Chapter 15

Positional Proteomics at the N-Terminus as a Means of Proteome Simplification

Gemma R. Davidson, Stuart D. Armstrong, and Robert J. Beynon

Abstract

One strategy to reduce complexity in proteome analysis is through rational reduction of the proteolytic peptides that constitute the analyte for mass spectrometric analysis. Methods for selective isolation of C- and N-terminal peptides have been developed. In this chapter, we outline the context and variety of methods for selective isolation of N-terminal peptides and detail one method based on negative selection through differential removal of internal peptides.

Key words: Proteomics, proteome simplification, N-terminal peptide isolation, biotinylation, positional proteomics, mass spectrometry.

1. Introduction

It is rare for proteome analysis to be conducted on unfractionated, complex mixtures of intact proteins. Some strategies aim to reduce complexity at the protein level (by, for example, gel-based separation). In other instances, the first analytical step in many experimental workflows in 'bottom-up' proteomics is based on selective hydrolysis into an even more complex mixture of peptides. Usually, the fragmentation reagent is trypsin, which cleaves peptide bonds C-terminal to lysine and arginine residues. Together, these two amino acids constitute about 8–10% of the amino acids in a proteome, and thus, tryptic fragments are short (typically about 15 amino acids long). Since each protein (~50 kDa average) will generate on the order of 40 tryptic peptides, this is a substantial increase in analyte complexity. This might therefore seem counterproductive, but against this

K. Gevaert, J. Vandekerckhove (eds.), *Gel-Free Proteomics*, Methods in Molecular Biology 753,
DOI 10.1007/978-1-61779-148-2_15, © Springer Science+Business Media, LLC 2011

increase in complexity are tensioned two positive factors: the more uniform and predictable behaviour of the peptides on high-resolution chromatographic separation and the reduction in size relative to the parent protein, which bring peptides into the optimal mass range for most mass spectrometers. This increase in complexity brings further advantages – identification or quantification of the parent protein can be attained by tandem mass spectrometric analysis of relatively few of the peptides. Indeed, it can be argued that many proteomic analyses suffer from 'over-determinism'; we analyse more peptides than are required to attain the goal of the experiment. This is particularly true not only for abundant proteins but for those proteins that are less abundant; not only are the peptides present at lower concentrations, but their analysis can be impeded by crowding or ion suppression in the analyte stream. It is not surprising, therefore, that there have been multiple attempts to achieve a reduction in proteome complexity. Some, based on the amino composition of each protein, are directed at specific chemistries associated with particular amino acid residues (for example, cysteine residues). Others attempt a rational reduction in proteome complexity by targeting one of the features that is common to every protein in the cell – an N-terminus and a C-terminus – so-called positional proteomics. Such proteomic simplification strategies have the aim of enhancement of proteome analysis by the selective removal or isolation of specific peptides, leading to a reduction in sample complexity without compromising information for analysis (1).

Positional simplification strategies for global proteome characterization are based upon the concept that peptide subsets provide substantial information with respect to the parent protein. Utilizing one peptide as a surrogate for its parent protein requires a degree of confidence in the information provided by the peptide of interest. Against this must be tensioned the resistance of the proteomics community to the use of a single peptide hit to confirm protein identification, thus the term 'one hit wonders' has been coined. Much of the controversy associated with 'one hit wonders' relates to the origin of the peptide and its connectivity to the parent protein (2). Traditional proteomic workflows often begin with protein separation of a sample by SDS-PAGE followed by in-gel proteolysis of a single protein and often mass spectrometric analysis. This technique generates a set of peptides, each of which can assume connection to the starting protein. However, when dealing with a complex biological sample, such as human plasma, an in-solution/shotgun proteolytic approach will yield an even more complex peptide mixture where connectivity between peptides and parent proteins can no longer be assumed. Therefore, it becomes increasingly difficult to confirm protein identifications from a complex biological sample based on a single peptide. However, the general resistance to single peptide hits is

based on an experimental workflow in which the peptide could have been derived from anywhere in the protein. Simplification strategies in which the positional location of a peptide within the parent protein is known gain assurance. Effectively, the identification becomes a question of matching a peptide to the positionally defined single peptide derived from each protein. For a search against a 6,000 protein database, the search is no longer a single peptide against 300,000 possibilities in the entire digested proteome but a single positional peptide against a predicted set of 6,000.

