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Analysis of peptide hormone maturation and processing specificity using isotope-labeled peptides

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Summary

Many peptide hormones and growth factors in plants, particularly the small post-translationally modified signaling peptides, are synthesized as larger precursor proteins. Proteolytic processing is thus required for peptide maturation, and additional post-translational modifications may contribute to bioactivity. To what extent these post-translational modifications impact on processing is largely unknown. Likewise, it is poorly understood how the cleavage sites within peptide precursors are selected by specific processing proteases, and whether or not post-translational modifications contribute to cleavage site recognition. Here we describe a mass spectrometry-based approach to address these questions. We developed a method using heavy isotope labeling to directly compare cleavage efficiency of different precursor-derived synthetic peptides by mass spectrometry. Thereby, we can analyze the effect of post-translational modifications on processing, and the specific sequence requirements of the processing proteases. As an example, we describe how this method has been used to assess the relevance of tyrosine sulfation for the processing of the Arabidopsis CIF4 precursor by the subtilase SBT5.4.

1. Introduction

Plant peptide hormones and growth factors, particularly the small post-translationally modified signaling peptides, are typically synthesized as larger precursor proteins. These precursors are proteolytically processed to release the mature peptides, which often require additional post-translational modifications for bioactivity, including tyrosine sulfation, proline hydroxylation, and glycosylation of hydroxyproline residues (*1*). The cleavage of peptide precursors by processing proteases can be analyzed conveniently by liquid chromatography (LC) coupled to mass spectrometry (MS), using synthetic peptide substrates that encompass the mature peptide sequence, extended by several precursor-derived amino acids at either end. For example, N-terminally extended IDA (Inflorescence Deficient in Abscission) peptides were used in LC-MS assays to identify the cleavage site, and to characterize cleavage specificity of IDA precursor-processing subtilases (*2*). Likewise, PSK (phytosulfokine) precursor processing by phytaspase, and its reliance on Asp for cleavage site recognition, were analyzed by MS using synthetic PSK peptide variants extended by five precursor-

derived residues at their N-termini (3).

While the identification of cleavage sites in synthetic peptides is rather straight forward, comparing processing efficiency between different sequence variants, or between post-translationally modified versus unmodified peptides is not, since MS analysis is not inherently quantitative. A direct comparison of ion intensities for different cleavage products is hampered by the fact that ionization properties may vary widely, even for closely related peptides. Further, label-free approaches for quantification based on peak intensity from extracted ion chromatograms suffer from sample variation, from run-to-run variability in instrument performance, and from suppressive effects of other ions (4). These problems have been circumvented in quantitative proteomics by the use of stable isotope-labeled peptide standards. Isotopologs of proteotypic peptides of the target protein are spiked into the sample prior to LC-MS analysis. They share the same physicochemical properties, including chromatographic co-elution, ionization efficiency, and fragmentation pattern, but can clearly be distinguished by their mass difference (5). Simultaneous measurement of ion intensities for the proteotypic and the isotope-labeled peptides compensates run-to-run variations in LC-MS analyses, caused by differences in injected sample volume or concentration, ion-suppression effects of co-eluting ions, as well as limitations in intrinsic dynamic range of MS, thereby permitting reliable quantification of the target protein (4).

We have employed this approach to analyze cleavage site selectivity of precursor processing proteases, and to assess the effect of post-translational modifications on cleavage site recognition. The rationale is outlined in Fig. 1a-c. In Fig. 1a, the relevance of a certain amino acid residue in P₁ or P₂ (the first and second residue N-terminal to the cleaved bond; nomenclature according to (6)) of a given precursor protein for cleavage by its processing protease, or the effect of a post-translational modification at one of these residues, is assessed with synthetic peptides encompassing the cleavage site. An equimolar mixture of the native peptide and a P₁ or P₂ substituted peptide, in which one of the prime-side residues (C-terminal of the cleavage site) is heavy isotope-labelled, is digested with the processing protease. Relative processing efficiency, and therefore, the effect of the amino acid substitution (or post-translational modification) on cleavage of the two peptides is analyzed by LC-MS. The abundance only of the prime-side cleavage product is compared, as it is identical for the two

