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Peptidomic approaches to study proteolytic activity

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Abstract

Peptidomics, the analysis of the peptide content of cells or tissues, can be used to study proteases in several ways. First, nearly all of the peptides detected in cells and tissues are proteolytic fragments of proteins. Analysis of the peptides therefore provides information regarding the proteolytic activities that occurred to generate the observed peptides. The use of quantitative peptidomic approaches allows the comparison of relative peptide levels in two or more different samples, which enables studies examining the consequences of increasing proteolytic activity (by enzyme activation or overexpression) or reducing proteolytic activity (by inhibition, knock-down, or knock-out). The quantitative peptidomics method can also be used in studies directly testing the cleavage specificity of purified proteases. For this, peptides are purified from the tissue/cell line of interest, incubated in the presence of various amounts of protease or in the absence of protease, and then analyzed by the quantitative peptidomics approach. This reveals which peptides are preferred substrates, which are products, and which are not cleaved. Collectively, these studies complement conventional approaches to study proteolytic activity and allow for a more complete understanding of an enzyme's substrate specificity.

Introduction

Proteases are involved in almost every cellular and molecular process, including the production of bioactive peptides, the activation of proteins and enzymes through propeptide cleavage, and the degradation of proteins. Recent peptidomics experiments have shown the existence of a large number of peptides in mouse brain and other tissues, and in cultured cell lines (Fricker, 2010; Gelman et al., 2010). While some of these peptides represent known neuropeptides which are produced from their precursors by selective endo- and exopeptidases, the majority of the identified peptides represent fragments of cytosolic, mitochondrial, or nuclear proteins. Many of these protein fragments correspond to the N- or C-termini of the proteins, suggesting that selective proteolytic activity generated the peptide, rather than protein degradation. In support of this, a recent proteomics study reported that the vast majority of identified proteins had N-terminal sequences that did not correspond to the N-terminus encoded by the mRNA (Mahrus et al., 2008). While some of the protein N-termini were expected, based on cleavages known to occur (including removal of the initiator methionine, signal or transit peptides, or any other propeptide processing sites), 50-80% of the N-termini did not match either the RNA-encoded sequence or a known cleavage site (Mahrus et al., 2008). Therefore, it is likely that proteolytic cleavages are much more common within the cell than previously thought.

Peptidomics is the analysis of the peptide content of a biological sample, with peptides usually defined as amino acid polymers less than 10,000 Da. Although some peptides are derived from small coding RNAs, most are produced by proteolytic cleavage of larger

proteins. Therefore, analysis of the peptide content of a sample provides information on the proteolytic activities required to generate the observed peptides. Additional information can be obtained from quantitative peptidomics in which the relative levels of peptides are compared among two or more samples (Figure 1A,B). This allows for direct studies to test the effect of modulating protease activity on the resulting peptidome. For example, overexpression of proteases in a cell line should lead to an increase in the levels of the enzyme's products and a decrease in the levels of the substrates, as shown for the overexpression of thimet oligopeptidase in HEK293 cells (Berti et al., 2009). Conversely, inhibition of a protease by classic inhibitors, RNA knock-down, or gene knock-out approaches should lead to an increase in levels of the substrates and a decrease in the levels of the products, as shown for the knock-out of prohormone convertase 1/3 and 2 (Wardman et al., 2010; Zhang et al., 2010). Thus, analysis of the peptidome provides a direct read-out of the proteolytic activities that occur within a cell or tissue.