Two positional locations are consistent amongst all proteins: the extremities (N-terminus and C-terminus). N-terminal and C-terminal peptide isolation can reduce sample complexity and also provide sequence information essential for understanding key processing events, such as in vivo modifications, removal of signal peptide and cleavage of methionine during protein maturation. Terminal sequence information is extremely valuable for protein identification, which can take place when four or five amino acids within the N-terminal sequence are known (3). Complementary methods have been developed for C-terminal analysis and isolation but these will not be discussed here.

A complexity in the analysis of protein N-termini lies in their diversity, a consequence of the substantial post-translational processing that can succeed de novo biosynthesis. When first translated, a nascent polypeptide chain can undergo extensive N-terminal processing (4), removal of signal peptides (endoproteolytic), excision of single amino acids (exoproteolytic) and N-terminal amino group modification by, for example, N-α-acetylation. Of course, most protein sequences in databases are derived from in silico translation of the corresponding genomic or cDNA sequence, and thus, the analyte, which is the true N-terminus in all its complexity, is likely to fail to be precisely represented in any sequence database, which poses a significant challenge for post-analytic bioinformatics. Indeed, positional proteomics can enhance protein databases by defining, unambiguously, the true N-terminus of an uncharacterized open reading frame.

There is a strong historical precedent to the analysis of the N-terminus of proteins based on the early development of Edman degradation. As mass spectrometry has reached into the arena of protein and peptide chemistry, several strategies for specific exploration of protein termini have been published. In this chapter, we address the methodologies for analysis of the N-terminus, more extensively developed as the chemical reactivity of the free amino group makes it easier to derivatize. One method in particular is detailed here.

In an analytical workflow for positional proteomics, the N-terminal peptide will be excised almost inevitably from the

parent protein by a proteolytic enzyme, and this will usually be trypsin. Moreover, many protocols block lysine residues (for example, by acetylation) and trypsin is not able to cleave peptide bonds adjacent to N-ε-acetyl lysine residues, reducing the cleavage sites to those adjacent to arginine residues only. The number of sites that can be digested in any protein is thus reduced by a factor of 2. Even then, the disposition of arginine residues is random and there will be some N-terminal peptides that are too small for analysis and do not contain sufficient sequence specificity to permit identification of the source proteins. Other peptides will be too large for chromatographic separation and mass spectrometric analysis. Inevitably then, all positional methods are selective, and greater coverage of the proteome would probably require orthogonal methods (e.g. multiple proteinases of differing specificities or a combination of N-terminal and C-terminal isolation).

Although there are a large number of published methods for analysis and isolation of N-terminal peptides, they reduce to simple analytical principles that predominantly make use of the nucleophilicity of the α-amino group as a reactive centre. The second source of amino groups in proteins is the side chain of lysine residues that are only marginally different in reactivity when compared to α-amino groups. However, targeting to N-terminal α-amino groups or side chain ε-amino groups is feasible (by careful control of pH and reaction time and by selective chemistries such as guanidinylation).

Proteomic strategies employed for the analysis of protein N-termini often involve chemical derivatization, affinity enrichment and labelling or selective tagging (for recent reviews *see* 5–7). Most derivatization and enrichment strategies are based upon the manipulation of differences induced by the addition or removal of chemical moieties. It is estimated that up to 80% of eukaryotic proteins have an acetylated α-amino group (8, 9), the function of which is not yet fully understood (10). Typically, N-α-blocked peptides will exhibit a single positive charge at acidic pH, if histidine is not present, unlike tryptic peptides that have at least two positive charges due to free α-amino group and C-terminal lysine or arginine. Cation exchange exploits the difference in charge between blocked N-terminal peptides and internal tryptic peptides for isolation (11, 12). Further development of cation exchange methods for isolation of N-α-blocked peptide using successive endoproteolysis and exoproteolysis has been reported in the literature (13). Strong cation exchange has proven beneficial for the simultaneous analysis of blocked (for example, by acetylation) N-terminal peptides and protein C-terminal peptides, due to their reduced basicity when compared to non-acetylated tryptic peptides (11, 14). Coupled with novel database searching strategies (15), strong cation exchange remains one of

the few methods observed in the literature for the simultaneous enrichment of C-terminal and blocked N-terminal peptides.

