_C13_heavy (KR)
WARP-LC Method	Numerator x	2
WARP-LC Method	Denominator y	1
Sequence editor	Modification type AA	SILAC_light (KR)
Sequence editor	Modification type AA	SILAC_C13_heavy (KR)
Sequence editor	Elemental Formula	SILAC_light : Loss 0; Gain 0
Sequence editor	Elemental Formula	SILAC_C13_heavy : Loss C6; Gain Ct6

* The mass shift of 6.02 Da was defined in one modification tab for both amino acids.

difference of 6.02 Da per arginine and lysine, and a relative ratio exceeding factor 1.5) after smoothing and baseline subtractions were generated automatically by the WARP-LC 1.1 program (Bruker Daltonics). Peptides were identified by database searches (SwissProt database, 20050621, human, 12359 sequences) of the combined MS/MS peaklists using BioTools 3.0 (Bruker Daltonics) in conjunction with Mascot in-house version 2.0. (Matrix Science). The specifications and definitions to perform a SILAC experiment workflow are shown in table 1. A tolerance of 100 ppm was used for the precursor mass and 0.4 Da for MS/MS fragments. Furthermore, trypsin was selected as enzyme considering up to one missed cleavage site and variable protein modifications were allowed such as SILAC_light (KR), SILAC_C13_heavy (KR), Met-oxidation and N-terminal protein acetylation.

WARP-LC Definitions and Quantitative Analysis

The double labeling strategy using arginine and lysine required new definitions in the Mascot mod-file, the Sequence editor of BioTools 3.0 and the WARP-LC SILE method tab. The heavy isotopes of arginine-$^{13}C_6$ and lysine-$^{13}C_6$ were defined as a two in one modification, called SILAC_C13_heavy, including both amino acids (KR). Furthermore, peptides including arginine-$^{12}C_6$ or lysine-$^{12}C_6$ were defined as SILAC_light. The identical definition for heavy peptide modification (SILAC_C13_heavy) was applied to the SequenceEditor and the WARP-LC SILE tab. To quantify the peptide pairs, SILAC_light and SILAC_C13_heavy were selected in the modification list of the SILE Chemistry tab. The mass difference between $^{13}C_6$-labeled and $^{12}C_6$-labeled (i.e., unlabeled) peptides of 6.02 Da (± 0.04) per arginine and lysine residue was used for the detection of modified pairs. Proteins were considered to be modified if at least two tryptic peptide pairs with an ion score of at least 20 and a relative ratio exceeding factor 1.5 was found with a total Mascot score of at least 53. All pairs with a determined ratio > 100 were considered to be singlets, i.e., highly regulated peptides without a particular quantitative value. All mass spectrometry data of regulated proteins were manually validated using the protein and peptide lists in the ProteinBrowser and MS and MS/MS data in the WARP-LC SurveyViewer and BioTools, respectively.