peptides, but can be distinguished based on the mass difference. *Vice versa* in Fig 1b, by placing the isotope label on the non-prime side of the hydrolyzed bond, the effect of prime-side substitutions can be analyzed. The approach is particularly useful also with respect to the effect of post-translational tyrosine sulfation (Fig 1c). In contrast to the phosphate ester, the sulfoester bond in sulfo-tyrosine is extremely labile at low pH, and the sulfate may thus be partially lost during LC. During MS and MS/MS, particularly in positive ion mode, sulfur trioxide is easily eliminated to produce the unmodified tyrosine, and sulfated y- and b-ions are not detected in fragment (MS/MS) spectra (7, 8). Therefore, cleavage products of the sulfated peptide and of the unmodified peptide cannot be distinguished. However, peptide quantification can be achieved by adding a heavy-isotope label to one of the residues in either one of the cleavage products (Fig. 1c).

Here we used this approach to analyze the effect of tyrosine sulfation on the processing of the *Arabidopsis thaliana* CIF4 (Casparian strip Integrity Factor 4) precursor by the processing subtilase SBT5.4. In **Section 3.1** we describe the *in vitro* cleavage assay, using a synthetic CIF4 peptide extended by three precursor-derived amino acids at either end, and its tyrosine-sulfated and glycine-^[13C2, 15N]-substituted isotopolog, as substrates for recombinant SBT5.4. The protocol for the identification of cleavage products by nanoLC-ESI-MS/MS is given in **Section 3.2**. Results are presented in Figs. 1d and 2, showing that the CIF4 precursor peptide is cleaved by SBT5.4 only at the C-terminal but not at the N-terminal processing site. The data further indicate that tyrosine sulfation does not affect processing, neither at the N-terminal nor at the C-terminal site.

2. Materials

2.1 Equipment

1. Desiccator (5 l) with vacuum pump.
2. End-over-end shaker.
3. Incubation shaker for microfuge tubes.
4. Vacuum concentrator.
5. High resolution tandem mass spectrometer (e.g., Orbitrap Exploris 480; Thermo Fisher Scientific,

Germany) linked to a nano-high performance liquid chromatography (nano-HPLC) system (e.g., Ultimate 3000 RSLCnano; Dionex, Thermo Fisher Scientific).

6. Trap (e.g. μ -precolumm C18 PepMap100, 300 μ m, 100Å, 5 μ m x 5mm; Thermo Fisher Scientific) and analytical (e.g. NanoEase M/Z HSS C18 T3, 1.8 μ m 100 Å 75 μ m x 250 mm, Waters, Germany) reverse-phase chromatography columns suitable for the used nano-HPLC system.

7. Personal computer with software package for peptide identification by MS/MS (e.g. Mascot 2.6; Matrix Science Ltd, London, UK), peptide quantification (e.g. Xcalibur; Thermo Fisher Scientific), and data visualization (e.g. Scaffold; Proteome Software, Portland, OR, U.S.A.).

2.2 Synthetic peptides

Custom-synthesized synthetic precursor peptides comprising the mature peptide sequence with at least three additional amino acids flanking the processing sites derived from the precursor sequence (*see Note 1*). In the described experiment, we used a N- and C-terminally extended CIF4 peptide (LYG/DYGFWNPSPVYGGGFPYPGPVPH/GSL) and its sulfated and Glycine-[¹³C₂,¹⁵N]-substituted analog (LYG/**Ds**YGFWNPSPVYGGGFPYPGPVPH/GSL); extensions underlined; substitutions in bold; processing sites indicated by “/”) as a specific case example (*see Note 2*).

