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Method for Qualitative Comparisons of Protein Mixtures Based on Enzyme-Catalyzed Stable-Isotope Incorporation

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Determining which proteins are unique among one or several protein populations is an often-encountered task in proteomics. To this purpose, we present a new method based on trypsin-catalyzed incorporation of the stabile isotope ^{18}O in the C-termini of tryptic peptides, followed by LC−MALDI MS analysis. The analytical strategy was designed such that proteins unique to a given population out of several can be assigned in a single experiment by the isotopic signal intensity distributions of their tryptic peptides in the recorded mass spectra. The method is demonstrated for protein−protein interaction analysis, in which the differential isotope labeling was used to distinguish endogenous human brain proteins interacting with a recombinant bait protein from nonbiospecific background binders.

Keywords: ^{18}O labeling • stable isotope • protein interactions • comparative proteomics • mass spectrometry • MALDI • nano-liquid chromatography

Introduction

The ability to detect minute differences between protein populations is a key issue in proteome research, for example when comparing protein pools representing different physiological cell states as well as when comparing samples, subjected to selective enrichment of targeted components, to control samples. The use of differential stable isotope incorporation in conjunction with mass spectrometry is one of the most promising approaches for comparative analysis. Several methods are available for introducing stable isotope labels into proteins, i.e., metabolically,[1] by chemical derivatization,[2] or enzymatically.[3,4] On the basis of these methods, a broad range of analytical strategies has been developed for comparative proteomics.[5]

Trypsin-catalyzed incorporation of ^{18}O in the C-terminus of tryptic peptides formed during and after proteolysis leads to the exchange of two oxygen atoms,[3,4] resulting in a molecular mass difference of +4 Da. This reaction is simple to perform and has found diverse use for mass spectrometric protein analysis. Proteolysis of a protein in a mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$ was used for determination of the protein's C-terminal peptide in the resulting proteolytic digest:[6] The C-terminal peptide is recognized by being the only peptide that does not exchange any oxygen atoms, and thus appears as a single signal in the mass spectrum while all other peptides appear as paired signals. A similar strategy was used for the identification of cross-linked peptides in mass spectra of proteolytic peptide mixtures:[7] The cross-linked peptides exchange a total of four oxygen atoms each while all other peptides exchange only two. Partial ^{18}O labeling was also used to assist the interpretation of fragment ion mass spectra:[8] C-terminal fragment ions can be distinguished from *N*-terminal ions by their representation by doublet signals in the spectra. The use of ^{18}O labeling was also reported for protein quantification:[9] A mixture of internal reference peptides was produced by tryptic digestion of the protein in $H_2{}^{18}O$. Lately, several different analytical strategies for quantitative differential analysis of protein mixtures have been reported.[10−12]

Stable isotope labeling is useful also when the aim is not to determine precisely the relative concentration of sample components, but also for qualitative comparisons, i.e., when the aim is to identify sample components that are unique to one protein population out of two or several. This is not a trivial task, particularly when the analyte abundance is close to the detection limit: small variations in analyte separation, sample preparation and instrument performance renders comparisons between data sets acquired from different samples unreliable.

The method reported here addresses this problem. Tryptic peptides derived from two or more protein populations are differentially labeled by trypsin-catalyzed ^{18}O incorporation. Samples of the peptide mixtures are combined and analyzed by LC−MALDI MS. Peptides unique to one sample can be readily distinguished from peptides present also in the other sample(s) by their distinctive isotopic peak patterns in the mass spectra, and identified by MS/MS analysis. This is achieved in a single experiment, and therefore the accuracy of the result does not rely on the reproducibility of the LC−MS analysis. In the current study, this method was evaluated in the analysis of protein−protein interactions by affinity pull-down experiments, to distinguish specifically enriched sample components

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from experimental artifacts. Proteins, for which interaction partners were previously known, were expressed as GST fusion proteins in *E. coli* and used as baits in affinity pull-down experiments in protein extracts from human brain.

Experimental Section

Chemicals. The peptide calibration standards, angiotensin I and ACTH 18−39 were purchased from Bachem (Heidelberg, Germany). Acetonitrile (HPLC Gradient Grade) was purchased from Carl Roth GmbH (Karlsruhe, Germany). Trifloroacetic acid (TFA), tetrahydrofuran (THF), *n*-octylglucopyranoside (nOGP), α-cyano-4-hydroxycinnamic acid (CHCA), and water used for HPLC solvents and MALDI matrix solutions were purchased from Fluka Chemie (Buchs, Switzerland). Porcine trypsin was purchased from Promega (Mannheim, Germany), dithiotreitol (DTT), iodoacetamide (IAA), and bovine serum albumin (BSA), from Sigma (Sigma-Aldrich, St. Louis, MO), and citric acid from Aldrich (Sigma-Aldrich, St. Louis, MO).

