Published in final edited form as:

Anal Chem. 2015 August 04; 87(15): 7987–7995. doi:10.1021/acs.analchem.5b01929.

Pronase E-Based Generation of Fluorescent Peptide Fragments: Tracking Intracellular Peptide Fate in Single Cells

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Abstract

The ability to track intracellular peptide proteolysis at the single cell level is of growing interest, particularly as short peptide sequences continue to play important roles as biosensors, therapeutics, and endogenous participants in antigen processing and intracellular signaling. We describe a rapid and inexpensive methodology to generate fluorescent peptide fragments from a parent sequence with diverse chemical properties, including aliphatic, nonpolar, basic, acidic, and non-native amino acids. Four peptide sequences with existing biochemical applications were fragmented using incubation with Pronase E and/or formic acid and in each case a complete set of fluorescent fragments was generated for use as proteolysis standards in chemical cytometry. Fragment formation and identity was monitored with capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-MS) to confirm the presence of all sequences and yield fragmentation profiles across Pronase E concentrations which can readily be used by others. As a pilot study, Pronase E-generated standards from an Abl kinase sensor and an ovalbumin antigenic peptide were then employed to identify proteolysis products arising from the metabolism of these sequences in single cells. The Abl kinase sensor fragmented at $4.2 \pm 4.8 \text{ zmol } \mu\text{M}^{-1} \text{ s}^{-1}$ and the majority of cells possessed similar fragment identities. In contrast, an ovalbumin epitope peptide was degraded at $8.9 \pm 0.1 \text{ zmol } \mu\text{M}^{-1} \text{ s}^{-1}$ but with differential fragment formation between individual cells. Overall, Pronase E-generated peptide standards were a rapid and efficient method to identify proteolysis products from cells.

Graphical Abstract

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Additional information as noted in the text. This material available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interest.

INTRODUCTION

Intracellular proteolytic processing of short peptides is an important biological phenomenon, with demonstrated functions in antigen presentation,¹ pheromone secretion,² gene regulation³ and modulation of signal transduction.⁴ Proteins and peptides are metabolized by complex multi-protein systems such as the proteasome, producing oligopeptides subsequently transported in the cytoplasm, secretory granules, or nucleus.^{5,6} These short sequences can then continue on to one of many fates, for example, reduction to their component amino acids for use as synthetic building blocks, aggregation into disease-inducing amyloid plaques⁷, or transportation to the extracellular membrane to act as antigens. Additionally, exogenous loading of peptidic sequences into cells has been used to study peptide processing⁸ and intracellular signaling^{9–12} as well as to deliver drugs and to enhance imaging contrast.¹³ The ability to assess cells individually for specific peptidolytic phenotypes is also increasingly desirable such as for the development of engineered T-cell therapies or the assay of peptidase heterogeneity for targeted tumor therapeutics. These recent advances for the design and delivery of peptide-based therapeutics further fuel the growing interest in tracking intracellular peptide stability and fate, particularly at the single-cell level. Thus tools to enhance the ability to directly monitor peptide reactions within cells will have tremendous value in a wide range of areas from sensor development to therapeutics and basic physiology.

Regardless if sequences are designed to undergo systematic degradation as sensors of protease activity, are delivered as a vaccine,¹⁴ or used for other purposes, the ability to study peptide fragmentation patterns is critical to tracking peptide fate. Measurements of intracellular peptide metabolism particularly that incorporate a separation step such as chemical cytometry also require a set of fragment standards to unambiguously detect peptide fragments.^{15,16} Facile generation of a full fragment set would enable rigorous characterization of the intracellular fate of peptides used as reporters for kinases, phosphatases and other enzymes in chemical cytometry as well as applications in mass spectrometry-based proteomics and peptide sequencing. Rapid generation of peptide fragments may also accelerate the determination of biologically active sequences from larger peptides or proteins, such as in the delineation of specific peptide binding motifs for the prediction of T-cell epitopes. Currently, this process involves screening all potentially active sequences from a parent peptide, requiring the laborious synthesis of all fragments.¹⁷

Methods to generate fragments from a peptide sequence center around three main approaches: solid phase peptide synthesis (SPPS), chemical fragmentation, and enzymatic digestions. While SPPS is unparalleled at guaranteeing the presence of all desired

sequences, the synthesis of fragment mixtures from complex cyclized, cross-linked or stapled peptides may be highly difficult and can require lengthy chromatographic purification.¹⁸ Chemical cleavage of peptides has been performed with a variety of reagents such as cyanogen bromide, 3-Bromo-3-methyl-2-(2-nitrophenylthio)-3H-indole, formic acid and hydroxylamine, which clip C-terminally of methionine, tryptophan, aspartic acid and asparagine, respectively.¹⁹ However, the nature of chemoselective fragmentation in combination with the relatively low abundance of the specified amino acid loci often result in the generation of long peptides. Thus, for the generation of all sequences from a peptide, concatenated cleavage steps with multiple reagents may be required and do not guarantee the presence of all fragments.

