Review Article

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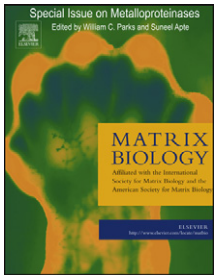
Review



Proteomic approaches to uncover MMP function

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Abstract

Proteomics has revolutionized protease research and particularly contributed to the identification of novel substrates and their sites of cleavage as key determinants of protease function. New technologies and rapid advancements in development of powerful mass spectrometers allowed unprecedented insights into activities of matrix metalloproteinases (MMPs) within their complex extracellular environments. Mass spectrometry-based proteomics extended our knowledge on MMP cleavage specificities and will help to develop more specific inhibitors as new therapeutics. Quantitative proteomics and N-terminal enrichment strategies have revealed numerous novel MMP substrates and shed light on their modes of action *in vitro* and *in vivo*. In this review, we provide an overview of current proteomic technologies in protease research and their application to the functional characterization of MMPs.

Introduction

The term ‘proteomics’ covers all approaches and methods that are applied to study and characterize proteins on a large scale. Ideally, the employed methods allow analysis of the entire set of proteins present in a sample, *i.e.* the proteome. Due to technical advancements and rapid developments in recent years, mass spectrometry (MS)-based proteomics has become the method of choice for identification and quantification of proteins, as well as their post-translational modifications (PTMs) [1,2]. Thereby, protease researchers are particularly interested in one highly specific and irreversible type of PTM: the limited hydrolysis of peptide bonds of substrate proteins by a protease that generates shorter but still defined protein chains. These highly specific processing events often alter biological activities of protease targets, ultimately determining the biological function of the protease, *e.g.* activation or inactivation of cleaved substrates [3,4].

Mass spectrometry-based proteomics has become a very powerful tool in MMP research [5] (Fig. 1). The first developed proteomics approaches enabled identification and quantification of MMPs, their natural inhibitors and the characterization of MMP active site specificities as well as proteolytic activity [6]. Later, the development of more and more sophisticated methods focusing on natural and protease-generated protein termini led to a breakthrough in MMP substrate discovery [7]. Due to the unbiased approach of proteomics, the latter facilitated uncovering of many novel biological roles for many members of the MMP family and expanded our knowledge about these proteases tremendously.

This review provides an overview of recent mass spectrometry-based proteomics approaches and their successful application in MMP research, which helped to uncover many new MMP functions.

Proteomic analysis of MMP active site specificity

Protease active site specificities allow prediction of substrates based on consensus sequences and can

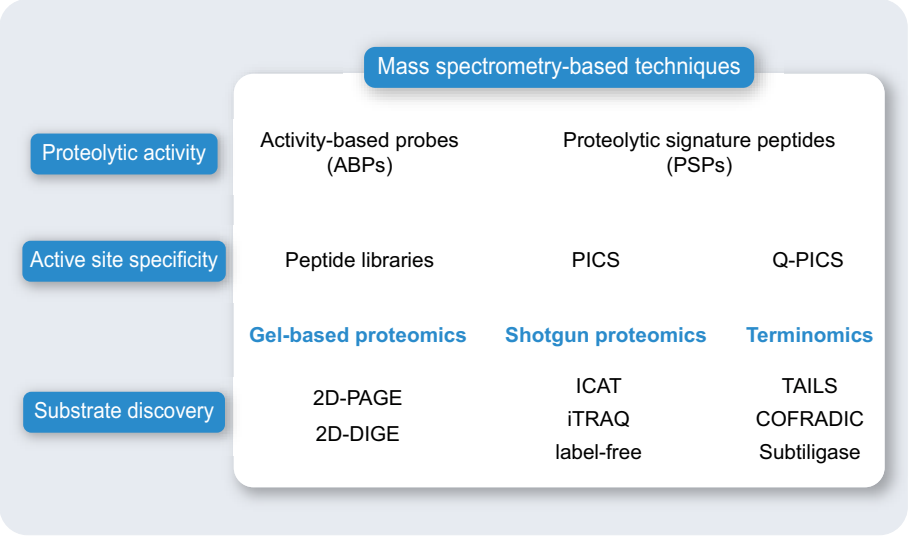

Fig. 1. Overview of MMP proteomics. Activity-based probes (ABPs) and Proteolytic Signature Peptides (PSPs) allow detecting active MMPs in complex samples. Peptide libraries, PICS and Q-PICS are powerful techniques for profiling active site specificities. An array of methods has been developed for MMP substrate discovery. 'Terminomics' approaches, *i.e.* technologies specifically aiming at protein termini, provide information on substrates and cleavage sites and have been successfully applied *in vitro* and *in vivo*. PICS, proteomics identification of cleavage sites; Q-PICS, quantitative PICS; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis; ICAT, isotope-coded affinity tag; iTRAQ, isobaric tags for relative and absolute quantitation; TAILS, Terminal Amine Isotopic Labeling of Substrates [45]; COFRADIC, COmbined FRActional DIagonal Chromatography [46]; Subtiligase, enzymatic labeling of N-terminal peptides for selective enrichment [43].

