# Peptide and protein *de novo* sequencing by mass spectrometry

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Although the advent of large-scale genomic sequencing has greatly simplified the task of determining the primary structures of peptides and proteins, the genomic sequences of many organisms are still unknown. Even for those that are known, modifications such as post-translational events may prevent the identification of all or part of the protein sequence. Thus, complete characterization of the protein primary structure often requires determination of the protein sequence by mass spectrometry with minimal assistance from genomic data — *de novo* protein sequencing. This task has been facilitated by technical developments during the past few years: 'soft' ionization techniques, new forms of chemical modification (derivatization), new types of mass spectrometer and improved software.

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**Current Opinion in Structural Biology** 2003, **13**:595–601

This review comes from a themed issue on
Biophysical methods
Edited by Brian T Chait and Keith Moffat

0959-440X/$ – see front matter


**DOI 10.1016/j.sbi.2003.09.005**

**Abbreviations**

| | |
|---|---|
| **CID** | collision-induced dissociation |
| **ECD** | electron capture dissociation |
| **ESI** | electrospray ionization |
| **FTICR** | Fourier transform ion cyclotron resonance |
| **MALDI** | matrix-assisted laser desorption/ionization |
| **MS** | mass spectrometry |
| **MS/MS** | tandem mass spectrometry |
| ***m/z*** | mass/charge |
| **QqTOF** | quadrupole/time-of-flight |
| **TOF** | time-of-flight |

## Introduction

The advent of large-scale genomic sequencing has greatly simplified the task of determining the primary structures of peptides and proteins in many organisms, because open reading frames in the nucleotide sequence serve as templates for the construction of the corresponding proteins. The masses of peptides produced by proteolytic digestion of an unknown protein can be compared with those predicted to arise from each protein in the database; this 'mass mapping' is often sufficient to identify any protein whose full-length sequence is contained therein [1–3,4••].

Nevertheless, the genome sequences of most organisms are still unknown. Even for those that are known, modifications such as post-translational events may prevent the identification of all or part of the protein sequence, or at least the definition of the modifications. Thus, complete characterization of the protein primary structure often requires determination of the protein sequence with minimal assistance from genomic data — *de novo* protein sequencing. Early *de novo* protein sequencing measurements relied on Edman degradation of the protein [4••], but mass spectrometry (MS) has reduced the need for this technique because it is more sensitive and provides higher sample throughput. It can also cope better with protein mixtures and with modifications to the protein N terminus.

*De novo* protein sequencing by MS dates back more than 30 years [5,6,7•], first in combination with Edman degradation and then on its own. Early measurements were mostly made on sector mass spectrometers or triple quadrupoles, and with electron ionization, which almost completely fragmented the proteins (normally involatile and labile compounds), unless they were chemically modified (derivatized) [8]. However, more recent determinations have been greatly facilitated by subsequent technical developments (see [1–3,4••]). First, 'soft' ionization techniques have been developed, particularly electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which produce ions from peptides and proteins with much less fragmentation. Second, workers now have available other types of mass spectrometer, such as ion traps, time-of-flight (TOF) devices and Fourier transform ion cyclotron resonance (FTICR) instruments, each of which has advantages for sequencing measurements. Third, more efficient derivatization reactions have been developed. Finally, there have been great improvements in software to enable automated descrambling of mass spectra by computer, thus reducing the demands on manual interpretation of the data.

These developments in *de novo* peptide and protein sequencing by MS are reviewed in the following sections.

## Peptide sequencing

Straightforward MS measurement of the mass spectrum of the collection of proteolytic peptides resulting from enzymatic digestion of an unknown protein is often sufficient for protein identification, as noted above. Along with a MALDI measurement of the overall protein mass, it is also the usual first step in *de novo* sequencing of the protein, yielding the masses of the individual peptides.

The next step is to determine the sequences of the peptides. The MS measurement itself may yield considerable information about the amino acid compositions of these peptides, especially when it includes accurate measurements of low mass ions, such as immonium ions [6,9–11]. However, it cannot determine the order of the amino acid residues. Consequently, *de novo* peptide sequencing requires tandem mass spectrometry, usually denoted MS/MS [12]. In this technique, a given parent (precursor) ion is selected in one mass spectrometer and then broken up, usually by collisions [13•]. The *m/z* (mass/charge) values for the resulting daughter (product) ions are measured in a second mass spectrometer. Under favorable conditions, this procedure may yield a series of ions that contains sufficient information to determine the peptide sequence. Even more information can be obtained in certain instruments, ion traps for example, by breaking up the daughter ions themselves to yield a spectrum of granddaughter ions.