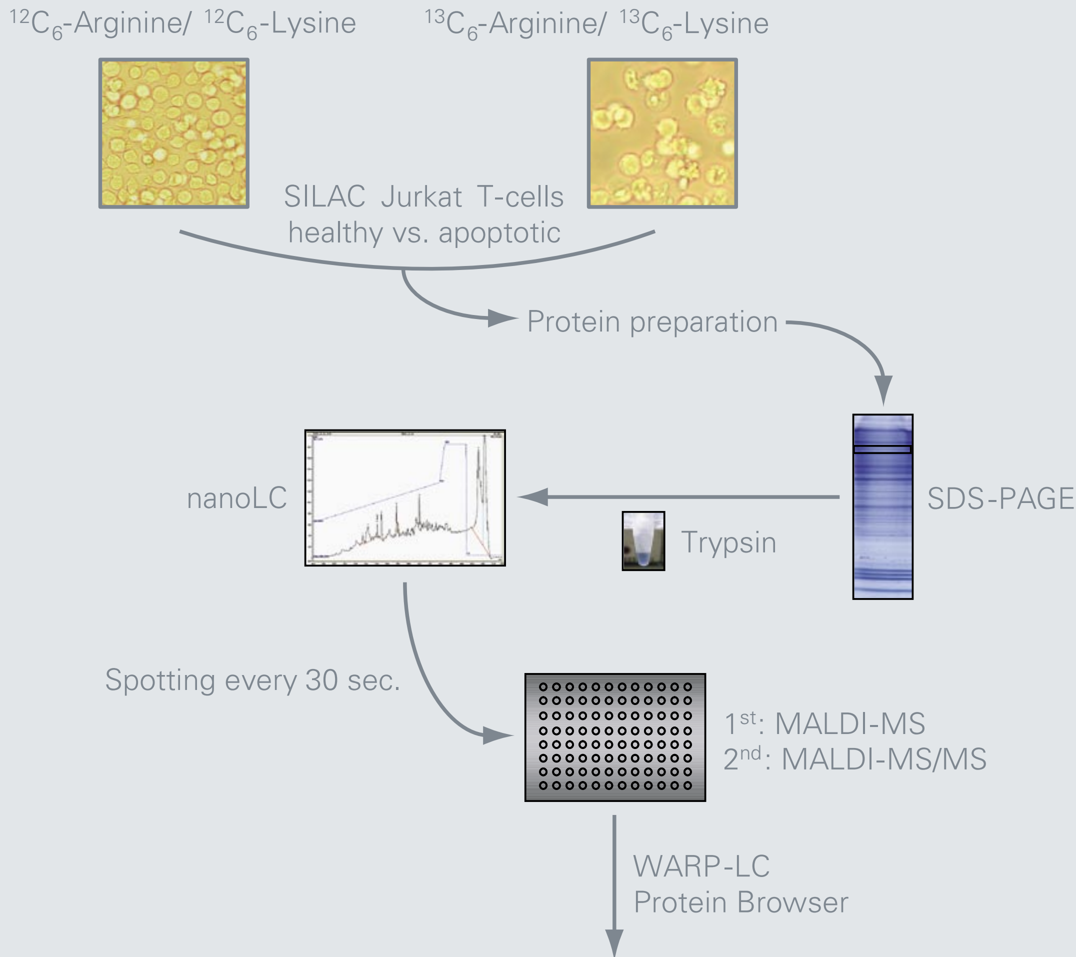
Results

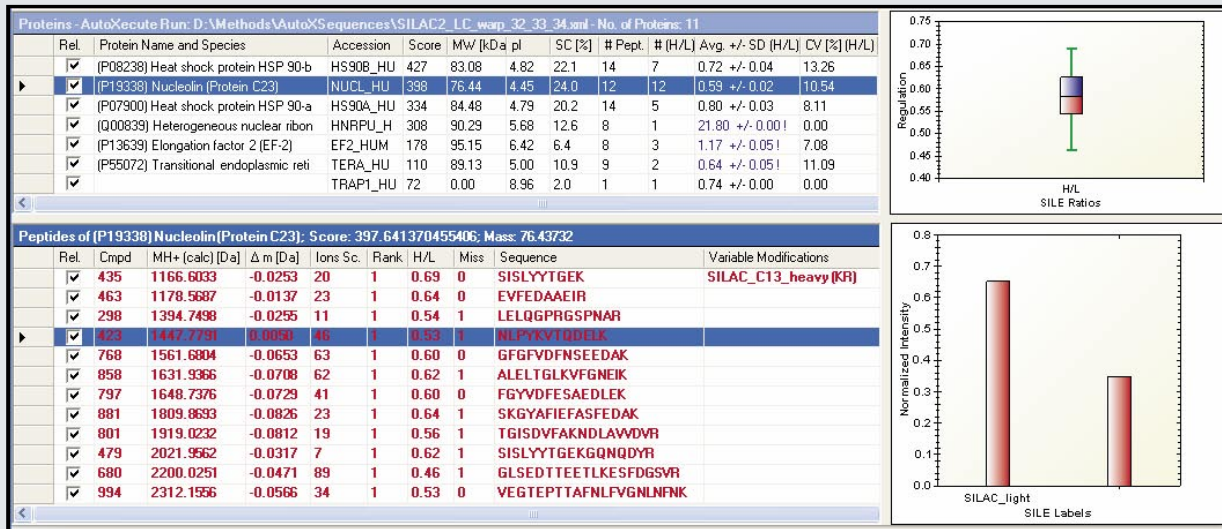
More than 250 peptide pairs were detected by LC-MALDI-TOF/TOF-MS with a mass shift of 6.02 Da or 12.04 Da within a gel band at about 80 kDa (Fig. 2). The average pair ratio (H/L) was 0.86 with an average standard deviation of 0.27 and a p-value of 0.042 (measure for the normal distribution of the H/L ratios). A relative quantity of more than factor 1.5 (H/L or L/H) was calculated for more than one hundred of the mass pairs and automatically selected for MS/MS analysis within this gel slice. One example for such a modified protein is given in (Fig. 3). Eight peptide pairs corresponded to nucleolin with an H/L ratio of the peptides varying between 0.46-0.69. Strikingly, the isotopic pattern of the peptide pair with m/z 2199.98 and 2212.02 (Fig. 3G) showed a significant deviation and revealed the lowest H/L value of 0.46 probably due to a peptide with an overlapping mass at m/z 2211. Considering the incorporation of labeled amino acids more closely, arginine-$^{13}C_6$ and lysine-$^{13}C_6$ were confirmed to be completely incorporated into the proteins. However, additional peaks with a mass shift of 5.02 Da (* in Fig. 3C and 2H) were detected and identified as proline-$^{13}C_5$ peptides, which were produced by metabolic conversion from arginine-$^{13}C_6$. Therefore, the correct peak intensity of the heavy state is the sum of both the proline-$^{12}C_5$ and the proline-$^{13}C_5$ mass peaks. Considering this into the calculation, the same ratios were detected as for non proline-containing peptide pairs. Notably, the average CV of the determined H/L values after this correction was 8.7 % - well within the typically observed range for the ICPL labeling technology (1) as one other example for current non-isobaric labeling chemistries.