Selective isolation and enrichment of N-terminal peptides routinely requires derivatization. Derivatization using acetylation blocks the α-amino group of the N-terminal peptide and ε-amino group of lysine residues. Upon enzymatic cleavage internal peptides yield free α-amino groups. Affinity removal of the internal peptides containing free α-amino groups using amine reactive matrices such as NHS-activated Sepharose (16), cyanogen bromide-activated Sepharose (17, 18) or isocyanate-coupled resin (19) leads to enrichment of α-amino blocked (N-terminal) peptides. Derivatization of free amino groups using biotinylating reagents followed by affinity removal with avidin or streptavidin (20) has been used to remove internal peptides for N-terminal peptide enrichment (21) and for N-terminal peptide removal and enrichment (22).

Gevaert and Vandekerckhove (23) pioneered a technique known as combined fractional diagonal chromatography (COFRADIC), based on the principle of chemical derivatization with a sorting regent that elicited a change in chromatographic behaviour of a subset of peptides. This approach, developed for selective isolation of α-amino blocked peptides has been reported in the literature (7, 24, 25). After reversed-phase chromatographic separation of a peptide mixture the blocked α-amino peptides or N-terminal peptides are segregated from internal peptides by the reaction of the free amino groups of the internal peptides with 2,4,6-trinitrobenzenesulfonic acid (TNBS) that induces a strong hydrophobic shift allowing separation of internal peptides from N-terminal peptides in a second chromatographic separation step. Recent developments of this method involve the use of strong cation exchange for the reduction of peptide noise observed by internal peptides and enzymatic treatment to enhance exposure of true N-terminal peptides (25).

Utilizing positional proteomics for the selective enrichment of N-terminal peptides of proteins found within complex biological samples has some advantages over global, ‘bottom-up’ proteomic workflows. The reduction in sample complexity observed following the isolation of N-terminal peptides reduces the peptide ‘noise’ and could therefore solve the challenge imposed by analysis of complex biological samples using shotgun strategies. The protocol described in this chapter selectively enriches N-terminally blocked peptides via the selective removal of internal peptides (16, 21) with changes in the chemical derivatization and removal protocols. Reagents are readily available and can be easily modified for the study of naturally acetylated N-terminal peptides. From sample preparation to collection of mass spectrometric data requires 3–4 days (*see* **Fig. 15.1** for workflow outline).

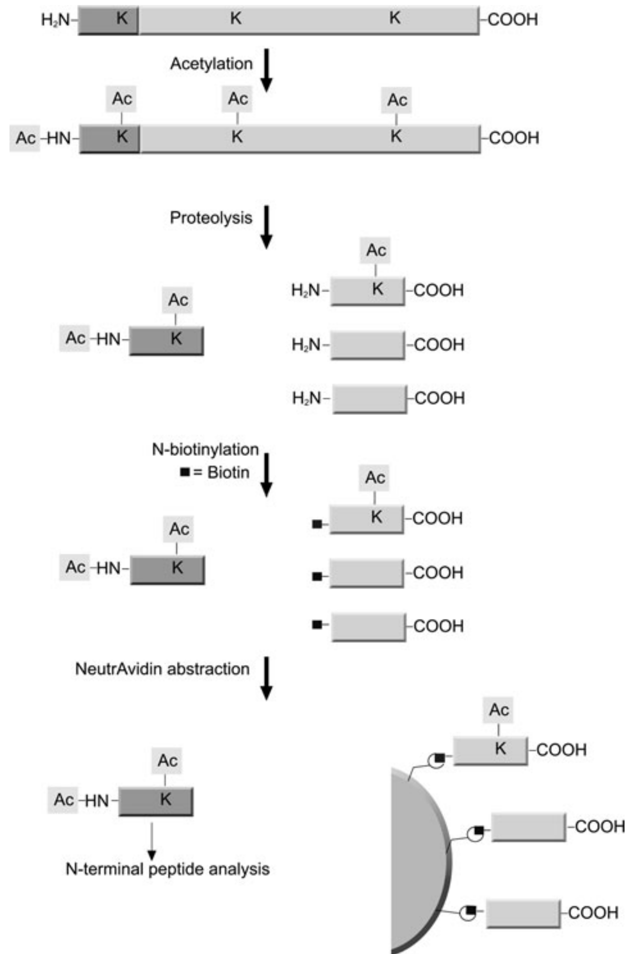

Fig. 15.1. Overview of N-terminal simplification strategy.