2.3 Plant Material, Constructs, Bacteria and Media

1. *Nicotiana benthamiana* plants six to eight weeks old (*see Note 3*).
2. *Agrobacterium tumefaciens*, strain C58C1 (Rif^R, Tet^R)(*see Note 4*).
3. *Agrobacterium tumefaciens*, strains C58C1 with p19 suppressor of silencing (Kan^R, Rif^R, Tet^R)(**9**).
4. Expression constructs for the protease of interest (here SBT5.4) in a binary vector. We use pART27 (Spec^R), (**10**), other binary vectors are equally possible.
5. 100 μ g/ml rifampicin; 1000x stock in DMSO.
6. 25 μ g/ml tetracyclin; 1000x stock in 70 % (v/v) ethanol.
7. 50 μ g/ml kanamycin; 1000x stock in dd H₂O.

- 100 µg/ml spectinomycin; 1000x stock in ddH₂O.
- LB (Lysogeny Broth)-medium: add 10 g tryptone, 5 g yeast extract and 5 g NaCl to 1 l H₂O. For solid media, add 15 g/l agar. Autoclave for 20 min. Cool down to about 60 °C before adding antibiotics from 100x stocks and pouring plates.

2.4 Transient Expression in *N. benthamiana* and purification of SBT5.4

1. Infiltration buffer: 10 mM MES pH 5.6, 10 mM MgCl₂.
2. 0.15 mM acetosyringone.
3. 15 ml and 50 ml culture tubes.
4. 1 ml and 20 ml PP/PE syringes.
5. Extraction buffer: 50 mM Na₂HPO₄/NaH₂PO₄ pH 7.0, 200 mM KCl, 1 µM pepstatin, 10 µM E64.
6. Ni-NTA agarose: 50 % slurry in 30 % (v/v) ethanol.
7. Binding buffer: 50 mM Na₂HPO₄/NaH₂PO₄ pH 7.0, 200 mM KCl, 4 mM imidazole.
8. Elution buffer: 50 mM Na₂HPO₄/NaH₂PO₄ pH 7.0, 200 mM KCl, 200 mM imidazole.
9. Bradford protein quantification assay.
10. Reaction buffer: 50 mM Na₂HPO₄/NaH₂PO₄ pH 6.0, 300 mM NaCl.

2.5 Sample preparation for MS analysis

1. PTFE (polytetrafluorethylene; TeflonTM) membranes with embedded C18 beads (e.g. Supelco EmporeTM solid phase extraction discs; Sigma-Aldrich/Merck, Darmstadt, Germany).
2. Solvent A: 0.5 % (v/v) glacial acetic acid, 80 % (v/v) acetonitrile in HPLC-grade water.
3. Solvent B: 0.5 % (v/v) glacial acetic acid in HPLC grade water.
4. Solvent C: 0.5 % (v/v) glacial acetic acid, 50 % (v/v) acetonitrile, in HPLC-grade water.

2.5 MS analysis

1. Solvent D: 0.1 % (v/v) formic acid, in HPLC-grade water.

2: Solvent E: 0.1 % (v/v) formic acid in 80 % (v/v) acetonitrile, in HPLC-grade water.

