Video Article

Protease- and Acid-catalyzed Labeling Workflows Employing ^{18}O-enriched Water

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Abstract

Stable isotopes are essential tools in biological mass spectrometry. Historically, ^{18}O-stable isotopes have been extensively used to study the catalytic mechanisms of proteolytic enzymes[1-3]. With the advent of mass spectrometry-based proteomics, the enzymatically-catalyzed incorporation of ^{18}O-atoms from stable isotopically enriched water has become a popular method to quantitatively compare protein expression levels (reviewed by Fenselau and Yao[4], Miyagi and Rao[5] and Ye *et al.*[6]). ^{18}O-labeling constitutes a simple and low-cost alternative to chemical (*e.g.* iTRAQ, ICAT) and metabolic (*e.g.* SILAC) labeling techniques[7]. Depending on the protease utilized, ^{18}O-labeling can result in the incorporation of up to two ^{18}O-atoms in the C-terminal carboxyl group of the cleavage product[3]. The labeling reaction can be subdivided into two independent processes, the peptide bond cleavage and the carboxyl oxygen exchange reaction[8]. In our PALeO (**p**rotease-**a**ssisted **l**abeling **e**mploying **^{18}O**-enriched water) adaptation of enzymatic ^{18}O-labeling, we utilized 50% ^{18}O-enriched water to yield distinctive isotope signatures. In combination with high-resolution matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS/MS), the characteristic isotope envelopes can be used to identify cleavage products with a high level of specificity. We previously have used the PALeO-methodology to detect and characterize endogenous proteases[9] and monitor proteolytic reactions[10-11]. Since PALeO encodes the very essence of the proteolytic cleavage reaction, the experimental setup is simple and biochemical enrichment steps of cleavage products can be circumvented. The PALeO-method can easily be extended to (i) time course experiments that monitor the dynamics of proteolytic cleavage reactions and (ii) the analysis of proteolysis in complex biological samples that represent physiological conditions. PALeO-TimeCourse experiments help identifying rate-limiting processing steps and reaction intermediates in complex proteolytic pathway reactions. Furthermore, the PALeO-reaction allows us to identify proteolytic enzymes such as the serine protease trypsin that is capable to rebind its cleavage products and catalyze the incorporation of a second ^{18}O-atom. Such "double-labeling" enzymes can be used for postdigestion ^{18}O-labeling, in which peptides are exclusively labeled by the carboxyl oxygen exchange reaction. Our third strategy extends labeling employing ^{18}O-enriched water beyond enzymes and uses acidic pH conditions to introduce ^{18}O-stable isotope signatures into peptides.

Video Link

The video component of this article can be found at http://www.jove.com/video/3891/

Protocol

The presented LeO-workflows allow for the stable isotope labeling of protein digests and synthetic peptides. These time course experiments (**Figure 1**) are applicable to comparative and quantitative proteomics studies as well as protease research. Each workflow consists of two experimental steps (**Figure 2**): A) The time resolved sampling of the respective ^{18}O-stable isotope-encoded reaction (protease-catalyzed peptide cleavage; protease-catalyzed carboxyl oxygen exchange reaction; acid-catalyzed carboxyl oxygen exchange reaction) and B) analysis by mass spectrometry and graphical representation of ^{18}O-incorporation kinetics.

A. TimeCourse Experiments

I. PALeO-TimeCourse: Protease-catalyzed labeling of proteolytic cleavages

1. (Optional) Disulfide bonds of proteins (10 µM) and peptides (250 µM) are reduced with DTT (final concentration 2.5 mM) in 25 mM NH_4HCO_3 (both freshly prepared) by incubation for 30 min at 50 °C.
2. (Optional) Free cysteines are alkylated with iodoacetamide (final concentration 10 mM) in 25 mM NH_4HCO_3 by incubation for 30 min at room temperature in the dark.
3. Depending on protease of interest, protein/peptide solutions need to be cleaned-up to remove residual buffer and alkylation agent. Use PepClean C-18 spin columns (Thermo) for peptide cleanup and Vivaspin Centrifugal Concentrators (Sartorius) to exchange buffers for protein samples.

