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Limited Proteolysis Via Millisecond Digestions in Protease-Modified Membranes

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Abstract

Sequential adsorption of poly(styrene sulfonate) (PSS) and proteases in porous nylon yields enzymatic membrane reactors for limited protein digestion. Although a high local enzyme density (~30 mg/cm³) and small pore diameters in the membrane lead to digestion in < 1 s, the low membrane thickness (170 μm) affords control over residence times at the ms level to limit digestion. Apomyoglobin digestion demonstrates that peptide lengths increase as the residence time in the membrane decreases. Moreover, electron transfer dissociation (ETD) tandem mass spectrometry (MS/MS) on a large myoglobin proteolytic peptide (8 kD) provides a resolution of 1–2 amino acids. Under denaturing conditions, limited membrane digestion of bovine serum albumin (BSA) and subsequent ESI-Orbitrap MS analysis reveal large peptides (3 kD–10 kD) that increase the sequence coverage from 53 % (2-s digestion) to 82 % (0.05-s digestion). With this approach we also performed membrane-based limited proteolysis of a large Arabidopsis GTPase, ROOT HAIR DEFECTIVE 3 (RHD3), and showed suitable probing for labile regions near the C-terminus to suggest what protein reconstruction might make RHD3 more suitable for crystallization.

Proteolysis is a vital initial step in most analyses of proteins by MS. Traditional in-solution digestion employs a dilute protease solution to avoid sample contamination and self-digestion of the enzyme, but the low protease concentration leads to extended digestion times. To increase throughput and facilitate online MS analysis, a number of research groups now employ immobilized enzymes for proteolysis.^{1–3} Immobilization greatly reduces self-digestion even at high local enzyme density (e.g. 30 mg/cm³),^{4–6} and such high densities allow protein digestion in as little as a few seconds. In addition, adsorption stabilizes the enzyme in many cases.¹ Protease immobilization can occur via covalent,⁷ hydrophobic^{4, 6} or electrostatic interactions,⁸ and supports for these enzymes include resins,⁹ monoliths,⁵ polymeric microfluidic channels^{4, 10} and membranes.⁶ All of these substrates provide relatively high surface areas for protease binding. However, porous membranes are unique in that their minimal thickness (typically 10–170 μm) should afford fine control over proteolysis time in flow-through digestion. When coupled with simple syringe pumps, membranes provide digestion times ranging from a few milliseconds to several seconds.

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SUPPORTING INFORMATION AVAILABLE

Details on chemicals, membrane modification and mass spectrometry procedures, analysis of pepsin immobilization, mass spectra of digested proteins, and tables of peptides identified from mass spectra. This information is available free of charge via the Internet at <http://pubs.acs.org>.

This work exploits short residence times in enzyme-modified membranes to perform limited proteolysis for applications such as “middle-down” proteomics or low-resolution studies of protein structure. We hypothesized that decreasing membrane residence times would lead to longer peptides with more charge (Figure 1), and this is particularly the case for pepsin-modified membranes. Unlike solution digestion, where partially digested peptides continuously compete with intact proteins for enzyme sites that catalyze additional proteolysis, convective flow in membranes rapidly separates digested peptides from immobilized enzymes. Large proteolytic peptides often contain more basic residues than small tryptic peptides and are particularly amenable to ETD MS/MS analyses with high amino acid resolution, which is important for studies of hydrogen/deuterium exchange (HDX) to determine amino acid accessibility.^{1-3,11-14} Current methods for creating large proteolytic peptides employ expensive enzymes such as Asp-N and Glu-C that cut proteins only before or after one type of amino acid.¹⁵ However, these methods work only for a few proteins that contain a limited number of cleavable sites.