A popular route to generating peptide fragment ladders is directed enzymatic cleavage, a technique borrowed from bottom-up mass spectrometry-based proteomics in which proteins are digested with trypsin or chymotrypsin to generate short peptide sequences.²⁰ However, these two enzymes have well-defined specificity and only yield peptide fragments by hydrolysis at five of the twenty amino acids. In cases where nonspecific peptide cleavage is desired, proteases such as Pepsin A and Proteinase K which cleave peptides with limited substrate specificity are often employed, although sequence coverage with these two enzymes is not 100%.²¹ Pronase E from the extracellular fluid of *Streptomyces gresius* is a commercially available, broad-specificity mixture of at least ten enzymatic components, including five serine-type proteases, two Zn²⁺-endopeptidases, two Zn²⁺-leucine aminopeptidases and a Zn²⁺-carboxypeptidase.^{22,23} The reported ability of Pronase E to hydrolyze peptide bonds internal to a sequence as well as sequentially remove amino acids from both the N and C terminus make this enzyme mixture an attractive alternative to enzymatic digestion with trypsin or chymotrypsin, whose regulated activity would deliver only specific fragments.²⁴

It was hypothesized that the broad range of proteolytic activities afforded by Pronase E would affect cleavage at each peptide bond, resulting in a standard mixture of all possible fluorescent fragments of a full-length peptide. Herein, we demonstrate the utility of Pronase E as a nonspecific, rapid, and inexpensive method to generate fluorescent peptide fragment ladders. We also introduce the use of secondary formic acid digestion as a post-enzymatic step to promote the formation of acidic fragments. Fragment formation and identity were synergistically monitored with CE-LIF and MALDI-MS in parallel. Finally, peptide fragment standards generated by Pronase E were then applied to identification of degradation products from peptide-based probes in single cell assays. Peptide ladders generated by Pronase E should have wide applicability in the identification of degradation products in single cell assays, for peptide sequencing purposes, and for the generation of biologically active sequences when identifying immunogenic epitopes.

EXPERIMENTAL SECTION

Supplemental Information regarding peptide synthesis, identification of peptide fragments, and cell culture is available online.

Pronase E Peptide Digestions

Pronase E from *Streptomyces gresius* was prepared in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM HCl, 1.75 mM KH₂PO₄, pH 7.4) to 2 mg/mL. Assessment of peptide degradation was performed in triplicate by combining peptide (10 μM) with various concentrations of Pronase E (0.001 μg/mL-1000 μg/mL) and incubating at 37°C. Aliquots were removed at time points and inactivated at 90°C for 4 min. Negative controls without peptide were concurrently sampled and heat-inactivated. Samples were diluted 100X in electrophoretic buffer prior to separation and detection with CE-LIF.

Supplemental Formic Acid Assisted Digestion

Additional fragments of Peptide III were generated by incubating partially digested samples from Pronase E assays in 1–5% formic acid solution at 37°C. Aliquots were neutralized with an equal volume of 200 mM NaOH to bring the pH to 6.5–7.5.

Capillary Electrophoresis

For separations not involving single cells, CE-LIF (488 nm) was performed using a Proteome-Lab PA 800 (Beckman Coulter, Fullerton, CA) equipped with 30 cm fused silica capillaries of 30 or 50 μm inner diameter, 360 μm outer diameter (Polymicro Technologies, Phoenix, AZ). Electrophoretic buffers for Peptides I, II, III, and IV were 100 mM tris, 100 mM tricine, pH 8.1; 140 mM borate, 70 mM sodium dodecyl sulfate (SDS), pH 9.5; 300 mM borate, pH 7.5; and 100 mM tris, 100 mM tricine, 5 mM sodium cholate, pH 8.1, respectively. Electropherograms were integrated using customized software²⁰ constructed using MATLAB (Natick, MA).