4. Redissolve/exchange peptides/proteins in 20 µl protease reaction buffer (ECE-1: 50 mM MES-KOH, pH 5.5; trypsin: 25 mM NH_4HCO_3, pH 8.0) containing 1:1 (v/v) final $H_2{}^{18}O$ (95%, Sigma Isotec).
5. Withdraw a zero time point sample prior to the addition of the protease: Mix 0.5 µl of the reaction mixture and 0.5 µl alpha cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% acetonitrile, 0.1% TFA). Spot sample on an Opti-TOF 384 MALDI target plate (AB SCIEX) and leave the solvent droplets at room temperature until dry (about 5 min).
6. Split the reaction solution into two aliquots. The first aliquot will be incubated with the protease of interest (ECE-1: 75 nM; trypsin: 0.04 nM) at room temperature or the recommended temperature for the particular enzyme. The second aliquot will be incubated without protease and will serve as control sample. The first aliquot represents a standard sample for protease-catalyzed ^{18}O-labeling and can be used for peptide and protein identification, detection of proteolytic activities and monitoring of proteolytic cleavage reactions. The second aliquot is used to show that ^{18}O-incorporation is induced by the protease; therefore, neither labeling nor cleavage is expected for this sample.
7. Follow the reaction by removing reaction aliquots and spotting them as described under step 5 at time intervals as seem fit. The reaction conditions described above are used to monitor a proteolytic reaction for up to four days (approx. 24 spots). Start sampling every 5 min until 30 min, then spot every 30 min until 2 hr, then every hr until 8 hr and every 8 hr until 4 days. Cleavage products should appear within the first 12 hr and the substrate should be completely hydrolyzed after 24 hr. Extended reaction incubation times allow to define stable reaction products that are discrete from reaction intermediates, which are further processed. Depending on research question and given enzyme-substrate pairs, spotting times and incubation temperature have to be modulated to assure optimal reaction sampling.
8. After the final reaction time point is spotted or in between extended spotting intervals, the MALDI target plate is submitted to MALDI-TOF/TOF MS/MS analysis as described below (Section B).

II. PALeO-TimeCourse: Postdigestion labeling of proteolytic termini

1. (Optional) Disulfide bonds of proteins (10 µM) and peptides (250 µM) are reduced with DTT (final concentration 2.5 mM) in 25 mM NH_4HCO_3 (both freshly prepared) by incubation for 30 min at 50 °C.
2. (Optional) Free cysteines are alkylated with iodoacetamide (final concentration 10 mM) in 25 mM NH_4HCO_3 by incubation for 30 min at room temperature in the dark.
3. Depending on the protease of interest, protein/peptide solutions need to be cleaned-up to remove residual buffer and alkylation agent. Use PepClean C-18 spin columns for peptide cleanup and Vivaspin Centrifugal Concentrators for protein buffer exchange.
4. Redissolve/exchange the cleaned-up peptide products/proteins in 20 µl protease reaction buffer (*e.g.* trypsin: 25 mM NH_4HCO_3, pH 8.0) and digest with protease of interest to completion (*e.g.* trypsin: 0.04 nM; 37 °C; 12 hr).
5. Cleanup cleavage products with PepClean C-18 spin columns. This step will eliminate residual protease activities.
6. Redissolve/exchange the cleaned-up peptide products in 20 µl protease reaction buffer (trypsin: 25 mM NH_4HCO_3, pH 8.0) containing 1:1 (v/v) final $H_2{}^{18}O$.
7. Withdraw a zero time point sample prior to the addition of enzyme: Mix 0.5 µl of the reaction mixture and 0.5 µl alpha cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% acetonitrile, 0.1% TFA). Spot sample on a MALDI target plate and leave the solvent droplets at room temperature until dry (about 5 min).
8. Split the reaction solution into two aliquots. The first aliquot will be incubated with protease of interest (*e.g.* trypsin: 0.04 nM) at room temperature or the recommended temperature for the particular enzyme. The second aliquot will be incubated without protease and will serve as a control. The first aliquot represents a standard sample for protease-catalyzed ^{18}O-postdigestion labeling and can be used for peptide and protein quantification. The second aliquot is used to show that ^{18}O-incorporation is catalyzed by the protease; therefore, no labeling is expected for this sample.
9. Follow the reaction by removing reaction aliquots and spotting them as described under step 7.) at time intervals as seem fit. For example, we spotted initially every 5 min for up to 30 min and every 15 min after that to monitor the carboxyl oxygen exchange reaction catalyzed by trypsin.
10. After the final reaction time point is spotted or in between extended spotting intervals the MALDI target plate is submitted to MALDI-TOF/TOF MS/MS analysis as described below (Section B).

III. ALeO-TimeCourse: Acid-catalyzed labeling of carboxyl groups

1. Incubate individual peptides (50 nM) with 1:1 (v/v) ^{18}O-enriched water in the presence or absence (control) of 0.1% (v/v) final trifluoroacetic acid (total volume 30 µl).
2. Sample the reaction products daily for 48 days by co-spotting a 0.5 µl aliquot of the mixture with 0.5µl of alpha cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% acetonitrile, 0.1% TFA) directly onto a MALDI target plate.
3. Between spotting intervals and after spotting of the final reaction time point submit MALDI target plate for MALDI-TOF/TOF MS/MS analysis as described below in Section B.