2. Materials

2.1. Equipment

1. StageTips, C18 pipette tips (Proxeon)
2. Microcentrifuge tubes, 0.5 and 1.5 ml
3. Glass vials, 0.1–1 ml
4. MALDI-TOF mass spectrometer
5. High-resolution nanoflow chromatography system, fitted with a reversed-phase C18 column, coupled to an electrospray ionization tandem mass spectrometer (optional)

2.2. Reagents

1. Coomassie Plus® protein assay reagent (Thermo Scientific)

2. HPLC-grade water
3. HPLC-grade acetonitrile
4. Dithiothreitol
5. Iodoacetamide
6. Formic acid
7. Dimethylformamide
8. Acetylation reagent: acetic anhydride
9. Acetylation buffer: 1 M sodium carbonate, pH 8.5 (store at room temperature)
10. Quenching reagent: Tris(2-aminoethyl)amine, polymer bound (Aldrich)
11. Trichloroacetic acid
12. Diethyl ether (flammable, use in fume hood)
13. Digestion buffer: 20 mM sodium phosphate buffer, pH 7.5 (store at 4°C)
14. Trypsin (or Arg C), sequencing grade (Roche), 0.1 μg/μl reconstitute in 50 mM acetic acid (store at 4°C)
15. EZ-Link NHS-biotin (Thermo Scientific, store desiccated at room temperature)
16. NeutrAvidin (or streptavidin) agarose, high capacity (Thermo Scientific, store at 4°C in ethanol)
17. Binding buffer: 20 mM sodium phosphate buffer, pH 7.5 (store at 4°C)
18. MALDI matrix: 10 mg/ml α-cyano-4-hydroxycinnamic in 50% acetonitrile, 0.2% formic acid (v/v)
19. HPLC Buffer A: 0.1% formic acid (v/v)
20. HPLC Buffer B: 90% acetonitrile, 0.1% formic acid (v/v)

3. Methods

3.1. Selective Enrichment of the N-Terminal Peptides of Proteins

1. Measure the protein concentration of the sample using, for example, the Coomassie Plus® protein assay. The protein concentration should ideally be 2–4 mg/ml. Ensure the protein sample is in a buffer compatible with the protocol, i.e. is non-amine containing. If not, dialyse the protein preparation against a suitable buffer such as sodium carbonate, sodium phosphate or HEPES (pH 7–9) (*see* **Note 1**).

2. If required, reduce and alkylate the protein sample using dithiothreitol and iodoacetamide at final concentrations of 3 and 9 mM, respectively (*see* **Note 2**). Incubate with dithiothreitol for 30 min at 50°C, followed by incubation with iodoacetamide for 1 h in the dark at room temperature.
3. Acetylate intact proteins by adding 50 μl of 1 M Na_2CO_3, pH 8.5, to 50 μl of 100 μg of the soluble protein solution (this buffers acid formation) in a 0.5 ml microcentrifuge tube. Add 1 μl of acetic anhydride to the 100 μl solution. Vortex for 20 s. Incubate at room temperature for 1 h. Add an additional 1 μl of acetic anhydride (>600-fold molar excess of acetic anhydride, reduce accordingly for less complex protein mixtures), vortex and incubate a further 1 h at room temperature (*see* **Note 3**).
4. Add approximately 5 mg of the free amine quenching reagent (Tris(2-aminoethyl)amine, polymer bound, 10-fold molar excess) directly to the acetylated protein solution, vortex for 30 s and incubate with gentle agitation at room temperature for 1 h (*see* **Note 4**). Recover acetylated proteins by using spin columns; using a needle carefully pierce a small hole in the bottom of the 0.5 ml microcentrifuge tube containing the sample and place inside a larger 1.5 ml microcentrifuge tube. Centrifuge at 2,000×*g* for 1 min at room temperature. Discard resin and retain flow-through (acetylated proteins).
5. Add 150 μl of 30% TCA (w/v) to the acetylated protein sample, vortex for 20 s and incubate for 2 h on ice (*see* **Note 5**).
6. Centrifuge at 13,000×*g* for 10 min at room temperature to pellet acetylated proteins. Remove TCA using a pipette and discard. In a fume hood wash the protein pellet with 200 μl of diethyl ether, centrifuge for 10 s at 13,000×*g* at room temperature. Using a pipette, carefully remove diethyl ether without disturbing the protein pellet. Repeat diethyl wash steps a further two times. Allow pellet to air-dry.
7. Re-solubilize the protein pellet in 50 μl of 20 mM sodium phosphate, pH 7.5.
8. Digest the acetylated protein with sequencing-grade trypsin (50:1 substrate:enzyme) overnight at 37°C. This is effectively an endopeptidase ArgC digest as lysine residues are acetylated and not compatible with the specificity of trypsin.
9. If there is enough protein digest available, verify that complete protein digestion has occurred using SDS-PAGE (*see* **Note 6**). To check acetylation, dilute a small aliquot of