A second distinct approach to study proteases using peptidomics involves *in vitro* assays with purified proteases (Figure 1C). In conventional studies, purified proteases are incubated with synthetic substrates and the products detected using chromogenic, fluorogenic, or other approaches. Typically, these studies test peptides separately and while they provide important information, it is prohibitively expensive to purchase and test hundreds of peptides using these approaches. A peptidomics approach allows the analysis of hundreds of naturally-occurring peptides in a single experiment. For this, peptides are first extracted from the tissue, cell line, or other biological sample of interest. Then, the mixture of peptides is incubated in the presence or absence of protease and the products analyzed by mass spectrometry. If different incubation times and/or concentrations of enzyme are tested, the resulting information provides lists of preferred substrates, weak substrates, non-substrates, and products, as shown for studies testing the activities of purified thimet oligopeptidase, carboxypeptidase A4, and carboxypeptidase A6 (Berti et al., 2009; Lyons and Fricker, 2010; Tanco et al., 2010).

A key technique necessary for both of the above peptidomic approaches is the quantitative analysis of relative levels of peptides in two or more samples. Some mass spectrometry analyses estimate peptide levels by consideration of the signal strength (spectral counts) during the liquid chromatography/mass spectrometry run (LC/MS). However, more accurate relative levels of peptides can be obtained using isotopic labels. One common stable isotopic-labeling reagent is the iTRAQ series of labels, available in 8 distinct forms (iTRAQ is an acronym derived from "isobaric tag for relative and absolute quantitation"). However, peptides labeled with the iTRAQ reagents do not show mass differences in mass spectrometry until fragment ions are produced (MS/MS ions), thereby limiting the amount of information that can be obtained from each run. The trimethylammonium butyrate (TMAB) labeling reagents do not suffer from this disadvantage and provide relative quantitative levels of any peptide detected, even if no MS/MS information is available. Originally described as two isotopic forms (Zhang et al., 2002), simple modifications of the synthesis provide five different isotopic reagents shown in Figure 1A (Morano et al., 2008; Lyons and Fricker, 2010). Here we describe the use of quantitative peptidomics in the analysis of the biological peptidome as well as in the *in vitro* analysis of peptidase activity.

Materials

Milli-Q distilled water system (Millipore)

0.1M Hydrochloric Acid, 6 N, sequanal grade, constant boiling (Pierce)

0.4 M NaH₂PO₄ (Sigma-Aldrich)

Ultrasonic processor W-380 (Ultrasonic Inc., Farmingdale, NY, USA)

Low retention microcentrifuge tubes (Eppendorf)

TMAB-NHS compounds. The synthesis of these compounds has been previously described (Morano et al., 2008).

1.0 M NaOH (Sigma-Aldrich)

Dimethylsulfoxide (Sigma-Aldrich)

NH₂OH HCl (Sigma-Aldrich)

Glycine (Sigma-Aldrich)

Hydron pH Papers, 8.0-9.5 (Micro Essential Laboratory)

Amicon Ultra 4 mL Ultracel 10,000 molecular weight cut-off Centrifugal Filter Devices (Millipore)

PepClean™ C-18 spin column (Pierce)

Acetonitrile, HPLC grade (Fisher Scientific)

Trifluoroacetic acid (Pierce)

Extract Peptides

1. Sacrifice mice by cervical dislocation. To reduce postmortem proteolytic degradation, immediately place the tissue of interest in a conventional microwave oven and heat at full power until the temperature of the tissue reaches 80°C. Alternatively, tissue can be placed into tubes and heated in a hot water bath to >80°C.

This heating of tissues results in inactivation of endogenous proteases. In our microwave it takes 8 seconds for a decapitated mouse head to reach 80°C; however, each microwave must be carefully calibrated as the strength of microwave ovens varies considerably. After heat-inactivation, tissue may be further dissected and frozen at -80°C until analysis. Low retention tubes, washed with double distilled water before use, should be used for tissue storage and further steps to reduce the adherence of peptides to the tube walls.

2. Sonicate tissues in an appropriate amount of ice-cold water. We use 5 µl of water per milligram of tissue, with a minimum of 200 µl of water per sample, and sonicate two times for 20 seconds at 1 pulse/second, duty cycle 3, 50% output.

After sonication, rinse the tip of the sonicator with a small amount of water to ensure collection of all peptides.