B. MALDI-TOF/TOF MS/MS Data Acquisition and Analysis

1. Mass spectra are acquired on a 4800 MALDI TOF/TOF Analyzer (AB SCIEX).
2. Prior to analysis, the instrument is calibrated with a mixture of peptide standards (Mass Standards Kit for Calibration of AB SCIEX TOF/TOF instruments) with a maximum mass measurement error tolerance of ± 50 ppm and a minimum number of six peaks to match.
3. MS spectra (mass range 400 - 4,000 *m/z*) are acquired in triplicate using positive ion mode with an adjustable laser intensity (3,400 - 3,800; step size 50) with an acceptable base peak intensity range of 2,000 - 45,000. Single shots are acquired for sub-spectra, with 400 total shots/spectrum, stop conditions come into effect after 800 sub-spectra are acquired (pass or fail) or 400 sub-spectra pass acceptance criteria. In case of low-abundant samples or in the presence of complex biological backgrounds the lower end of the MS detection range should be raised to 800 m/z. In addition, it may be necessary to remove salt and other interfering compounds with a sample cleanup step as described earlier or by LC separation.
4. MS data files (.t2d files) are exported from the 4000 Series Explorer data acquisition software and imported into our in-house laboratory information system, which utilizes MASCOT Distiller software (Matrix Science) for spectral processing and peak detection. Isotopic envelopes are deconvoluted and ^{18}O-incorporation ratios automatically determined using an algorithm similar to the one described by Mason *et al.*[12] and adapted by our group[9]. Alternatively, software tools such as ZoomQuant[13] and Viper[14], as well as commercial software packages such as

BioWorks Xpress (Thermo Fisher Scientific) and Mascot Distiller Quantitation Toolbox (Matrix Science) can deconvolute ^{18}O-type data[15,16]. ^{18}O-incorporation ratios are expressed as the relative contributions of individual peptide isotope species (*i.e.,* peptides containing ^{16}O, $^{18}O_1$ or $^{18}O_2$) to the entire isotopic envelope.

5. For each TimeCourse experiment, the molecular masses ($[M+H]^+$) for all detected peptide species are extracted from the associated MS data files and the values binned at a 100 ppm mass width.
6. At least three $[M+H]^+$ are set to be required to populate a bin. In case of known substrates, the filtered bin list is compared to a list of proteolytic cleavage products predicted from the substrate peptide sequence using the ExPASy FindPept tool[17] (http://au.expasy.org/tools/findpept.html) and a 200 ppm mass error acceptance tolerance.
7. MS/MS spectra are acquired for all mass values of cleavage products predicted by the FindPept tool and for bin values that have ^{18}O-incorporations associated with them. MS/MS data are acquired on the 4800 MALDI TOF/TOF Analyzer in 1kV reflector positive ion mode, with a fixed laser intensity of 4200 and CID-gas in off mode. 50 shots are acquired in a randomized pattern per sub-spectra up to a total of 40 sub-spectra per spot (yielding a total of 2,000 shots/spot).
8. MS/MS data files (.t2d files) are exported from the 4000 Series Explorer data acquisition software and imported into our in-house laboratory information system, peaks are detected and MS/MS peak lists are associated with the corresponding binned MS data.
9. For peptide identification, MS/MS peak lists are searched against the SwissProt database using the MASCOT search engine with the following search parameters: no enzyme specificity, 150 ppm precursor ion and 0.2 Da fragment ion mass tolerances.
10. Peptide identifications can additionally be validated using the Data Explorer software (AB SCIEX) by confirming the characteristic ^{18}O-incorporation patterns across y-series fragment ions as described by Shevchenko *et al.*[18].

C. Preparation of Spectral Time and ^{18}O-incorporation Plots

Spectral time plots: MS data files (.t2d files) for each reaction time point are exported from the Data Explorer software as ASCII-files using a macro and imported into a data analysis and graphic software program (*e.g.* Origin by OriginLab) and displayed as waterfall plots (**Figure 3**).

^{18}O-incorporation plots: For each binned peptide cleavage product, the relative contributions of individual peptide isotope species (^{16}O, $^{18}O_1$ or $^{18}O_2$) are extracted across all reaction time points and plotted against time (**Figure 4**).