sample 1:20 with matrix. Spot 1 μl onto a clean MALDI-TOF target and allow to air-dry. Analyse the peptide mixture using MALDI-TOF over the range of *m/z* 800–3,500. The spectra obtained will vary according to the complexity of the initial protein sample. For more complex samples (e.g. *Escherichia coli* lysates, plasma) nLC-MS/MS analysis is preferable and will allow for a more extensive and definitive survey of the extent of peptide acetylation. N-terminal- and/or lysine-containing peptides will shift mass by +42 Da in the acetylated digest (**Fig. 15.2**, *see* **Note 7**). This acetylated peptide mixture can be stored frozen for several weeks.

10. Add 7 μl of EZ-Link NHS-biotin (1 mg in 50 μl DMF, prepared in a glass vial) to the acetylated peptide mixture (gives approximately 7 nmol of biotin/μl digest, a 20-fold molar excess). Vortex 20 s. Incubate at room temperature for 1–2 h or overnight at 4°C (*see* **Note 8**).
11. To check biotinylation, dilute the digest with matrix solution (1:20) and spot 1 μl onto a MALDI-TOF target and analyse as in Step 9. Again, more complex digests will require nLC-MS/MS analysis. Peptides with a free α-amino group ('internal' peptides) will have shifted mass by +226 Da (due to incorporation of biotin). N-terminal peptides will have no mass shift (**Fig. 15.2**). Once biotinylation is confirmed the 'internal' peptides can be removed using neutrAvidin (*see* **Note 9**).
12. Take 6 μl of the digest and dilute with 100 μl of 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.5.
13. Add digest mixture to approximately 350 μl of neutrAvidin (washed three times with 200 μl 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.5, remove excess buffer from resin by using spin columns as described in Step 4) in a 0.5 ml microcentrifuge tube. Ensure resin is just swollen, with no excess buffer. Incubate at room temperature for 1 h.
14. Spin neutrAvidin mixture as described in Step 4 and collect the flow-through (N-terminal peptides). Wash the neutrAvidin resin with an additional 100 μl of binding buffer. Pool N-terminal peptide eluates in a 0.5 ml Eppendorf tube.
15. Use a StageTip or a similar C18 reversed-phase column to concentrate and desalt the N-terminal peptide mixture (*see* **Note 10**).
16. Using the entire N-terminal preparation in one injection, separate N-terminal peptides using a reversed-phase column in line with an ESI-MS/MS capable mass spectrometer (*see* **Note 11**).

