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From top-down to bottom-up: Time-dependent monitoring of proteolytic protein degradation by LC-MS

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ABSTRACT

The understanding of proteolytic processes includes manifold aspects, ranging from the characterization of proteases and their corresponding substrates to the localization of cleavage sites. The analysis of protease-catalyzed reactions at a single time-point in many cases excludes the identification of intermediate cleavage products of potential biological function. To overcome this problem, proteolysis has to be monitored over time.

For that purpose, we established a straight-forward two-step approach. First, Tricine-SDS-PAGE separation of the proteolytic products is applied to survey the proteolytic reaction. In the second step, the reaction mixture is analyzed by an LC-MS set-up. An optimized chromatographic separation coupled to electrospray Orbitrap mass spectrometry allowed the simultaneous monitoring of intact substrates, intermediates and cleavage products of lower molecular weight. The applicability of the strategy was shown on the example of the gastric protease pepsin and its physiologically relevant substrate hen egg white lysozyme, one of the major egg allergens. While lysozyme-derived cysteine-free peptides were cleaved comparatively fast, disulfide bonds protected connected peptides from rapid peptic proteolysis. Two previously identified potential IgE-binding motifs were observed as disulfide-linked cleavage products.

In summary, the presented approach is not only ideally suited for the simulation of gastro-intestinal digestion, which is of high interest in food research, but can be transferred to any protease-substrate pair of interest. Furthermore the strategy can be exploited to deduce the effect of post-translational modifications on proteolysis.

1. Introduction

The protease-catalyzed hydrolysis of peptide bonds represents a key event in a variety of biological processes, such as blood-clotting, tissue development, immune responses and gastrointestinal digestion. Beside the knowledge concerning the nature and identity of a protease and its corresponding substrates, the identification of cleavage sites is one of the major goals in degradomics [1] as these determine the molecular properties of the cleavage products and hence the biological function thereof. If there is more than one cleavage site within a protein, new questions regarding intermediate proteolytic products with kinetically preferred cleavage sites arise as these can exhibit other biological activities. To unravel this issue it is necessary to monitor proteolytic reactions over time.

Several studies have performed time-course analyses of proteolytic reactions in the context of highly complex samples employing N-terminomics approaches. Three time-points of human granzyme B catalyzed proteolysis on Jurkat cell lysates were investigated employing SILAC (stable isotope labeling by amino acids in cell culture) and the N-terminal COFRADIC (combined fractional diagonal chromatography) protocol [2]. Caspase-dependent proteolysis on Jurkat cell lysates was studied by SRM (selected reaction monitoring) of approximately 1000 peptides [3]. An 8plex-iTRAQ-TAILS (terminal amine isotopic labeling of substrates) approach was applied to explore degradation kinetics of MMP-10 (matrix metalloproteinase-10) on fibroblast secretomes [4]. However, besides long and error-prone sample preparation steps, which include chemical derivatization or subsequent enrichment strategies to reduce sample complexity, N-terminomics approaches do not allow for the identification of full length cleavage products as only N-terminal peptides are monitored after digestion of cleavage