Affinity Pull-Down. GST-fusion proteins and GST were expressed in *E. coli* SCS1 at 30 °C overnight as described.[13] To prepare cell lysates, the cell pellets were resuspended in lysis buffer (1 mg/mL lysozyme, 0.5% NP 40, 1% Triton X-100, 1 mM PMSF, 25 U/mL benzonase and 150 mM NaCl in PBS) and incubated for 90 min on ice under shaking. The unsoluble cell debris was removed by centrifugation. Supernatants containing GST-fusion proteins or GST were collected for subsequent affinity pull-down (AP). Human brain extract was prepared by homogenizing 11.3 g human cortex in 17 mL AP−buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM Mg Cl_2, 1 mM EGTA, 20 mM NaF, 10% Glycerol, 1% NP-40) with protease inhibitors. Insoluble material was removed by centrifugation at 20 500 rpm for 30 min. The protein concentration in the extract was 5.3 mg/mL as determined by the Bradford assay. For affinity pull-down experiments 30 μL glutathione sepharose (Amersham Biosciences) were pipetted into each well of a 96-well filter plate (Corning Lifesciences) and washed 5 times with 200 μL ice-cold PBS. The lower side of the filter plate was sealed with adhesive foil (peQLab, nos. 82−026). Then, 50 μL PBS and 100 μL of *E. coli* cell lysate containing the GST-fusion proteins or GST (for Control 1) were added to each well. The upper side of the filter plate was sealed with adhesive foil and the samples incubated 30 min at 4 °C. The foil was removed, and the wells were washed 5 times with 200 μL PBS and once with 200 μL AP-buffer. After sealing of the lower side 50 μL AP-buffer and 100 μL brain extract or 150 μL AP-buffer (for Control 2), respectively were added to the filter plates. The plates were sealed on top again and incubated overnight at 4 °C. Then the wells were washed 3 times with ice-cold PBS. For SDS-PAGE affinity pull-downs and control samples, proteins were eluted from the respective well positions by addition of 2 × 50 μL SDS sample buffer.

Trypsinolysis on the Beads. The protein samples were equilibrated with 50 μL digestion buffer (10 mM tris-HCl buffer, pH 8.1). The buffer was removed by vacuum and the bottom of the filter plate was sealed with adhesive foil. Trypsinolysis on the beads was performed by addition of 50 μL digestion buffer containing 0.5 μg trypsin to the respective wells. A polyethylene foil was welded onto the filter plate and the samples were incubated overnight at 37 °C. Tryptic peptides were collected in a polypropylene microtiter plate by centrifugation at 2000 rpm for 5 min. Peptides from a second elution with 50 μL 0.1% TFA were collected in the same microtiter plate. The samples were lyophilized by vacuum centrifugation, redissolved in 10 μL 5 mM DTT and incubated for 30 min at 50 °C. After letting the samples cool, 10 μL 10 mM iodoacetamide was added and the sample was incubated for 30 min at room temperature, after which additional 10 μL 5 mM DTT were added.

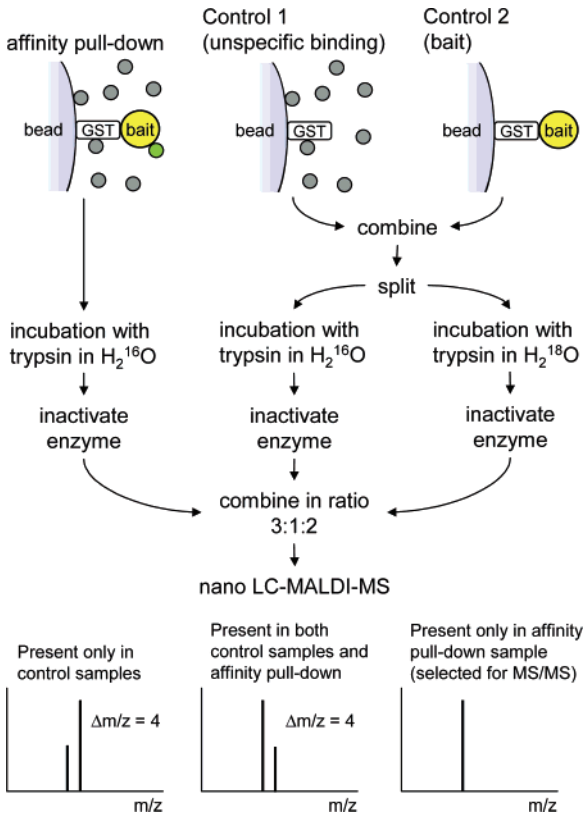

Figure 1. Analytical strategy: Three protein samples (in this case protein isolates obtained from one affinity pull-down and two control experiments) are compared with the objective to identify the proteins that are only present in one of the samples (the affinity pull down isolate). The affinity pull-down isolate is incubated with trypsin in $H_2{}^{16}O$. The two controls are pooled, then split in two parts, which are incubated separately with trypsin, one part in $H_2{}^{16}O$, and the other in $H_2{}^{18}O$. The resulting peptide mixtures are mixed in the ratio 3:1:2 and analyzed by nano LC−MALDI MS. Tryptic peptides of proteins present only in the control samples or in the control samples and the affinity pull down isolate are detected as paired signals with Δ*m*/*z* 4 Da, while peptides detected only in the affinity pull-down isolate are detected as unpaired signals.