The study was performed to identify apoptosis-modified proteins. Proteins grown with light arginine-$^{12}C_6$ and lysine-$^{12}C_6$ in untreated Jurkat T cells and proteins labeled with heavy arginine-$^{13}C_6$ and lysine-$^{13}C_6$ in apoptotic Jurkat T cells were combined and analyzed. The complete analysis of the SDS-PAGE gel revealed more than 30 regulated proteins using the described approach, thereof 20 already known and 10 unknown to be involved in apoptosis (details to be published elsewhere). The example presented here, nucleolin (Fig. 2, bottom), is known to be cleaved by caspases at least at two sites during apoptosis (2). The molecular mass according to SDS-PAGE indicated that we have identified full-length nucleolin. Consequently, the degradation of full-length nucleolin during apoptosis was revealed by an H/L ratio < 1 (0.59 as shown in
. 3).

The WARP-LC 1.1 software simplified the analysis of quantification data generated by the LC-MALDI approach and saved us a great deal of analysis time. Manually generated quantification results were compared with the results from the software and yielded equivalent propositions. Furthermore, the software works stable, fast and reliable if the criteria that control the selection and calculation of SILAC pairs were set in a strict way. However, it is advisable to check the results manually due to the high complexity of the data sets. Here the direct links from the quantification result table to the SurveyView and the sequence annotated raw spectra (MS/MS) in BioTools were a great help.