Representative Results

We used the PALeO-TimeCourse workflow to dynamically monitor the incorporation of ^{18}O-stable isotopes into peptide cleavage products generated by proteolytic enzymes. The presented approach is a versatile tool to comparatively study proteolytic processing pathways for different substrate and protease combinations. By sampling proteolytic reactions repeatedly over the course of the reaction, the PALeO-TimeCourse experiment provides time-resolved snapshots of substrate and product abundances and processing details. Co-spotting samples with acidic matrix solution on a target plate stops the enzymatic reaction and matrix crystallization further preserves sample composition. Therefore, time points can retrospectively be analyzed by MALDI-TOF/TOF MS/MS after conclusion of the enzymatic reaction. To accommodate the data-rich nature of these experimental workflows, we implemented a semi-automatic bioinformatics system that calculates ^{18}O-incorporation ratios for each peptide. **Figure 3** shows a representative spectral time course plot of the processing of peptide Endokinin C by ECE-1. PALeO-TimeCourse data is multidimensional: The expanded view of the MS data across the entire mass range simultaneously shows the abundances of the peptide substrate as well as the abundances of the peptide products. The temporal arrangement allows deciphering the sequence of cleavage events and determining which peptide fragments are stable cleavage products. The zoomed-in view of the MS data reveals the isotope envelopes of each peptide and cleavage-induced ^{18}O-labeling is readily identified by its characteristic isotope distribution. ^{18}O-labeling allows for positive selection of cleavage products for subsequent identification by MS/MS. Altogether, the PALeO-TimeCourse assay captures the dynamics of proteolytic processing and allows evaluating preferred cleavage sites of proteases. Yet another level of information can be obtained depending on the enzymatic mechanism of the utilized protease. Certain proteases such as the serine protease trypsin are capable of covalently rebinding their peptide cleavage products. The hydrolysis of the acyl-enzyme intermediate results in the incorporation of a second ^{18}O-atom in the C-terminal carboxyl group. **Figure 4** shows the resulting shifts in the relative contribution of the different isotopomers over time: The initial peptide bond cleavage reaction results in a 1:1 split between the ^{16}O and $^{18}O_1$ peptide fractions. The carboxyl oxygen exchange reaction results in the increase of the $^{18}O_2$ fraction to the 25% ratio at equilibrium with a concomitant decrease of the ^{16}O fraction. Proteases capable of catalyzing the carboxyl oxygen exchange reaction can therefore be used for an additional ^{18}O-labeling workflow, the postdigestion labeling of proteolytic termini. In these types of experiments, substrates are initially digested in the absence of $H_2{}^{18}O$, peptide products cleaned up and incubated with a fresh batch of protease, but this time in the presence of 50% $H_2{}^{18}O$. **Figure 4** shows the differences in isotope incorporation that can be observed in these two experimental workflows and the differences in incorporation speed between individual peptide substrates. In the postdigestion labeling workflow, the rate for both ^{18}O-incorporations is determined by the carboxyl oxygen exchange reaction. The reaction rate depends on how readily the protease interacts with its cleavage products. The affinity for the reaction product may indirectly be used to infer the affinity of the protease to the original peptide substrate. Such information could be useful to determine which peptide sequences are ideal substrates for example for tryptic digests in quantitative proteomics studies. Peptides that are readily cleaved and double-labeled by trypsin are likely to be proteotypic peptides that are reproducibly and quantitatively formed and therefore ideally suited for comparative proteomics studies.

At low pH, oxygens of carboxylic acid groups exchange with the aqueous solvent[19-20]. This reaction can be used as an alternative approach to protease-catalyzed labeling strategies to introduce stable isotopes into peptides. In the ALeO-TimeCourse (acid-catalyzed labeling employing ^{18}O-enriched water) workflow, we monitored the slow incorporation of ^{18}O-atoms into synthetic peptides. The acid-catalyzed oxygen exchange stopped after co-crystallization with the matrix solution on the MALDI target plate, effectively "freezing" the isotope distribution state. We used Angiotensin 1 as a model peptide and examined its isotope envelope over time (**Figure 5**). An uptake of two ^{18}O-atoms for each carboxyl group was observed. Under the current experimental conditions, the acid-catalyzed carboxyl oxygen exchange reaction was much slower than the protease-catalyzed exchange[9] and reached equilibrium only after 48 days. However, the ALeO approach offers the advantage of labeling peptides that are not recognized by proteases. In addition, peptides incorporate multiple ^{18}O-atoms depending on the number of acidic side chains, which can result in full separation of the isotope envelopes of unlabeled and labeled species. The overall mass shift provides information on the number of acidic residues in a given peptide and their location can be derived by the analysis of MS/MS fragment ion mass shifts. ^{18}O-labeled peptides display near identical chromatographic behavior as their unlabeled counterparts, which enables their comparative analysis

at identical elution time. In summary, acid-catalyzed ^{18}O-labeling can serve as an alternative to chemical, enzymatic and metabolic labeling approaches commonly used in quantitative proteomics. One particular promising application of this technique is the use of ^{18}O-labeled peptides as stable isotope standards.