Limited digestion can also provide low-resolution structural information when digesting proteins in their native configuration. In this case proteolysis only occurs in target protein regions that are readily accessible to the enzyme. Traditional limited protein digestion employs a solution-based approach, which includes varying the incubation time, enzyme to substrate ratio, or reaction temperature to control the extent of proteolysis. This approach is laborious and sometimes difficult to reproduce.^{16, 17} With protease-containing membranes, similar experiments can be performed by simply varying the flow rate of a protein solution through the membrane. This method can greatly decrease the experimental time (~15 min) and sample consumption (~100 µg) in limited digestion studies, and fine control of the flow rate using a syringe pump may increase reproducibility. We demonstrate this concept by investigating which regions of recombinant RHD3 are flexible and susceptible to hydrolysis. Based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and MS data, we find three or four readily accessible regions at the protein C-terminus and suggest that truncation of the most labile site may increase the likelihood of RHD3 crystallization.

EXPERIMENTAL SECTION

Modification of Membranes with Proteases

The unmodified nylon membranes (25 mm-diameter, 0.45 µm nominal pore size) were obtained from Millipore (HNWP02500). Modification of UV/ozone-cleaned (10 min) nylon membranes with trypsin was reported previously, but we increased the trypsin concentration in the adsorption solution from 0.6 to 1.0 mg/mL.⁸ To fabricate pepsin- and chymotrypsin-modified membranes, 10 mL of a 0.02 M poly(styrene sulfonate) (PSS), 0.5 M NaCl solution (pH=2.3) was first passed through the membrane at 2 mL/min, followed by 30 mL of deionized water at the same flow rate. Subsequently, 4 mL of 2 mg/mL pepsin in 5% v/v formic acid (FA) or 5 mL of 1 mg/mL α-chymotrypsin in 2.7 mM HCl, 2 mM CaCl₂ was circulated through the membrane at 1 mL/min for one hour. After protease deposition, the membrane was rinsed with 30 mL of 5% v/v FA for pepsin modification or 1 mM HCl for α-chymotrypsin modification. Modified membranes were dried and stored in a desiccator. Using a Bradford assay with calibration curves, the amount of pepsin in the membrane was determined from the decrease in pepsin concentration in the loading solution after circulation through the membrane. (See Figure S1. Figure and Table numbers beginning with S refer to the supporting information). The amounts of trypsin and α-chymotrypsin immobilized in membranes were similarly determined from direct UV absorbance at 280 nm using a NanodropTM UV/Vis Spectrometer (Thermo).

Controlled Apomyoglobin Digestion with a Pepsin-Modified Membrane

Without urea denaturation or reduction, 100 μg of apomyoglobin was dissolved in 1 mL of 5% v/v FA, and this solution was passed through a small membrane (Figure 1) at flow rates from 0.1 to 30 mL/h. We calculated the residence time by dividing the membrane pore volume (estimated assuming 50% porosity, a 170- μm thickness, and for the small holder 0.02 cm^2 of external area) by the flow rate. Consequently, the residence times for 0.1 and 30 mL/h flow rates were 6 s and 0.02 s, respectively. Fifty μL of each digest was collected, dried with a Speed-Vac and reconstituted in 50 μL of 1% acetic acid/49% H_2O /50% methanol. The same experiments were also carried out with a trypsin-modified membrane, except that the apomyoglobin digestion buffer was 10 mM NH_4HCO_3 . For in-solution peptic digestion, 100 μg of apomyoglobin in 200 μL 5% v/v FA was mixed with 5 μg of pepsin and incubated at 37 $^\circ\text{C}$ for 5 min or 20 s. Two hundred μL of acetonitrile was added to stop the reaction, and the solution was immediately frozen with liquid nitrogen and lyophilized with a Speed-Vac before reconstitution.