Schematic presentation of quantitative proteomics by SILAC, SDS-PAGE, and nano-LC-MALDI mass spectrometry





Rel.	Protein Name and Species	Accession	Score	MW [kDa	pI	SC [%]	# Pept.	# (H/L)	Avg. +/- SD (H/L)	CV [%] (H/L)
✓	(P08238) Heat shock protein HSP 90-b	HS90B_HU	427	83.08	4.82	22.1	14	7	0.72 +/- 0.04	13.26
✓	(P19338) Nucleolin (Protein C23)	NUCL_HU	398	76.44	4.45	24.0	12	12	0.59 +/- 0.02	10.54
✓	(P07900) Heat shock protein HSP 90-a	HS90A_HU	334	84.48	4.79	20.2	14	5	0.80 +/- 0.03	8.11
✓	(Q00839) Heterogeneous nuclear ribon	HNRPU_H	308	90.29	5.68	12.6	8	1	21.80 +/- 0.00 !	0.00
✓	(P13639) Elongation factor 2 (EF-2)	EF2_HUM	178	95.15	6.42	6.4	8	3	1.17 +/- 0.05 !	7.08
✓	(P55072) Transitional endoplasmic reti	TERA_HU	110	89.13	5.00	10.9	9	2	0.64 +/- 0.05 !	11.09
✓		TRAP1_HU	72	0.00	8.96	2.0	1	1	0.74 +/- 0.00	0.00

Rel.	Cmpd	MH+ (calc) [Da]	Δ m [Da]	Ions Sc.	Rank	H/L	Miss	Sequence	Variable Modifications
✓	435	1166.6033	-0.0253	20	1	0.69	0	SISLYYTGEK	SILAC_C13_heavy (KR)
✓	463	1178.5687	-0.0137	23	1	0.64	0	EVFEDAAEIR	
✓	298	1394.7498	-0.0255	11	1	0.54	1	LELQGPRGSPNAR	
✓	423	1447.7791	0.0050	46	1	0.53	1	NLPYKVTQDELK	
✓	768	1561.6804	-0.0653	63	1	0.60	0	GFGFVDFNSEEDAK	
✓	858	1631.9366	-0.0708	62	1	0.62	1	ALELTGLKVFGNEIK	
✓	797	1648.7376	-0.0729	41	1	0.60	0	FGYVDFESAEDLEK	
✓	881	1809.8693	-0.0826	23	1	0.64	1	SKGYAFIEFASFEDAK	
✓	801	1919.0232	-0.0812	19	1	0.56	1	TGISDVFAKNDLAVVDVR	
✓	479	2021.9562	-0.0317	7	1	0.62	1	SISLYYTGEKGQNQDYR	
✓	680	2200.0251	-0.0471	89	1	0.46	1	GLSEDTTEETLKESFDGSVR	
✓	994	2312.1556	-0.0566	34	1	0.53	0	VEGTEPTTAFNLFVGNLNFNK	

Fig. 2: Jurkat T cells were grown with light ($^{12}C_6$) or heavy ($^{13}C_6$) arginine and lysine. Apoptosis was induced into cells grown with heavy ($^{13}C_6$) labeled amino acids. The proteins of healthy and apoptotic cells were combined and separated by SDS-PAGE. 60 gel slices were cut and proteins digested with trypsin. For each gel slice, tryptic peptides were separated by nano-LC and spotted onto a MALDI plate every 30 seconds for MS spectra acquisition. Peptide pairs with a relative ratio > 1.5 were selected automatically by the WARP-LC program for MS/MS spectra acquisition and protein identification. Peptide and protein lists were displayed in the ProteinBrowser (bottom) after automatic outlier detection for further manual validation.