provide valuable information for the design of specific inhibitors by exploiting small differences in cleavage site preferences. With this intention, several methods have been developed for the analysis of active site specificities using array- and library-based approaches [8–10]. However, many of these techniques are cumbersome and use artificial peptide substrates with random sequences.

Proteomic Identification of protease Cleavage Sites (PICS) addresses this limitation by the use of database-searchable peptide libraries that are derived from the natural proteome of the organism of interest [11,12]. First, a peptide library is generated by tryptic digestion of the test proteome and then cysteine thiol groups as well as primary amines (N-terminal α-amines and lysine ε-amines) are blocked. After the exposure of the peptide library to the test protease, protease-generated unblocked N termini are labeled with biotin, affinity purified and analyzed by mass spectrometry. Finally, in contrast to all previous approaches, mass spectrometry and bioinformatics analyses are used to infer both the prime and the non-prime sides of the cleavage site, making PICS a comprehensive method. Later, PICS was further extended by the use of isobaric mass tags that enable active site specificity analysis of multiple proteases within a single PICS experiment [13]. Active site specificities of some MMPs have already been characterized by PICS [12,14], and very recently the family-wide characterization of MMPs of *Arabidopsis thaliana* was reported [15]. It can be expected that family-wide studies for other organisms will follow in the near future, which will provide invaluable information for all MMP researchers. A disadvantage of PICS is the presence of artificially blocked lysine and cysteine residues that might influence active site specificities of test proteases. PICS is also not suitable for protease substrate discovery, since the relatively short peptides of the library neglect exosite contributions as well as the impact of protein folding. Targeted terminomics techniques address these limitations, and, although originally developed for substrate discovery, can also be applied for the analysis of active site specificities using a native proteome [16,17]. This, however, is only possible if sufficient numbers of cleavages are detected that are inherently higher in PICS approaches. Thus, both types of methods are complementary in the analysis of protease cleavage site specificities [18].

Proteomic analysis of MMP proteolytic activity

In addition to identification and quantification of MMPs, mass spectrometry-based proteomics facilitates analyzing their proteolytic activity in complex proteomes. For this purpose, activity-based probes (ABPs) were developed, comprising a warhead that

covalently binds to the active site of proteases and that is attached to a reporter group, *e.g.* a fluorophore or an affinity handle like biotin. This concept was successfully employed to study many proteases, which form covalently bound substrate intermediates, such as serine and cysteine proteases [19,20].

Following the same principles researchers adapted ABPs for activity profiling of metalloproteases [21,22]. Since metalloproteases use a zinc-activated water molecule for catalysis and do not form covalently bound substrate intermediates, zinc-chelating hydroxamates are coupled to a photocrosslinker that covalently binds only to active but not to inactive metalloproteases. Next, the biotin group of the ABP is utilized for affinity purification of attached enzymes, which are subsequently analyzed by mass spectrometry. This strategy allowed creation of a whole library of metalloprotease-directed ABPs that was applied as a 'cocktail' to cancer cell lines for concomitant monitoring of twenty metalloproteases, including six different MMPs [23].

Very recently, a different concept for the proteomic analysis of MMP proteolytic activity was reported by Christopher Overall and co-workers who introduced proteolytic signature peptides (PSPs) in combination with isobaric tags [24]. PSPs are spiked into the proteome of interest and allow for absolute quantification of active MMPs by monitoring removal of propeptides from corresponding zymogens. Although PSPs are currently available for only a few MMPs, they enable the simultaneous analysis of multiple proteases and their proteolytic activity in complex proteomes with high specificity and sensitivity.