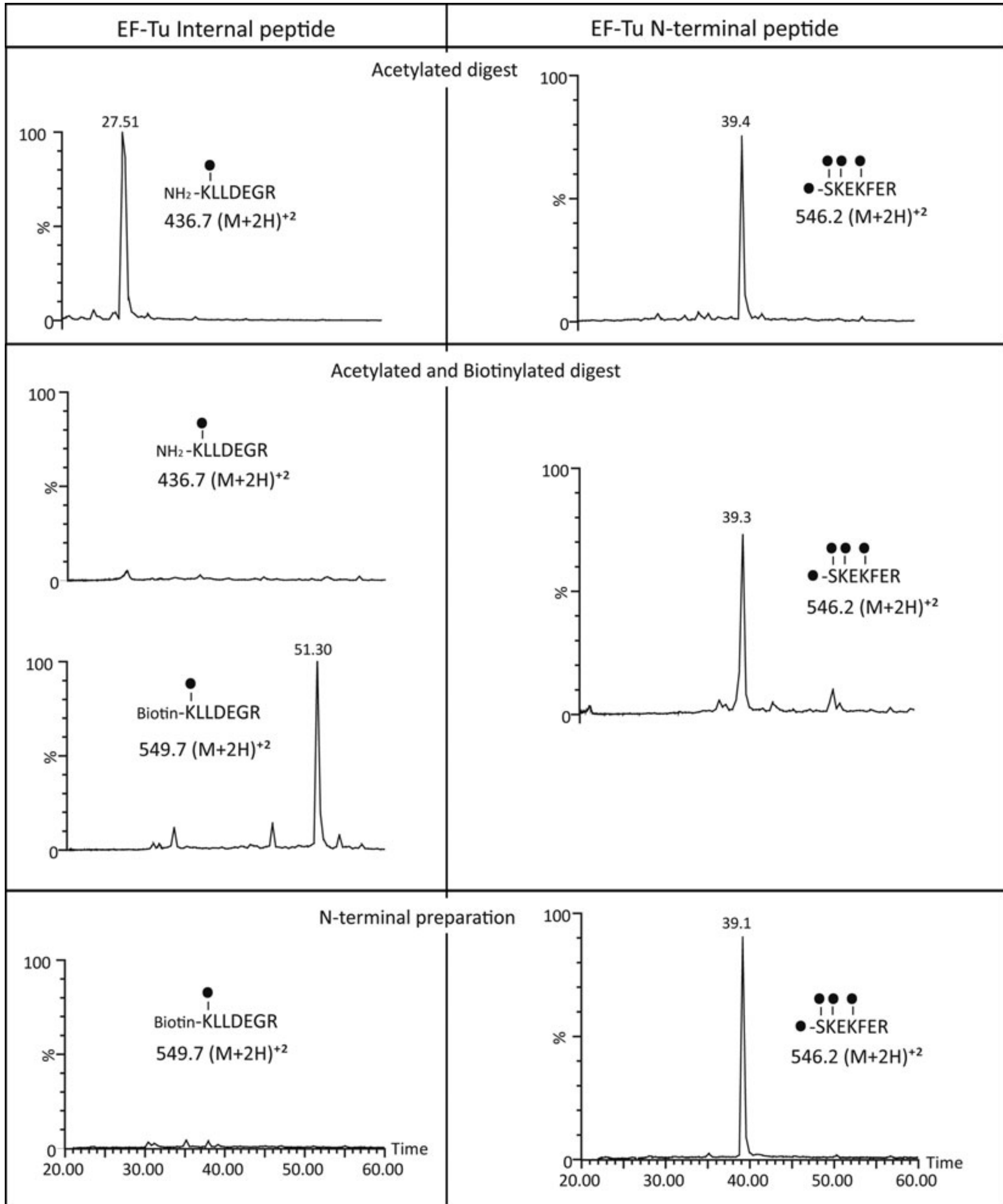

Fig. 15.2. Isolation of N-terminal peptides. A protein sample from *Escherichia coli* was passed through the N-terminal peptide protocol (**Fig. 15.1**). At each stage, samples were removed and analysed on a Waters Synapt QTOF instrument coupled to a nanoAcquity chromatography system. Extracted ion chromatograms were prepared to show the recovery of the N-terminal peptide (*right-hand panes*) and loss of an internal peptide (*left-hand panes*) for an abundant protein (elongation factor Tu) through the process. All chromatograms are normalized to the same scale.

17. Search the tandem mass spectra against Swiss-Prot (or database of choice) using MASCOT. Use search parameters that include fixed modifications of N-terminal acetylation and lysine acetylation (and carbamidomethyl if sample was reduced and blocked) and the variable modifications of O-acetylated serine (*see* **Note 12**) and methionine oxidation. To check that no internal peptides have managed to leak through the system use search parameters that include the fixed modifications of lysine acetylation and N-terminal biotinylation and the variable modifications, O-acetylated serine and methionine oxidation.