Controlled BSA Digestion with a Pepsin-Modified Membrane

BSA was digested with urea denaturation and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) reduction. Thus, 100 μg of BSA was dissolved in 10 μL of 6 M urea and mixed with 1 μL of 0.5 M TCEP for 30 min at 56 $^\circ\text{C}$. To make a 0.1 mg/mL BSA solution, 989 μL of 5% v/v FA was added to the above 11 μL samples. This solution was passed through a small membrane at 0.3, 1.5, 3, 6 and 12 mL/h (a 5-mL syringe was used for the last two flow rates). Two hundred μL of each digest was collected, dried with a Speed-Vac and reconstituted in 200 μL of 1% acetic acid/49% H_2O /50% methanol. A similar experiment occurred with a trypsin-modified membrane, except that we applied a non-ionic acid labile surfactant (NALS) as the denaturation agent (Cat. no. ALS-410, Proteabio, Morgantown, WV).¹⁸ A 100 μg BSA sample was dissolved in 20 μL 0.1 % NALS at pH 7.8 (50 mM NH_4HCO_3), reduced with 5 μL 10 mM 1,4-dithio-DL-threitol (DTT) for 30 min at 56 $^\circ\text{C}$ and allowed to react with 10 μL 100 mM iodoacetamide in the dark for 30 min. Prior to digestion, this sample was diluted to 0.1 mg/mL BSA with 10 mM NH_4HCO_3 .

RHD3 Expression and Purification

Production of RHD3(1–676)-HIS6 subcloned in pET28b vector was induced in *Escherichia coli* (BL21 λDE3). The bacteria were cultured at 37 $^\circ\text{C}$ in LB medium containing 100 $\mu\text{g}/\text{mL}$ of kanamycin until reaching an optical density of 1.0 at 600 nm. Protein production was induced by addition of 1 mM isopropyl 1-thio- β -D-galactopyranoside for 5 h at 30 $^\circ\text{C}$. Harvested cells from 1 L of cell culture were suspended in 50 mL of buffer A (30 mM Tris-HCl, pH 8.0, 200 mM NaCl, 4 mM MgCl_2 , 2 mM β -mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride) and lysed with 4 min of sonication at an output of 48 watts with 10 s pulses (Sonicator Misonix 3000). Following a centrifugation step at 20,000 $\times g$ (4 $^\circ\text{C}$) for 20 min, the clarified lysate was applied to a 7-mL nickel-nitrilotriacetate (Qiagen) gravity flow column, which was pre-equilibrated in buffer A containing 25 mM imidazole. After extensive washing with buffer A, fractions were eluted over 2 column volumes using buffer A containing 150 mM imidazole. Fractions containing RHD3 were pooled and concentrated to about 1 mL, and 500 μL volumes were applied at 0.4 mL/min to a Superose 6 10/300 GE gel filtration column (GE Healthcare), which was pre-equilibrated with buffer B (30 mM Tris-HCl, pH 8.0, 200 mM NaCl, 4 mM MgCl_2 , 1 mM EDTA, and 1 mM DTT). Peak fractions containing target proteins were concentrated to ~ 15 mg/mL based on UV/Vis absorbance at 280 nm. Analytical gel filtration chromatography was also used to estimate the molecular size of purified RHD3, which eluted at a position corresponding to a homo dimer with a molecular weight of 150 kD.

Limited Proteolysis of RHD 3 with either Trypsin- or α -Chymotrypsin-Modified Membranes

The gel filtration-purified RHD3 was diluted with 10 mM NH_4HCO_3 to make 1 mL of 0.1 mg/mL RHD3, and this solution was passed through a small trypsin- or α -chymotrypsin-modified membrane at 0.6, 3 and 13 mL/h. The digests were analyzed by MALDI-MS and ESI-MS, and 3 μg of each digested protein and intact protein were also analyzed by SDS-PAGE with Coomassie Blue staining.