Post-Digest ^{18}O Labeling. The ^{18}O exchange was performed in a separate step after tryptic digestion of the sample proteins. Although it is not always necessary to separate these tasks in two steps, it was required in this particular case because trypsin was also used as the eluting agent in the affinity pull-down experiment.

A 10-μL portion of each tryptic digest was used for enzymatic ^{18}O exchange. After addition of 1 μL of 1 M ammonium bicarbonate and 2 μL of trypsin solution (0.05 μg dissolved in 0.1 M HCl), samples were lyophilized by vacuum centrifugation. The lyophilized samples were redissolved in 10 μL of either $H_2{}^{18}O$ or $H_2{}^{16}O$. The sample vials were then flushed with argon, closed and additionally sealed with Parafilm. For the oxygen exchange reaction, the samples were incubated at 37 °C for 48 h. The ^{18}O- and ^{16}O-labeled digests were mixed according to the scheme shown in Figure 1 and described in the Result

section. To ensure that no oxygen back-exchange would occur after mixing the samples, trypsin was first deactivated by addition of 0.5 μL acetic acid (final concentration 5% v/v) to each sample and boiling for 15 min.

Nano-LC−MALDI MS. Nano LC-MALDI MS was performed as described recently.[14] In brief, peptide samples were analyzed on an 1100 Series Nanoflow LC system (Agilent Technologies, Waldbronn, Germany). The mobile phases used for the RP separation were Buffer A: 1% acetonitrile (v/v), 0.05% TFA (v/v) and Buffer B: 90% acetonitrile (v/v), 0.04% TFA (v/v). The LC effluent was deposited onto preformed microcrystalline layers of CHCA, prepared on prestructured MALDI sample supports (AnchorChip 600/384, Bruker Daltonics, Bremen). The CHCA layers were prepared by spreading 200 μL of matrix solution (100 g/l CHCA in 90% tetrahydrofuran, 0.001% TFA (v/v), 50 mM citric acid), containing the two calibration standards angiotensin I (1 pmol/μL) and ACTH 18−39 (2 pmol/μL) with a Teflon rod.[15]

Mass analysis of positively charged peptide ions was performed on an Ultraflex LIFT MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Positively charged ions in the m/z range 500−4500 Da were analyzed automatically in the reflector mode. Sums of 30 single-shot spectra were acquired from 10 different sample spot positions (300 in total from each sample). Fixed laser attenuation was used, the optimal value of which was determined prior to analysis by evaluation of a few fractions. MALDI-TOF/TOF analysis was performed on the Ultraflex instrument operated in LIFT mode, by operator-controlled data acquisition. Automatic detection of the peptide monoisotopic signals was performed using the algorithm SNAP,[16] implemented in the FlexAnalysis software (Bruker Daltonics, Bremen, Germany).

Data Processing. The spectra were calibrated externally using a previously described procedure based on a polynomial function.[17] Internal mass correction was performed using the signals of two peptides (Angiotensin I, MH^+: 1,296.6853 (monoisotopic mass), and ACTH (18−39), MH^+ 2,465.1989) included in the MALDI matrix solution, as reference masses. Filtering of background signals and grouping of signals into a peptide profile was performed as previously described.[14] Protein identification was performed using the Mascot software (Matrixscience, London, UK) using the SwissProt and NCBI protein sequence databases. The m/z values of all detected unpaired signals combined with all fragment ion peak lists were used as input data. The following settings were used for the searches: mass error tolerance for the precursor ion: 30 ppm; mass error tolerance for the fragment ions: 0.8 Da; fixed modification: carbamidomethylation; variable modification: methionine oxidation; number of missed cleavages: 1; type of instrument: MALDI-TOF−PSD.

Results and Discussion

Analytical Strategy. Our analytical strategy is outlined in Figure 1. An affinity pull-down experiment was performed in which a bait protein, bound to glutathione-sepharose beads, was incubated with a protein extract containing putative ligands. In parallel, two blank control experiments were performed. In one (Control 1), the linker protein, glutathione-S-transferase (GST) bound to the beads, was incubated with the protein extract. This experiment serves to isolate proteins from the extract that interact with GST and proteins that adhere nonbiospecifically to the surface of the beads. In the second (Control 2), beads loaded with bait protein were subjected to the affinity pull-down procedure with the protein extract substituted by sample buffer. In this experiment, the bait protein itself is isolated, and any impurities that may remain from its preparation.

After tryptic hydrolysis of the affinity pull-down- and control-samples, the hydrolysates from the two control experiments were combined. The mixture was then divided in two equal parts, which were separately incubated with trypsin in either $H_2{}^{16}O$ or $H_2{}^{18}O$ for post-digest ^{18}O exchange.

