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Separation of peptide fragments of a protein kinase C substrate fused to a β -hairpin by capillary electrophoresis

Imola G. Zigoneanu^{1,2}, Christopher E. Sims³, and Nancy L. Allbritton^{1,2,3}

Nancy L. Allbritton: nlallbri@unc.edu

¹Department of Biomedical Engineering, University of North Carolina, Chapel Hill, NC 27599, USA

²North Carolina State University, Raleigh, NC 27695, USA

³Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, USA

Abstract

Synthetic peptides incorporating well-folded β -hairpin peptides possess advantages in a variety of cell biology applications by virtue of increased resistance to proteolytic degradation. In this study, the WKpG β -hairpin peptide fused to a protein kinase C (PKC) substrate was synthesized, and capillary-electrophoretic separation conditions for this peptide and its proteolytic fragments were developed. Fragments of WKpG-PKC were generated by enzymatic treatment with trypsin and Pronase E to produce standards for identification of degradation fragments in a cellular lysate. A simple buffer system of 250 mM H_3PO_4 , pH 1.5 enabled separation of WKpG-PKC and its fragments by capillary electrophoresis in less than 16 min. Using a cellular lysate produced from Ba/F3 cells, the β -hairpin-conjugated substrate and its PKC α -phosphorylated product could be detected and separated from peptidase-generated fragments produced in a cell lysate. The method has potential application for identification and quantification of WKpG-PKC and its fragments in complex biological systems when the peptide is used as a reporter to assay PKC activity.

Keywords

Capillary electrophoresis; β -Hairpin; Peptide separation; PKC; Protease resistance; Reporter

Introduction

Synthetic peptides serve numerous biomedical applications by providing models for understanding protein folding and stability [1–5], being useful tools for studying antigen presentation [6], and acting as substrates and inhibitors for kinases, peptidases and phosphatases [7, 8]. Despite their importance as bioactive molecules and as tools for biological studies, the use of peptides in the presence of cellular components, either in intact

Correspondence to: Nancy L. Allbritton, nlallbri@unc.edu.

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cells or cell lysates, is limited by rapid proteolysis since most peptides are hydrolyzed to individual amino acids by intracellular peptidases within minutes in the absence of peptidase inhibitors. A number of approaches have been explored to increase peptide stability in the cytosolic environment, including incorporation of unnatural amino acids at defined positions in the peptide [7, 9], cyclization [10], and addition of polyethylene glycol (PEG) moieties [11]. In addition, peptides composed exclusively of β - and γ -amino acids have demonstrated resistance to degradation when exposed to 15 different peptidases in vitro [12, 13]. However, modifications such as the presence of unnatural amino acids or peptide cyclization may impede binding and recognition of the modified peptide compared to that in its native form. The addition of multiple PEG moieties also significantly increases the molecular mass and alters chemical properties of the peptide, which may impact its performance for its designed role.

An alternative approach to increase cytosolic stability is the use of peptides that form secondary structures such as helices, β -sheets, and hairpins [14]. Peptides possessing β -turns that mimic antimicrobial α -peptides have shown increased stability in the presence of proteolytic enzymes while retaining their antimicrobial properties [15]. β -Hairpin peptides comprising only α -amino acids have also demonstrated increased resistance to proteases in vitro [14]. More recently, well-folded β -hairpins that exhibit increased resistance to protease and peptidase degradation have been developed [16–18]. The presence of the turn-inducing sequence, typically Asn-Gly or DPro-Gly, and the cross-strand interactions, especially aromatic and hydrophobic interactions, play an important role in stability of the β -hairpin and consequently in its resistance to peptidases [14, 17, 18]. A peptide having the amino acid sequence Ac-RWVKVNGOWIKQ-NH₂ (here-in referred to as “WKWK”) has proven to be highly resistant to degradation by trypsin, α -chymotrypsin and Pronase E compared to a scrambled peptide control [14]. In comparison to WKWK, a related peptide Ac-RWVKVpGKWIKQ-NH₂ (WKpG) having D-Pro-Gly (pG) in place of Asn-Gly (NG) shows enhanced folding by increasing the cross-strand interactions and thus further stabilizing the β -hairpin [14, 19]. The WKpG peptide has also been demonstrated to confer greater resistance than WKWK to enzymatic cleavage by trypsin, proteinase K, subtilisin, elastase, and collagenase [14, 19].

The concept of conjugating a β -turn peptide to a kinase substrate peptide has been demonstrated to significantly increase proteolytic resistance of the conjugated peptide in cell lysate and intact cells while maintaining its properties as a substrate [10]. The β -turn peptides termed “protectides” were strategically conjugated to the N-terminus of the substrate in order to impede entry of the substrate domain into the catalytic site of peptidases. In those studies, all conjugates containing β -turn peptides exhibited increased lifetime in cellular lysates compared to the native substrate peptide while retaining the ability to be phosphorylated by the kinase [10].