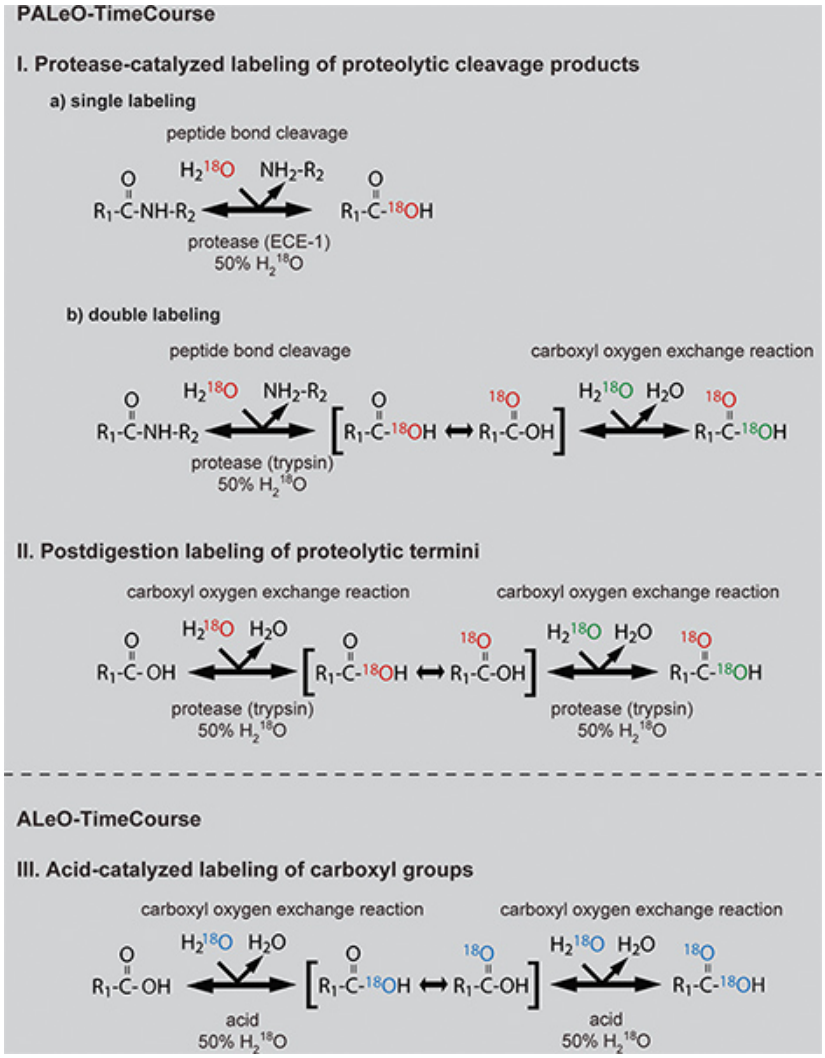

Figure 1. ^{18}O-based labeling offers a variety of time course workflows. (I) The protease-catalyzed PALeO-TimeCourse workflow allows for the monitoring of the dynamics of proteolytic cleavage reactions. Depending on protease, these reactions result in **(a)** single (*e.g.* in the case of certain metalloproteases) or **(b)** double ^{18}O-incorporation (*e.g.* in the case of certain serine proteases). **(II)** Double ^{18}O-labeler such as trypsin can also be used in postdigestion labeling workflows, in which ^{18}O-incorporation is solely mediated by the protease-catalyzed carboxyl oxygen exchange reaction. **(III)** In contrast, the ALeO-TimeCourse workflow relies on the acid-catalyzed carboxyl oxygen exchange reactions of acidic peptide side and terminal groups. Click here to view larger figure.