Following inactivation of trypsin by boiling in 5% acetic acid, the tryptic peptide mixture from the affinity pull-down experiment, the combined controls incubated in $H_2{}^{16}O$, and the combined controls incubated in $H_2{}^{18}O$, were mixed in the ratio 3:1:2. An aliquot of this peptide mixture was analyzed by nano LC−MALDI-TOF MS. In the mass spectra resulting from the analysis of all LC-fractions, peptides derived from proteins that are significantly more abundant in the affinity pull-down sample compared to the controls, i.e., specifically binding protein ligands, are represented by unpaired signals. All other peptides, i.e., those present only in the control(s) and those present both in the control(s) and in the affinity pull-down sample, are representded by paired signals ($\Delta m/z = 4$). Only the unpaired signals are selected for MALDI MS/MS fragment ion analysis and the proteins from which they were derived identified by database searching.

Detecting paired signals is easier the closer the signal intensity ratios are to 1. For protein identification it is desirable to load as high amount as possible of the affinity pull-down sample. The chosen ratio of 3:1:2 (affinity pull-down: control ^{16}O: control ^{18}O) is a good compromise between these two criteria. The reason for dividing the control sample into two parts which are incubated with $H_2{}^{16}O$ and $H_2{}^{18}O$ separately and then mixing them, instead of incubating the control sample in a mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$, was to facilitate the recognition of the $^{16}O/^{18}O$ pairs in the spectra. Incubation in a mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$ results for each peptide in three populations, incorporating zero, one, or two ^{18}O atoms. The isotopic signal distribution of such peptides is in many cases difficult to distinguish from unpaired signals, particularly for large peptides. By doing it in the described manner, we obtain essentially two populations, incorporating zero or two ^{18}O atoms, leading to distinct signal pairs separated by 4 Da, which are easily recognizable in the spectra.

Analysis of Interactions between Recombinant Bait Proteins and Native Human Brain Proteins. Two proteins were used as baits to evaluate the method: the carboxy-terminus of Hsp70-interacting protein (CHIP), and Microtubule-associated protein 1A/1B light chain 3A (MAP1A/1B LC3A). CHIP is a well-characterized co-chaperone that is known to interact with Hsp 70, Hsc 70 and Hsp 90, both in vitro and in vivo.[18,19] MAP1A/1B LC3A is involved in the filamentous cross-bridging between microtubules and other skeletal elements and can associate with MAP1A and MAP1B proteins.[20,21]

The bait proteins were expressed recombinantly in *Escherischia coli*, as GST fusion proteins. For the affinity pull-down experiment, each bait protein bound to glutathione-sepharose beads was incubated with a protein extract of human brain. To distinguish true interactants from proteins binding to the glutathione-sepharose beads and the GST domain, or from impurties of the bait protein itself, two corresponding control samples were prepared.

For one set of samples, elution was performed by adding SDS sample buffer to the washed beads, resulting in the

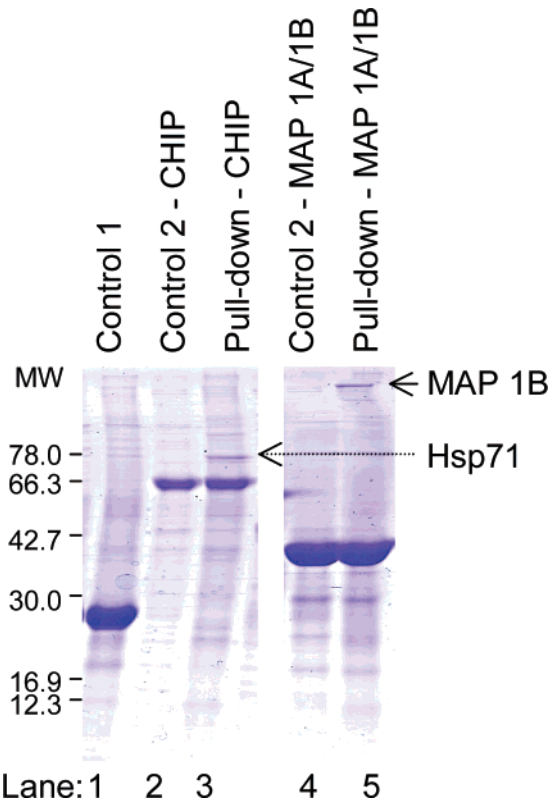

Figure 2. SDS-PAGE image of affinity pull-downs and controls. Lane 1 (Control 1): Beads with immobilized GST incubated with protein extract. Lane 2 (Control 2-CHIP): Beads with immobilized CHIP-GST incubated with buffer. Lane 3 (Pull-down CHIP): Beads with immobilized GST-CHIP incubated with protein extract. Lane 4 (Control 2 − MAP 1A/1B): Beads with immobilized MAP 1A/1B-GST incubated with buffer. Lane 5 (Pull-down − MAP 1A/1B): Beads with immobilized MAP 1A/1B-GST incubated with protein extract.