3. Methods

3.1 Digestion of the CIF4 precursor peptide and its sulfated isotopolog by SBT5.4

3.1.1 Transient expression of SBT5.4 by agro-infiltration of *N. benthamiana* leaves

1. Resuspend a single colony of *A. tumefaciens* strains carrying the expression construct for (i) C-terminally His-tagged SBT5.4 (or the empty pART27 vector as a control) and (ii) the p19 silencing suppressor in 500 μ l LB, and streak them out evenly on LB plates with appropriate antibiotics (100 μ g/l rifampicin + 25 μ g/l tetracycline + 100 μ g/l spectinomycin for (i), 50 μ g/l kanamycin instead of spectinomycin for (ii)). Grow for 48 h at 28 °C.
2. For each plate, resuspend the bacteria in 6 ml infiltration buffer, wash it off the plate, and collect the suspension in a 15 ml culture tube.
3. Spin down at 4000 \times g for 15 min, discard supernatant, resuspend in 6 ml infiltration buffer.
4. Perform two more washes as in 3, but resuspend in 1 ml infiltration buffer after the final wash.
5. Determine optical density (OD) at 600 nm.
6. Prepare bacterial infiltration solution by mixing the strains carrying the expression constructs (i) and (ii) at an OD₆₀₀ ratio of 0.7_(i):1.0_(ii) in a total volume of 150 ml infiltration buffer. The dilution factor Y for (ii) is OD_{600(ii)}/1.0= Y . The dilution factor Z for (i) is OD_{600(i)}/0.7= Z . To prepare X ml of Infiltration solution add X/Y ml of suspension (ii) and X/Z ml suspension (i).}}
7. Add acetosyringone to a final concentration of 150 μ M, and incubate for 1 to 3 h at room temperature.
8. Use a needle-less 1 ml syringe to pressure-infiltrate the bacterial suspension into *N. benthamiana* leaves by gently pressing the syringe onto the abaxial side, supporting the leaf with one finger on the opposite side. Continue to grow the plants as described (see **Note 3**).
9. Leaves are harvested at 5 days post infiltration (see **Note 5**).
10. Remove the central vein and place the remaining leaf material facing upside down into ice-cold extraction buffer.
11. Use a desiccator for vacuum infiltration at 75 mbar for 2 min, then slowly release the vacuum.

12. Blot the leaves dry and place them into a syringe barrel plugged with glass wool, within a centrifuge tube.
13. Spin for 7 min at 1500 x g and 4 °C to collect the apoplastic wash.
14. Transfer the apoplastic wash to a new tube, remove cellular debris by centrifugation, and collect the supernatant into a new tube.
15. Add elution buffer to adjust imidazole concentration to 4 mM.

3.1.2 Purification of recombinant SBT5.4

Metal chelate affinity chromatography of His-tagged SBT5.4 is performed on Ni-NTA agarose in a batch procedure.

1. Sediment 500 µl Ni-NTA agarose (50 % slurry) in a 15 ml culture tube by gentle centrifugation (about 500 x g; we use a manual centrifuge for this purpose).
2. Wash 3 times with 10 ml binding buffer, resuspend in 1 ml binding buffer after final wash.
3. Add the apoplastic wash from step 13 above; incubate for 1 h at 4°C in an end-over-end shaker.
4. Sediment the matrix by gentle centrifugation and wash twice with 12 ml binding buffer.
5. Add 600 µl elution buffer, sediment by centrifugation, recover the supernatant. Repeat twice.
6. Determine protein concentration using the Bradford assay.
7. Add 50 % (v/v) glycerol and store at -20 °C.

3.1.3 Peptide digest

1. Resuspend the lyophilized peptides in ddH₂O and determine the concentration using the molar extinction coefficients for tyrosine ($\epsilon_{280} = 1490 \text{ M}^{-1}\text{cm}^{-1}$) and sulfo-tyrosine ($\epsilon_{260} = 283 \text{ M}^{-1}\text{cm}^{-1}$; **(II)**).
2. Set up the *in-vitro* digestion reaction (*see Note 6*) in 100 µl reaction buffer containing the two peptides at 10 nM each, and SBT5.4 at 3.4 pM (*see Note 7*). As a control, set up a second digest, in which the SBT4 protease is replaced by the corresponding volume of a sample that was the mock-purified from empty-vector-infiltrated plants, following the same procedure as described in 3.1.1 and 3.1.2.
3. Incubate at 25 °C and 300 rpm in an orbital incubation shaker for microfuge tubes.

4. Take 20 μl aliquots at 15 min, 1 h, 2 h and 5 h, and stop the reaction by addition of 1% (v/v) TFA.

3.2 Analysis of cleavage products by MS

3.2.1 Sample preparation (desalting) for MS analysis (*see Note 8*)

C18 ZIP tips or StageTips (**12**) are used for desalting and concentration of peptide samples.

