

Mini Review

Trypsin as a Biochemical Tool for the Characterization of Ras-Related Protein Structure and Function

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Abstract

Bovine trypsin is a well-characterized serine protease used as a vital tool in biochemistry, particularly proteomics, as it cleaves peptides or proteins, with high specificity, at the carboxy-terminus of lysine (Lys) and arginine (Arg) amino acid residues. Moreover, the fragment peptides are typically in mass ranges that are amenable to detection by mass spectrometry techniques other than Matrix Assisted Laser Desorption Ionization (MALDI). In addition, Trypsin is found in the digestive system of many vertebrates, and is stable under diverse chemical conditions. The RAS (Rat Sarcoma) families of proteins are involved in several cell-signaling activities that are vital to processes such as cell proliferation and differentiation. If not properly regulated, these signaling processes can result in diseases such as cancer, underlying their importance as targets for drug discovery efforts to control Ras-stimulated abnormal cell signaling. The activity of Ras-related proteins is dictated by their nucleotide-bound state where the GTP-bound is active, and the GDP-bound state inactive. The regulation of this nucleotide-cycling activity is dictated by protein-protein interactions with effectors that may induce conformational changes. Here, we highlight recent advancements showing the use of trypsin in the characterization of Ras-related protein structure and function in cell signaling and regulation.

INTRODUCTION

Bovine trypsin is a classical and well-characterized serine protease [1,2]. Trypsin furthermore is a widely used and vital tool in biochemistry, particularly proteomics, as it cleaves peptides or proteins, with high specificity, at the carboxy-terminus of lysine (Lys) and arginine (Arg) amino acid residues. This high specificity makes trypsin a valuable tool in proteomics studies, as many high-scale mass spectrometry-based proteomics studies are done through the use of trypsin to cleave protein mixtures into specific peptide fragments [3]. In addition, the fragment peptides typically occupy mass ranges that are amenable to detection by mass spectrometry techniques other than Matrix Assisted Laser Desorption Ionization (MALDI) [3]. Nevertheless, it should be noted that trypsin's rate of cleavage is slowed when Lys or Arg is adjacent to acidic residues or disulfide bridges, and cleavage does not occur when Lys or Arg is followed by a proline (Pro) residue [4]. Trypsin is naturally found in the digestive system of many vertebrates, and is stable under diverse chemical conditions [5]. The high specificity of trypsin is also responsible for its efficient use in biochemical and structural biological approaches.

As an example, experiments using trypsin can be helpful for understanding Ras-related (Ras) proteins. These proteins are involved in various cell-signaling activities, and, if not properly regulated, aberrant cell-signaling can lead to cancers or other developmental pathologies [6]. This underlies their importance as targets for drug discovery efforts to control Ras-stimulated abnormal cell signaling [7]. Moreover, the frequency of Ras gene mutations in selected human cancers has continued to highlight

why these proteins are important in cell transformation. The activity of Ras-related proteins is dictated by their nucleotide-bound state where the GTP-bound is active, and the GDP-bound state inactive. The regulation of this nucleotide-cycling activity is dictated by protein-protein interactions with effectors [8], which often induce conformational changes in both the Ras-related protein and effector proteins. Indeed, mutations leading to altered protein-protein interactions and/or abnormal function that result from conformational changes can be probed by proteolytic digestion using proteases such as trypsin. Here, we highlight recent studies showcasing the use of trypsin in the characterization of Ras-related protein structure and function in cell signaling and regulation.