MALDI-MS Analysis

MALDI-MS analyses were performed in high-resolution reflectron mode with an AB SCIEX 4800 Plus instrument equipped with a 337 nm laser. Peptide standards and assay samples were diluted in deionized water to 3 μM. Samples were prepared by spotting a 500 nL sample volume onto a 364-spot MALDI sample plate immediately followed by 500 nL of saturated α-cyano-4-hydroxycinnamic acid matrix solution. Spots were air-dried under laminar flow. The laser intensity was set to 4200 mW and 50 shots were averaged for the peptide spectra.

Single Cell Capillary Electrophoresis

Custom glass chambers for housing cells were placed on the stage of a customized single-cell CE system with LIF detection (488 nm).²⁵ Cells were microinjected with 100 μM peptide (I or IV) using a Transjector 5246 microinjection system (Eppendorf AG, Hamburg, Germany). Cells were maintained at 37°C with a constant flow of warmed extracellular buffer (ECB; 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4) throughout incubation and analysis. At 2 min post microinjection, the cell was lysed with a focused Nd:YAG laser pulse as previously described²⁶ and the contents injected into the capillary by applying a negative potential to the capillary outlet reservoir (5 s at 79 V/cm). For separations of single cells microinjected with Peptides I or IV, electrophoresis was performed in respective buffers at –171 and –263 V/cm, respectively.

RESULTS AND DISCUSSION

Experimental Overview

The goal of this work was to develop a simple strategy to generate fluorescent peptide fragments regardless of their sequence or structure. To that end, a small collection of chemically diverse peptides with existing biochemical applications was selected (Fig 1A), and the efficacy of Pronase E in generating fragments as a function of substrate chemistry and enzyme concentration was examined. Each peptide under investigation was incubated with Pronase E at varying concentrations for up to 60 min (Fig 1B,C). Samples were then divided into two equivalent fractions and subjected to parallel analysis by CE-LIF and MALDI-MS in order to determine how many fluorescent fragments were generated (Fig 1D). The number of fragments formed and the rate of peptide degradation was determined from the number and area of peaks appearing in electropherograms (see Fig 1A for fragment nomenclature for Peptides I–III, Table S1 for Peptide IV). MALDI-MS identified which fragments formed under the different conditions. To validate the identity of fragments in electropherograms, all fluorescent fragments of Peptides I–III were synthesized using SPPS to identify unique electrophoretic and mass footprints. We then applied the assay to the formation and identification of fragments from Peptide IV, an ovalbumin epitope sequence without synthesized standards. Pronase E-generated standards were then employed to identify peptides formed due to protease activity in single cells.

Enzymatic Generation of Peptide I Standards

Peptide I (5FAM-GGAYAAPFKKKA-NH₂), a sequence employed as an Abl kinase sensor, contains primarily hydrophobic and basic residues and was well matched to the reported specificity of Pronase E.^{22,23} All fragments of Peptide I were readily separated in an optimized¹⁰ buffer system (Fig 2A) and efficiently detected by MALDI-MS (Fig 2B). Due to moderate salt levels in the sample matrix, Peptides I-k, I-g, I-f, and I-e often ionized as sodium or potassium adducts, which are denoted in mass spectra by asterisks (*). After verifying that all fragments possessed unique electrophoretic and mass footprints, Peptide I was incubated with Pronase E and the assays sampled over time by CE-LIF to assess the formation of fluorescent fragments. Complete degradation into all representative fragments was observed after incubation with 0.02 µg/mL enzyme for 15 min, represented by the appearance of 11 new peaks in electropherograms (Fig 2C) and the identification of each fragment mass by MALDI-MS (Fig 2D). Fragments I-j ($23\pm4.2\%$) and I-l ($30\pm5.6\%$), resulting from cleavage at Ala-Tyr and Gly-Gly bonds, respectively, were among the predominant fragments formed, although all 11 were detected by both CE-LIF and MALDI-MS (Fig 2C,D). Taken with data from incubation of Peptide I with more concentrated solutions of Pronase E (Fig 3A), these results indicated that the enzyme mixture first hydrolyzes multiple peptide bonds within a sequence before reducing the peptide to the shortest peptide fragments. Overall, Pronase E was capable of generating all fluorescent fragments of Peptide I in as short as 15 min, which, at minimum, is 50 fold faster than by SPPS.

