Research Techniques Made Simple: Mass Spectrometry for Analysis of Proteins in Dermatological Research

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Identifying previously unknown proteins or detecting the presence of known proteins in research samples is critical to many experiments conducted in life sciences, including dermatology. Sensitive protein detection can help elucidate new intervention targets and mechanisms of disease, such as in autoimmune blistering skin diseases, atopic eczema, or other conditions. Historically, peptides from highly purified single proteins were sequenced, with many limitations, by stepwise degradation from the N-terminus to the C-terminus with subsequent identification by UV absorbance spectroscopy of the released amino acids (i.e., Edman degradation). Recently, however, the availability of comprehensive protein databases from different species (derived from high-throughput next-generation sequencing of those organisms’ genomes) and sophisticated bioinformatics analysis tools have facilitated the development and use of mass spectrometry for identification and global analysis of proteins, summarized as mass spectrometry-based proteomics. Mass spectrometry is an analytical technique measuring the mass \((m)\)-to-charge \((z)\) ratio of ionized biological molecules such as peptides. Proteins can be identified by correlating peptide-derived experimental mass spectrometry spectra with theoretical spectra predicted from protein databases. Here we briefly describe how this technique works, how it can be used for identification of proteins, and how this knowledge can be applied in elucidating human biology and disease.


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Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:
- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.
- Describe the potential impact of these techniques.

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INTRODUCTION
Basic dermatological research that uses genetic and cellular techniques has resulted in significant advances, allowing for precise diagnosis and optimized therapy of skin disease, as illustrated for autoimmune blistering diseases (Kasperkiewicz et al., 2017). Only recently has a more global proteomic picture in dermatologic (and other) conditions emerged, allowing new insights of clinical relevance. For example, for pemphigus vulgaris, it was shown how various monoclonal anti-desmoglein 3 autoantibodies contribute to the polyclonal serum response and how the amount of each monoclonal antibody (Ab) changes over the course of disease (Chen et al., 2017). In another study, proteomics was used to identify differentially expressed proteins relevant to filagrin-deficient atopic eczema (Elias et al., 2017), potentially yielding new therapeutic targets. Additionally, previously unknown interaction partners of autoantibodies in dermatologic and other autoimmune conditions were successfully identified by proteomics (Miske et al., 2016; Schepens et al., 2010).

In this review, we focus on use of liquid chromatography tandem mass spectrometry (LC-MS/MS) for protein identification because it is currently the most practical means of direct and global protein identification (Domon and Aebersold, 2006).

SUMMARY POINTS
What mass spectrometry for analysis of proteins does:
- Enables direct analysis of protein amino acid sequences, allowing for identification of unknown proteins (e.g., new autoantigens in disease)
- Enables analysis of changes in global protein expression, for example, in epidermis or other organs under different experimental conditions

LIMITATIONS
- Limits in the detection of proteins in very complex samples, requiring reduction in complexity of samples of interest (e.g., by affinity purification)
- Nondetection of a protein of interest in complex samples does not exclude presence of the protein, and detection of a peptide characteristic for one protein may not be specific for this protein because peptides can be shared between proteins (i.e., protein interference problem)
- Experienced bioinformaticians are needed to interpret the complex MS results

MS-based proteomics consists of the following stages, which will be briefly described: (i) isolation of the protein
sample, (ii) mass spectrometric analysis, and (iii) analysis and interpretation of MS data using bioinformatic tools. The general steps of a typical LC-MS/MS experiment are summarized in Figure 1.

**ISOLATION AND FRACTIONATION OF THE PROTEIN SAMPLE OF INTEREST**

A major advantage of LC-MS/MS is that it can identify unknown proteins. Potential sources of such proteins are theoretically unlimited and depend on the research question under investigation. For example, unknown reaction partners of antibodies (e.g., autoantigens in autoimmune diseases) can be immunoprecipitated from cell lysates and subjected to LC-MS/MS for identification and validation (Miske et al., 2016; Schepens et al., 2010). A single protein band can be stained after separation by SDS-PAGE, cut out of the gel, and subjected to analysis by LC-MS/MS. However, samples of higher complexity can be studied (and compared) as well to gain a more global view of proteins expressed under stable or different experimental conditions or at different time points over the course of disease. Examples of use of this type of analysis include plasma membrane isolates from whole keratinocyte lysates (Blonder et al., 2004), homogenized human epidermal living skin equivalents in atopic eczema (Elias et al., 2017), or affinity-purified autoantibodies from serum (Chen et al., 2017). These examples are discussed in more detail.