displacement of the GST fusion protein from the glutathione beads, along with its bound ligands. The eluates were analyzed by SDS−PAGE (Figure 2). The proteins isolated in the affinity pull-down experiments with CHIP and MAP1A/1B LC3A were separated in Lanes 3 and 5 on the gel, respectively. Lanes 2 and 4 correspond to the bait protein control experiments, respectively, in which each bait protein was incubated with buffer instead of protein extract. Lane 1 corresponds to the control experiment in which beads loaded with GST were incubated with protein extract. The predominating protein bands on all lanes of the gel were those corresponding to the bait proteins. In the lanes corresponding to the affinity pull-down experiments, it is possible to detect additional bands, indicating proteins that are ligands to the respective bait protein.

For the second set of samples, elution was performed by adding trypsin solution to the washed beads, resulting in the release of tryptic peptides from the proteins bound to the beads. The obtained tryptic peptide mixtures from the affinity pull-down experiment and the two corresponding control samples were subjected to differential ^{18}O-labeling, as described above, followed by nano LC−MALDI MS analysis.

The obtained peptide profile for the analysis of CHIP interacting proteins is shown in Figure 3a. In the plot, the fraction number and detected m/z value of each assigned signal is indicated by a black dot. By grouping the detected signals according to their retention times and m/z values, a peptide profile was produced. For each peptide, the isotopic signal intensity distribution of each peptide was evaluated. Out of the 303 detected peptides, 103 were unpaired (indicated in blue in the figure). The remaining 200 peptides were paired (indicated in red in the figure). The two cases can be distinguished visually in the spectra as can be seen in the example given in Figure 3b, which shows the mass spectrum acquired from Fraction 88. In the mass spectrum, only the peptide signal of m/z 1253.62 is unpaired. The fragment ion spectrum acquired from this peptide, shown in Figure 3c, identified Heat shock cognate 71 kDa protein, which is a previously known interaction partner of CHIP.

The acquired fragment ion spectra were submitted to database searches as described in the Methods section. Searches were performed both with restriction to the cleavage specificity of trypsin (C-terminal to lysine and arginine, unless adjacent to proline), and without restriction of cleavage specificity. *E. coli* proteins in the Swiss-Prot database were also searched, in case of contaminating proteins from the bait protein expression system. The significant search results are summarized in Table 1. Several heat shock proteins received high scores. However, because of the high homology among many of these proteins the data is not in all cases sufficient to determine unambiguously which are present or not. The highest-ranking candidate, Heat shock cognate 71 kDa protein (P11142), has two matching MS/MS spectra that are unique to this candidate (precursor ion m/z 1199.67 and 1481.79), whereas the other five matching fragment ion spectra could also be assigned to some of the following four candidates, which are homologous heat shock proteins. These all share one matching fragment ion spectrum (precursor ion m/z 1183.63) that distinguishes them from the Heat shock cognate 71 kDa protein. Thus, while Heat shock cognate 71 kDa protein is identified with high certainty, the four Hsp 70 proteins are identified as potential interacting partners; however, based on the obtained data it is not possible to say whether only one Hsp 70 proteins or all four of them are involved in the interaction with CHIP.

Database searches performed without restricted enzyme cleavage specificity resulted, in addition to the previously identified heat shock proteins, in high probability scores for CHIP (the bait protein) and Myelin basic protein (Table 2). The latter was detected in affinity pull-down experiments with several different bait proteins, and appears to be an abundant background protein (data not shown). Because nontryptic peptides do not undergo trypsin-catalyzed ^{18}O-exchange, these appear as unpaired signals in the mass spectra. No significant identification results were obtained from searching among *E. coli* proteins.

The identification results for the affinity pull-down experiment using MAP1A/1B LC3A as bait are shown in Table 3. A database search using the MS/MS spectra of all unpaired signals, and cleavage specificity set to trypsin, resulted in the identification of Microtubule-associated protein 1B (MAP 1B), a previously known interaction partner of MAP1A/1B LC3A. [20,21] Searching the same data without restricted cleavage specificity resulted in addition in identification of the bait protein and Myelin basic protein, as in the previous case (Table 4).