3. To extract peptides, incubate homogenates at 70°C for 20 minutes, cool on ice for 15 minutes, and acidify homogenates by the addition of ice-cold 0.1M HCl to a final concentration of 10 mM. Mix by vortexing and further incubate on ice for 15 minutes. Spin homogenates at 13,000 g for 40 minutes at 4°C. Transfer supernatants to new low retention tubes.

Incubation of homogenates at 70°C reproducibly enhances peptide extraction without inducing postmortem cleavages. Acidification of the sample prior to centrifugation reduces the amount of proteins in the extract; most proteins are not soluble under acidic conditions. It is important to be sure extracts are ice-cold before adding acid to prevent acid-labile bonds from breaking. The supernatants obtained from this step can be frozen and concentrated to a lower volume in a vacuum centrifuge. This may be necessary if large volumes of peptide extracts are used, as the total volume of all labeled extracts should be under 4.0 ml to fit in filters used in a subsequent step.

4. Adjust the pH of peptide extracts to 9.5 by the addition of 0.4 M sodium phosphate buffer, pH 9.5, to a final concentration of ~60 mM buffer.

Extracts can be stored at -80°C until further processing.

Digest peptides with enzyme (optional – depending on purpose of experiment)

If the goal of the experiment is to compare peptides isolated from different tissues or different cell treatments (as shown in Fig. 1B), then skip these steps and continue with peptide labeling in the next section. If the goal is to perform *in vitro* analysis of protease substrate specificity (as shown in Fig. 1C), then complete the following three steps.

5. Adjust the pH of the peptide extracts to a pH appropriate for the enzyme to be investigated. For example, if the enzyme to be tested has a neutral pH optimum, neutralize peptide extracts using 100 mM borate buffer.

Do not use Tris-HCl buffers, as the primary amines in these buffers will compete for label in the following steps.

6. Aliquot 100 μl peptide extract into five low retention tubes (or less, depending on the number of labels that will be used). Add 5-10 fold dilutions of enzyme to each tube (e.g. 100 nM, 10 nM, 1 nM, 0.1 nM, and buffer only), and incubate at an appropriate temperature for one hour.

Use sufficient amounts of enzyme such that the best substrates are completely cleaved by the higher concentrations, but only partially cleaved by the lower amounts of enzyme. This will allow for weak substrates to be identified (i.e. those peptides that require higher concentrations of enzyme to cleave a detectable amount of the peptide).

7. Stop the reaction by the addition of a specific inhibitor and/or by heating.

Do not use inhibitors that will interfere with the subsequent MS analysis. Most proteases are heat sensitive, and this provides a simple method to stop the reaction. We typically use 80°C for 10 minutes, but vary this if your enzyme is heat-stable.

Label Peptides with TMAB Isotopic Tags

8. Dissolve TMAB-NHS labels in DMSO to a concentration of 0.4 mg TMAB-NHS/ μl . Typically 5 mg TMAB-NHS/50 mg wet weight of original tissue or 5 mg TMAB-NHS/brain region is used. Mix until fully dissolved.

Prepare labeling solutions as well as glycine and hydroxylamine solutions fresh each day. It is extremely important to prepare all solutions (HCl, NaOH, glycine, phosphate buffer and desalting solutions) with ultrapure deionized water to avoid contamination from small organic molecules that can interfere with mass spectrometry.

9. Adjust the pH of samples to 9.5 with 1.0 M NaOH, measuring pH by blotting 1 μl extract onto pH paper. Add one-seventh of total TMAB-NHS label to sample. Incubate samples at room temperature for 10 minutes before adjusting pH to 9.5 with 1.0 M NaOH and adding another one-seventh of total TMAB-NHS label. Repeat this process until all TMAB-NHS label has been added (seven rounds of TMAB-NHS label addition and pH adjustment). After the final addition of TMAB-NHS label, incubate samples for an additional 30 minutes at room temperature.