Due to the growing utility of peptides possessing β -turns, there exists a need for analytical methods to quantify and characterize them, especially in cytosolic lysates and intact cells. The most commonly used tool for their separation and quantification has been high-performance liquid chromatography (HPLC) coupled with fluorescence detection, while matrix-assisted laser desorption/ionization mass spectrometry time-of-flight (MALDI-MS

TOF), circular dichroism (CD), and nuclear magnetic resonance (NMR) have also been used for characterization of the β -hairpins and β -turns [3, 14, 20–25]. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been shown to be well-suited for investigation of peptides and other analytes sampled from a cellular environment, particularly as the single-cell level [7, 10, 26] due to its high separation efficiency and superb detection limits ($\sim 10^{-20}$ mol) [7, 8, 10, 27–31]. Enzymatic activity in cell lysates and single cells has been quantified using CE-LIF [7, 32], and phosphorylation of an Abl kinase substrate conjugated to β -turn peptides has been quantitatively assayed in the complex biological matrices of cellular lysates and intact cells [10].

In the current work, method development was undertaken to establish conditions to enable separation of the proteolytic degradation fragments of a highly positively charged peptide composed of a fluorescently labeled WKpG β -hairpin conjugated to the N-terminus of a protein kinase C (PKC) substrate through a PEG₂ linker (WKpG-PKC). This separation was required to assess the potential metabolic products of the peptide prior to use as a substrate for PKC in cell-based assays. The peptide was synthesized by solid phase peptide synthesis, and the proteolytic fragments of the peptide were generated using trypsin and Pronase E. While trypsin is a serine endopeptidase that cleaves predominantly at bulky aromatic and basic residues, Pronase E is a mixture of proteases and cleaves non-specifically [14]. These two proteolytic conditions were employed to create standards for the current and future work. Mass spectrometry was used to identify the fragments generated under both conditions. The optimal buffer composition and CE conditions for separation of these fragments were then investigated and separation of the standards in buffer and from a complex biological sample matrix, a cell lysate, was tested. The peptide was screened in vitro to determine whether it served as a substrate for PKC α . The WKpG-PKC construct was also exposed to a cell lysate and its resistance to degradation characterized.

Materials and methods

Chemicals and reagents

CLEAR-amide resin and 6-chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyClock) were purchased from Peptides International (Louisville, KY). All natural Fmoc-protected amino acids and *N*-hydroxybenzotriazole (HOBt) were received from Advanced ChemTech (Louisville, KY). The lysine protected with ivDde (K-ivDde) and PEG₂ was procured from Chem-Impex International (Wood Dale, IL). Trypsin, Pronase E, and 85 % m/m H₃PO₄ solution in water were purchased from Sigma-Aldrich (St. Louis, MO), and 6-carboxyfluorescein (6-FAM) was obtained from AnaSpec (Fremont, CA). All other chemicals were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA) unless otherwise specified. Roswell Park Memorial Institute media (RPMI-1640) was obtained from Cellgro, penicillin and streptomycin from Gibco (Carlsbad, CA) and fetal bovine serum from Atlanta Biologicals (Flowery Branch, GA).

Enzymatic degradation reactions

The procedure adapted from Cline and Waters [14] was used for trypsin and Pronase E degradation assays. The assay buffer used was 10 mM Na₂HPO₄, 140 mM NaCl, pH 7.6.

For the trypsin assay, 54 μL of 5 μM WKpG-PKC was incubated at 37 $^{\circ}\text{C}$ with 3 μL of 50, 250, and 500 ng/mL trypsin. Aliquots of 10 μL each were collected at 5, 10, 15, 20, and 30 min, and the reactions were terminated by incubation at 95 $^{\circ}\text{C}$ for 4 min. MALDI-MS in the positive ion mode was utilized to confirm the presence of the expected fragments. The aliquots were combined and stored at -20°C for further studies. For the Pronase E assay, 5 μM peptide was incubated at 37 $^{\circ}\text{C}$ with 1, 3, and 5 $\mu\text{g/mL}$ Pronase E, and aliquots were collected at 3, 10, 15, 20, 30, 45, and 60 min. Reactions were also terminated by incubating the aliquots at 95 $^{\circ}\text{C}$ for 4 min. To ensure assay reproducibility and to prepare a large stock of peptide fragments, 162 μL of 10 μM peptide was incubated at 37 $^{\circ}\text{C}$ with 9 μL of 6 $\mu\text{g/mL}$ Pronase E, 55 μL /aliquots were collected at 15, 20, and 30 min, and heat inactivated by incubation at 95 $^{\circ}\text{C}$ for 4 min. MALDI-MS was used to confirm the presence of the fragments. The 3 aliquots were combined and stored at -20°C . The trypsin- and Pronase E-formed WKpG-PKC fragments were diluted in 10 mM Na_2HPO_4 , 140 mM NaCl, pH 7.6 buffer prior to electrophoresis.