Proteomic discovery of MMP substrates

In order to better understand the roles of MMPs in physiological processes and disease, many efforts have been made to define the substrate repertoire of individual MMPs (Fig. 2). One of the first MMP substrate screens employed the yeast two-hybrid system using the MMP2 hemopexin domain as bait and exploiting exosite interactions outside the catalytic domain as important determinants for substrate recognition [25]. Thereby, McQuibban et al. identified monocyte chemoattractant protein-3 (MCP-3) as a physiological MMP2 substrate that upon cleavage turns into a chemokine antagonist and dampens inflammation. These and other findings dramatically changed the view on MMP function from simple tissue degradation to specific regulation of pivotal biological processes, such as the inflammatory response. These break-through results stimulated subsequent substrate screens that helped to define many novel biological roles of MMPs.

Gel-based approaches

One of the first proteomics screens for MMP substrates was conducted to identify MMP14 targets in human plasma by employing two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with mass spectrometry analysis [26]. Thereby, human plasma proteins were first incubated with the catalytic domain of MMP14, and then 2D-PAGE was utilized to resolve the complex protein mixtures. By comparing protein spot patterns of treated and untreated samples, selected protein spots that were altered in intensity in protease-treated samples were selected, and candidate proteins were further analyzed by mass spectrometry. Combination of 2D-PAGE and MS allowed identification of nine novel MMP14 substrates as well as validation of six already known substrates in a complex biological sample. A similar 2D gel-based proteomic approach was used to explore the substrate repertoire of MMP7 in the medium of colonic myofibroblasts [27], and later gel-based approaches were also applied to search for *in vivo* MMP substrates, *e.g.* for MMP7 and MMP9 in mouse models of myocardial infarction [28,29].

A complication of 2D-PAGE is the reliability of protein identification, because two samples have to be analyzed on two separate gels but under very reproducible conditions. This led to the introduction of two-dimensional difference gel electrophoresis (2D-DIGE), which allows analysis of multiple samples on a single gel [30]. Thereby, samples are labeled with different fluorescent dyes that upon 2D-PAGE facilitate densitometric image analysis and improve sample quantification. This approach was applied for an *in vivo* substrate screen in bronchoalveolar fluids from double knockout mice deficient for MMP2 and MMP9 and wild-type animals [31].

In general, 2D-PAGE is an inexpensive and robust method with broad applicability, but it has several drawbacks, since gel-based separation complicates the analysis of proteins with very small or very large molecular weight, extreme pI values and/or high hydrophobicity (*e.g.* membrane proteins). In addition, insufficient separation can result in migration of multiple proteins within a single spot, and the resolution does not allow for dissolving very small differences in molecular weight, which might be of particular importance for protein function [25]. Moreover, the sensitivity of gel-based approaches is limited by the applied staining procedure that might prevent detection of low-abundance proteins, and densitometry analysis of protein spots only detects major changes in intensity.

Solution-based 'shotgun' approaches

In recent years, 2D-PAGE was mainly replaced by gel-free approaches, also referred to as 'shotgun' proteomics [32]. Thereby, proteome samples are

denatured in solution, disulfides reduced, cysteines alkylated and proteins digested preferentially with trypsin. Next, resulting peptides are separated in multiple dimensions by liquid chromatography (LC) and identified by combining information on peptide precursor masses in MS1 and corresponding peptide fragmentation patterns in MS2 (MS/MS).

Since shotgun proteomics data do not inherently provide quantitative information, several stable isotopic tags were introduced that enable quantitative comparison of multiple samples (*e.g.* protease-treated versus untreated samples). Among the first available tags were isotope-coded affinity tags (ICATs) that covalently bind to cysteine residues and comprise a cleavable biotin moiety for isolation of labeled peptides [33]. ICAT labels contain differential linker regions with either "light" or "heavy" carbon isotopes, resulting in the same chemical properties of both tags but introducing a mass difference of 9 Da between peptides derived from different conditions. In a typical comparative experiment, two protein samples are first digested with trypsin followed by labeling of cysteine residues with either the light or the heavy tag. Then, the samples are combined in a one-to-one ratio, and labeled peptides are positively enriched by affinity purification prior to LC-MS/MS analysis. Due to the mass difference of the two tags, each labeled peptide is represented as a pair of peaks in MS1, whereby the areas of the two peaks are used to determine the relative abundances of the corresponding peptides and assigned proteins in the original sample. Tam et al. applied this quantitative proteomics strategy to explore the substrate repertoire of MMP14 in breast carcinoma cells [34]. This study led to the identification of numerous novel bioactive MMP14 substrates, such as tumor necrosis factor α (TNFα), connective tissue growth factor (CTGF) and death receptor-6, thereby emphasizing the importance of MMPs in signaling processes. Additional ICAT-based substrate screens identified numerous novel MMP2 substrates [35] and further extended the MMP14 substrate degradome by pharmacoproteomics using a potent MMP inhibitor [36].