Because accurate sequence assignment of MS/MS spectra can be achieved only for short linear peptides (~7–50 amino acid residues), the purified (often SDS-PAGE-separated) proteins are usually treated with proteolytic enzymes (e.g., trypsin) before loading them into the LC-MS/MS instrument (Figure 1a). To allow for higher resolution in LC-MS/MS, the digested peptide pools are first separated on a liquid chromatography (LC) column, usually by reversed-phase chromatography. This separation technique is based on a column with a hydrophobic stationary phase, with high affinity for hydrophobic peptides (Figure 1b). By applying a
mobile phase that consists of an increasing gradient of nonpolar solvents over polar solvents (e.g., acetonitrile over water) with time, hydrophilic peptides are eluted first and hydrophobic peptides last. This elution can take place over time (such as 1–2 hours), with the resultant eluate continuously loaded into the MS/MS analyzer.

MS ANALYSIS
The instrument used for MS analysis consists of an ionizer, a mass analyzer, and a detector. MS analysis of peptides (usually derived from trypsinization of a protein) after ionization is based on their migration in an electromagnetic field, which is a function of their mass (m) and charge (z). To reliably differentiate distinct peptides with equal mass and charge (i.e., with same m/z), reversed-phase LC is used first, because such peptides will most likely elute at different retention times through the LC column based on their hydrophobicity (Figure 1b). As each peptide comes off the column, it is ionized and analyzed in the first mass analyzer of a tandem-in-space mass spectrometer. Then each precursor peptide with a defined m/z is fragmented by collision-induced dissociation in a collision cell (Figure 1a). The resulting fragment ions of that precursor ion are then analyzed in a second mass analyzer, and a fragment ion spectrum is recorded. Alternatively, a tandem-in-time mass spectrometer can perform both MS scans in one trapping mass analyzer. This process is repeated throughout the LC separation process to allow amino acid sequence determination of most of the peptides in the digest (Boström, 2014). During collision-induced dissociation fragmentation, the most common bonds cut are the peptide bonds (Figure 2). From each cleavage, two ions result: the C-terminal fragment, called the y-ion, and the N-terminal fragment, called the b-ion. To be detected by MS, the fragments must be charged. The usual site of charge is at the cleaved peptide bond of the fragment, which results in one charge (i.e., z = 1), but additional charges may be introduced on other parts of the peptide (z > 1), such as the amino group side chain of lysine and arginine and the imidazole ring of histidine. For ions with the same charge, the differences between the peaks in the ion spectrum measures the difference in mass of the ions. Because the mass of each amino acid is known, the amino acid cleaved off the peptide by collision-induced dissociation can be deduced from the loss of that given mass, allowing sequence determination (Figure 3a and 3b).

BIOINFORMATIC ANALYSIS AND INTERPRETATION OF MS DATA
Because resulting fragment ion spectra rarely contain all possible fragment ions and de novo interpretation of such spectra is time consuming and error prone, experimental spectra are searched by bioinformatic means and with the help of search engines, such as Mascot, Sequest, or Andromeda, against theoretical spectra generated from in silico digestion of theoretical input proteins to identify matches of experimental to theoretical spectra. If those input proteins are unknown, public databases that include all known relevant proteins (e.g., all human proteins) (see Figure 4 as an application example) can be used by the software to generate theoretical spectra (see www.uniprot.org for an example of such databases and Magrane and UniProt Consortium, 2011). In some studies, such as studies of antibodies that differ in each individual, custom-made databases must be produced. For example, next-generation sequencing of B cell-derived transcripts coding for Abs can be used to deduce a database of possible antibody amino acid sequences (Figure 5). Peptides identified by matching the LC-MS/MS spectra to such databases are reported with a probability score as a measure of the reliability of their identification (Boström, 2014). By spiking in internal standards to the sample being analyzed, confirmation of the spectra identifying that particular peptide, and even its absolute quantitation, becomes possible. This is accomplished by using synthetic heavy isotope-labeled peptides with the same sequence as the deduced light peptide from the sample (Domon and Aebersold, 2006).

RESEARCH TECHNIQUES MADE SIMPLE
EXAMPLES OF APPLICATIONS OF MS IN IMMUNOLOGY AND INVESTIGATIVE DERMATOLOGY

In an attempt to globally characterize plasma membrane proteins of human epidermal keratinocytes, LC-MS/MS has been successfully used to identify 496 proteins, including many of those already previously identified (by genetic methods, immunoprecipitation, and other approaches), thus independently confirming their expression and membrane localization (Blonder et al., 2004) (Figure 4). LC-MS/MS of whole epidermis extracts has led to identification of new proteins potentially relevant to the pathogenesis (and, importantly, novel therapeutic options) of atopic eczema by comparing normal versus laggrin-deficient skin. (Expression changes of some of these proteins were not correlated to changes in mRNA expression profiles, highlighting the importance of complementing genetic analyses using proteomic approaches) (Elias et al., 2017).