Mass Spectrometry and Data Analysis

An LTQ XL ion trap mass spectrometer equipped with a vMALDI source (Thermo Fisher) was used to perform MALDI-MS and CID-MS/MS analyses. The matrix was 2,5-dihydroxybenzoic acid. For ESI-MS analyses, 40 μL samples in 1% acetic acid, 49% H_2O and 50% methanol were loaded into a Whatman multichem 96-well plate (Sigma Aldrich) and sealed with Teflon Ultra Thin Sealing Tape (Analytical Sales and Services, Prompton Plains, NJ). Then, using an Advion Triversa Nanomate nano-electrospray ionization (nESI) source (Advion, Ithaca, NY) with a spray voltage of 1.4 kV and a gas pressure of 1.0 psi, samples were introduced into a high resolution accurate mass LTQ Orbitrap Velos mass spectrometer (Thermo Fisher) equipped with a dual pressure ion trap, Higher-Energy Collisional Dissociation (HCD) cell and ETD. High resolution mass spectra, as well as all ETD-MS/MS spectra, were acquired in positive ionization mode using the FT analyzer operating at 100,000 resolving power. All mass spectra show relative intensities on the Y-axis. Identification of peptides resulting from protein digestions was performed manually by mass fingerprinting or MS/MS. ETD MS/MS signals for an 8 kD peptide were manually assigned by matching MS/MS product ions with *in silico* peptide fragments generated using ProteinProspector. See the supporting information for more details on MS and MS fingerprinting.

RESULTS AND DISCUSSION

Membrane Modification with Protease

We previously showed that alternating adsorption of PSS and trypsin effectively immobilizes trypsin in nylon membranes.⁸ In 2.7 mM HCl, the protonated trypsin (isoelectric point, pI, of 10.2) adsorbs to negatively charged PSS.^{8, 19, 20} Chymotrypsin has a pI of 8.7 and adsorbs similarly. Pepsin has a much lower pI (2.8), so we both adsorbed this protease and performed peptic digestion using solutions containing 5% v/v FA (pH=2). Based on analyses of protease adsorption solutions, the protease/PSS-modified nylon membranes contain ~30 mg of chymotrypsin or trypsin per cm^3 of pores, and ~60 mg pepsin per cm^3 (Figure S1). These values assume a membrane porosity of 50%. Control experiments show that pepsin and trypsin do not adsorb to the bare membrane without PSS. The high local protease density in modified membranes, compared to 0.025 mg/cm^3 for in-solution digestion, leads to membrane-based protein digestion in times ranging from milliseconds to seconds, depending on the protein. Presumably because of immobilization, we observed no peptides due to specific pepsin self-digestion, but occasionally we found two autolytic peptides (sequence coverage of 18%) when using immobilized trypsin.

Apomyoglobin Digestion with Different Membrane Residence Times

Due to its simple secondary structure and high purity, apomyoglobin served as the first model protein to test our hypothesis that decreasing the membrane residence time will give longer proteolytic peptides. Figure 2 shows manually deconvoluted ESI-Orbitrap mass spectra of myoglobin digested by passing solutions through pepsin-modified membranes at flow rates of 0.1, 1, 10 and 30 mL/h, which correspond to residence times of 6, 0.6, 0.06 and 0.02 s, respectively. As the residence time decreases, large peptides emerge and the strongest signals shift to a higher m/z region. (Figure S2 shows the original spectra, and

Table S1 lists peptides identified from the spectra along with their monoisotopic masses, amino acid positions and cleavage sites). With the 6-s residence time, all detected peptides have masses less than 5 kD, consistent with fewer missed cleavages at longer digestion times. In-solution digestion for 5 min or 20 s and membrane digestion for 6 s give similar mass spectra that yield 100 % sequence coverage with a minimum of 5 peptides (**2**, **7**, **8**, **11** and **13**) (Figure S3). For the 0.6 and 0.06-s membrane-based digestions, covering the entire 153 amino acid sequence requires 4 peptides (**2**, **7**, **8**, and **16**) and only 3 peptides (**7**, **15** and **16**), respectively. As the residence time decreases, the signal for peptide **16** replaces the signals for peptides **11** and **13** due to missed cleavage. Similarly, signals for peptides **2** and **8** decrease in absolute intensity, and the signal for peptide **15** (the combination of peptides **2** and **8**) grows. For the 0.02-s digestion, signals for the intact protein (**20**) and peptide **19** (amino acids 30–153) dominate the spectrum, indicating incomplete digestion.