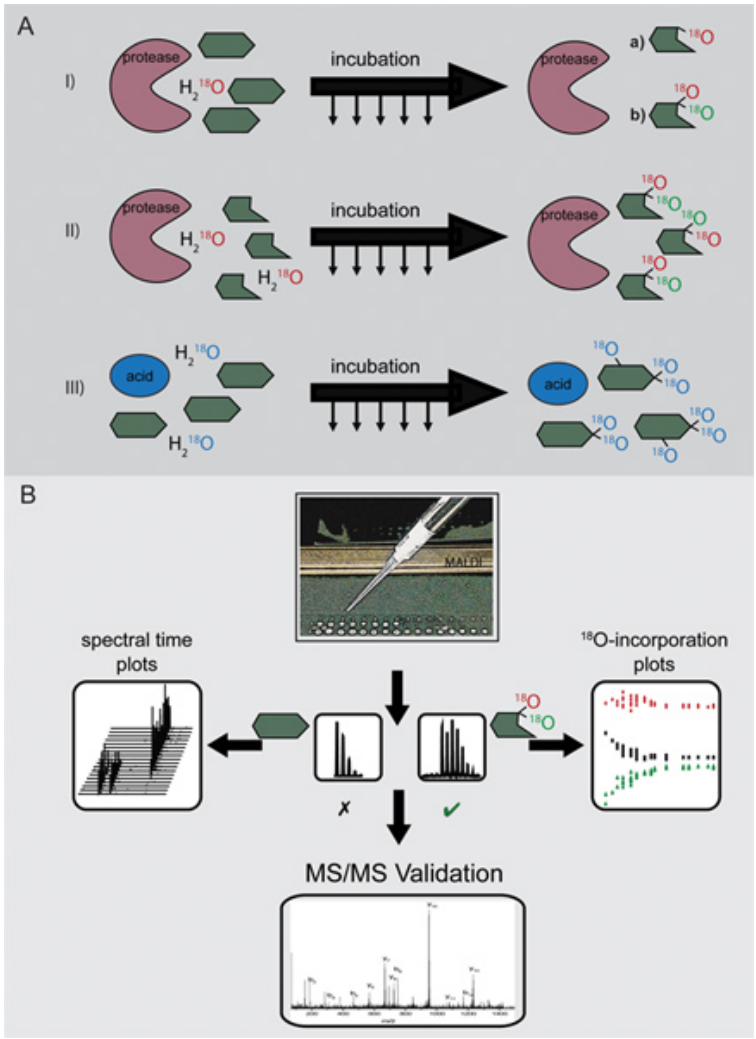

Figure 2. Experimental workflows of the PALeO- and ALeO-TimeCourse strategies. A) (I) In a protease-catalyzed PALeO-TimeCourse experiment, substrates are incubated with a protease in the presence of $H_2{}^{18}O$ resulting in the incorporation of up to two ^{18}O-atoms depending on the protease. (II) In a PALeO postdigestion labeling experiment, cleavage products from a previous digestion are re-incubated with a protease of interest in the presence of $H_2{}^{18}O$. Proteases capable of double labeling (in workflow I) will catalyze the incorporation of two ^{18}O-atoms. (III) In an acid-catalyzed ALeO experiment, all acidic functional groups incorporate ^{18}O-atoms. **B)** TimeCourse Analysis: At timed intervals, aliquots of the reaction mixtures are co-spotted with matrix on MALDI-target plates. **Upon MS-data acquisition, spectral time plots of the peptide cleavage reactions as well as ^{18}O-incorporation plots of individual peptide species are generated and cleavage products are selected for MS/MS-based sequence identification.** Click here to view larger figure.

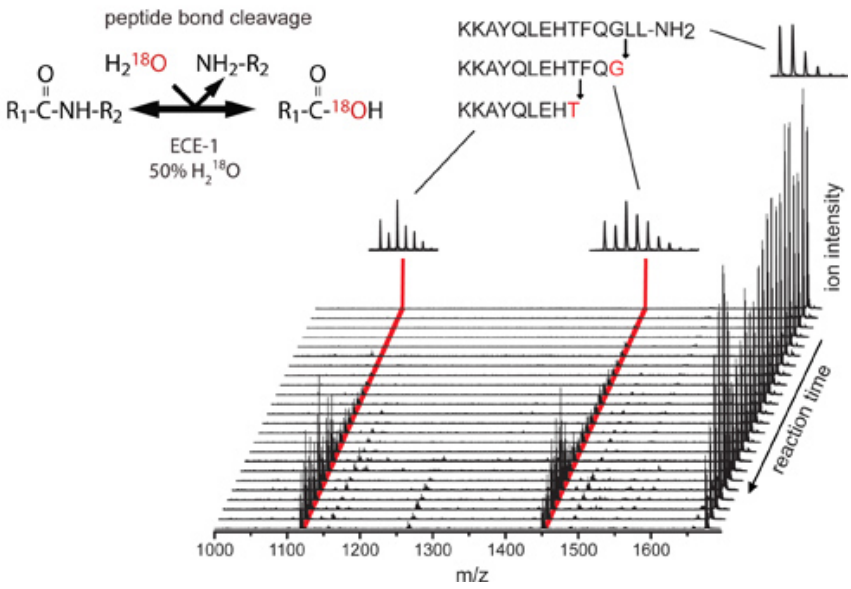

Figure 3. Spectral time plots display the dynamics of proteolytic cleavage reactions. By plotting MS spectra of PALeO-TimeCourse experiments in a waterfall arrangement, it is possible to simultaneously monitor the degradation of the substrate and the emergence of intermediate and final cleavage products. Here, the cleavage of the bioactive peptide Endokinin C by Endothelin-converting enzyme-1 (ECE-1) is shown. Cleavage products were identified by MS/MS and their isotope envelopes displayed the characteristic ^{18}O-incorporation signatures (highlighted in red).