Discussion

Determining which proteins are unique among one or several protein populations is an often-encountered task in proteomics, a prominent example being the discrimination of

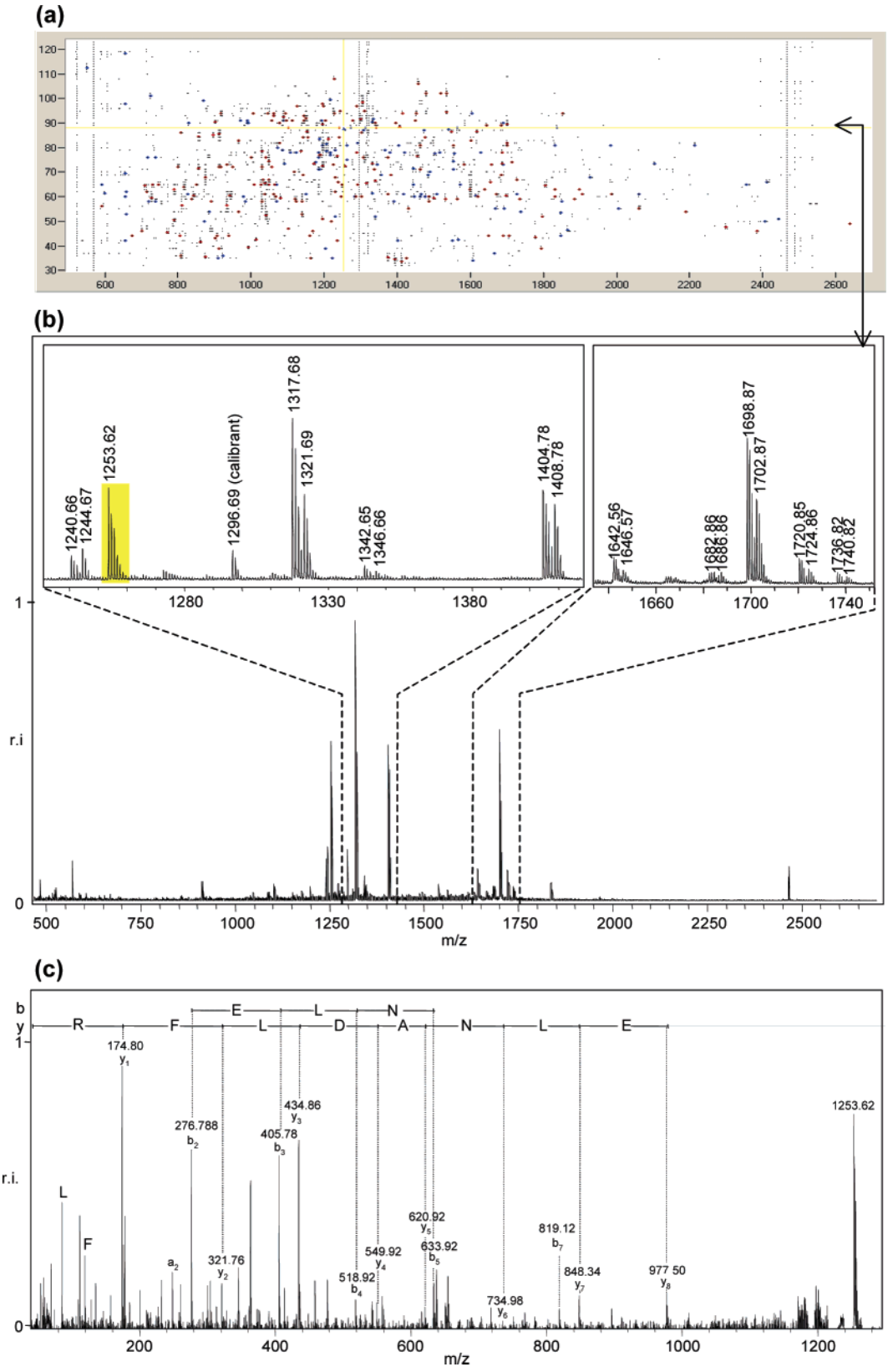

Figure 3. (a) Peptide profile obtained from the LC−MALDI MS analysis of the protein isolate obtained from the affinity pull-down experiment with CHIP as the bait protein. Blue dots: unpaired peptides, red dots: paired signals: black dots: all detected monoisotopic peaks. (b) Mass spectrum acquired from Fraction 89. The signal of *m*/*z* 1253.62 is, apart from the internal calibrants, the only unpaired signal in the spectrum. (c) MS/MS spectrum of *m*/*z* 1253.62. A database search identified the peptide FEELNADLFR of Heat Shock Cognate 71 kDa Protein.

experimental artifacts in affinity pull-down experiments as shown in this study. To date this task is usually addressed by separating and visualizing the proteins in the different samples by SDS-PAGE, and based on the gel image, it is determined