Figure S4 summarizes these results in terms of number of peptides, average peptide length and average charge state. Overall, shorter residence times generate larger peptides with more charge. ETD analysis of peptide sequences requires multiple charges to generate abundant fragments,²¹ and Coon and coworkers suggest that a low residues/charge ratio and three or more charges are the ideal conditions for effective ETD MS/MS.²² Short membrane digestion (0.06 s) gives a myoglobin peptide (**16**, amino acids 30–106) with a mass of 8764.93 Da and a +10 charge state, and Figure 3 shows the ETD MS/MS spectrum for this peptide. We identified 46 c ions and 52 z ions, which represent cleavage of 61 out of 76 total amino acid junctions. The average spatial resolution is less than two amino acid residues, equivalent to the results carried out by more complex instrumentation in Top-Down FT-ICR ECD analysis.²³

This high spatial resolution coupled with rapid, acidic peptic digestion may be particularly attractive for HDX studies that provide structural information.^{11, 12} Short, acidic digestion should reduce either back-exchange or over-exchange in HDX, and high spatial resolution will increase the confidence in exchange site localization. Additionally, acidic digestion might facilitate characterization of membrane proteins that are insoluble at neutral pH. Other MS-based methods for investigating protein structure and dynamics including hydroxyl radical labeling²⁴ and protein–ligand interaction by mass spectrometry, titration, and H/D exchange (PLIMSTEX)²⁵ may also benefit from controlled membrane digestion to increase spatial resolution.

We also explored residence time-dependent digestion of apomyoglobin using trypsin/PSS modified membranes and observed large tryptic peptides at faster flow rates. However, a high level of intact apomyoglobin co-exists with these peptides (data not shown). The presence of intact protein confirms that convection rapidly removes protein from the membrane, in contrast to a previous study that employed trapping in nanopores to enhance digestion.²⁶ In conventional in-solution digestion with several proteases, apomyoglobin undergoes slow proteolysis at pH 7–8.²⁷ Apomyoglobin denaturation at pH 2 might explain why at low residence times peptic digestion is more efficient than tryptic digestion for this protein. Thus, pepsin is superior to trypsin for creating large proteolytic peptides from apomyoglobin.

In-Membrane BSA Digestion under Denaturing Conditions

Sequence coverage is a critical parameter for MS-based protein characterization, particularly for comprehensive examination of posttranslational modifications. With a molecular mass of 66 kD (583 amino acids) and 17 disulfide bonds, BSA is a reasonable model for examining how membrane-based digestion time affects sequence coverage for a medium-sized, denatured protein. The 17 disulfide bonds make denaturation and reduction prerequisites for membrane digestion.

Figure 4 shows the ESI-Orbitrap MS spectra (m/z 540–1440) of BSA digested in membranes using flow rates of 0.3 and 12 mL/h, or residence times of 2 and 0.05 s, respectively. The 0.05-s residence time represents limited digestion, and the 2-s residence time usually provides nearly complete digestion. Peak labels indicate identified BSA peptides in order of increasing mass of $(M+H)^+$ from 628.414 for peptide **1** to 9204.38 for peptide **52**. Comparing the 2-s (Figure 4a) and 0.05-s (Figure 4b) digestions, the shorter time reveals more peptides at a high m/z range. Figure 5 presents the peptide size distributions in the two digestions. Remarkably, short digestion yields 10 peptides with masses >5 kD, whereas the 2-s digestion shows only 1 peptide with a mass greater than 3 kD. With 0.05-s digestion, 12 peptides (**17, 21, 33, 35, 39, 44, 45, 46, 48, 50, 51** and **52**) cover 82 % of the 583 amino acids, while the 2-s membrane digestion needs 19 peptides (**1, 2, 8, 9, 10, 11, 17, 18, 20, 22, 26, 27, 28, 29, 30, 31, 32, 33** and **36**) to cover just 53 % of the sequence. (The inclusion of other identified peptides does not increase sequence coverage, and we included only peptides with signals >1.5% of the highest signal in the spectrum.) The low ionization efficiency of some small peptides may contribute to the low sequence coverage with the 2-s digestion time, whereas longer peptides with more basic residues (H, K, and R) provide higher ionization efficiency in positive-ion mode.²⁸