1. In order to prepare StageTips, use a hypodermic needle to punch out small disks from PTFE membranes with embedded C18 beads, and place them into one 200 μl pipet tips. Use two discs per tip.
2. To condition the StageTips, add 50 μl solvent A and spin in a microfuge tube for 1 min at 2300 \times g, 4 $^{\circ}\text{C}$.
3. Wash twice by adding 100 μl solvent B and spin as above.
4. Apply the sample from the final step in 3.1.3, spin for 1 min at 800 \times g, 4 $^{\circ}\text{C}$.
5. Wash twice by adding 150 μl solvent B and spin for 1 min at 2300 \times g, 4 $^{\circ}\text{C}$.
6. Transfer the StageTips to new microfuge tubes and elute twice by adding 20 μl solvent C (*see Note 9*) and centrifugation for 1 min at 800 \times g, 4 $^{\circ}\text{C}$.
7. Dry samples in a vacuum concentrator and continue with nanoLC-ESI-MS/MS analysis or store at -20 $^{\circ}\text{C}$ until further analysis.

3.2.2 nanoLC-ESI-MS/MS

The exact procedure will have to be adjusted to the equipment available at the local mass spectrometry facility. Here we used an Ultimate 3000 RSLCnano system (Dionex, Thermo Fisher Scientific) with a precolumn (μ -precursor C18 PepMap100, 300 μm , 100 \AA , 5 μm \times 5mm, Thermo Fisher Scientific) and a NanoEase analytical column (NanoEase M/Z HSS C18 T3, 1,8 μm 100 \AA 75 μm \times 250mm column, Waters) operated at constant temperature of 35 $^{\circ}\text{C}$. The RSLCnano system was coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) using a NanosprayFlex source (Thermo Fisher Scientific). The Orbitrap Exploris 480 was operated under the control of Xcalibur software (version 4.4., Thermo Fisher Scientific). Internal calibration was performed using lock-mass ions from

ambient air (**13**).

1. Resuspend samples (from 3.2.1 step 7) in 20 μ l solvent D.
2. Adjust the flow rate to 300 nl/min solvent D.
3. Inject samples (1 μ l) directly into the precolumn.
4. Perform gradient elution with the following profile: 2 % - 55 % solvent E in 30 min, 55 % - 95 % solvent E in 10 min, 5 min isocratic at 95% solvent E, 10 min from 95 % to 2 % E and then re-equilibration for 10 min with 2 % E.
5. Collect survey spectra ($m/z = 200-2000$) at a resolution of 60.000 at $m/z = 200$.
6. Generate data-dependent MS/MS mass spectra for the 30 most abundant peptide precursors using high energy collision dissociation (HCD) fragmentation at a resolution of 15000 with normalized collision energy of 30.

3.2.3 MS Data analysis

1. Use the Mascot 2.6 (Matrix Science)(*see Note 10*) search algorithm for peptide identification, by searching spectra against a custom specific protein database (here the sulfated and unmodified CIF4 precursor peptide) including a suitable Uniprot database as background (here the *Arabidopsis thaliana* protein database) to allow the search algorithm to calculate false discovery (FDR) rates.
2. As search parameters, do not specify a specific enzyme (*see Note 10*), set mass tolerance at 5 ppm for peptide precursors and 0.02 Da for fragment ions. The mass tolerance settings may vary dependent on the resolution and mass accuracy of the mass spectrometer used for LC-MS analysis. Allow methionine oxidation, tyrosine sulfation, and [$^{13}\text{C}2,^{15}\text{N}$]-labeling of glycine (M+3) as variable modifications.
3. Transfer Mascot search results to ScaffoldTM 4.10.0 (Proteome Software) for visualization (*see Note 11*).
4. Compare results from database search with the expected cleavage products (here the peptides resulting from N- and/or C-terminal maturation of CIF4). m/z values of product peptides should match the expectation within the specified mass accuracy. Validate results using MS/MS spectra recorded for

product peptides.

5. Peptide quantification is accomplished by integration of ion intensities of the two peptides over their chromatographic elution profile. Use the MS-signal intensity of the area under the chromatographic peak of the peptide precursor in the extracted ion chromatogram.