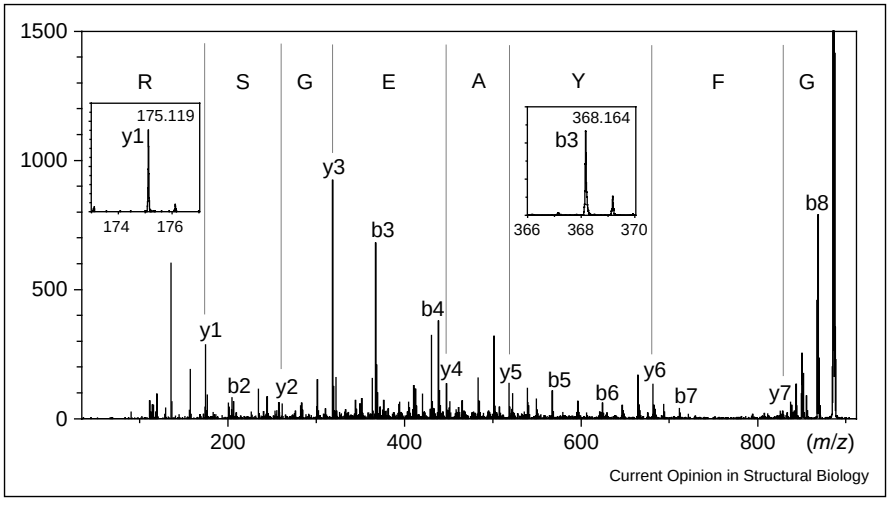
For underivatized and unlabeled peptides, ESI *de novo* sequencing measurements have been carried out following collision-induced dissociation (CID) in the triple quadrupole mass analyzer [14,15,16•] or in the quadrupole/TOF (QqTOF) instrument [15,16•,17–22], and also after resonant excitation in the quadrupole ion trap [23–25]. MALDI *de novo* sequencing has been carried out using CID and metastable decay in single- [26] and double-stage [27–29,30••] TOF instruments, using CID in QqTOF devices [31•,32,33,34••,35] and using resonant excitation/CID in ion traps [36••].

CID fragmentation mechanisms are extensively discussed in [12,13•,37•,38–45]. Extraction of the peptide sequence from the daughter ion spectra depends on the completeness of the observed series, the accuracy with which the mass differences between the relevant ion peaks are determined and the extent to which the fragmentation spectrum can be correctly interpreted. A peptide resulting from tryptic digestion normally has a basic residue (arginine or lysine) at its C terminus and it yields a prominent doubly charged ion peak when ionized by ESI. If this ion is chosen as the parent ion for an MS/MS measurement, the production of a series of y ion daughters (ions resulting from cleavage at the amide bonds and containing the C terminus) is favored and the resulting spectrum is likely to be easy to interpret [12,13•,14,15,16•,17–25,37•,38–42]. For this reason, ESI has been a popular choice for *de novo* peptide sequencing.

On the other hand, CID of MALDI ions (predominantly singly charged) tends to yield daughter ion spectra that include both y and b ions, the latter corresponding to cleavage at the peptide bond and containing the N terminus [26–29,30••,31•,32,33,34••,35,36••]. They also may be dominated by a few preferred fragmentation pathways [31•,43–45], particularly in ion traps. However, the presence of both b and y ions means that the spectra are rich in information, provided that the spectra can be interpreted. In this regard, sensitivity and accuracy of the instrument used for mass analysis are particularly important. A recent *m/z* spectrum obtained in our laboratory on a QqTOF instrument is shown in Figure 1 and compared with calculated mass values in Table 1. The good agreement between calculated and observed masses lends confidence to the assignments.

MALDI ionization has several attractive properties for the analysis of peptides. In particular, it has a relatively high tolerance to impurities and common biochemical

**Figure 1**




MS/MS *de novo* sequencing of the 886.407 Da (residues 170–177) GFYAEGSR peptide from SARS virus nucleocapsid protein. Series of y and b fragments are labeled. (Note that 886.407 was the measured mass during the HPLC MS run, but the calculated $MH^+$ is 886.406 Da.)