Quantification and identification of nucleolin by MALDI-TOF/TOF-MS

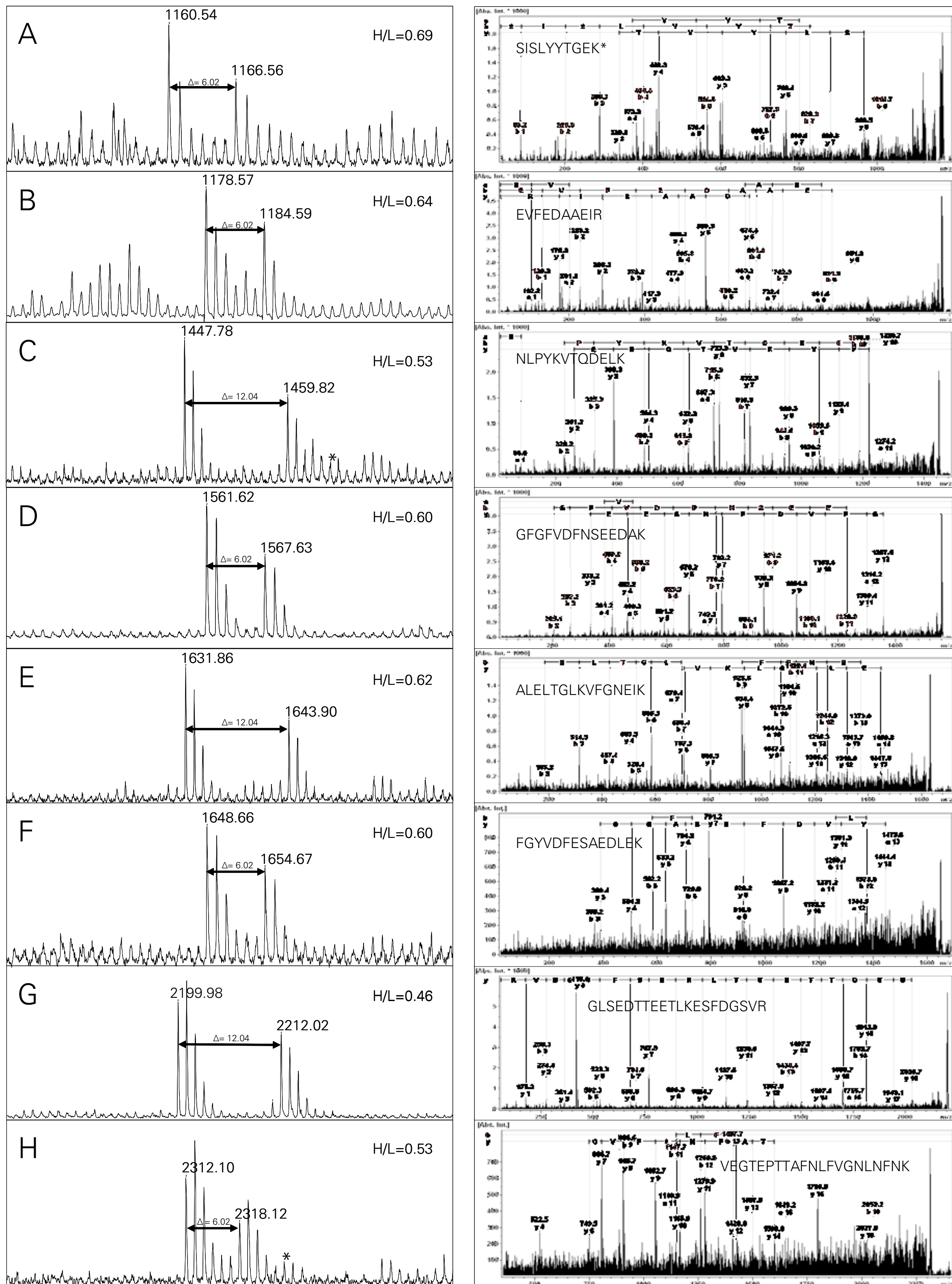


Fig. 3: Eight peptides with an average ratio of 0.59 (heavy-to-light) determined by nano-LC-MALDI-MS were automatically selected by the software WARP-LC 1.1 for analysis by MALDI-MS/MS analysis and corresponded to nucleolin (derived from SDS-PAGE gel slice at approximately 80 kDa). Predominantly, the labeled fragments in the MS/MS spectra corresponded to the matched b- and y-ions of the identified peptides.