4. Notes:

1. Tyrosine-sulfated and isotope-labeled peptides produced by Fmoc-chemistry-based solid-phase synthesis are available from most vendors. High quality peptides should be used at high purity (> 95%). The peptides used here were obtained from Peptide Specialty Laboratories (Heidelberg, Germany).

2. The mass shift caused by the isotope-labeled amino acid should be sufficiently large in order to distinguish the isotopomer clusters of the labeled and unlabeled forms of the peptide. Consequently, longer peptides require larger mass shifts (**14**). The 3 Da mass shift caused by substitution of Glycine- [¹³C2, ¹⁵N] was sufficient for the peptides under study. Other labeled amino acids can also be used if there is no glycine residue in a suitable position (*see* Fig. 1a-c). Deuterated peptides should be avoided because peptide deuteration causes reversed-phase LC retention times to shift (**15**).

3. We grow the plants on seeding substrate (Plagron Seeding and Cutting Soil), at 25°C and 16/8 h day/night cycle. Plants older than three weeks are watered with 1,48 g/l N (20%) P(20%) K (20%) universal fertilizer including micronutrients. Expression levels are much reduced in flowering plants. Therefore, plants have to be used before they start to bolt.

4. Other strains of *Agrobacteria* may also be suitable; we also used strains GV3101 and LBA4404.

5. The optimum time for transient expression needs to be determined experimentally. If degradation of the expressed protease is observed, or tissue necrosis as a result of protease overexpression, earlier time points may have to be chosen for maximum yield.

6. Use high quality microfuge tubes (e.g. the original Eppendorf tubes) and tips to avoid any plasticizer leaking into the sample, which is detrimental to subsequent MS analysis.

7. The peptide substrates were used here in a 3000-fold molar excess over the protease. However, this cannot be generalized. The amount of protease in the assay and the required substrate concentration depend on the properties of the protease under study.

8. nanoLC-ESI-MS analysis is sensitive to high salt concentrations. Therefore, whenever the concentration of salt or buffer ions in the digest exceeds 20 mM, peptides need to be desalted on C18 ZIP tips or StageTips (*12*). This is particularly important when the LC-MS system is operated without a pre-column.

9. The apoplastic wash contains high-molecular-weight proteins including the subtilase SBT5.4. Therefore, it is recommended to use only 50% ACN in the elution buffer C for ZIP tips or StageTip sample preparation. This is sufficient for the elution of peptides, but avoids elution of larger proteins which adhere strongly to C18 material and may thus damage analytical nano-LC-columns. This is particularly important when the LC-MS system is operated without a pre-column.

10. Mascot Server is a commercial product from Matrix Science Ltd. (London, UK). For the analysis of low complexity samples like peptide mass fingerprints, Mascot Server can be used on the company's server without purchasing a license. However, this is of limited value for the LC-MS experiments described here, because the "no enzyme" function is only available in the licensed version of Mascot. MS data analysis can also be performed using other commercial search algorithms (Sequest (Thermo Fisher Scientific), Byonic (Protein Metrics)) or freeware programs like Andromeda/MaxQuant, X! Tandem, or MSFragger/FragPipe.