4. Notes

1. Amine-containing buffers (e.g. Tris or ammonium bicarbonate) will compete with proteins for acetylation. In the preparation of any protein lysate ensure that a proteolytic inhibitor cocktail (e.g. complete inhibitor cocktail tablets, from Roche Diagnostics) is included to limit degradation by endogenous proteases. This is important as uncontrolled proteolytic trimming may create artefactual protein N-termini.
2. Acetylation of some primary amino groups may be impaired by protein structure. Increasing the acetylation incubation time or using reagents that denature the protein structure, e.g. chaotropes, detergents and reducing agents, may increase primary amino group accessibility. These reagents may need to be removed prior to the acetylation reaction and/or to any mass spectrometry steps.
3. If a precipitate forms on addition of acetic anhydride, check that the correct buffer was added prior to the acetylation reaction. It is important to keep the pH at about 8.5 for the reaction.
4. This step ensures that any excess acetylation reagent is removed before the digestion step. Make sure that mixing is continuous by using a rotating or end-over-end mixer. If the proteins are subsequently precipitated using TCA, a Tris buffer solution (approximately 50 μl of 1.5 M Tris solution) can also be used to mop up excess acetylation reagent.
5. The precipitation step will denature proteins and will also remove unwanted reagents (e.g. reducing agents, any remaining acetylation reagent), prior to proteolysis, biotinylation and neutrAvidin steps.

6. Take an aliquot of digest (approximately 10 μg) and analyse using SDS-PAGE. No protein bands should be visible on the Coomassie stained gel. Incomplete digestion can be due to incorrect pH of the reaction mixture due to inadequately washed protein pellets. Wash pellets carefully to remove all traces of TCA.
7. If poor MALDI-TOF spectra are obtained, desalt the peptide mixture using a StageTip before analysis. If incomplete acetylation is observed it could be due to several factors. The reaction needs a longer incubation time. The acidification of the protein sample by acetic anhydride shifts the pH from the optimal range. The accessibility of amine groups and the presence of competing amines will also hinder acetylation (*see* **Notes 1** and **2**).
8. Use NHS-ester solutions immediately as they readily hydrolyse and become un-reactive. Do not store or reuse the solution. Ensure organic solvent concentration does not exceed 20% of final reaction volume. DMF may strip polymers from plastic tubes and result in contaminant peaks in mass spectrometry analysis. Use glass vials to contain the NHS-biotin stock solution and perform the biotinylation reaction.
9. If biotinylation is incomplete, make sure that the NHS-biotin solution is fresh and/or increase the incubation time.
10. The relatively low capacity of neutrAvidin for biotin means that a large amount is needed to bind excess NHS-biotin as well as the 'internal' biotin-labelled peptides. Another approach is to load a greater amount (above the biotin-binding capacity of the neutrAvidin) of the acetylated and biotinylated peptide preparation onto a neutrAvidin packed column and slowly pump the peptide sample through (e.g. 100 μl/h with a syringe pump), collecting fractions. Monitor each fraction for N-terminal peptides using MALDI-TOF or nLC-MS/MS. When biotinylated peptides elute, cease taking fractions. Desalt and concentrate the N-terminal fraction(s) using StageTips and analyse using nLC-MS/MS.
11. If biotinylated peptides are present in the N-terminal peptide mixture, increase the volume of neutrAvidin used and increase the coupling time.
12. There are a number of methods reported in the literature for the reversal of O-acetylation. Popular strategies include treating the sample with hydroxylamine (25) or heating the sample in a hot water bath.