**Table 1**

**Calculated and measured masses of y and b series of fragments from MS/MS spectra of the 886.407 Da (residues 170–177) GFYAEGSR peptide from SARS virus nucleocapsid protein.**

| b fragments | | Residue | y fragments | |
|---|---|---|---|---|
| [MH$^+$] calculated | *m/z* (observed) | | [MH$^+$] calculated | *m/z* (observed) |
| 58.029 | – | G | 886.406 | 886.407 |
| 205.098 | 205.098 | F | 829.384 | 829.386 |
| 368.161 | 368.164 | Y | 682.316 | 682.318 |
| 439.198 | 439.204 | A | 519.253 | 519.247 |
| 568.241 | 568.247 | E | 448.216 | 448.217 |
| 625.262 | 625.270 | G | 319.173 | 319.177 |
| 712.294 | 712.297 | S | 262.152 | 262.152 |
| 868.395 | 868.398 | R | 175.120 | 175.119 |

additives and salts, reducing the demands on sample purification compared with ESI. Also, the MALDI sample is usable until it is depleted, so data can be collected from many different ion species produced from a single peptide mixture and until the desired quality of spectra has been obtained. These qualities have stimulated recent interest in MALDI sequencing, particularly after pretreatment of the sample by derivatization or isotopic labeling, as described below.

## Derivatization and labeling

As mentioned above, the use of electron ionization required chemical modification (derivatization) of the peptide to produce an informative mass spectrum. Although this is no longer an absolute requirement, the procedure may still yield marked improvement in mass spectral quality. Derivatization methods used until the late 1990s are reviewed in [8], but other techniques have been developed more recently [25,46••,47–55]. A notable example is sulfonation of the peptide N terminus, originated by Keough and his collaborators [46••,47–53]. This modification usually leads to the production of a series of C-terminal y daughter ions from singly charged parents and thus transfers the advantages of ESI to MALDI. Moreover, the required reagents can be obtained conveniently as a commercially available kit (the CAF MALDI sequencing kit from Amersham Bioscience). The main disadvantage of the method is a reduction in sensitivity by a factor ~10.

An alternative technique calls for isotopic labeling [56–63,64•,65–72], which helps to distinguish C-terminal from N-terminal ions, and also may provide information on quantitation. Particularly noteworthy is the use of labeling by $^{18}O$ [60–63,64•,65–72], usually carried out by doing the proteolytic digestion in a mixture of ordinary water and $H_2{}^{18}O$. Under these conditions, the C-terminal residue of each proteolytic fragment (except the one containing the C terminus of the protein) contains a mixture of $^{16}O$- and $^{18}O$-containing ions. Consequently, the C-terminal daughter ions alone are identified by this label and the N-terminal ions are unlabeled. A major advantage of the technique is that the normal digestion protocol is maintained and no sensitivity is lost. In addition to possible ambiguities arising from incorporation of either one or two $^{18}O$ atoms [69], the main disadvantage is the high cost of the $H_2{}^{18}O$.

## Mass accuracy

The accuracy with which *m/z* values are determined has a pronounced effect on the reliability of sequence assignments [9]. The 20 common amino acid residues have distinctive elemental compositions and consequently have distinctive masses, except for the L/I pair. However, a low accuracy measurement may be incapable of discriminating between D and N, or between E and Q/K (cases where $\Delta m \sim 1$ Da), and a much higher accuracy is needed to distinguish Q from K, where $\Delta m = 36$ mDa. In addition, there are combinations of amino acid residues that yield the same mass number [6] or even the same elemental composition [e.g. (G+G) = N = $C_4H_6N_2O_2$; (G+A) = Q = $C_5H_8N_2O_2$], and these may create ambiguities if the intermediate daughter ion is not seen. When modified peptides are considered, there are many additional possibilities.

However, there are several instrument types that are sufficiently accurate to resolve some of these uncertainties. Recent examples of success in QqTOF instruments include K versus Q, and VV versus PT (both with $\Delta m = 36$ mDa) [36••], as well as R–G versus V ($\Delta m = 11.2$ mDa) [33]. Phosphorylation has also been distinguished from sulfation ($\Delta m = 9.5$ mDa) by FTICR [73•,74].

## From peptide to protein sequence

The peptide sequences provide the raw material for determining the overall protein sequence. The problem is then one of ordering the individual peptide sequences. A purely MS method is to search for proteolytic fragments that span the initial ones. These may arise in the initial digestion (usually by trypsin) because of missed cleavages