Enzymatic Generation of Fragments from Peptide II

After verifying that Pronase E could be used to generate fragments of a peptide containing residues similar to its reported preference, a longer and more charge diverse sequence was evaluated. Peptide II (6FAM-RAHEEIYHFFFAKKK-NH₂) is a substrate of epidermal growth factor receptor (EGFR) tyrosine kinase and has found widespread utility for *in vitro* enzymatic characterization of EGFR.^{27,28} Containing aromatic, aliphatic, acidic, and basic residues, Peptide II represents a chemically-diverse testing ground for the formation of fragments by this enzyme mixture. 0.1 µg/mL Pronase E was required to form all 15 fragments within 15 min (Fig 3B). Although fragments II-l and II-m formed less than 2% of the total fragments detected by CE-LIF, these were readily identified with CE-LIF and also with MALDI-MS (Fig S2). While a moderate concentration of Pronase E was sufficient to generate all fragments, it should be noted that pooling samples from differing concentrations which contain overlapping sequence coverage is a method in which the desired fragments can be obtained.

Pronase E digestion of degradation resistant Peptide III

In order to assess limitations of Pronase E to generate all fluorescent fragments of a peptide, a degradation resistant sequence was identified and synthesized, along with its corresponding fluorescent fragments. Peptide III (6FAM-LEDDYEDDNle-NH₂, where Nle is Norleucine) was adapted from a substrate of protein tyrosine phosphatases (PTPs).⁹ In single cells, the median half-life of the peptide was 35 min; 70-fold longer than that of Peptide I.^{11,29} Though truncated from its original 13 amino acid sequence, Peptide III possesses 80% of the glutamic and aspartic acid residues to which its degradation resistance was attributed. This peptide also contains a non-native amino acid, which confers resistance to degradation, presumably due to the limited ability of peptidases to recognize non-coding amino acids.^{30,31}

Peptide III was resistant to enzymatic degradation 60 min after incubation with 50 µg/mL Pronase E, a 500-fold more concentrated solution than that required to generate all fluorescent fragments of Peptide II in 15 min. However, by incubating the peptide with 500 µg/mL Pronase E for 15 min all fluorescent fragments were generated and detected with CE-LIF (Fig 4A). As expected, the majority of fragments formed during digestion were the result of cleavage between native amino acids. The three fragments forming in the smallest percentages were all generated by cleavage between two acidic residues (III-c, e, g), which falls in agreement with reports of the resistance of peptide bonds between anionic residues to hydrolysis.²⁹ Due to the highly sensitive nature of CE-LIF, several fragments formed in small quantities detectable by CE but were not identified through MALDI-MS analysis (Fig 4B). These included III-b, c and e, all fragments resulting from cleavage involving acidic or non-native amino acids, which were identified with fragment standards. Increasing the Pronase E concentration to 1000 µg/mL had the effect of generating a higher fraction of short (<5 residues) peptides and fragment III-b due to cleavage of Nle (Fig 3C), but fragments such as III-c, III-e and III-g were never formed in amounts greater than 3% of the total fragments detected in electropherograms, and were rarely detected with MALDI-MS.

Formic Acid-Assisted Degradation of Peptide III

We hypothesized that the acidic nature of Peptide III contributed to the limited formation of several fragments and our resultant inability to detect some fragments with MALDI-MS. Thus, we sought a method which could be used in combination with Pronase E to generate fragments in a more equivalent fashion. Given that conditions for the selective cleavage of peptides and proteins at the carboxyl side of acidic residues such as aspartic acid using formic acid had been reported,³² we selected formic acid for digestions. Formic acid treatment alone was insufficient to generate all fragments (Fig S3). However, if Peptide III was first partially hydrolyzed with 500 µg/mL Pronase E for 15 min, heat-inactivated, and then exposed to 2% formic acid for 15 min at 37°C, all 8 fragments were detected along with native peptide. The fraction of peptides produced due to cleavage at the C-terminal end of aspartic acid residues each improved to a minimum composition of 6%, and was easily detected with CE-LIF (Fig 4C). The use of formic acid also resulted in improved sensitivity and better representation of true peptide stoichiometries in MALDI-MS spectra (Fig 4D), presumably due to improved ionization garnered after protonation by formic acid.^{33,34} Overall it was found that the combination of enzymatic and chemical peptide hydrolysis produced a simple, rapid, and novel method to nonspecifically generate fragments of an acidic, degradation-resistant peptide.