In studies with synthetic peptides, incomplete labeling was obtained when equimolar amounts of TMAB-NHS and peptide were used. For complete labeling, it was necessary to use a several hundred-fold molar excess of the TMAB-NHS

reagent. Because the reagent is labile in water, it is preferable to divide the addition of reagent into multiple steps. Initially we used 5 additions but found some cases where labeling was incomplete, and therefore increased this to 7 steps which has worked consistently to fully label the vast majority of endogenous peptides.

10. Quench any unreacted TMAB-NHS reagent by the addition of 10 μ l 2.5 M glycine per 5 mg TMAB-NHS reagent used. Incubate at room temperature for 40 minutes.

Purify peptides

11. Combine all labeled peptide samples to be compared (e.g. with different TMAB labels) and apply pooled samples to washed 4 ml Amicon Ultra Ultracel 10 kDa cut-off filters and spin according to usage guidelines in a refrigerated centrifuge at 4°C. The flow-through is retained for use in further steps.

This filtration will remove all proteins larger than 10 kDa. Before using the filters, they should be washed by centrifugation with 2 ml water to remove any glycerol that remains on the filter from the manufacturer. Some brands of filters can cause problems, either because they bind the peptides or due to contaminants that leach from the filter and obscure the signal from the peptides during subsequent MS analysis.

12. Adjust the sample pH to 9.0 with 1.0 M NaOH. Prepare 3 μ l hydroxylamine solution for every 10 mg TMAB-NHS label that was used in the initial labeling. Add one-third of the hydroxylamine solution to the sample, mix, and incubate at room temperature for 10 minutes. Adjust the pH to 9.0 with 1.0 M NaOH, and repeat the addition of a third of the hydroxylamine two more times. Filtrates can be stored at -80°C before desalting.

For example, if 5 mg of two different labels were used, then prepare 3 μ l hydroxylamine solution. Treatment with hydroxylamine is performed in order to remove any tyrosine TMAB labeling and therefore ensure that all peptides are labeled on free amino termini and lysines only.

13. Desalt samples on PepClean™ C-18 spin columns according to the manufacturer's instructions using acetonitrile and trifluoroacetic acid solutions. Elute peptides with 80 μ l 70% acetonitrile and 0.1% trifluoroacetic acid in water. Freeze eluates and concentrate to 10-20 μ l in a vacuum centrifuge. Store aliquots of samples at -80°C until mass spectrometry analysis.

For the amount of sample described in the preceding steps, we typically use two PepClean™ C-18 spin columns per sample. You can either use two separate columns or simply combine the resin from two PepClean™ C-18 spin columns in one column, which cuts down on the number of separate columns.

Separate and detect peptides by liquid chromatography and mass spectrometry (LC/MS)

Separation of peptides is performed by reverse phase chromatography followed by detection and quantification by direct electrospray ionization mass spectrometry on a quadrupole time-of-flight (q-TOF) instrument. Other instruments have been used, but generally with suboptimal results. For example, ion traps produce substantially less data and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) results in the partial decomposition of the isotopic TMAB tag which precludes the ability to quantify the results. A typical protocol using either nanospray or microspray LC/MS analysis on a q-TOF mass spectrometer is described below.

14. Thaw samples and briefly spin to remove particulates.

15. Inject an aliquot (typically 2-5 μ l) onto a C18 trapping column.

We typically use a Symmetry C18 trapping column (5 μ m particles, 180 μ m i.d. $\times$ 20 mm, Waters, USA).

16. Desalt material online for 15 minutes.

17. Separate peptides through a C18 column, eluting with a gradient of acetonitrile in 0.1% formic acid at a flow rate appropriate for the column and mass spectrometer.

A typical gradient is a linear increase from 5% to 45% solvent B over 50 min, with solvent A = 2% acetonitrile/0.1% formic acid in water and solvent B = 98% acetonitrile/0.1% formic acid in water. Many columns have been used and work well for this purpose. One typical column is the BEH 130 – C18 column (1.7 μ m particles, 100 μ m i.d. $\times$ 100 mm, Waters, USA) with a flow rate of 1 μ l/min.