Survey view from a single SDS-PAGE gel slice

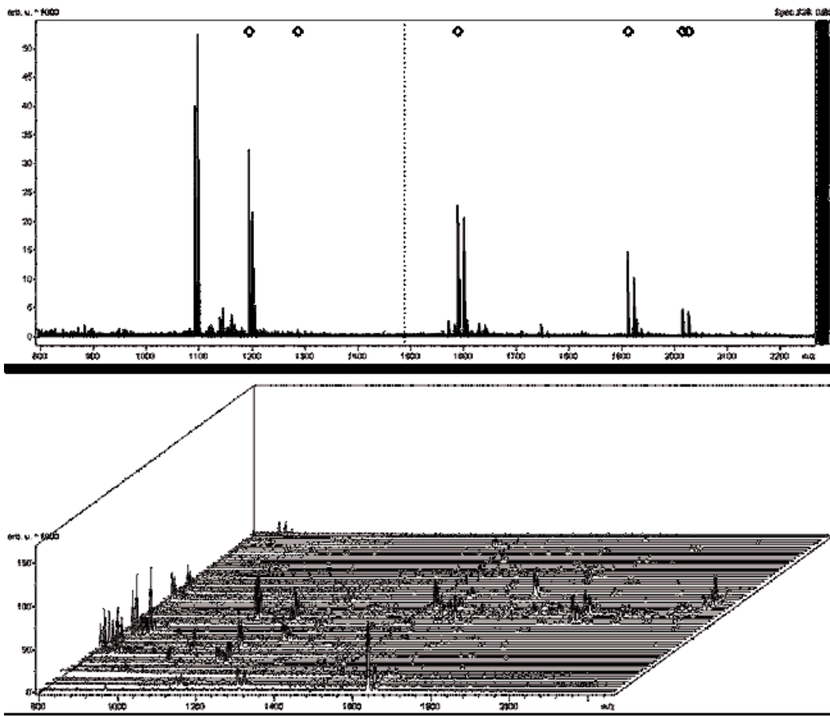


Fig. 4: A single band was cut out the gel (MW 80 kDa range) and digested with trypsin. More than 50 peptides were selected for MALDI-MS/MS analysis from this digest. The Survey-View shows the entire LC-MS/MS data from this gel band (bottom), example spectra with analyzed precursor masses are highlighted on top.