Capillary electrophoresis

A commercial CE-LIF system (ProteomeLab PA800, Beckman Coulter, Fullerton, CA, 488 nm) was used for separation of the WKpG-PKC fragments. Fused-silica capillaries of 50 μm inner diameter and 360 μm outer diameter (Polymicro Technologies, Phoenix, AZ) with a total length of 30 cm and an effective length of 20 cm were used for the separation. Sample was loaded into the capillary by applying 0.5 psi (3.4×10^3 Pa) for 5 s to the capillary inlet. A negative voltage applied to the outlet initiated sample electrophoresis. A separation voltage of -6 kV at 80 μA was used to prevent Joule heating unless otherwise specified. A series of buffers was tested for separation of the trypsin and Pronase E generated fragments. The samples were co-mixed with the undigested WKpG-PKC for identification of the peak corresponding to the parent. Tested basic buffers included: 50 mM Na_2PO_4 , pH 8.8; 100 mM Tris, 100 mM Tricine, pH 8.1; 100 mM Tris, 100 mM Tricine, 5 mM SDS, pH 8.1; and 300 mM sodium borate, pH 7.5. Assayed acidic buffers included: 170 mM sodium citrate, pH 4.8; 170 mM sodium acetate, pH 4.0; and 100 to 300 mM Na_2HPO_4 , pH 1–4.8 (see Electronic Supplementary Material (ESM) Table S2). The capillary was washed between runs as follows: NaOH for 2 min; H_2O for 2 min; H_3PO_4 , pH 1.5 for 2 min.

Data analysis

The electrophoretic data were plotted, analyzed and fitted using a Gaussian fit as described previously [33] in Origin 9.0 (OriginLab Corporation, Northampton, MA). Resolution between peaks (R) was calculated based on the approximation $R=2(\mu_2-\mu_1)/1.7(w_{0.5,1}+w_{0.5,2})$ [34] and the number of theoretical plates (N) for each peak as $N=5.54(\mu_1/w_{0.5,1})^2$ [35] where μ_1 and μ_2 are the migration times of the two peaks of interest while $w_{0.5,1}$ and $w_{0.5,2}$ are the peak widths measured at half the peak height. The half-height method was selected as it is commonly used to analyze peaks that are not baseline-resolved and for which it is not possible to measure the width at the baseline [34].

Separation of fragments in a biological matrix

To mimic the sample matrix of a biological experiment, a cellular lysate produced from Ba/F3 cells was used as a sample matrix in separating trypsin or Pronase E generated fragments. Ba/F3 cells, a murine leukemic B lymphocyte cell line, were cultured in RPMI-1640 media supplemented with 10 % fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) and maintained in a humidified atmosphere of 5 % CO₂ and 37 °C. The cells were collected by centrifugation at 0.8×g for 2 min and rinsed twice with phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 27 mM KCl, 1.75 mM KH₂PO₄, pH 7.4). The cell pellet was re-suspended in 0.5 mL 10 mM Na₂HPO₄, 140 mM NaCl buffer, pH 7.6 and lysed through 3 freeze-thaw cycles. The membranes were removed by centrifugation at 14,000×g for 10 min, and the supernatant was collected and stored on ice. A fluorescamine-based assay was used to determine total protein concentration in the cellular lysates [36]. Briefly, the cellular lysates were mixed with 3 mg/mL fluorescamine in a volume ratio of 3-to-1 and incubated at 25 °C for 5 min. A fluorescence plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) with 390 nm excitation and 475 nm emission wavelengths was utilized for quantification. Bovine serum albumin (Calbiochem, Billerica, MA) was used to prepare the standard curve. Aliquots of 3.33 mg/mL total protein concentration of Ba/F3 cell lysates were prepared and stored at -20 °C. For separation of the Pronase E-generated fragments in the cellular lysates, the lysates were heat inactivated at 95 °C for 4 min prior to adding the WKpG-PKC pre-formed fragments. Pronase E fragments (1.7 or 3 µM final concentration) were added to the heat-inactivated Ba/F3 cytosolic lysate to a final concentration of ~3 mg/mL total protein. The samples were analyzed by CE as described in Section 2.4.

In vitro phosphorylation of WKpG-PKC

The PKCα enzyme (Life Technologies, Carlsbad, CA) was pre-diluted in enzyme dilution buffer (20 mM MOPS, 1 mM DTT, 1 mM CaCl₂, pH 7.2) to a final concentration of 1.4 µM. The reaction mixture comprising 140 nM PKCα, 1 µL lipid activator (EMD Millipore Corporation, Temecula, CA), 3 µM WKpG-PKC, 10 mM MgCl₂, and 1 mM ATP was prepared and incubated at 30 °C while aliquots were collected at different time points and analyzed by CE-LIF. The enzyme was inactivated by incubating the mixture at 95 °C for 4 min. The separation of phosphorylated and non-phosphorylated WKpG-PKC was performed on undiluted sample using a CE-LIF system equipped with a 50 µm inner diameter capillary and by applying a voltage of -6 kV to the capillary outlet. The separation of the two species was accomplished using the optimized separation buffer (250 mM phosphoric acid, pH 1.5).