Table 1. Protein Identification Results in Interaction Experiment with CHIP as the Bait Protein[a]

significant id results	MH+	Δ *m*/*z*	ion score	aa pos	sequence
Heat shock cognate 71 kDa protein	1197.66	−0.01	12	459−469	K FELTGIPPAPR G
P11142	1199.67	−0.01	33	160−171	K DAGTIAGLNVLR I
total score: 414 (17)	1228.63	0.01	39	26−36	K VEIIANDQGNR T
database: Swiss-Prot	1253.62	0	55	302−311	R FEELNADLFR G
	1481.79	−0.02	95	329−342	K SQIHDIVLVGGSTR I
	1487.67	−0.03	93	37−49	R TTPSYVAFTDTER L
	1691.68	−0.05	87	221−236	K STAGDTHLGGEDFDNR M
Heat shock-related 70 kDa protein 2	1183.63	−0.01	25	462−472	K FDLTGIPPAPR G
P54652	1228.63	0.01	39	27−37	K VEIIANDQGNR T
total score: 299 (17)	1253.62	0	55	305−314	R FEELNADLFR G
database: Swiss-Prot	1487.67	−0.03	93	38−50	R TTPSYVAFTDTER L
	1691.68	−0.05	87	224−239	K STAGDTHLGGEDFDNR M
Heat shock 70 kDa protein 1-	1183.63	−0.01	25	461−471	R FDLTGIPPAPR G
P34931	1197.69	0	14	162−173	K DAGVIAGLNVLR I
total score: 170 (17)	1228.63	0.01	39	28−38	K VEIIANDQGNR T
database: Swiss-Prot	1487.67	−0.03	93	39−51	R TTPSYVAFTDTER L
Heat shock 70 kDa protein 1	1183.63	−0.01	15	459−469	R FELSGIPPAPR G
P08107	1197.69	0	14	160−171	K DAGVIAGLNVLR I
total score: 160 (17)	1228.63	0.01	39	26−36	K VEIIANDQGNR T
database: Swiss-Prot	1487.67	−0.03	93	37−49	R TTPSYVAFTDTER L
Heat shock 70 kDa protein 6	1183.63	−0.01	15	461−471	R FELSGIPPAPR G
P17066	1228.63	0.01	39	28−38	R VEILANDQGNR T
total score: 146 (17)	1487.67	−0.03	93	39−51	R TTPSYVAFTDTER L
database: SwissProt					
cleavage specificity: trypsin					

[a] The certainty of each matched peptide is characterized by an ion score (−10 * log *P*, where *P* is the probability that the observed match is a random event), which for all peptides matching one protein are combined to a total score. The score that corresponds to a 95% confidence interval for identity or extensive homology is given within parentheses.

Table 2. Additional Identifications in the Interaction Experiment with CHIP as the Bait Protein When Performing the Database Search without Restriction to Tryptic Peptides[a]

significant id results	MH+	Δ *m*/*z*	ion score	aa pos	sequence
Myelin basic protein	988.47	−0.02	38	217−224	D ENPVVHFF K
P02686	1332.62	−0.01	78	214−225	R TQDENPVVHFF
total score: 115 (46)					
database: SwissProt					
Carboxy-terminus of	883.41	−0.01	17	256−262	K DIEEHLQ R
Hsp70-interacting protein					
gi|4928064	992.47	−0.01	42	155−162	R IHQESELH S
total score: 560 (46)	1204.58	−0.01	97	130−140	L NFGDDIPSALR I
database: NCBI	1329.6	−0.01	88	156−166	I HQESELHSYLS R
	1355.64	−0.01	72	155−165	R IHQESELHSYL S
	1442.66	−0.03	95	155−166	R IHQESELHSYLS R

[a] The certainty of each matched peptide is characterized by an ion score (−10 * log *P*, where *P* is the probability that the observed match is a random event), which for all peptides matching one protein are combined to a total score. The score that corresponds to a 95% confidence interval for identity or extensive homology is given within parentheses.

Table 3. Protein Identification Results in the Interaction Experiment Using MAP1A/MAP1B LC3 A (Q9H492) as the Bait Protein[a]

significant id results	MH+	Δ *m*/*z*	ion score	aa pos	sequence
Microtubule-associated					
protein 1B (MAP 1B)					
	1028.60	0.00	65	2400−2409	R AVLDALLEGK A
P46821	1055.62	0.00	29	55−64	R AIGNIELGIR S
total score: 314 (17)	1241.65	−0.01	67	204−213	R HNLQDFINIK L
database: SwissProt	1646.89	−0.01	10	867−880	K LKETEPVEAYVIQK E
	1698.88	0.00	72	853−866	K DIKPQLELIEDEEK E
	2081.13	−0.03	18	348−366	K NLISPDLGVVFLNVPENLK N
	2372.22	0.00	53	104−125	R SDVLETVVLINPSDEAVSTEVR L

[a] The certainty of each matched peptide is characterized by an ion score (−10 * log *P*, where *P* is the probability that the observed match is a random event), which for all peptides matching one protein are combined to a total score. The score that corresponds to a 95% confidence interval for identity or extensive homology is given within parentheses.

which proteins are unique to a given sample. Gel bands of interest are then excised and the contained proteins identified by mass spectrometry. While this strategy has been successfully used in several studies, distinction of true protein ligands by gel image analysis is a limitation: low-abundant protein ligands and weakly interacting proteins are often not detected on the gel above the level of the background, and the low resolving power of SDS-PAGE in many cases makes matching of gel bands uncertain. Using the method described here, it is possible to omit the SDS-PAGE step and instead distinguish