18. Acquire data in data-dependent mode and dissociate selected peptides with argon.

In order to obtain optimal fragmentation through collision-induced dissociation in MS/MS analysis, it is often helpful to use higher collision energy values than typically used for non-labeled peptides. (Gelman et al., 2010).

Identify peptides

Instrument-specific software is used to analyze the data obtained from LC/MS. For example, Waters mass spectrometers use MassLynx software, Applied Biosystems instruments use Analyst software, and Agilent instruments use MassHunter software. Data are analyzed by manual scanning and identification of peak sets, which can be identified by the presence of the appropriate number of peaks (determined by the number of isotopic labels used) evenly separated from each other and co-eluting. TMAB-labeled peptides typically elute over a 20-30 minute range and have mass-to-charge ratios of 300-1400.

Peptides are identified by analysis of the fragmentation ions produced in tandem mass spectrometry (MS/MS). In addition to considering the fragmentation ions, it is important that the observed peptide mass, charge state, and number of TMAB tags attached match to the theoretical values for the candidate peptide, as described below.

19. Determine the charge of a peptide by comparing the mass of the monoisotopic peak with the ^{13}C -containing peaks.

For example, if a mass spectrum indicates a mass/charge (m/z) difference of 0.33 between the monoisotopic and ^{13}C -containing peaks, the difference in m/z is $1/3$ and the charge is 3. This can be seen in the representative data shown for chromogranin B 438-454 (Fig. 2B). Peptides shown in Fig. 2A, C and D have an m/z difference of 0.5, indicating that the charge is 2.

20. Determine the number of labels incorporated into the peptide from the separation of peaks within a peak set.

For example, if two peaks for a peptide with a charge of 3 and labeled with D0- and D3-TMAB are being compared and their respective monoisotopic peaks are separated by an m/z difference of 1, the mass difference is 3 Da, consistent with the addition of one TMAB label (see Fig. 2B). If, however, the m/z difference is 2 for a peptide with a charge of 3, the total mass difference is 6 Da which indicates that the peptide was labeled with 2 TMAB labels. Similarly, peptides with 3 or more tags will appear with mass differences of 9 Da or more, in multiples of 3 Da.

21. Determine the mass of the unmodified peptide (no tags or protons) using the following formula: mass of unmodified peptide = $(m/z \cdot z) - (c \cdot T) - (1.008 \cdot (z-T))$, where m/z is the observed mass to charge value for the monoisotopic peak, z is the charge state, c is the mass of the TMAB tag (128.118 for D0-TMAB, 131.133 for D3-TMAB, 134.155 for D6-TMAB, 137.170 for D9-TMAB, and 140.190 for D12-TMAB), T is the number of tags incorporated, 1.008 is the mass of a proton, and $(z-T)$ is the calculation of the number of protons.

The last part of the equation $(z-T)$ is necessary because each TMAB tag adds a positive charge to the peptide due to the quaternary amine group and therefore the charge state is not equal to the number of protons.

This information can now be used to search peptide databases for peptides with the same mass, charge, and potential sites for TMAB incorporation. However, the conclusive identification of a peptide requires amino acid sequence information derived from MS/MS analysis.

Although it is possible to do manual analysis of MS/MS data, this analysis is usually performed using a program to search protein databases with the MS/MS data. A number of programs are available and we have found Mascot to be ideal for TMAB-labeled peptides. This program has four of the five TMAB labels included as options (it does not include D12-TMAB). Mascot also considers the neutral loss of trimethylamine (TMA) from the peptides during collision-induced dissociation; this causes the loss of 59 Da from peptides labeled with the D0-TMAB tag, 62 Da from the D3-TMAB tag, 65 Da from the D6-TMAB tag, 68 Da from the D9-TMAB, and 71 Da from the D12-TMAB.