References

1. Gevaert, K., Ghesquiere, B., Staes, A., Martens, L., Van Damme, J., Thomas, G. R., and Vandekerckhove, J. (2004) Reversible labeling of cysteine-containing peptides allows their specific chromatographic isolation for non-gel proteome studies, *Proteomics 4*, 897–908.
2. Veenstra, T. D., Conrads, T. P., and Issaq, H. J. (2004) Commentary: What to do with "one-hit wonders"? *Electrophoresis 25*, 1278–1279.
3. Wilkins, M. R., Gasteiger, E., Tonella, L., Ou, K., Tyler, M., Sanchez, J. C., Gooley, A. A., Walsh, B. J., Bairoch, A., Appel, R. D., Williams, K. L., and Hochstrasser, D. F. (1998) Protein identification with N and C-terminal sequence tags in proteome projects, *Journal of Molecular Biology 278*, 599–608.
4. Meinnel, T., and Giglione, C. (2008) Tools for analyzing and predicting N-terminal protein modifications, *Proteomics 8*, 626–649.
5. Agard, N. J., and Wells, J. A. (2009) Methods for the proteomic identification of protease substrates, *Current Opinion in Chemical Biology 13*, 503–509.
6. Doucet, A., Butler, G. S., Rodriguez, D., Prudova, A., and Overall, C. M. (2008) Metadegradomics: Toward in vivo quantitative degradomics of proteolytic post-translational modifications of the cancer proteome, *Molecular & Cellular Proteomics 7*, 1925–1951.
7. Van Damme, P., Van Damme, J., Demol, H., Staes, A., Vandekerckhove, J., and Gevaert, K. (2009) A review of COFRADIC techniques targeting protein N-terminal acetylation, *BMC Proceedings 3*(Suppl 6), S6.
8. Brown, J. L., and Roberts, W. K. (1976) Evidence that approximately 80 per cent of soluble-proteins from Ehrlich ascites-cells are N-alpha-acetylated, *Journal of Biological Chemistry 251*, 1009–1014.
9. Polevoda, B., and Sherman, F. (2000) N-α-terminal acetylation of eukaryotic proteins, *Journal of Biological Chemistry 275*, 36479–36482.
10. Hwang, C. S., Shemorry, A., and Varshavsky, A. (2010) N-terminal acetylation of cellular proteins creates specific degradation signals, *Science 327*, 973–977.
11. Kawasaki, H., Imajoh, S., and Suzuki, K. (1987) Separation of peptides on the basis of the difference in positive charge – simultaneous isolation of C-terminal and blocked N-terminal peptides from tryptic digests, *Journal of Biochemistry 102*, 393–400.
12. Titani, K., Okunuki, K., and Narita, K. (1962) N-Terminal sequence in beef- and horse-heart cytochrome C, *Journal of Biochemistry 51*, 350–358.
13. Betancourt, L., Besada, V., Gonzalez, L. J., Morera, V., Padron, G., Takao, T., and Shimonishi, Y. (2001) Selective isolation and identification of N-terminal blocked peptides from tryptic protein digests, *Journal of Peptide Research 57*, 345–353.
14. Gorman, J. J., and Shiell, B. J. (1993) Isolation of carboxyl-termini and blocked amino-termini of viral-proteins by high-performance cation-exchange chromatography, *Journal of Chromatography 646*, 193–205.
15. Dormeyer, W., Mohammed, S., van Breukelen, B., Krijgsveld, J., and Heck, A. J. R. (2007) Targeted analysis of protein termini, *Journal of Proteome Research 6*, 4634–4645.
16. McDonald, L., and Beynon, R. J. (2006) Positional proteomics: Preparation of amino-terminal peptides as a strategy for proteome simplification and characterization, *Nature Protocols 1*, 1790–1798.
17. Akiyama, T. H., Sasagawa, T., Suzuki, M., and Titani, K. (1994) A method for selective isolation of the amino-terminal peptide from [alpha]-amino-blocked proteins, *Analytical Biochemistry 222*, 210–216.
18. Zhang, X., Ye, J., and Højrup, P. (2009) A proteomics approach to study in vivo protein N-α-modifications, *Journal of Proteomics 73*, 240–251.
19. Mikami, T., and Takao, T. (2007) Selective isolation of N-blocked peptides by isocyanate-coupled resin, *Analytical Chemistry 79*, 7910–7915.
20. Miller, B. T., Collins, T. J., Rogers, M. E., and Kurosky, A. (1997) Peptide biotinylation with amine-reactive esters: Differential side chain reactivity, *Peptides 18*, 1585–1595.
21. McDonald, L., Robertson, D. H., Hurst, J. L., and Beynon, R. J. (2005) Positional proteomics: Selective recovery and analysis of N-terminal proteolytic peptides, *Nature Methods 2*, 955–957.
22. Yamaguchi, M., Nakazawa, T., Kuyama, H., Obama, T., Ando, E., Okamura, T., Ueyama, N., and Norioka, S. (2005) High-throughput method for N-terminal sequencing of proteins by MALDI mass spectrometry, *Analytical Chemistry 77*, 645–651.
23. Gevaert, K., and Vandekerckhove, J. (2004) COFRADIC(TM): The Hubble telescope of proteomics, *Drug Discovery Today: TARGETS 3*, 16–22.

24. Gevaert, K., Goethals, M., Martens, L., Van Damme, J., Staes, A., Thomas, G. R., and Vandekerckhove, J. (2003) Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides, *Nature Biotechnology 21*, 566–569.

25. Staes, A., Van Damme, P., Helsens, K., Demol, H., Vandekerckhove, J., and Gevaert, K. (2008) Improved recovery of proteome-informative, protein N-terminal peptides by combined fractional diagonal chromatography (COFRADIC), *Proteomics 8*, 1362–1370.