11. This step is not essential, but recommended for best visualization results.

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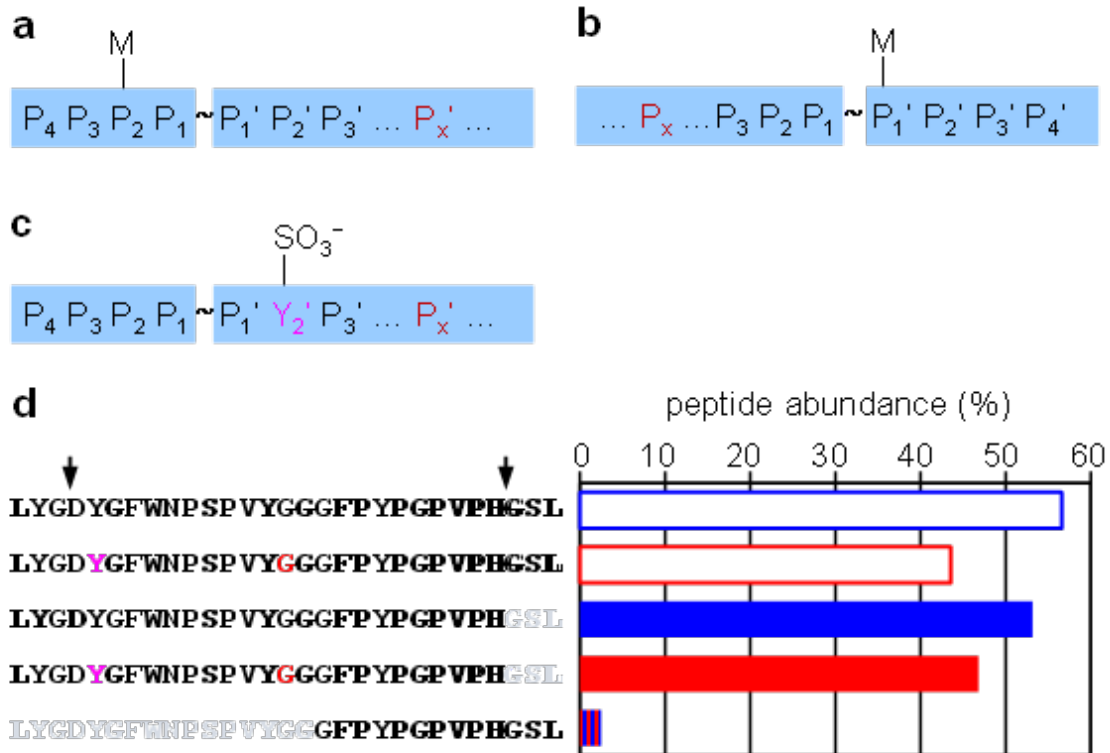


Fig. 1 Isotope-labeled peptides to assay the effect of post-translational modifications and amino acid substitutions on processing efficiency by MS. **(a)** The isotope-labeled amino acid (indicated in red) is placed downstream of the cleavage site (~) for the analysis of amino acid substitutions or post-translational modifications (M) in positions P₁ to P₄ (amino acid positions numbered according to (6)). **(b)** The label is placed upstream of the cleavage site for the analysis of prime-side (P', C-terminal) modifications. **(c)** The isotope label is used to distinguish the cleavage products generated from a tyrosine-sulfated vs. the non-modified peptide. **(d)** C-terminal cleavage during maturation of the CIF4 peptide by SBT5.4 is not affected by tyrosine sulfation. The unmodified extended CIF4 peptide and its tyrosine-sulfated and isotope-labeled derivative (highlighted in magenta and red, respectively) are shown in the upper two rows. Processing sites for CIF4 maturation are indicated by arrows. The bar graph shows the abundance of residual (not cleaved) peptides detected after 2 h digestion with SBT5.4 (unlabeled blue and labeled red, open bars). Peptides resulting from C-terminal cleavage are shown in the third and fourth rows (unlabeled blue and labeled red, filled bars). MS/MS analysis of these two cleavage products is shown in Fig. 2. Comparable amounts of C-terminal cleavage product are detected for the sulfated and the non-sulfated substrate peptides (compare rows three and four). Hence, there is no effect of tyrosine sulfation on C-terminal processing efficiency. Peptides resulting from cleavage at the N-terminal processing site are not observed, neither for the sulfated nor for the unmodified substrate peptides. SBT5.4 is thus not responsible for N-terminal maturation of CIF4. The minor cleavage product in row five was also observed in controls and cannot be attributed to SBT5.4 activity. It does not include the isotope label and, therefore, cannot be assigned unambiguously to either one of the two substrate peptides (indicated by the red and blue stripes). Peptides were quantified as the integrated peak intensity in the extracted ion chromatograms. Relative abundance is shown in percent of the normalized intensity for the sum of substrate and product peptides.