Mascot searches must be followed by manual interpretation to eliminate false positives and verify that the peptide meets the following criteria:

- 4. The isotopic form of TMAB matched by Mascot must correspond to the observed form, based on analysis of the peak set.

Mascot does not consider the peak set and know which of the individual peaks correspond to the D0, D3, D6, D9, or D12 forms. Instead, the program tries to match any of the observed peaks with whatever TMAB tag is specified in the search parameters. False positives are usually apparent because the Mascot result does match the isotopic form that is observed from the analysis of the peak groups (see Figure 2B and C for clear examples of peak groups). For example, if using the five isotopic forms of TMAB, there is a 1 in 5 chance that a false positive labeled with one tag is correct. If two tags, there is a 1 in 25 chance that a false positive has the correct tags. Therefore, a correlation of the isotopic TMAB form in the observed peak set with the predicted Mascot match is a simple and necessary step.

- 5. The number of tags incorporated into the peptide should match the number of free amines present on the peptide (N-terminus and lysine side chains).

Because an excess of TMAB labeling reaction is used, the labeling usually goes to completion and the major forms of all peptides have amines fully labeled with TMAB. Some sequences are more difficult to label than others and on occasion will show incomplete labeling; for example, a Lys adjacent to one or more Arg or Lys residues may show incomplete labeling. Also, N-terminal Lys residues often show incomplete labeling and take up only 1 TMAB tag instead of the expected 2 tags. If multiple tags are incorporated into a peptide, all tags should be the same isotopic

form on a particular peptide (i.e. all D0-TMAB, or D9-TMAB, and not one D0-TMAB and one D9-TMAB on the same form of a peptide).

- 6. The Mascot score is either the top score of all potential peptides, or the other peptides with comparable scores can be excluded by the above criteria, leaving only one peptide that matches all criteria.
- 7. The Mascot hit is likely to represent the predicted peptide, based on manual inspection of the MS/MS data. It is not sufficient to rely on score alone – some high scoring peptides are clearly incorrect (being excluded based on the above criteria) while some low scoring peptides show excellent matches to all of the above criteria and are likely to be correct. Several additional criteria that we include in our manual verification are:

- a. The majority (>80%) of the major MS/MS fragment ions match predicted a, b, or y ions, or precursor ions with loss of trimethylamine.

The b and y ions are usually strongest when peptides are fragmented by collision-induced dissociation. Other methods of fragmentation produce other types of fragment ions.

- b. The mass accuracy of the fragment ions is within the accepted specification for the mass spectrometer used for the analysis.

For modern q-TOF instruments, the accuracy is within several parts per million.

- c. A minimum of 5 fragment ions match b or y ions.

This can be a problem for small peptides that show few fragment ions.

- d. The charge state should be consistent with the peptide sequence.

Usually the charge state of a peptide is equal to the number of TMAB labels along with the number of arginine residues which are almost always protonated. In some peptides histidine is charged. In addition, some peptides can pick up another charge, although this is usually a minor species. Finally, some peptides lose protons (due to ionization of Glu or Asp side chains) and show charge states lower than the number of positive charges expected on the peptide. This is most common with large peptides that contain multiple Glu and/or Asp residues.

- e. The cleavage pattern of the peptide fits with expected collision-induced dissociation cleavages.

For example, cleavages to the N-terminal side of Pro residues are usually strong ions while cleavages to the C-terminal side of Pro are weak or undetectable. Cleavages on either side of Gly are also usually weak ions. The b2 and a2 ions are usually very strong unless the N-terminus is blocked or has a Pro or Gly in a position that would interfere with the cleavage to generate the b2/a2 ions.

COMMENTARY

Background Information

Peptides have a broad spectrum of functions in biological systems. Signaling in the brain is mediated by a large number of neuropeptides, which function in a manner similar to the

well-characterized neurotransmitters. A number of bioactive peptides circulate in the bloodstream and are involved in the regulation of blood pressure, clotting, and other physiological activities. Recently, it has come to light that proteolytic fragments of cytosolic peptides may have crucial roles both inside and outside of the cell (Ferro et al., 2004; Fricker, 2010). Methods for the identification and characterization of these peptides are necessary, both to understand the biological functions of the peptides themselves and of the enzymes which produce them.