**Table 2**

**Proteolytic fragments from the 'high plains virus' protein @ALSFKNSSGVLKAKTLKDGFVTSSDIETTVHDFSYEKPDLSSVDGFSLKS...***

| Enzyme | *m/z* (observed) | $MH^+$ (calculated) | Δm (mDa) | Fragment sequence |
|---|---|---|---|---|
| Trypsin | 3522.653 | 3522.644 | +9 | DGFVTSSDIETTVHDFSYEKPDLSSVDGFSLK |
| Asp-N | 3322.769 | 3322.753 | +16 | @ALSFKNSSGVLKAKTLKDGFVTSSDIETTVH |
| Glu-C | 2884.534 | 2884.531 | +3 | @ALSFKNSSGVLKAKTLKDGFVTSSDIE |
| Asp-N | 1834.080 | 1834.081 | –1 | @ALSFKNSSGVLKAKTLK |
| Trypsin | 1491.856 | 1491.854 | +2 | @ALSFKNSSGVLKAK |
| Trypsin | 1292.723 | 1292.722 | +1 | @ALSFKNSSGVLK |
| Trypsin | 607.350 | 607.346 | +4 | @ALSFK |

* The N-terminal portion of the original U60141 sequence is shaded. @A denotes acetylated alanine.

or they may be produced by digestion with other proteases. A simple example is shown in Table 2; a 14-residue peptide appeared, after tryptic digestion, in the same gel spot as peptides from a protein identified in the database as 'the putative protein of high plains virus'. Additional digestions then showed that this peptide was contained in an additional 18 amino acid residues that formed the actual N terminus of the putative protein.

The complete protein sequence can only be obtained by this method if the peptide coverage is also complete. This is difficult to obtain in a single digestion, but digestion by several different proteases is likely to improve the coverage. A useful check on the completeness of the coverage is provided by a MALDI measurement of the overall protein mass.

A combination of techniques may provide the complete protein sequence even if only partial peptide sequence information is available, as a single peptide sequence enables construction of a short oligonucleotide useful for the isolation of the gene that encodes the protein of interest. However, such cloning is most efficiently carried out if the peptide sequence is long, 100% accurate and encodes low degeneracy primers, that is, if it contains amino acids encoded by only one base triplet (Met, Trp) or two (Phe, Tyr, Gln, Glu, Asn, Asp, Cys); these requirements may not always be easy to fulfill.

## 'Top-down' sequencing

A recently developed method that circumvents some of the above difficulties is 'top-down' sequencing, pioneered by McLafferty and his collaborators [75–81]. The technique has been most frequently applied to measurements on FTICR mass spectrometers, because they provide very high resolution and mass accuracy. In this method, the ionized protein itself is introduced into the ICR cell. *M/z* measurements are then carried out in the same spectrometer both on the intact protein and on the products of its dissociation, thus avoiding the need to stitch together the products of peptide sequencing. In addition, the FTICR measurements are able to make use of various methods to manipulate the ions in the ICR cell, including CID and IR multiphoton dissociation. Most importantly, the ions can be broken up by the newly discovered electron capture dissociation (ECD) technique [76–82], which has only been available in FTICR instruments up to now. ECD induces far more general backbone cleavage than other methods, yielding extensive sequence information on proteins as large as approximately 40 kDa.

Nevertheless, although the fractional mass accuracy of the FTICR instrument is impressive, the measurements are made on very large ions, so the absolute mass accuracy, which determines the ability to distinguish between various sequence alternatives, is somewhat limited. This is one argument for using a combination of top-down sequencing with the usual 'bottom-up' method [83].

## Data interpretation and analysis

Several computer algorithms have been devised that seek to infer sequence *de novo* from MS/MS data [84–91,92••,93–95]. This work, together with methods that look for homology of the unknown protein with sequences of organisms that are already in the database, has been recently reviewed by Liska and Shevchenko [91,92••]. Clearly these techniques have great potential for improving *de novo* peptide and protein sequencing by MS.

## Conclusions

The recent developments summarized above have produced considerable improvements in our ability to carry out *de novo* peptide and protein sequencing by MS, although it is still not an easy task unless nature has designed the sequence to be particularly easy to unravel. Although mere protein identification will become more straightforward as more genomes are sequenced, complete protein characterization will continue to require *de novo* techniques; this will probably become more important as long lists of identifications are found to be inadequate for determining protein function. Fortunately, there is still room for ample improvement in all the methods described above. Thus, *de novo* peptide and protein sequencing by MS is likely to have a bright future.

## Acknowledgements

I thank Brian Chait and Andrej Shevchenko for helpful discussions and communication of their results before publication. I also thank Oleg Krokhin for supplying the data shown in Figure 1 and Table 1. Gideon Garland, James McNabb, Yi-min She and Victor Spicer provided invaluable technical support. This work was supported by grants from the US National Institutes of Health (GM 59240), and from the Natural Sciences and Engineering Research Council of Canada.

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