We also employed trypsin/PSS-modified membranes to digest BSA denatured with a NALS, but there is no obvious trend in peptide size with residence time. With a ~0.05-s digestion, the sequence coverage is 90%, even higher than for short-time peptic digestion. The relatively high sequence coverage with the tryptic digest may stem from a denaturing procedure that, in contrast to the denaturation for peptic digestion, included alkylation and no urea. However, out of 65 tryptic peptides, 63 have masses less than 5 kD and the remaining 2 peptides have masses between 5 and 6 kD. Large peptides are important for correlating neighboring posttranslational modifications.^{29, 30} Unfortunately, with the simple syringe pump system we could not achieve accurate flow control at higher rates where larger tryptic peptides might appear.

Although pepsin provides large peptides more easily than trypsin, low specificity is a drawback of pepsin. In our database search, we assumed peptic cleavage occurs at the C-terminus side of A, F, I, L, W and Y. For apomyoglobin digestion in pepsin-modified membranes, we observed at least some cleavage at 2/15 A, 1/7 F, 1/9 I, 9/17 L, 0/2 W and 0/2 Y residues. With BSA, the values were 8/47 A, 12/27 F, 2/14 I, 28/61 L, 0/2 W and 1/20 Y residues. In both cases pepsin cleavage occurred preferentially after L, and with BSA, peptic cleavage after F also occurred at nearly 50% of the sites. These data are consistent with a previous statistical analysis of the specificity of immobilized pepsin in the digestion of 39 proteins (13766 amino acids residuals). That study showed that F and L have a cleavage probability larger than 40 %, whereas cleavage is less than 20% at A, I, W, and Y sites.³¹ One particular challenge in working with pepsin is that other cleavage sites or N-terminus cleavage of A, L, I, F, W or Y could lead to unassigned peptide signals, but tandem mass spectrometry can potentially overcome this problem.³²

For the same conditions, the spectra from digestions with replicate membranes show similar patterns although the relative intensities for each peptide vary. Figures S5 and S6 show the mass spectra from 6-s apomyoglobin digestions and 2-s BSA digestions performed with two different pepsin-modified membranes. The shelf life for pepsin-modified membranes is around one month, as we observed reduced digestion efficiency for such membranes after two months of storage (see Figure S7). Digestions reported in this paper were performed within two weeks of fabricating the membranes.

Limited Membrane Proteolysis of RHD3 for Structural Information

Homologous to human atlastins, RHD3 is one of the largest membrane-bound GTPases in *Arabidopsis thaliana*.³³ This protein participates in the modeling of the endoplasmic reticulum and contains a GTPase domain, a helix bundle, two trans-membrane helices and one cytosolic tail.³⁴ Human atlastin may be involved in homotypic fusion of the membrane of the endoplasmic reticulum, and much of the information in the mechanisms that could underpin such a fusion has been gathered from crystallographic analyses of the soluble domain (cytosolic) of atlastin.³³ The biological role of RHD3 is unknown,³⁵ and a crystal structure of this protein would most likely offer valuable insights into RHD3's role in plant cells.

This work aims to use limited membrane-based proteolysis to map flexible protein regions that may restrict crystallization (Scheme 1).^{36, 37} Flexible regions should contain digestion sites that are much more accessible to proteolysis than similar sites in the globular region of the protein. Subsequently, proteins expressed without the flexible regions would be more stable and compact, and consequently have a much higher probability of crystallization.^{16, 17, 36, 38} Because the globular regions of the protein resist digestion, we could employ trypsin and α -chymotrypsin in limited proteolysis. These enzymes are more specific than pepsin and operate at non-denaturing conditions (pH 7.5 NH_4HCO_3 buffer), which is vital for structural studies.