Degradation of an immunogenic peptide with an internal fluorophore

Intracellular peptide metabolism gives rise to many compounds of biological importance, including epitopes recognized by cytotoxic T-cells (CTL). Use of these antigens as immunotherapy is a promising new treatment for patients with advanced stage oncological malignancies, but antigen immunotherapy is limited by the paucity of suitable peptide antigens that are recognized by CTLs,³⁵ necessitating laborious epitope mapping screens in which full length peptides and all possible truncated active sequences must be synthesized. Thus, a mixture of all possible fragments from a potential epitope-containing peptide or protein could be useful, especially since it has been shown that pooling of peptide fragments does not cause masking of the active sequences in high-throughput screens.³⁶ Peptide IV (SIINFE(K-5FAM)L-OH) is the 256–264 residue class I (Kb)-restricted peptide of ovalbumin, a frequently modeled epitope in the context of the major histocompatibility complex (MHC) Class I allotype. It has been demonstrated that the peptide can be used to elicit strong CD8+ cytolytic T cell responses.³⁷ Although presentation of Peptide IV requires no further proteolytic processing, this sequence acted as proof of principle for the enzymatic generation of all possible fluorescent fragments of an active epitope with Pronase E.

To establish whether all fragments of a peptide with an internal fluorophore could be generated without the use of synthesized standards, Peptide IV was incubated with varying amounts of Pronase E until the maximum number of fluorescent fragments (nomenclature; Table S1) were identified with MALDI-MS (Fig S4). Compared to Peptides I-III, an intermediate concentration of Pronase E (5 µg/mL) was required to generate all fluorescent fragments of this sequence (Fig. 3D). Three pairs (IV-d/e, IV-f/g, and IV-h/i) of Peptide IV fragments possessed similar or identical masses (Table S1) and could not be unambiguously identified by MALDI-MS alone, making identification of these fragments a unique

challenge. One species, fragment IV-n, could not be identified by MALDI-MS, potentially due to poor ionization under the conditions tested. To ensure that all fluorescent fragments were indeed formed, the same sample was subjected to separation with CE-LIF. 15 unique peaks were identified, each presumably representing a fragment. Since sequence-specific identities could not be assigned to peaks appearing in electropherograms, fragments of Peptide IV detected by CE-LIF were labeled with roman numerals according to migration time (Fig 5D). While the sequence of each fragment cannot be determined by migration time alone, the combination of MALDI-MS and CE-LIF data indicates that each fragment is present. Based on the MALDI-MS analysis, we can speculate that fragments formed in greater quantities, such as IV-a and IV-k, may correspond to species generating a large peak area in CE-LIF analysis, such as IV-xiii and IV-ii, respectively. Peptide IV was identified as IV-ix based on co-migration with the synthetic full length sequence. Interestingly, the contents of the slowest migrating peak in Fig 5D and Fig S5 co-migrated with the free fluorophore (5FAM) which was not observed in Pronase E assays with Peptides I–III, which are fluorescently labeled on the N-terminus rather than an internal residue. Overall, Pronase E was a rapid and simple method to generate fluorescent fragments regardless of the fluorophore placement. Thus, this technique finds applications in comprehensive fragment profile generation with the exact sequence-specific peptides being unknown. However, in such cases where comprehensive fragment profiles are generated without the use of synthesized standards, a secondary method of peptide sequence generation or a combination of separation and mass spectrometry may be required if identification of sequence-specific fragments is desired.

Tracking proteolysis of peptides in single cells

Single-cell CE-LIF permits highly sensitive (10^{-20} mole)^{9,25} enzyme activity measurements within individual cells. However in order to identify species arising from reporter or peptide degradation, it is necessary to generate a set of peptide standards which represent all possible degradation products for use as migration standards. This is especially true when the degradation products of peptides within individual cells are unknown and may co-migrate with other peptide modifications such as phosphorylation or acetylation. Although mass spectrometric methods have the capability to directly assign species identities, the combination of high-salt physiological buffers, limited sensitivity, variable analyte ionization, and small sample volumes have proven generally incompatible with mass spectrometry to directly identify products from single cells.^{15,37–39} To demonstrate the utility of a simple and rapid method to generate all fluorescent fragments in tracking single-cell peptide metabolism, the intracellular fate of peptides was tracked in single-cells by CE-LIF.