Proteolytic digestion assays have yielded low-resolution structural information on Ras proteins for many years. A 1989 study by Grant and colleagues showed how structural features of N-Ras could be affected by the binding of the guanine nucleotide and metal ions to the protein. Mg²⁺, Ca²⁺, K⁺, GDP were added to apo-Ras construct(s). The proteins were then subjected to trypsin and chymotrypsin digestion to determine how the presence of Mg²⁺, Ca²⁺, K⁺ or GDP influenced protein resistance to hydrolysis [9]. These results paved the way for more sophisticated structural biological, as well as proteomics studies on Ras proteins. Recently, proteomics studies of Ras isoforms expressed in colorectal and pancreatic cell lines by Mageean et al. [10], showed the biological contributions of the isoforms expressed in the cells. In these types of studies, trypsin digestion resulted in Ras peptides that were analyzed by the in-

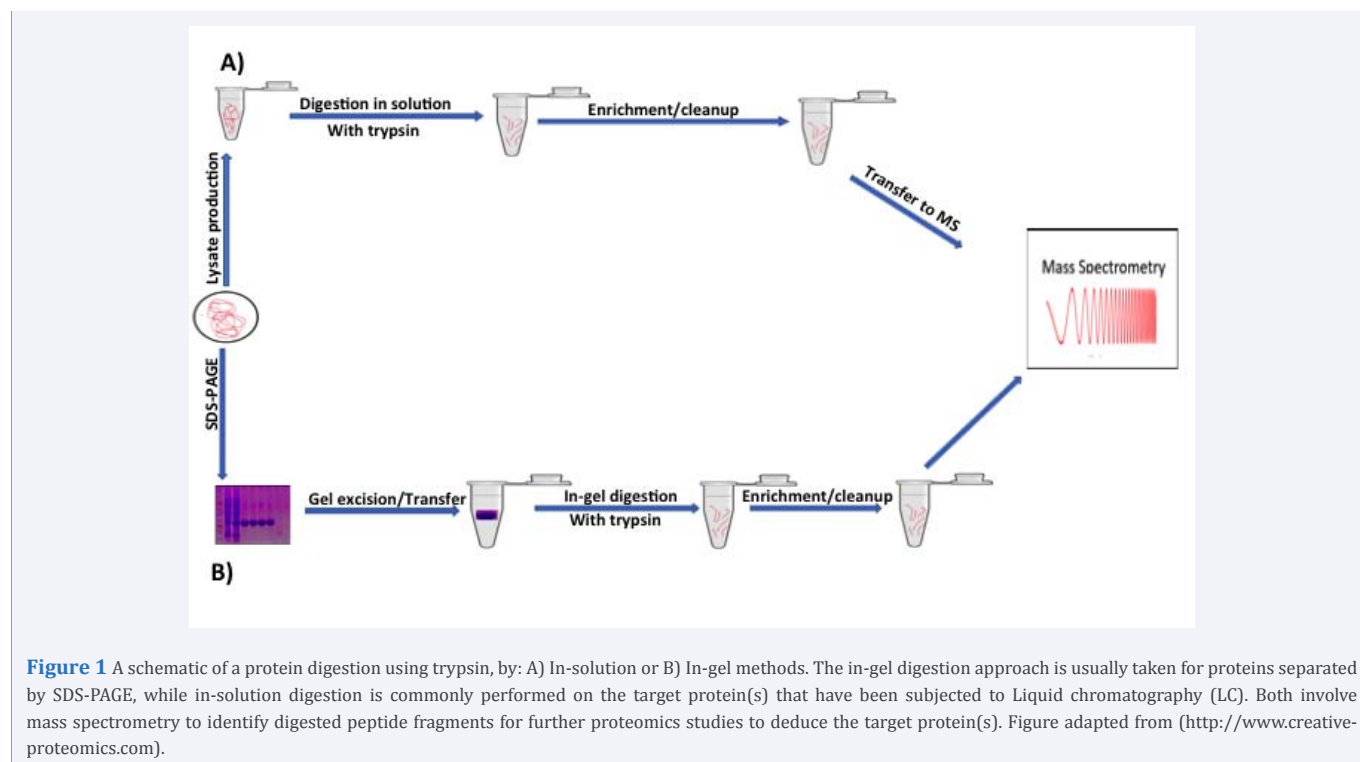


Figure 1 A schematic of a protein digestion using trypsin, by: A) In-solution or B) In-gel methods. The in-gel digestion approach is usually taken for proteins separated by SDS-PAGE, while in-solution digestion is commonly performed on the target protein(s) that have been subjected to Liquid chromatography (LC). Both involve mass spectrometry to identify digested peptide fragments for further proteomics studies to deduce the target protein(s). Figure adapted from (<http://www.creative-proteomics.com>).