To obtain different digestion patterns, we selectively passed RHD3 solutions through either trypsin- or chymotrypsin-modified membranes at 0.6, 3 and 13 mL/h. Figure 6 shows the SDS-PAGE analysis of the proteolytic digests. In both images, lane 1 is the protein ladder, lane 2 is the intact protein, and lanes 3–5 are the digests at the different flow rates, which correspond to residence times of 0.045, 0.2 and 1 s, respectively. After tryptic digestion, three major protein bands appear with molecular masses around 70, 65, and 60 kD. We performed the chymotrypsin membrane digestion one week after the trypsin membrane digestion, and the protein itself degraded slightly, presumably because of trace protease not removed during the purification process. The SDS-PAGE after chymotrypsin digestion reveals a dominant intermediate digestion product with a molecular weight around 65 kD.

We applied both MALDI-LTQ MS and ESI-Orbitrap MS to investigate the most abundant peptides in the RHD3 digests. Figures 7a, b and c show the MALDI mass spectra of RHD3 digested in a trypsin-modified membrane for different residence times. Peak labels show peptide sequences determined from comparison of masses with theoretical masses obtained from *in silico* digestion. The recombinant RHD3 protein has 691 amino acids including its polyhistidine tag, and the most intense peak at the shortest digestion time corresponds to the peptide with amino acids 673–691, suggesting a flexible polyhistidine tag region. The mass spectra also indicate cleavage after amino acids R542, R560, R569, K600, K617, K632, K637, K646 and R650. Chymotrypsin also shows cleavage sites in these regions (Table S3). In the predicted secondary structure, all of these peptides lie within the last 4 α -helices or regions between them. Comparison of the three spectra in Figure 7 suggests that cleavage is most rapid at R672, as relative to the peptide 673–691, signals for other peptides typically increase with increasing digestion time. This suggests that longer digestion times result in digestion deeper into the protein, which is consistent with the SDS-PAGE results.

The ESI-orbitrap mass spectrum (Figure 7d) confirms MALDI-MS results and shows dominant cleavage sites after R576, K617, R650, and R672. (Because ESI gives multiply charged ions, we detect peptides with masses larger than 4 kD. These peptides do not appear in MALDI mass spectra because ions are predominantly singly charged and the upper limit of our mass analyzer is $m/z = 4000$.) The signal for peptide 673–691 is especially strong. Overall, the mass spectra suggest there are 4 highly accessible regions around R576, K617,

R650, and R672. Figure S8 (ESI mass spectra of RHD3 digested in a trypsin-modified membrane for different residence times) shows that peptide 618–691 is present primarily at low digestion times, suggesting that the K617 site is more accessible than R650. This Figure also reveals a small signal for peptide 1–71 from the N-terminus, showing limited access to this part of the protein. Table S3 lists the most abundant peptides in limited trypsin and α -chymotrypsin digests. In Figures 7a, b and c there is a signal that seems to correspond to peptide 251–263. This peptide is in the globular region of the protein where the GTPase domain and helical region connect, and this linker region may also be accessible to the enzyme.

Overall, these data suggest that the region around R672 is highly flexible and that truncating the protein at R672 may increase the probability of crystallization. (Notably, we did not detect cleavage at K679). In fact a new construct up to R672 shows no degradation over several weeks, but we have not yet successfully crystallized this protein. Compared to in-solution, limited digestion methods to suggest truncated protein sequences, the membrane method is rapid and convenient and provides finer control over the digestion time.