The identification of the p170 paraneoplastic antigen as A2ML1 was possible by analyzing an unknown band in an SDS-PAGE gel after immunoprecipitation (Schepens et al., 2010). Similarly, in a neurologic patient who had a suspected autoimmune condition, serum was incubated with cryosections of nerve tissue, followed by extraction and precipitation of immunocomplexes and LC-MS/MS, resulting in identification of the autoantigen (Miske et al., 2016). By using skin cryosections, this histoimmunoprecipitation approach could be translated to a subgroup of dermatological patients that show skin blisters and bound, keratinocyte-specific autoantibodies in direct immunofluorescence studies on skin biopsy samples but that do not show reactivity of serum autoantibodies by routine ELISA or blotting techniques with any of the major keratinocyte autoantigens described and extensively validated so far (e.g., desmogleins 3/1, BP180/230, laminin 332, collagen VII), resulting in diagnostic difficulties and uncertain final diagnoses (Giurdanella et al., 2016; personal observation by CMH).

LC-MS/MS also allows characterization of serum Abs. Ab responses have historically been analyzed mostly by
MULTIPLE CHOICE QUESTIONS

1. In analysis and interpretation of tandem mass spectrometry (MS/MS) data, which of the following statements is not correct?
   A. Theoretical MS/MS spectra are generated from in silico analysis of predicted digestion products of known proteins.
   B. To interpret MS spectra of human antibodies (and their clonalities), a custom database or de novo interpretation is required.
   C. Precursor ion spectra are correlated with theoretical MS/MS spectra generated from protein databases.
   D. Public protein databases serve as input to guide in silico analysis of proteins into predicted digestion products.

2. In the field of immunology and investigative dermatology, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been successfully used to
   A. identify an unknown protein from immunoprecipitation.
   B. characterize plasma membrane proteins within the human epidermis.
   C. characterize the circulating antibody response in an autoimmune disease and/or after immunization.
   D. All of the above

3. Which of the following statements about potential limitations of LC-MS/MS is not correct?
   A. Ambiguity in protein inference can be introduced by the use of proteolytic enzymes and by redundancy phenomena in the databases used for comparison.
   B. Unknown proteins can be easily identified without the use of protein databases.
   C. Different structural labilities of peptide bonds can make interpretation of MS/MS spectra difficult.
   D. De novo interpretation of fragment ion spectra is time consuming and prone to error.

4. What are the main underlying principles that allow for separation of peptides by reverse-phase liquid chromatography (LC) and mass spectrometry (MS), respectively?
   A. Peptide hydrophobicity, only the charge of the peptide
   B. Peptide’s charge and mass in both LC and MS
   C. Only the mass in LC, the mass and the charge in MS
   D. Peptide hydrophobicity, the mass and the charge of the peptide

5. Which of the following statements is correct?
   A. The proteolytic enzyme trypsin cuts proteins after amino acids arginine and lysine.
   B. The heavy-chain complementarity determining region 3 (H-CDR3) is a unique identifier of an antibody and can be detected by LC-MS/MS experimentation.
   C. Collision-induced dissociation describes fragmentation of precursor ions in a collision cell and does not always result in all potential fragment ions (e.g., b- and y-ions of a given peptide).
   D. All of the above

LIMITATIONS

As outlined, LC-MS/MS is a powerful and versatile technique that directly identifies proteins/peptides produced by cells. By contrast, immunohistochemistry approaches need well-characterized Abs against known proteins, and RNA sequencing or microarray techniques identify genetic sequences that may or may not be produced as proteins in cells.

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This field is advancing and improving rapidly, but there remain substantial limitations that should be taken into account, as highlighted in the following (not comprehensive) examples. In most cases, MS protein identification involves enzymatic digestion of protein samples into peptides and subsequent analysis of the resulting peptides by tandem MS. This peptide-centric approach results in the fundamental issue of protein inference, especially for complex proteomes. The presence of degenerate peptides, that is, identical peptide sequences that are found in multiple homologous proteins or protein isoforms, makes it difficult to accurately reassemble peptides to proteins for identification. The protein inference issue is exacerbated by significant protein sequence redundancy in databases that is caused by polymorphisms and DNA sequencing errors that produced partial or nearly identical sequences. Therefore, it can be difficult to determine whether all related protein isoforms are present in a sample or only some are truly present, and it is important to differentiate those because these related isoforms often have distinct structural or functional roles in vivo.

Current quantitative LC-MS/MS proteomic approaches also rely on the assumption that proteins are completely digested into peptides that are all reproducibly detected by MS analysis. In practice, this is not always true, because incomplete digestion or recovery can occur, and unpredictable interferences from sample matrix can result in ion suppression or variable peptide signal intensity. In addition, peptide bonds have different structural labilizations, and fragmentation may thus be skewed toward more labile bonds such as the N-terminal side of proline, resulting in poor MS/MS spectra that are difficult to interpret. Finally, some amino acids have the same mass (leucine, isoleucine) or nearly exactly half of the mass of others (glycine with 57.02146 Da vs. asparagine with 114.04293 Da), and peptides containing combinations of these residues will be impossible to distinguish, resulting in ambiguous sequence assignment. These examples point to the need for skilled scientists trained specifically in proteomics analysis. Successful proteomics analysis requires collaborative efforts between cell biologists, biochemists, and bioinformaticians.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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