We have developed a relatively cheap and accurate method to label peptides and compare peptides within multiple samples. This labeling method makes use of reagents that react with the free amines found on the N-termini and lysine side chains of peptides (Julka and Regnier, 2004). Similar labeling methods are available commercially, such as succinic anhydride with four hydrogens or deuteriums, and the iTRAQ reagents. However, we find that TMAB-NHS, originally developed by Regnier and colleagues (Zhang et al., 2002), is superior to the other labeling reagents for several reasons. The TMAB-NHS reagents can be easily synthesized from gamma-aminobutyric acid and iodomethane containing various numbers of hydrogen and deuterium (Morano et al., 2008), and also with iodomethane containing deuterium and ^{13}C . Altogether, five different isotopic forms of TMAB-NHS reagents can be produced differing in mass by 3 Da each, allowing the comparison of peptide levels from five different samples (Lyons and Fricker, 2010). Peptides labeled with different isotopic forms of the TMAB-NHS reagents elute from reverse phase liquid chromatography columns at the same time, unlike isotopic forms of succinic anhydride (Che and Fricker, 2005). This is crucial for accurate comparison of different samples. Since TMAB labels are positively charged, peptides labeled with TMAB maintain the positive charges associated with the N-termini and lysine residues of peptides which are important for detection by mass spectrometry in positive ion mode. In contrast, succinic anhydride labeling of free amines converts the positive charge to a negative charge and may result in the inability to detect the peptide if no other positive charge is present on the peptide. TMAB labels have shown stronger signals in mass spectrometry-based analyses than succinic anhydride labels (Che and Fricker, 2005). While the iTRAQ reagents are available in 8 different isotopic forms, quantitation requires MS/MS data and so the majority of peptides in a complex sample will not be quantifiable because of the absence of MS/MS data. In contrast, the TMAB-labeled peptides are quantified based on the MS data, and so anything that is detectable above the background (and which doesn't co-elute with another peptide of the same m/z) can be readily quantified with the TMAB reagents.

The combination of differential isotopic labels and mass spectrometry analysis presents many possibilities for peptide discovery and enzyme characterization. We have used this method in the analysis of the cellular peptidome and the discovery of novel secreted and intracellular peptides (Fricker et al., 2006; Berezniuk et al., 2010; Gelman et al., 2010; Wardman et al., 2010). This has enabled a number of predictions to be made regarding bioactive peptides and the enzymes that produce them. We have also used this method in the characterization of enzyme substrate specificity. Brain peptides were incubated with two different purified metallocarboxypeptidase enzymes allowing the identification of not only optimal P1' substrate residues for these enzymes, but also P1 residues (Lyons and Fricker, 2010; Tanco et al., 2010). Similarly, an analysis of the *in vivo* activity of an endopeptidase, thimet oligopeptidase, was performed using the same methodology (Berti et al., 2009).

Critical Parameters and Troubleshooting

In order to extract only those peptides that are biologically relevant, rather than proteolytic break-down products following death, it is critical that the tissue is rapidly heat-inactivated. A simple way to achieve this is by using microwave irradiation, either as a means of sacrifice (Svensson et al., 2003) or using a conventional microwave oven to irradiate tissues

immediately upon sacrificing the animals. Extracting peptides in ice-cold HCl, rather than in hot acid, also helps to avoid peptide artifacts that are a result of labile peptide bonds.

Throughout the process, it is critical to avoid contaminants. Small molecules and polymers can substantially interfere with the MS analysis. Contaminants can arise from water as well as many plastics. Some brands of centrifuge tubes and filtration devices have polymeric contaminants that appear as polyethylene glycol-related compounds on MS; these contaminant signals usually overwhelm the signals from the tissue-derived peptides. Whenever possible, rinse tubes and tips with ultrapure deionized water before use.