Conversion of arginine-$^{13}C_6$ into proline-$^{13}C_5$

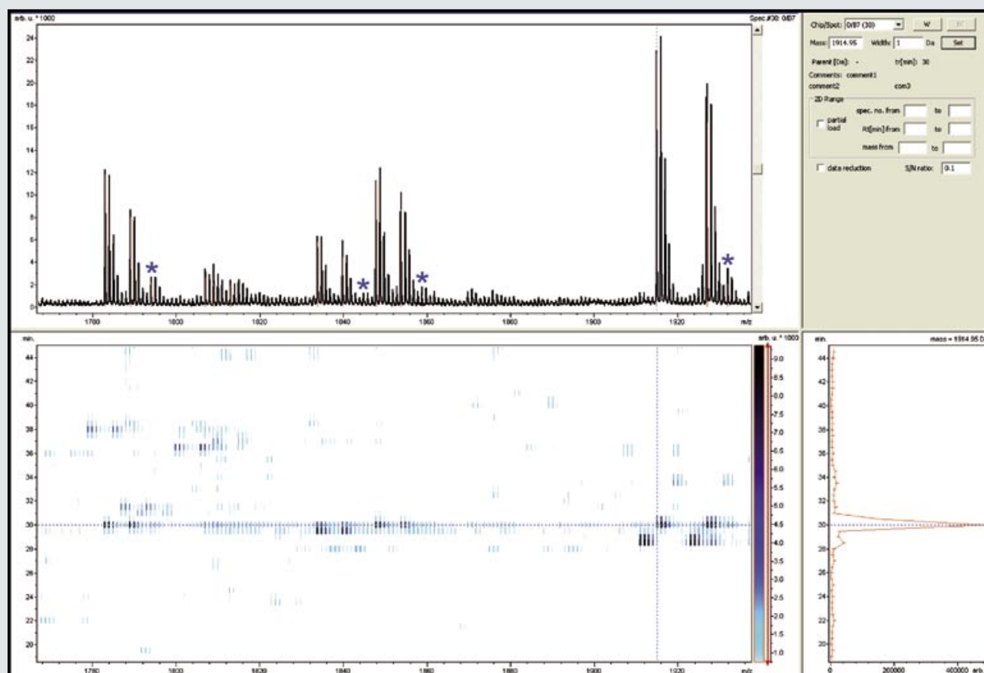


Fig. 5: Some cell lines convert arginine-$^{13}C_6$ into proline-$^{13}C_5$, which results in the formation of two distinct peak clusters for proline-containing peptides. The correct peak intensity of the heavy state is therefore the sum of both the arginine-$^{13}C_6$ or lysine-$^{13}C_6$/proline-$^{12}C_5$ and the arginine-$^{13}C_6$ or lysine-$^{13}C_6$/proline-$^{13}C_5$ mass peaks. The mass spectrum presented here included four proline-containing peptides within the range of 1770-1930 Da. The additional mass peaks of proline-$^{13}C_5$ containing peptides (marked with *) displayed approximately 15% of the intensity of the corresponding $^{13}C_6$-labeled peptides with proline-$^{12}C_5$. Thus, the H/L ratios of the four peak pairs were close to 1.

Conclusion

Different proteomic approaches were applied to study proteins involved in apoptosis. The proteome analyses enabled the identification of more than 100 different apoptosis-modified proteins (3). Thereby, mainly protein degradation was observed beside translocation, altered synthesis rates and other modifications. Most of the identified proteins were unknown to be involved in apoptosis. Approaches that combine protein separation and molecular mass determination by SDS-PAGE and labeling on the protein level prior to the separation are well-suited to identify apoptosis-modified proteins. The resulting large number of LC-runs that are generated on such proteomics experiments creates a huge workload in particular for LC-MALDI-MS/MS analysis. Here the non-isobaric nature of SILAC allowed assigning H/L values to peptide pairs, and the quantification-dependent data acquisition - as controlled by WARP-LC - provides an efficient answer to this challenge. The MS/MS workload is cut to less then 10 % of the detected peptide pairs and the MS/MS analysis focuses on the regulated peptides and proteins. Notably, the partial conversion of arginine-$^{13}C_6$ into proline-$^{13}C_5$ must be considered for maximum accuracy using SILAC approaches. A CV of < 10 % can be expected for these experiments under such conditions.

Keywords:
Quantitative Proteomics
apoptosis
Online LC-ESI-MS
offline LC-MALDI-MS
double labeling strategy
SILE
ProteinBrowser
SurveyViewer
ICPL
SILAC

Instrumentation & Software
ultraflex™ II MALDI-TOF/TOF WARP-LC 1.1 BioTools 3.0 (all Bruker Daltonics)
UltiMate™ 3000 nanoLC system Probot (both LC-Packings)
SwissProt database
Mascot 2.0. (Matrix Science)

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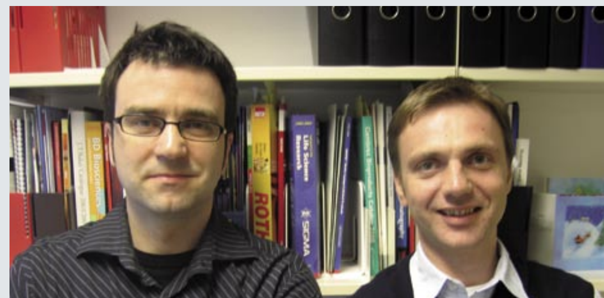

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