ICAT limits the number of identified proteins by only including cysteine-containing peptides that are labeled and thus quantifiable. In order to increase the coverage of quantified proteins, a new generation of stable isotopic labels was developed that are named isobaric tags for relative and absolute quantitation (iTRAQ) and attach to primary amines in lysine side chains as well as at each peptide N terminus [37]. Currently, there are eight different iTRAQ labels available, allowing to test eight different experimental conditions in a single proteomics experiment [38]. Thereby, relative peptide quantification is based on intensities of so-called reporter ions that are generated in MS2 upon fragmentation of iTRAQ reporter groups attached to labeled peptides. The iTRAQ labeling strategy was successfully applied in a substrate screen for MMP2 in murine fibroblasts [39], which showed a 9-fold increase in numbers of identified proteins compared to the ICAT labeling strategy using the same type of samples [35].

Label-free quantitative proteomics based on spectral counting is an alternative to the relatively expensive isotopic labeling approaches [40]. Due a relationship between the level of sampling observed for a certain protein and the relative abundance of that protein in the sample mixture, the number of recorded spectra for peptides assigned to each protein can be used for semi-quantitative analysis of abundances. This label-free strategy was successfully applied to reveal novel MMP9 substrates in macrophages [41] and in prostate cancer cells [42].

Terminal enrichment strategies

A shortcoming of comparative analyses of protein abundances in protease-exposed and control samples to infer protease substrates is the lack of information about the exact cleavage site, since the complexity of peptide mixtures often hinders detection of neo-N-terminal peptides generated by the test protease. Therefore, a new generation of techniques for the system-wide discovery of protease substrates was introduced that exploit the generation of neo-N termini and neo-C termini upon cleavage, which can be specifically enriched and facilitate determination of exact cleavage sites. For N-terminal peptides this can be achieved by selective labeling with affinity handles and subsequent binding to corresponding resins. A major challenge in these procedures is to find a chemistry that favors α-amines on protein N termini over ε-amino groups in lysine side chains that are almost identical in reactivity against amine reactive labels [43,44]. In contrast to these techniques for positive selection of N-terminal peptides, negative selection procedures, such as COmbined FRActional DIagonal Chromatography (COFRADIC) and Terminal Amine Isotopic Labeling of Substrates (TAILS) ignore the lysine problem but selectively remove internal peptides upon whole protein amine labeling and tryptic digest [45,46]. Current positive and negative enrichment approaches for N- and C-terminal peptides have been recently comprehensively covered in excellent reviews [47,48].

TAILS has been the most frequently applied method for the system-wide discovery of new MMP substrates. In TAILS, natural protein N termini, lysine residues and protease-generated neo-N termini are labeled using amine-reactive isotopic reagents on the protein level followed by digestion with trypsin. Unblocked trypsin-generated internal peptides are then bound to an amine-scavenging polymer, which leads to negative enrichment of natural and neo-N termini [45,49]. This inherently reduces the complexity of the sample and thus allows detection of low-abundance proteins. Quantitative comparison of N termini in protease-treated and control samples

discriminates between natural N termini and neo-N termini derived from basal proteolysis with equal abundances in both samples and neo-N termini generated by the test protease that are only present in the protease-treated proteome. Bioinformatics analysis determines exact cleavage sites extending to both the prime and the non-prime side, which enables the determination of cleavage site specificities in the presence of all natural cofactors in a native proteome. With CLIPPER a custom data analysis pipeline is available for TAILS that alleviates the interpretation of results by automatic annotation of identified N termini and their statistical assignment to groups of substrates and non-substrate proteins [17,50].

TAILS was originally developed using heavy and light isotopic forms of formaldehyde for reductive dimethylation of primary amines to identify hundreds of novel MMP2 substrates in pairwise comparisons [45]. By use of iTRAQ TAILS was extended to a multiplex analysis platform that allowed comparative analysis of substrate degradomes of the closely related gelatinases MMP2 and MMP9 [16] and revealed novel substrates for the yet poorly characterized neutrophil-specific MMP25 [14]. Multiplexing by iTRAQ-TAILS also allowed simultaneous identification of MMP12 substrates in culture supernatants and cell lysates in a recent study that correlated proteolytic processing with a novel transcriptional role for MMP12 in antiviral defense [51]. Importantly, TAILS is not only applicable to *in vitro* or cell-based systems, but was already successfully employed to reveal a new crosstalk between MMP2, the complement and the kinin–kallikrein systems in control of vascular permeability and complement activation by multiplexed analysis of normal and inflamed skin tissues from MMP2 knockout and wild-type mice [52]. Similarly, iTRAQ-TAILS defined a new role for MMP12 in inflammation by analysis of peritoneal inflammatory exudates in wild-type and MMP12-deficient mice and identified MMPs as highly enriched in porcine wound exudates [53,54]. Recently, the multiplex capabilities of iTRAQ-TAILS were further exploited for the time-resolved analysis of the MMP10 substrate repertoire in murine fibroblasts [55]. Together with a conceptually new data analysis strategy, this novel approach enhanced confidence in substrate identification and enabled categorization of cleavage events by specificity and structural accessibility of the cleavage site.

