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Rheostatic control of tryptic digestion in a microscale fluidic system

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ABSTRACT

Integrated fluidic systems that unite bottom-up and top-down proteomic approaches have the potential to deliver complete protein characterization. To circumvent fraction collection, as is conducted in current blended approaches, a technique to regulate digestion efficiency in a flow-through system is required. The present study examined the concept of regulating tryptic digestion in an immobilized enzyme reactor (IMER), incorporating mixed solvent systems for digestion acceleration. Using ovalbumin, cytochrome c, and myoglobin as protein standards, we demonstrate that tryptic digestion can be efficiently regulated between complete digestion and no digestion extremes by oscillating between 45 and 0% acetonitrile in the fluid stream. Solvent composition was tuned using programmable solvent waveforms in a closed system consisting of the IMER, a sample delivery stream, a dual gradient pumping system and a mass spectrometer. Operation in this rheostatic digestion mode provides access to novel peptide mass maps (due to substrate unfolding hysteresis) as well as the intact protein, in a reproducible and stable fashion. Although cycle times were on the order of 90 s for testing purposes, we show that regulated digestion is sufficiently rapid to be limited by solvent switching efficiency and kinetics of substrate unfolding/folding. Thus, regulated digestion should be useful in blending bottom-up and top-down proteomics in a single closed fluidic system.

1. Introduction

The field of proteomics has evolved tremendously over the past decade, not only in the scope of analyses but also in the analytical techniques employed. The goal of proteomics research is to completely characterize the proteome in cells, tissues, or organisms at the level of component interactions, co-/post-translational modifications, protein conformation and to determine the influence of these on function within a biological organism [1,2]. To supply the necessary data on a proteomics scale, an array of mass spectrometry (MS)-based methodologies have been developed [3–5].

Included in these methods are standard bottom-up proteomic strategies, which couple up-front tryptic digestion of proteins with peptide separation and mass detection. Variations on this theme have led to improvements in such figures of merit as peak capacity and spectral resolution, which has in turn successfully supported identification and quantitation of proteins in very complex mixtures [6–8]. Although proteolytic digestion is usually performed in-solution and off-line, a closed system approach to protein mixture analysis, wherein all elements of the experimental workflow are conducted on-line with minimal manual sample handling, has considerable analytical appeal. Such a system could process individual samples with less loss and greater speed, enabling enhanced detectability and sequence coverage.

Despite the merits of bottom-up approaches, two notable shortcomings exist. First, they fail to provide properties of the intact protein (*e.g.* the molecular weight, distribution of modifications), which can impede the identification of an unknown protein in a biological sample. Second, they seldom provide deep sequence coverage for anything other than abundant proteins. The reasons behind this are many, but include selective peptide retention in the LC column and variable ionization efficiency, the impact of which can be altered by incomplete protein digestion. Thus, incomplete sequence coverage usually prohibits the complete cataloging of mutations or co-/post-translational modifications, and prevents the full characterization of a protein state.

Given these limitations, there is increasing interest in top-down proteome characterization strategies [9,10]. These strategies analyze intact protein without prior chemical or enzymatic digestion and employ direct sequencing of mass selected proteins in the gas phase of the mass spectrometer [11]. Such strategies facilitate accurate protein mass and modification determinations, near-100% protein sequence coverage and the identification of

Abbreviations: Cyt c, cytochrome c; ESI, electrospray ionization; GRAVY, grand average of hydropathy; IMER, immobilized enzyme reactor; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; myg, myoglobin; ova, ovalbumin; p*I*, isoelectric point; TICs, total ion chromatograms; QqTOF, quadrupole time-of-flight; XICs, extracted ion chromatograms.

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protein isoforms including their relative abundances [12–14]. Notwithstanding, top-down proteomics methods suffer from limited sensitivity and throughput [12,15] and are challenged by high molecular mass (>100 kDa) and highly hydrophobic (*e.g.* integral membrane) proteins.

To achieve complementarity in the use of both analytical approaches, blended strategies that integrate intact protein molecular mass measurement with proteolytic fragment identification have been proposed [16–21]. For example, Wu et al. demonstrated the power of an integrative approach in the analysis of the Saccharomyces cerevisiae proteasome complex [16]. In their study, yeast cell lysate was fractionated followed by reversed-phase high-performance liquid chromatography (RP-HPLC) of the protein mixture for (i) peptide analysis by solution-based proteolytic digestion and subsequent RP-HPLC-MS/MS and (ii) protein analysis by direct infusion into a Fourier transform ion cyclotron resonance mass spectrometer. Overall, by combining bottom-up and top-down methodologies, a more complete protein characterization could be achieved.

The optimization of combined bottom-up and top-down proteomic strategies have largely focused on improving protein/peptide separation. Digestions in these workflows have been performed using traditional overnight solution-based protocols (i.e. ~12 h, 37 °C) and have not implemented recent innovations developed independently to enhance proteolytic digestion efficiency. These methods include microwave irradiation [22], ultrasound irradiation [23], elevated pressure [24,25], and mixed aqueous-organic solvents [26–29]. The elevated pressure approach shows promise for the integration of top-down and bottom-up strategies. For instance, after demonstrating that high pressures can enhance tryptic activity and accelerate proteolysis [24], Smith et al. developed an on-line pressurized digestor for rapid proteolysis in bottom-up proteomics experiments [25]. Another promising method involves mixed solvent digestion. Our lab has achieved high digestion efficiency of even proteolytically resistant proteins by digesting them under mixed solvent conditions in an immobilized enzyme reactor (IMER) [28]. These findings were extended to the development of an on-line protein nanoLC-digestor-MS system that demonstrated limits of identification similar to that achievable with gel-to-MS-based workflows [26].

