Biochimica et Biophysica Acta 1864 (2016) 130–142

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

Detection of protease activity in cells and animals☆

Martijn Verdoes [a], Steven H.L. Verhelst [b,c,1]

[a] Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Geert Grooteplein 26-28, 6525 GA Nijmegen, The Netherlands
[b] Leibniz Institute for Analytical Sciences, ISAS, e.V., Otto-Hahn-Str. 6b, 44227 Dortmund, Germany
[c] Laboratory of Chemical Biology, Department of Cellular and Molecular Medicine, University of Leuven, Herestraat 49, Box 802, B-3000, Belgium

ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form 21 April 2015
Accepted 28 April 2015
Available online 7 May 2015

Keywords:
Activity-based probes
Degradomics
Imaging
Proteases
Substrate reporters

ABSTRACT

Proteases are involved in a wide variety of biologically and medically important events. They are entangled in a complex network of processes that regulate their activity, which makes their study intriguing, but challenging. For comprehensive understanding of protease biology and effective drug discovery, it is therefore essential to study proteases in models that are close to their complex native environments such as live cells or whole organisms. Protease activity can be detected by reporter substrates and activity-based probes, but not all of these reagents are suitable for intracellular or *in vivo* use. This review focuses on the detection of proteases in cells and *in vivo*. We summarize the use of probes and substrates as molecular tools, discuss strategies to deliver these tools inside cells, and describe sophisticated read-out techniques such as mass spectrometry and various imaging applications. This article is part of a Special Issue entitled: Physiological Enzymology and Protein Functions.

1. Introduction

Peptidases, more frequently referred to as proteases, are a group of enzymes that cleave peptide bonds within proteins and peptides. They have a wide range of biological functions. These do not only include protein destruction, which occurs during food digestion and protein turnover, but also regulatory processes such as blood coagulation, inflammation and apoptosis. In order to prevent cleavage of substrates at unwanted times and locations, most proteases are synthesized as inactive zymogens and upon controlled activation, various post-translational mechanisms keep protease activity in check, for instance by inhibition, degradation or covalent modifications (Fig. 1A). Obviously, proteases and their substrates also need to be localized within the same cellular or extracellular environment in order to encounter each other. These multiple layers of regulation make protease biology very complex.

The human genome codes for more than 500 different proteases. Most proteases have distinct binding pockets around the active site for specific substrate recognition. Depending on the nature of these pockets, this may result in broad or narrow substrate specificity (Fig. 1B). Related proteases may have very similar substrate specificities and cleave overlapping sets of substrates. This is, for example, the case in the caspase family of cysteine proteases. Such similarities make the development of selective inhibitors difficult. In addition, when one particular protease is knocked out or inhibited, proteases from the same family may be up-regulated as a compensation mechanism. Therefore, inhibition of a single protease may not lead to an effect. To add another layer of complexity, the function of proteases can be different in different tissues or diseases.

Changes in active protease levels underlie many disorders including cancer and cardiovascular diseases. In addition, proteases play an essential role in the infection by viruses, bacteria, single- and multi-cellular parasites. For these reasons, proteases are considered viable targets for drug development and several protease targeting drugs are now used in the treatment of multiple myeloma, hypertension and HIV infection [1,2].

Traditionally, proteases are studied *in vitro* in recombinant and purified form. However, for all the reasons outlined above, it is now recognized that both the functional study of proteases as well as drug development efforts can benefit the most from studying proteases in models of the complex proteomes in which they function, such as cell or tissue lysates, live cells in culture or whole animals [3]. For experiments *in vivo* or in live cells, suitable methods need to be available in order to detect or monitor protease activity, which is the topic of this review. In the next paragraph, we will first describe the different molecular tools that are available to study protease activity. We will then

☆ This article is part of a Special Issue entitled: Physiological Enzymology and Protein Functions.

E-mail addresses: steven.verhelst@isas.de, steven.verhelst@kuleuven.be (S.H.L. Verhelst).
[1] Tel.: +49 231 1392 4236.