Some peptides bind to the walls of regular tubes and tips, and the use of low-retention centrifuge tubes and pipette tips is important to avoid this problem. Scaling up the procedure can also reduce losses of peptides.

Data analysis is the most time-consuming part of this procedure. Automated programs for the quantitation of TMAB-labeled peptides would be an important advance, provided that the programs were accurate in their identification of peak sets and quantitation. Because of the complexity of peptides in most biological samples, there are many peptides that show overlapping m/z spectra with other co-eluting peptides. However, it is often possible to manually obtain quantitative information by carefully considering the spectra and choosing ones in which the contaminating peptides are not present.

Anticipated Results

The quantitative peptidomics technique is able to identify hundreds of peptides and their specific processing forms from a single LC-MS/MS run. Many more peptides may be identified using newer mass spectrometers with their greatly increased sensitivity and dynamic range. Still, not all peptides can be seen by this technique. For example, some peptides lack an N-terminal free amine due to acetylation, pyroglutamylation, or another modification. If these peptides also lack internal lysines, they will not be labeled by the TMAB reagent and will show up on the m/z spectra as unquantifiable single peaks. In other cases, intrinsic factors can cause low ionization efficiency of certain peptides during mass spectrometry, and many peptides are outside of the optimal mass/charge ratios detected in these studies (typically 300-1700 m/z).

Cleavage of peptide mixtures with a purified peptidase results in the identification of many substrates and the determination of enzyme substrate specificity. For example, incubation of carboxypeptidase A6 (CPA6) with whole brain peptides allowed the identification of good substrates such as Little SAAS (Fig. 2A), weak substrates such as Chromogranin B 438-454 (Fig. 2B) and peptides that are not cleaved at all by this enzyme such as Chromogranin B 588-597 (Fig. 2C). In some cases the product of a cleavage can also be identified, as shown for Little SAAS (Fig. 2A, D)

Time Considerations

The entire procedure, outside of the data analysis, should take approximately 3 days. Peptide extraction and enzyme digestion (if performed) can be completed in one day, peptide labeling and purification on a second day, and LC/MS completed in a third day. Data analysis can become quite time consuming as much must be performed manually, and may take several weeks or more depending on the amount and quality of data obtained. However, considering the number of peptides analyzed and the accuracy of the data obtained with the quantitative peptidomics method, this approach is much faster than alternatives for peptide identification and characterization.

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Fig. 1.

Method of labeling peptides with TMAB tags. (A) The structure of the TMAB tags is shown. The N-hydroxysuccinimide (NHS) moiety is replaced by the N-terminal or lysine side chain amines upon reaction with peptides. Five different isotopic forms of the label can be made containing different numbers of hydrogen, deuterium, ¹²C or ¹³C in the methyl (Me) groups. (B) To identify peptides and compare their levels among multiple tissues or treatments, peptides are extracted from these tissues or cells and directly labeled with isotopic TMAB reagents. These peptides are then pooled and analyzed by LC/MS. (C) To directly test the ability of proteases to cleave a variety of peptides, the peptides are first extracted from an appropriate biological sample (such as a tissue or cell population) and aliquots are digested with different amounts of a proteolytic enzyme (CONC 1-4). These different digests are then labeled with the TMAB reagents, pooled, and analyzed by LC/MS.

Fig. 2.

Representative peptide substrates and products identified by quantitative peptidomics upon incubation with a peptidase. Peptides were extracted from mouse brain, digested with different amounts of purified carboxypeptidase A6, labeled with isotopic TMAB tags as indicated, and analyzed by LC-MS/MS. Examples of representative data are shown for (A) good substrates, (B) weak substrates, (C) non-substrates, and (D) products. Many additional substrates and products were detected in this analysis (Lyons and Fricker, 2010).