Peptide I (38 ± 9 amol, $n=4$ cells), or IV (46 ± 15 amol, $n=7$ cells) was microinjected into individual PANC-1 cells, a pancreatic adenocarcinoma cell line. This cell line was chosen based our observations that PANC-1 cells possess robust proteolytic activity and would likely generate fragments of the selected peptides.²⁵ Peptide was incubated for 2 min before cells were lysed with a pulse from an Nd:YAG laser and the contents loaded into a capillary directly above the cell. Intracellular metabolism of the peptide was identified using standard fragment mixtures obtained from incubation with Pronase E. With the exception of a peak

detected due to cell fluorescence (Fig 5D), each fragment formed in the cell co-migrated with a standard fragment suggesting that all cell-derived peaks were fragments formed by cleavage at peptide bonds and were not due to other metabolic processes such as phosphorylation or acetylation, which would likely shift the migration times observed in CE-LIF.

During a 2-min incubation, Peptide I, an Abl kinase sensor, underwent proteolysis within the cytosol of all intact cells analyzed, and all fragments were identified through the use of Pronase E-generated standards. The five-residue fragment I-h, a result of cleavage between the Ala-Ala residues (Fig 5A), was preferentially formed in all cases. Similar peptide cleavage sites were observed among the cells analyzed, with between 5–7 fragments formed within the 2 min incubation period, and the identities of fragments formed between cells varied only slightly (Fig 5E, S6). No fragments shorter than 3 amino acids were observed. The average rate of degradation of Peptide I within cells was $4.2 \pm 4.8 \text{ zmol } \mu\text{M}^{-1} \text{ s}^{-1}$, and resulted in 24–78% intact Peptide I after 2 min. While one of the four cells tested did not exhibit significant amounts of peptide fragment generation, the peptides generated possessed similar profiles between cells (Fig S6). The variability in absolute fragmentation rate observed in these studies combined with previous work displaying the rapid fragmentation of this peptide sensor in single cells¹⁰ indicates that in numerous cases this sequence is quickly degraded and requires fragmentation monitoring within the intracellular environment, even in cases where peptide phosphorylation is the desired readout.

Peptide IV was also rapidly metabolized in cells, with an average of $8.8 \pm 5.3\%$ intact remaining after 2 min (Fig 5B). The average rate of proteolysis was $8.9 \pm 0.1 \text{ zmol } \mu\text{M}^{-1} \text{ s}^{-1}$. Although Peptide IV requires no additional proteolytic processing for antigen presentation, its limited intracellular lifetime suggests rapid peptidase activity towards this sequence. This is consistent with advancement of degraded peptides to one of multiple fates, including binding to MHC class I molecules to become immunologically relevant or acting as substrates for various cytosolic peptidases which hydrolyze peptides into their component amino acids.⁶ The 7 individual cells produced between 3–11 unique fluorescent peptide sequences, with no two cells possessing the same profile of fragments (Fig 5F, S7). The constant incubation time combined with the negligible correlation between the number of fragments generated in individual cells and amount of Peptide IV delivered by microinjection ($R^2 = 0.15$, Fig S8) indicates that the cleavage site heterogeneity was not a function of the loading technique or duration. While additional single cell analyses will be required to provide further evidence of any subpopulations of cells with differential proteolysis phenotypes, these pilot studies show the ease and feasibility of tracking intracellular peptide fate with standards generated by Pronase E. The variation in fragments formed in single cells as identified by Pronase E standards may provide useful biological information even without certainty of peak assignments, particularly in cases where specific peaks act as bioindicators. Variations in fragment number or degradation rate can be a simple indicator of the activation or inhibition of enzymes or particular pathways upon drug treatment, sample handling practices, or upregulation as a result of a disease state.^{40,41}

CONCLUSIONS

Rapid digestion of peptide sequences with Pronase E and synergistic CE-LIF and MALDI-MS detection of fluorescent fragments was validated *in vitro* and implemented in the identification of fragments from single cells. A small but chemically diverse group of peptide sequences underwent proteolysis with Pronase E, and conditions for the hydrolysis of basic, polar, and nonpolar amino acids within a sequence were demonstrated. Synthesized fragments were employed to verify the identities of sequences in initial studies (Peptides I–III), and then all fragments from Peptide IV were generated and identified from assay mixtures alone. It was shown that limited hydrolytic capabilities of Pronase E towards acidic or non-native amino acids could be overcome by supplementing enzymatic degradation with formic acid-induced peptide cleavage. The formation of peptide fragments was tracked in single PANC-1 cells, and the simultaneous identification of degradation products as well as the rate of their formation was achieved with a combination of Pronase E-generated fragment standards and chemical cytometry. In cells incubated with Peptide IV, there was substantial variation in the identity of fragments formed between cells. This heterogeneity reflects the value of single cell analysis, as the unique fragment profiles formed in each cell would not be visible in population averages.