http://dx.doi.org/10.1016/j.bbapap.2015.04.029
1570-9639/

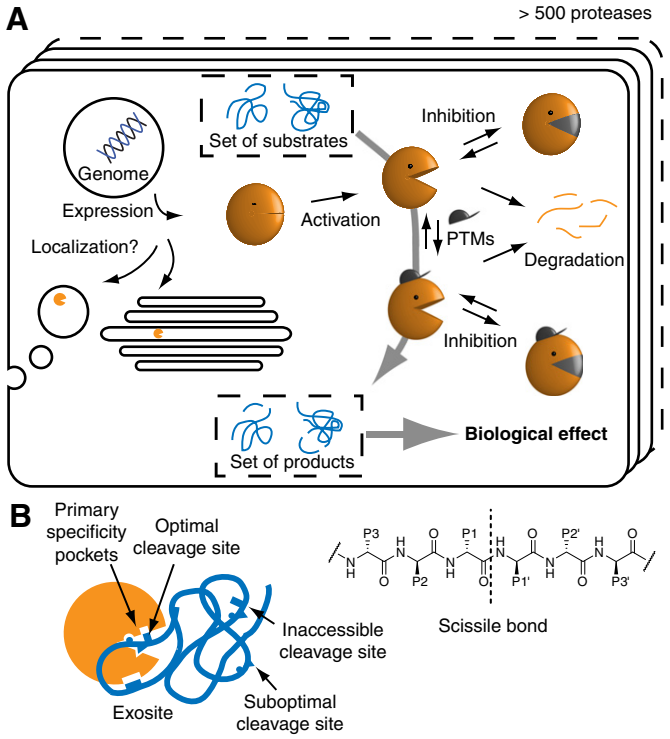

Fig. 1. The complex biochemistry of proteases. (A) Proteases are regulated at multiple levels. First of all, not all proteases are expressed in every cell. If expressed, proteases have a particular subcellular localization, such as the lysosome, the Golgi network or the plasma membrane. In this way, they only encounter a small set of potential substrates instead of the entire proteome of the cell. Most importantly, virtually all proteases are expressed as inactive zymogens. Only after the proteolytic removal of an inhibitory propeptide, the protease becomes active. In turn, the active protease is tightly regulated by a variety of processes: inhibition by endogenous proteins or post-translational modifications (PTMs) such as phosphorylation. Proteases will finally be degraded by the cellular protein turnover machinery (*e.g.* proteasomal degradation). Naturally, only active proteases will be able to cleave substrates. Cleavage of a substrate may lead to either activation or inactivation of the substrate protein, which subsequently leads to a downstream biological effect. Besides co-localization of protease and substrate, the cleavage heavily depends on the protease substrate specificity. (B) The substrate specificity is primarily determined by specificity pockets near the active site. Amino acid side chains around the scissile bond (…, P2, P1, P1′, P2′, …) can dock into the pockets designated S2, S1, S1′, S2′ etc. In some cases, binding sites distant from the active site cleft, so called exosites, improve binding of substrate proteins. Inaccessible cleavage sites will not be processed, while suboptimal cleavage sites will undergo processing with much slower kinetics.

summarize strategies to deliver those tools intracellularly, and we will finally describe a number of detection methods that are convenient for the study of intracellular or *in vivo* protease activity.

2. Tools for protease study

The tools that are applied in protease research can be divided into two categories: substrate reporters and activity-based probes (ABPs). The basic principles of these reagents will be discussed in the next two sections and are summarized in Fig. 2. In Section 2.1, we will successively summarize the use of unlabeled protein substrates, fluorescent proteins, fluorogenic peptides and copolymer substrates. In Section 2.2, ABPs, quenched fluorescent ABPs and photocrosslinking probes will be presented.

2.1. Substrate reporters

2.1.1. Unlabeled protein substrates

The use of substrates as reporters of protease activity is as old as the study of proteolytic enzymes and dates back to as early as 1836, when Theodor Schwann reported that a chemical entity other than hydrochloric acid is responsible for the digestive properties of gastric juice [4]. He named this entity 'pepsin', and his report marks the discovery of the first protease. During his research, Schwann determined the digestion of coagulated egg white by pepsin through simple visual inspection. Later in the 19th century, it was discovered that proteases do not only occur in the digestive tract, but also in other tissues, such as liver [5] and spleen [6]. The research tools were still limited to natural protein material, for instance fibrin or gelatin, and the digestion itself was measured by electrical conductivity [7] or weighing the amount of residual protein after precipitation with acid [8]. Because of the low costs, purified natural proteins are still used in protease assays nowadays (Fig. 2A). In protease zymography, for example, a protein substrate (generally casein or gelatin) is copolymerized with acrylamide before gel electrophoresis. Proteases will locally digest the protein and after gel staining their presence will be revealed as unstained bands [9,10].

2.1.2. Labeled protein substrates

Modification of purified proteins by fluorescent dyes enables spectrophotometric measurement of protease action. Incorporation of multiple fluorophores on a protein, such as in commercially available BODIPY-labeled casein, leads to fluorescent quenching. These quenched substrates display a gradual increase in fluorescence upon digestion, allowing the detection of protease activity in real time [11]. Interestingly, these substrates are not restricted to soluble proteases, but also applicable to intramembrane proteases [12]. However, they have limited application in the detection of intracellular proteases, as they may only enter the cell by endocytosis. In contrast, natural or recombinant proteins that are expressed within the cell can be used for the detection of proteases in the cytosol and other organelles (discussed in detail in Section 3.1).

2.1.3. Synthetic peptide substrates

The first synthetic substrates for proteases were published in 1934 by Max Bergmann [13]. He synthesized dipeptides, using the Cbz protecting group developed some years before [14], and showed that these were cleaved by a peptidase. Nowadays, a wide variety of chromogenic, fluorogenic and luminogenic substrates are commercially available or accessible by organic synthesis, allowing sensitive and high-throughput measurement of protease activity (Fig. 2B). The design of substrate reporters is based on the substrate specificity of the target protease, which itself can be uncovered by using libraries of synthetic substrates [15] or proteomics methods [16,17]. The design of a substrate that is only turned over by one particular protease is still a difficult task, since proteases from the same family often have overlapping substrate specificity. To tackle specificity issues, the use of non-natural amino acids can be helpful. The Drag laboratory has used this strategy in the design of highly selective aminomethylcoumarin (AMC) substrates for human neutrophil elastase [18] and different caspases [19]. The Wolan group has similarly applied unnatural amino acids for the development of a highly selective substrate for caspase-3 [20].

2.1.4. Synthetic polymer substrates

Another type of synthetic substrates comprises copolymers that display multiple substrate peptides attached to fluorophores (Fig. 2B) [21]. Similar to proteins labeled with multiple fluorophores, they display internal quenching, which is alleviated upon proteolysis. Copolymer substrates designed for a variety of proteases, including general tumor-associated proteases [22], cathepsin D [23], and matrix metallo proteases [24] have been applied in studies in live cells and *in vivo*. Using a similar autoquencing strategy, dendrimeric cathepsin S substrates have been synthesized [25] and applied to image cathepsin S activity in atherosclerotic mice [26]. Fluorescent peptide substrates have also been attached to nanoparticles, *e.g.* gold nanoparticles as fluorescent quenchers or quantum dots as FRET partners [27].

Generally, fluorogenic substrates are very sensitive tools to study the activity of proteases due to the amplification of the fluorescent signal.