While gains continue to be made toward maximizing digestion efficiency, one undeveloped aspect required for an efficient integrated top-down/bottom-up system involves "tunable" digestion. We define this as the ability to control the extent of digestion through rapid control over the digestion enhancer in software, analogous to a rheostat for tuning electrical resistance. This capability would support simultaneous bottom-up and top-down protein characterizations within a single experiment and remove the need for fraction collection, as used in the integrative approaches [16–21]. In a fully closed system, tryptic digestion should therefore be regulated, such that the fragment length of the digest products be tunable from full digestion (zero missed cleavages) to no digestion (intact protein).

With regulation as the ultimate goal, this study explores the switching properties of a previously described IMER [28] in greater detail, to determine the degree to which a peptide map can be rapidly tuned. Tuning was accomplished by varying the solvent composition emanating from a dual gradient system through programmable solvent waveforms. The limiting factors involved in switching from one level of digestion performance to another were determined using ovalbumin, cytochrome c, and myoglobin as protein standards. These proteins are useful test cases as they present a range of proteolytic resistivity and physicochemical properties. Our results demonstrate that while surface adsorption of substrate and products modulates the performance of a "rheostatic" digestion concept, tunable digestion is feasible in a fluidic system and ultimately limited by solvent switching efficiency and protein folding/unfolding behavior.

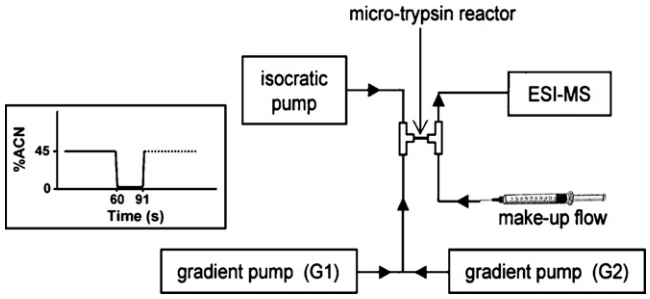

Fig. 1. Schematic of the experimental configuration used for regulating proteolytic tryptic digestion in an IMER for subsequent detection by electrospray mass spectrometry. The isocratic pump supplies the substrate or predigested control, while the gradient pumps supply the aqueous–organic mixture for tuning digestion. The inset illustrates one period of waveform 1, which is used to regulate digestion between 45 and 0% ACN.

2. Materials and methods

2.1. Chemicals, reagents, and solutions

Chicken egg albumin (ovalbumin, ova), horse cytochrome c (cyt c), horse heart myoglobin (myg), Tris base, ammonium bicarbonate (NH_4HCO_3), trifluoroacetic acid (TFA), porcine renin substrate, sinapinic acid (all >99% pure), ammonium hydroxide (NH_4OH, 29.1%), and hydrochloric acid (HCl, 36.8%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (FA, 98%) and ammonium acetate (NH_4CH_3COOH, >99%) were acquired from Fluka (Buchs, Switzerland). POROSzyme immobilized trypsin beads was purchased from Applied Biosystems (cat. #2-3127-00). HPLC-grade water/acetonitrile and acetone were obtained from Fisher Scientific (Fair Lawn, NJ, USA) while methanol was acquired from EM Science (Gibbstown, NJ, USA).

Aqueous stock solutions of all proteins were prepared to 300 μM and stored at −80 °C until use. Solutions for two gradient pump systems (G1 and G2, see Fig. 1) were prepared according to a previously described procedure [28]. This involved four solutions to deliver a low and high pH gradient of organic solvent. Briefly, acidic mobile phases A and B for the G1 system (referred to as G1-A and G1-B) consisted of 0.05% FA in H_2O and in 90% ACN, respectively. Basic mobile phases A and B for the G2 system (referred to as G2-A and G2-B) contained 20 mM NH_4CH_3COOH and 10% NH_4OH in H_2O and in 90% ACN, respectively. All mobile phases were vacuum degassed under sonication, prior to use, and prepared fresh weekly. The pH of a 1:1:1:1 mixture of all mobile phases was measured daily to ensure a pH of 8–8.2 (uncorrected for solvent effects).

2.2. Pumping systems

Solution delivery to the digestor was achieved using prototype splitless low-flow isocratic and gradient dispensing modules (Upchurch Scientific/IDEX, Oak Harbor, WA, USA) [26]. The individual pump modules consist of 250 μL positive displacement piston pumps, an integrated electronic stream selector valve, and an inline flow sensor for active feedback and flow control. The isocratic pump was used to supply an aqueous protein or peptide solution (5 μM at 0.75 μL min^{-1}), which was mixed with the combined output of the two gradient systems at the head of the trypsin IMER (Fig. 1). Each gradient system delivered mobile phase at 2 μL min^{-1} and were plumbed such that no differential gradient delay existed between the two gradient pumps. Although a single gradient (i.e. binary) pump system would be sufficient for testing the impact of regulat-