This approach for the generation of peptide fragments has several advantages over existing methodologies, primarily in its simplicity and flexibility. The relatively nonspecific nature of Pronase E is well suited to the generation of fragments from a wide range of peptides, and by varying the concentration of enzyme mixture, even degradation resistant sequences can be fragmented. Based on our success with post-enzymatic chemical peptide cleavage, it stands to reason that directed chemical cleavage with other reagents in cases where Pronase E is insufficient is possible, and will be the focus of further studies. Overall, the use of Pronase E to rapidly generate fragments should see wide applicability in peptide sequencing, rapid generation of biologically active peptides from larger proteins, and in generation of fragment standards for chemical cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Shan Yang, PhD, for preliminary studies leading to the development of this assay and Gregory Woss for artistic contributions in figure creation. Funding for this research was provided by the NIH (EB011763 and CA177993) to NLA.

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Figure 1.

Schematic of a Pronase E peptide degradation assay. A) Synthesized full length peptides for individual degradation by Pronase E. Uppercase letters represent single letter amino acid abbreviations, Nle is Norleucine, and NH₂ or OH represent amidated or carboxylated C-termini, respectively. Lowercase letters indicate cleavage locations that form the specified fluorescent peptide fragments. Fragment nomenclature for Peptide IV can be found in Supplemental Table S1. B) Each peptide was incubated with active Pronase E. Colored circles represent the different amino acids present in each sequence, and stars represent fluorescein (FAM). C) The resulting mixture containing fluorescent fragments. D) Results of parallel CE-LIF and MALDI- MS analysis identifying fluorescent fragments.

Figure 2.

Separation and detection of Peptide I fragments. A) CE-LIF separation of peptide fragments prepared by SPPS. B) Positive-ion MALDI-MS spectra of synthesized Peptide I fragments. C) CE-LIF separation of fragments generated by incubation of Peptide I with 0.02 µg/mL Pronase E for 15 minutes. D) Positive-ion MALDI-MS spectra of Pronase E assay sample separated in (C).

Figure 3.

Summary of Pronase E degradation profiles for Peptide A) I B) II C) III D) IV. Each plot represents the fragments formed during 15 minutes incubation with the indicated enzyme concentration (µg/mL). The blue layer signifies the Pronase E concentration that generated the most complete distribution of fragments. The percentage of fragment formation (0–100%) is scaled independently for each fragment. Fragment (I–IV)a in each plot indicates the full length peptide sequence. With the exception of Peptide IV (Fragment nomenclature in Supplemental Table S1) each subsequent letter represents the fragment resulting from removal of one residue from the C-terminus. The variation in fragment formation between replicates of each peptide's optimized Pronase E concentration (blue layer) is described in Fig S1.

Figure 4.

Digestions of Peptide III. A) Electropherogram of fragments generated with 500 µg/mL Pronase E and separated with CE-LIF. B) MALDI-MS spectra corresponding to digestion of Peptide III with Pronase E alone C) CE-LIF separation of Peptide III fragments generated with 2% formic acid-assisted Pronase E digestion D) MALDI-MS spectra of Peptide III fragments generated with 2% formic acid-assisted Pronase E digestion.

Figure 5. Identification of fragments formed due to peptide metabolism in single cells based on Pronase E-generated standards. PANC-1 cells were microinjected with Peptide I (A,C,E) or Peptide IV (B,D,F). (A,B) Electropherograms of a single PANC-1 cell 2 min after microinjection. The Pronase E generated standards of Peptide I (C) or IV (D) were loaded into the capillary immediately after loading the cell contents. Electrophoresis was then initiated by applying a voltage to the capillary, which resulted in concurrent separation of fragment standards and cellular contents. Radar plots for Peptide I (E) and Peptide IV (F) indicate the fragments formed in individual cells.