Table 4. Additional Identifications in the Interaction Experiment with MAP1A/MAP1B LC3 A (Q9H492) as the Bait Protein When Performing the Database Search without Restriction to Tryptic Peptides[a]

significant id results	MH+	Δ *m*/*z*	ion score	aa pos	sequence
MAP1A/MAP1B LC3A	886.39	−0.01	45	118−125	Y ASQETFGF
Q9H492	940.48	−0.01	34	56−63	K FLVPDHVN M
total score: 712 (40)	1031.54	−0.01	47	75−83	R LQLNPTQAF F
database: SwissProt	1087.52	−0.00	42	56−64	K FLVPDHVNM S + Ox.(M)
	1148.51	−0.01	61	116−125	M VYASQETFGF
	1291.69	−0.02	32	75−85	R LQLNPTQAFFL L
	1367.69	−0.01	77	58−69	L VPDHVNMSELVK I
Myelin basic protein	1185.55	−0.00	32	214−224	R TQDENPVVHF K
P02686	1332.62	−0.00	62	214−225	R TQDENPVVHFF N
total score: 94 (40)					
database: SwissProt					

[a] The certainty of each matched peptide is characterized by an ion score (−10 * log *P*, where *P* is the probability that the observed match is a random event), which for all peptides matching one protein are combined to a total score. The score that corresponds to a 95% confidence interval for identity or extensive homology is given within parentheses.

true protein ligands from background proteins, in a single experiment, by the isotopic signal intensity distributions of their tryptic peptides in the recorded mass spectra.

To generate two peptide pools of different isotopic distributions, trypsin-catalyzed incorporation of ^{18}O in the C-terminus of tryptic peptides was used. This reaction results in incorporation of two oxygen atoms, resulting in a molecular mass difference of +4 Da. The enzymatically catalyzed isotope exchange reaction has a number of advantages over chemical derivatization. For chemical derivatization, the reagent/substrate ratio and reaction time is often critical, and have to be carefully adjusted to achieve complete derivatization, without side reactions. For enzymatic reactions, the enzyme/substrate ratio and reaction time are not so critical due to the absence of the side reactions.

In the literature, there is a large variation in the incubation time reported for the enzymatic ^{18}O-exchange, ranging from 2 to 36 h.[12,10] Kinetic studies of enzyme-catalyzed exchange showed that the reaction rates varied significantly for different peptides, and that the time required for complete exchange ranged between 1.5 and 20 h.[22] Our experiments, using tryptic peptides from BSA as the substrates, showed that there was no disadvantage of long incubation times. To ensure complete exchange, 48 h incubation was used for all experiments.

The detection sensitivity of the used LC−MALDI MS system was evaluated previously.[14] For a tryptic digest of bovine serum albumin (BSA), the optimal loading amount was 50 fmol − 1 pmol (with regard to the digested protein). Below 50 fmol, the number of detected peptides decreased steeply but with 1 fmol loaded, still signals of sufficient intensity for protein identification by MS/MS were detected. However, while signals of low intensity can still yield good MS/MS spectra, the distinction between singlets and doublets becomes unreliable when the signal-to-noise ratio is too low. In the current study, signals with $s/n < 10$ were excluded from evaluation.

The strategy described here assumes that all peptides in the samples contain Lys or Arg in their C-termini. However, when performing the database searches without restriction to trypsin cleavage specificity, the bait proteins in the respective experiments were identified (Tables 2 and 4). As can be seen in the tables, most of the matching peptides were nontryptic peptides, which explains why they did not undergo ^{18}O-exchange, and thus appear as false positives in our MS data set. These peptides are most likely formed through nonspecific cleavage by trypsin, or possibly due to chymotrypsin contamination in the protease preparation. If the latter is the case, then the use of recombinant trypsin should alleviate this problem. It would be possible to distinguish these peptides from tryptic peptides by inverting the ^{18}O labeling (i.e., incubating the affinity pull-down sample in $H_2{}^{18}O/H_2{}^{16}O$ and the control in $H_2{}^{16}O$) at the cost of splitting the sample and performing an additional LC−MALDI MS analysis per sample. However, the presence of these signals did not impair the results in this study since their MS/MS spectra do not lead to protein identification when performing the database search with trypsin as cleavage specificity.

Recently, mainly due to the development of MALDI TOF-TOF instruments, LC−MALDI MS has emerged as a complementary technique to LC−ESI MS for the analysis of complex peptide mixtures. For the method described here, LC−MALDI MS is of particular advantage. Because of the off-line nature of the LC−MALDI interface, the acquisition of MS data and their processing can be separated from the acquisition of MS/MS spectra. Thus, based on the analysis of the entire MS data set, unpaired signals can be assigned and selected for MS/MS analysis from the fractions in which they appear with the highest abundance. Because there is no time-restraint, this selection can be verified by an expert user. This workflow allows for a conservative use of sample, and results in MS/MS data sets with low redundancy.

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