CONCLUSIONS

Immobilizing common proteases (trypsin, α -chymotrypsin and pepsin) in nylon membranes at high enzyme density (30–60 mg/cm³) conveniently creates membrane-based enzymatic reactors. These reactors catalyze limited proteolysis in <1 s. More importantly, tuning the flow rate through the membrane affords control over the size of proteolytic peptides. For apomyoglobin digestion in pepsin-modified membranes, shorter residence times yield longer peptides with more charge. A large peptide (8kD) with a +10 charge state provides a spatial resolution of <2 amino acid residues in ETD MS/MS analysis. Similarly, in membrane-based peptic digestion of denatured BSA, limited digestion yields many missed cleavages and creates long peptides that increase the sequence coverage from 53% to 82% on going from 2-s to 0.05-s digestion. One limitation of this technique is that the number and relative reactivities of cleavage sites vary from protein to protein. Thus the extent of digestion for a given residence time will depend on the protein. We can partly overcome this limitation by varying the residence time. Future work will focus on limited digestion of proteins with multiple posttranslational modifications to study correlations between different modifications. Limited, membrane-based proteolysis shows which regions of a non-denatured protein are readily accessible to enzymes. For RHD3, digestion in a membrane-based enzymatic reactor reveals rapid digestion at K672 and suggests that truncation of the protein at this amino acid will isolate a more conformationally compact version of the protein that is more suitable for crystallization. Future studies with other proteins will further demonstrate the value of limited digestion in protease-modified membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cartoon of controlled digestion using a membrane containing immobilized pepsin and a miniaturized holder with a syringe pump. (The apomyoglobin structure was generated from PDB 1MBN.)

Figure 2.

Manually deconvoluted ESI-Orbitrap mass spectra of 0.1 mg/mL (6 μ M) apomyoglobin digested with residence times of (a) 6, (b) 0.6, (c) 0.06 and (d) 0.02 s in a pepsin-modified membrane. Signals with intensities above 1% of the highest signal were assigned to a specific peptide if possible and converted to the m/z value for the +1 charge state. The normalized intensities in the spectra are the sum of signal intensities for all detected charge states of a given peptide. The inset table lists all peptides identified in the four spectra (AA represents the amino acids sequence).

Figure 3. ESI-Orbitrap ETD-MS/MS spectrum of the $[M+10H]^{10+}$ precursor ion at 878.00. The sequence shows cleavage site for formation of major c and z ions.

Figure 4. ESI-Orbitrap mass spectra of 0.1 mg/mL BSA (1.5 μ M in 0.06 M urea) digested in a pepsin-modified membrane using (a) 2-s and (b) 0.05-s residence times. The numbers refer to the identified BSA peptides where masses increase with the peptide number. (Table S2 lists all peptides along with their monoisotopic masses and sequences.)

Figure 5. Peptides size distributions in ESI-Orbitrap mass spectra of 0.1 mg/mL BSA digested in a pepsin-modified membrane for 2 s (blue, 32 peptides) and 0.05 s (red, 35 peptides).

Figure 6.

SDS-PAGE analysis of limited RHD3 digestions in a trypsin-modified membrane (left) and an α chymotrypsin-modified membrane (right). Three μg of either protein or digests were loaded onto each column, and lanes 1–5 are protein ladder, intact protein, digests at 13 mL/h (residence time of 0.045 s), digests at 3 mL/h (residence time of 0.2 s) and digests at 0.6 mL/h (residence time of 1 s), respectively.

Figure 7. MALDI mass spectra of 0.1 mg/mL (1.3 μ M) RHD 3 digested in residence times of (a) 1, (b) 0.2 and (c) 0.045 s by a trypsin-modified membrane, and (d) the ESI mass spectrum of the same sample as (c). Signals were assigned to peptides by mass fingerprinting and are labeled with the amino acid position of these peptides (protein full length 1–691). Compared to the C-terminus peptide (673–691, stars), the relative intensities of the smaller peptides cleaved after amino acids K600, K617 (red circles), R650 (green circle) or R560 (blue circles) increase as the residence time increases from (c) to (b) to (a), suggesting that longer digestion times result in deeper access into the protein.

Scheme 1.

Strategy for limited RHD3 digestion in membranes containing trypsin and α -chymotrypsin. The protein picture (top left) is that for a human atlastin dimer (made from PDB 3QOF and 3QNU using Pymol), which has a similar domain configuration to RHD3 but with less α -helix. The drawings represent RHD3 dimers (one GTPase domain and 9 α -helices on each monomer).