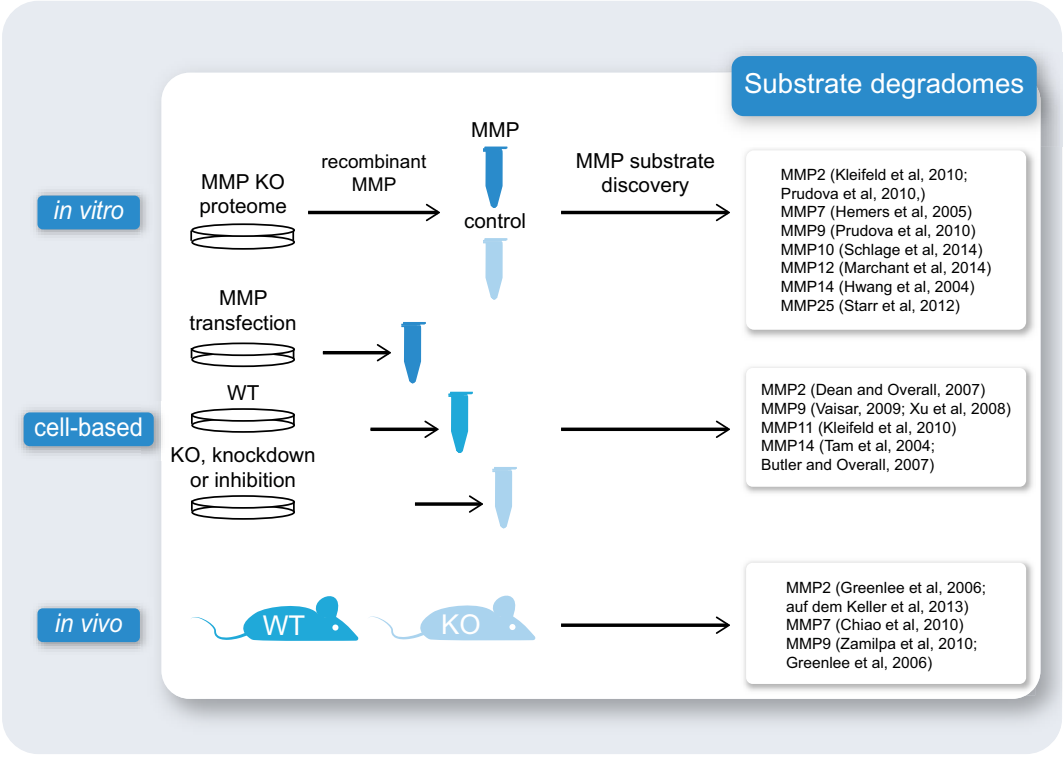

Fig. 2. MMP substrate discovery. Recent advances in mass spectrometry-based proteomics and the concomitant development of sophisticated substrate discovery approaches have strongly promoted the uncovering of MMP substrate degradomes. Analyses of proteomes from MMP-deficient cells incubated with the recombinant protease (*in vitro*), of culture supernatants from cells with normal and altered MMP activity (cell-based), or of tissue samples from MMP knockout and wild-type mice (*in vivo*) revealed substrates and novel biological functions for many members of the MMP family. KO, knockout; WT, wild-type.

Conclusions

Mass spectrometry-based proteomics has become an invaluable tool for MMP research. Theoretically, proteomics has the ability to analyze entire protease systems, including their endogenous inhibitors and their substrates in complex biological samples. More and more sophisticated and powerful mass spectrometry-based approaches enable the system-wide analysis of MMP expression, proteolytic activity, cleavage site specificity and substrate processing. Proteomics supports unbiased exploration of novel biological areas, leading to discovery of numerous new biological functions of MMPs in health and disease.

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