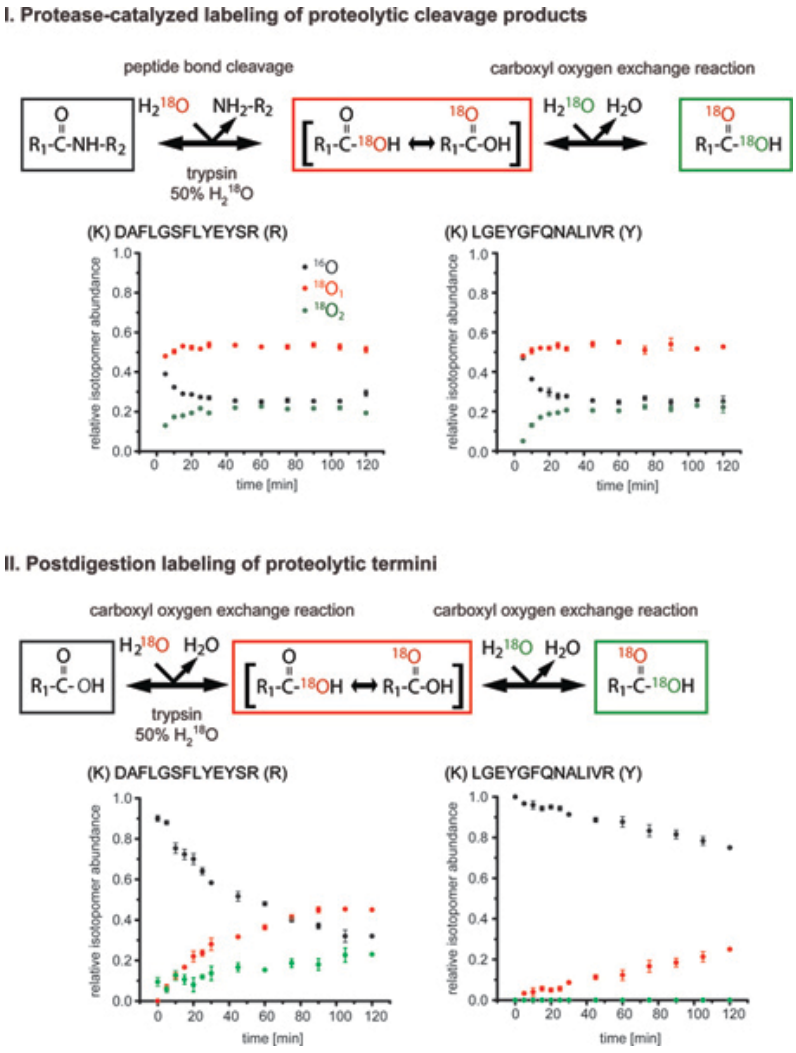

Figure 4. Serine proteases such as trypsin mediate the incorporation of up to two ^{18}O-atoms into peptide cleavage products. (I) In the protease-based labeling workflow, the peptide bond cleavage reaction results in a 50% single ^{18}O-incorporation ratio for freshly generated peptide cleavage products (black dots indicate unlabeled, red single-labeled peptide fractions). Proteases that rebind their reaction products (*e.g.* serine proteases such as trypsin) further catalyze the incorporation of a second ^{18}O atom via the carboxyl oxygen exchange reaction (green dots, double-labeled peptide fraction). At equilibrium, a label distribution of 0.25:0.5:0.25 (un-; single-; double-labeled) is reached. **(II)** In the postdigestion labeling of proteolytic termini workflow, no peptide bond cleavages occur. Instead, the incorporation of up to two ^{18}O-atoms is exclusively based on the carboxyl oxygen exchange reaction. Therefore, ^{18}O-incorporation only occurs with proteases that rebind their peptide cleavage products. Click here to view larger figure.