gel digestion approach (Figure 1). This approach allowed for an accurate measurement of endogenous protein copies of the RAS isoforms (K-Ras, H-Ras, and N-Ras). In a proteomics study by Gnad et al., (cellular phosphorylation, caused by constitutive KRas/Cdc42 signaling or PAK4 ablation, was characterized by digesting protein lysates from NIH3T3 cell lines with trypsin. Analysis by mass spectrometry revealed phosphorylation sites that were in agreement with expected sites [11]. Junemann J et al., described an MS-based approach to quantify the degree of glycosylation in Ras/Rho-related small GTPases generated by trypsin and chymotrypsin digestions. A multiplexed MRM (Multiple Reaction Monitoring-based) assay was used to characterize the degree of glycosylation of the small GTPases and allowed for the detection of glycosylated toxic proteins [12]. In a 2012 study by Ferreira et al., 2-Dimensional Electrophoresis (2DE), semi-quantitative image analysis, in-gel trypsin digestion and mass spectrometry were employed to characterize proteome changes amongst Ras isoforms (H, K, N). The results of this proteomic characterization study demonstrated that loss of function for 2 of the isoforms (H- and N-) could provide credible information on differences in protein expression and suggest possible therapeutic targets toward these proteins [13]. Studies examining the effect(s) of single-point mutations in important binding regions of Ras-related proteins, using trypsin digestion, have also been useful in highlighting the role(s) of key residues. One such study by Shin et al., highlighted how GTPase and translation functions of the eukaryotic translation initiation factor 5B (eIF5B) could be affected by a Gly to Ala mutation in one of the flexible “Switch” regions of the protein, the Switch2 region. WT eIF5B and eIF5B-G479A were subjected to limited trypsin proteolysis. Results showed differences in the number of protein fragments produced by the trypsin cleavage of the G479A variant as compared to WT,

suggesting that the G479A mutation fostered a conformational change in the Switch 2 region [14].

Ras-related proteins have been shown to often undergo conformational changes that lead to aberrant cell signaling and hyperactivity. An approach to combat this has included the characterization of small molecule targets with the potential to alter Ras-stimulated abnormal cell-signaling [15]. To this end, trypsin digestions have revealed important information on the influence small molecule targets can have on Ras-related protein conformation upon function. Recently, Patricelli et al., showed that a small molecule, ARS-853, could serve as a covalent inhibitor of a K-Ras variant, in a cellular environment, by binding to the GDP-bound inactive form of the protein and preventing its activation via GDP dissociation. Trypsin digestions of a control wild type K-Ras peptide were compared to that of the variant K-Ras^{G12C} in the presence of ARS-853. Mass spectroscopy revealed differences in the fragments from the digestions of the G12C variant in comparison to wild type, suggesting that the small molecule engaged the K-Ras wild type and variant peptides differently [16]. Moreover, the combination of stable isotope labeling with limited trypsin proteolysis has been used to monitor protein conformational changes upon the binding of small molecules [17]. In these studies, the limited proteolysis was analyzed by mass spectrometry showing how this novel combination approach could be used to observe specific structural changes induced by small molecule binding.

In summary, the use of trypsin to cleave proteins into peptide fragments for characterization has been a robust approach applied to basic proteomics studies, and its specificity has made it important for these experiments as well [3]. In addition, although there are more advanced techniques to probe protein conformational changes, such as spectroscopy and X-ray

crystallography, trypsin digestion can be used as a quick and relatively inexpensive method to generate preliminary, low-resolution structural data on Ras-related proteins. As Ras-related protein structure and function continues to be an important aspect towards a better understanding of these proteins' role(s) in stimulating various diseased states, approaches using proteolytic digestions with trypsin can be expected to continue to help uncover information on conformational changes that may be exploited for subsequent structure-based drug design.

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Citation

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