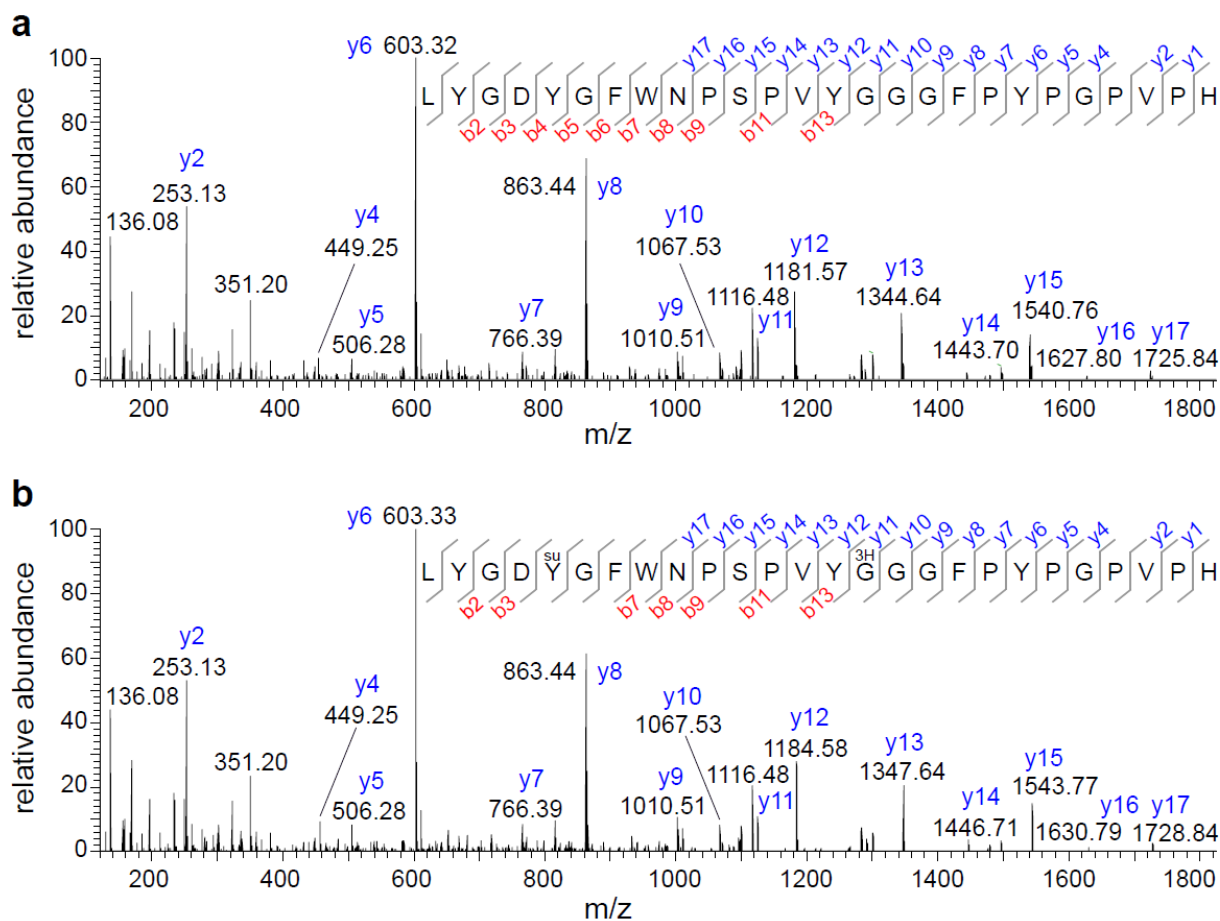


Fig. 2: MS/MS identification of the C-terminal cleavage products. The precursor ions of the major cleavage products of the extended CIF4 peptide (a) and its tyrosine-sulfated isotopolog (b) were characterized by MS/MS analysis. Identity and sequence of the two peptides were confirmed by the almost complete y-ion series (blue), and additional ions from the b series (red). Note the mass shift of 3 Da for all ions from the y series including and larger than y12, i.e. all those that comprise the isotope-labeled glycine.