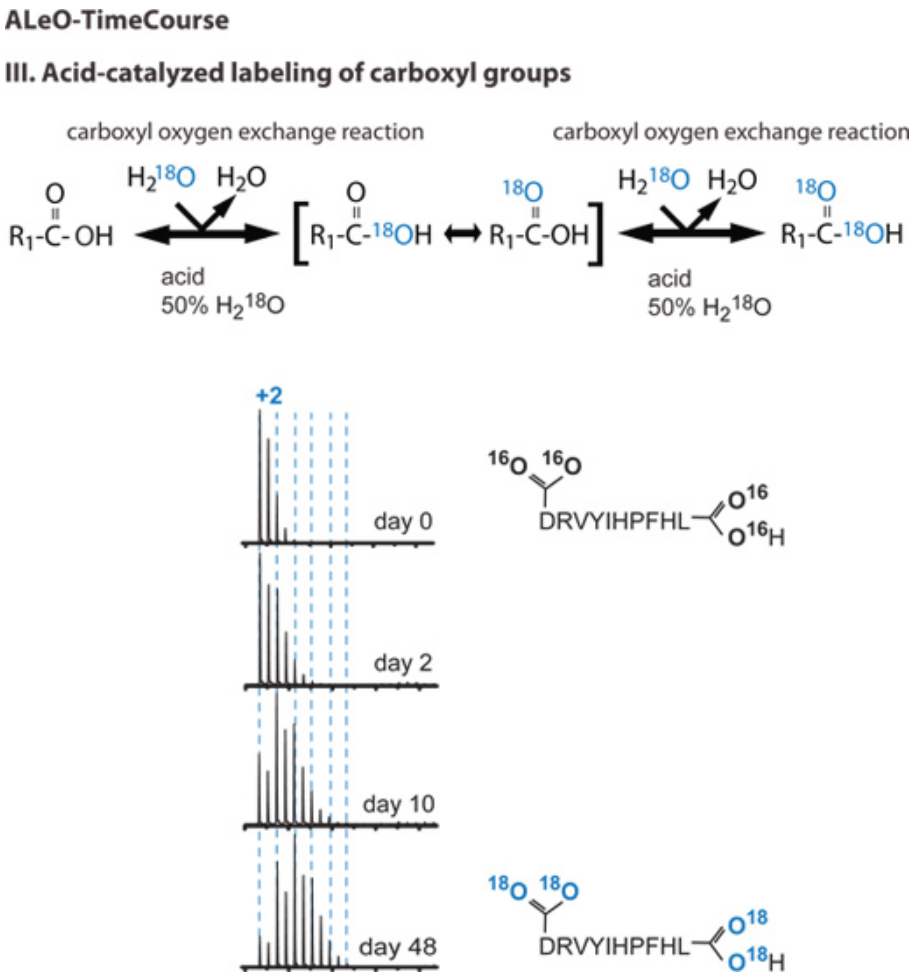

Figure 5. Acid-catalyzed ^{18}O-labeling leads to the incorporation of two ^{18}O-atoms per carboxyl group. Over the course of the experiment, the isotopic envelope of Angiotensin-1 underwent multiple +2 Da mass shifts (dashed lines) corresponding to the uptake of ^{18}O-atoms. The sites of ^{18}O-incorporation are highlighted in the amino acid sequence (blue).

Discussion

By combining stable isotope labeling and high-resolution mass spectrometry in a time-resolved manner, the PALeO-TimeCourse method allows for a dynamic analysis of the generation of peptide products. The assay can be used to generate stable isotopically labeled peptides for quantitative and qualitative proteomics studies and to evaluate the kinetics by which proteotypic peptides are generated. Furthermore, PALeO-TimeCourse is designed to evaluate proteolytic pathways under specific, physiologically relevant conditions *ex vivo.* Endogenous proteins, peptides and proteases as well as synthetic peptides and recombinant proteases can be utilized in the workflow. Depending on the particular proteolytic reaction to be investigated, assay conditions and spotting times can be adjusted to provide for optimal sampling of the reaction process. In our experience, it is helpful to slow proteolytic reactions down (*e.g.* by using low enzyme concentrations, room temperature) to allow for convenient manual sampling intervals**.** Like with other stable-isotope labeling methodologies, deviations for the experimental ^{18}O-incorporation ratio measurements are small - typically less than 5% for peaks with sufficient ion statistics. The overall protocol can be easily adapted to other assay formats, for example the screening of a large set of synthetic substrates or the characterization of the proteolytic processing of endogenous peptides from complex biological samples (*e.g.*, cell culture media, body fluids). We previously demonstrated how the PALeO-approach can be hyphenated with an LC-separation step, and used to define the dynamic composition of the human salivary peptidome[9]**.** Proteolytic signatures identified by the PALeO-TimeCourse provide important facts regarding the substrate specificity of the protease of interest. In addition, the time-resolved data also yields kinetic information. Such knowledge is particularly useful in the evaluation of proteotypic peptides for quantitative proteomics studies, where the choice of reproducible and highly abundant target peptides is essential[21]. Likewise, identifying rate-limiting steps in proteolytic pathways is of high interest for the development of novel protease-based drugs. Proteases are key factors in many physiological and pathological processes (*e.g.* cardiovascular disorders, neurodegenerative diseases, cancer) and such observations can open up new opportunities for therapeutic interventions. The ALeO-strategy - acid-catalyzed labeling of carboxyl groups - is easily applied to synthetic and endogenous peptides to produce stable isotopically encoded standards. The kinetics of the acid-catalyzed reaction is much slower than the enzyme-catalyzed reaction. Therefore, experiments have to be carefully planned ahead. ALeO-TimeCourse is a low-cost alternative to chemical, metabolic and synthetic labeling methods currently used in proteomics studies. The labeling process can be monitored to validate that ^{18}O-incorporation reached equilibrium, which can be an advantage over other stable isotope labeling methods. In conclusion, the LeO-

TimeCourse workflows described here are versatile tools that can creatively be employed in many qualitative and quantitative proteomics studies as well as protease research.

Disclosures

No conflicts of interest declared.

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