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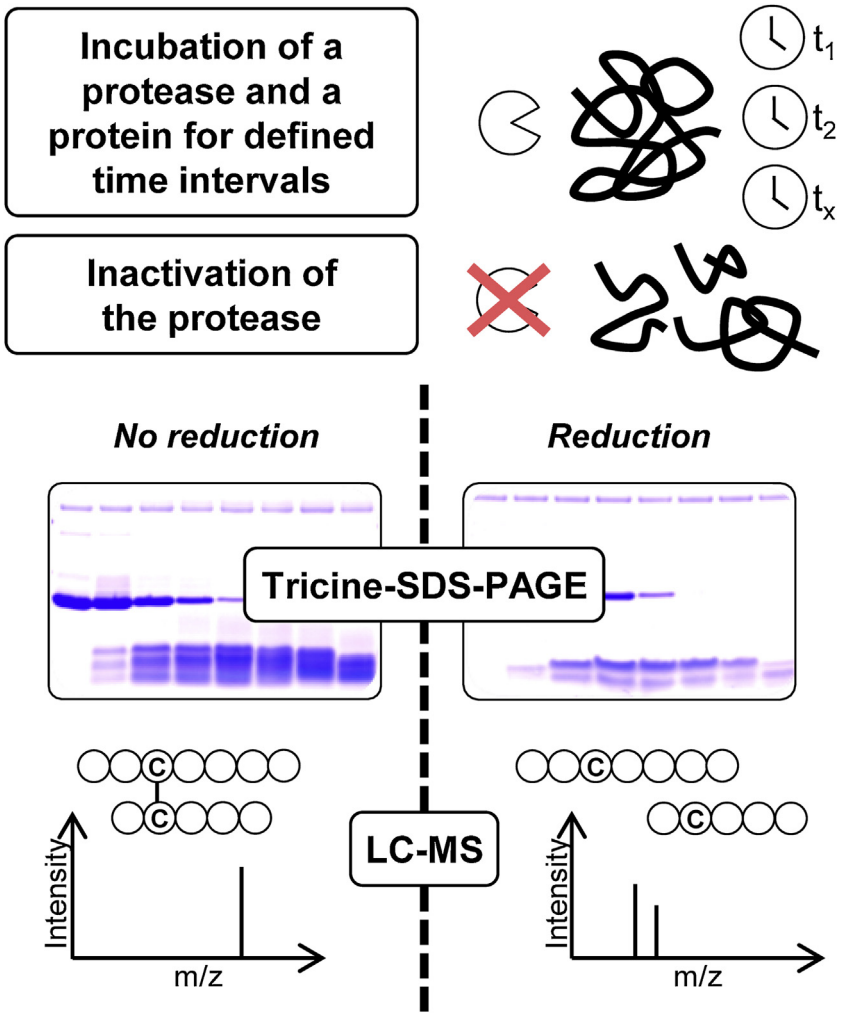

Fig. 1. Analytical strategy. The protease and substrate of interest are incubated at particular reaction conditions. After sampling at specific time-points, the protease is irreversibly inactivated and samples are subjected to Tricine-SDS-PAGE (non-reducing and reducing conditions) for a first survey. Relevant samples are further analyzed by LC-MS (disulfide linkages maintained and reduced).

products by a secondary (work) protease. An alternative method suitable for time-course analyses, PROTOMAP (protein topography and migration analysis platform) [5], is based on SDS-PAGE separation of protease treated samples of different incubation times, in-gel digestion and subsequent LC-MS analysis. The sequence coverage of substrates and cleavage products is displayed in peptographs including semi-quantitative data obtained by spectral counting. Although this strategy is able to identify full-length cleavage products, the assignment of N- and C-termini remains challenging as target protease specificities might be unknown and bottom-up approaches usually result in a loss of information. Top-down proteomics, based on LC separation and mass spectrometric analysis at the level of intact proteins, is an upcoming approach suitable to circumvent some of the aforementioned pitfalls. In particular, proteins up to around 25 kDa can be routinely analyzed with sufficient sensitivity [6].

In the present work, we developed a strategy for the simultaneous LC-MS analysis of the intact protein substrate and the cleavage products formed at different time-points: a combined top-down and bottom-up LC-MS approach without any derivatization steps. In order to determine the most relevant time-points, the LC-MS analysis is preceded by gel electrophoresis. The strategy offers the direct identification of cleavage sites and enables to monitor the influence of post-translational modifications, such as disulfide linkages, on proteolysis.

The applicability of the method was shown on the example of pepsin and hen egg white lysozyme (LYZ); a protease-substrate pair of biological significance. Pepsin, a protease located in the human stomach, catalyzes the first step of food protein degradation under acidic conditions. The proteolytic products subsequently enter the small intestine and are further decomposed by pancreatic proteases, such as trypsin and chymotrypsin. Hen egg white lysozyme is a 14.3 kDa hydrolytic enzyme of 129 amino acids stabilized by four disulfide bonds. It is not only found in hen egg white, but variants of this enzyme are also present in different biological fluids, such as human milk, saliva and tears and in numerous other organisms [7]. The enzyme decomposes the peptidoglycan layer of bacterial cell walls, in particular of Gram-positive bacteria, and consequently possesses antimicrobial activity. This feature is not only exploited in nature but also in food technology, e.g. as preservative on cheese rind. Additionally, LYZ was shown to contain antimicrobial peptide motifs [8–10]. On the other hand the protein is one of the major egg allergens, next to ovalbumin or ovomucoid, and particularly infants suffer from allergic reactions thereof [11,12]. However, in most cases the allergy disappears with growing age. One reason is the drop in pH of the stomach from approximately 4 to 2 [9], which increases the susceptibility of the allergen to peptic