Degradation of WKpG-PKC and PKC in a Ba/F3 cytosolic lysate

The Ba/F3 cell lysate prepared as described above were used for the degradation assay. The WKpG-PKC peptide and the control PKC substrate were incubated with 1.7 mg/mL total protein Ba/F3 cell lysate at 37 °C to a final concentration of 3.3 µM of the peptide. Aliquots were collected at various time points and analyzed by CE-LIF as described above. The sample corresponding to the 60 min time point was co-mixed with phosphorylated WKpG-PKC to confirm the identity of the phospho-peptide generated in the cell lysate.

Results and discussion

Design of the WKpG-PKC peptide

A peptide that incorporates domains containing a substrate for PKC and a β -hairpin region conjugated to the N-terminus of the substrate through a PEG₂ linker (Fig. 1) was designed with the purpose of creating a long-lived fluorescent reporter to measure the activity of PKC in cellular lysate and single cells by CE-LIF. It was hypothesized that this peptide would demonstrate increased resistance to proteolysis in cell-based assays due to the presence of the β -hairpin structure acting to impede the peptide from entering the peptidase active-site cleft. The amino acid sequence RFRRFQTLKIKAKA was selected as the substrate domain since it has been shown to serve as an efficient and specific substrate for PKC isoforms [37, 38]. The substrate and β -hairpin domains were linked through a two-unit PEG moiety to prevent steric hindrance at the kinase catalytic site by the β -hairpin domain. The presence of PEG₂ also increased the solubility of the peptide facilitating its purification and use. The peptide was labeled with 6-FAM at the lysine adjacent to the β -turn of the β -hairpin region to prevent interference with the β -hairpin folding [23, 39]. A control PKC peptide (controlPKC) was also synthesized and labeled with 6-FAM at the N-terminus (6-FAM-RFRRFQTLKIKAKA).

Enzymatic generation of WKpG-PKC standards

There are numerous advantages to using peptide substrates to gain insight into cellular enzymatic pathways [8, 9]; however, once in the presence of cytosolic components, such peptides may be rapidly degraded through proteolysis [9]. For quantification of enzymatic activities by CE-LIF, the substrate, product and degradation fragments must be separated to distinguish the activity of the desired enzyme from proteolysis [7, 8]. For this reason, standards of peptide-fragments were developed to optimize electrophoretic conditions for peptide separation. Generation of each individual fragment through standard solid phase peptide synthesis for a relatively long peptide such as WKpG-PKC would be labor intensive, time consuming and expensive; therefore, enzymatic digestion of WKpG-PKC was undertaken to generate the potential degradation products of the peptide. Digestion in the presence of trypsin or Pronase E was followed by characterization of the fragment mixture by MALDI-MS. The only fragments detected in the presence of these proteases under the tested enzyme concentrations and reaction times were those due to cleavage of the peptide within the PKC substrate domain. Cleavage within the PEG or WKpG motif was not observed (Fig. 2a, b, ESM Figs. S1, S2, respectively). These findings are consistent with a previous report that β -hairpin peptides are resistant to protease degradation unless very high concentrations of proteases are utilized [14]. Since trypsin cleaves peptides at specific sites on the C-terminal end of lysine and arginine [40], a total of 6 fragments and the intact peptide were expected at the concentrations of trypsin used (ESM Table S1). All 7 fluorescently labeled peptides were present in different ratios when varying concentrations of trypsin were employed (2.6, 13.2, and 26.3.0 ng/mL) (Fig. 2a and ESM Fig. S1).

WKpG-PKC fragments formed by incubation with Pronase E, a protease mixture, were also prepared through enzymatic degradation. Fourteen fragments and the intact parent were expected (ESM Table S1). Thirteen species, including the parent, were identified by

MALDI-MS after the parent peptide was incubated with Pronase E (0.3 µg/mL) for 15 and 30 min (Fig. 2b, ESM Fig. S2). All but two possible fragments attainable from hydrolyzing accessible peptide bonds were identified by MALDI-MS after incubation with Pronase E. The missing fragments may not have formed or may not be observable by MALDI-MS due to ion suppression or poor ionization under the conditions tested [41]. Aliquots collected after different incubation times with Pronase E were mixed to create standards for the CE analysis (ESM Fig. S2D).

Optimization of separation conditions

Separation of trypsin-generated fragments—Our goal was to employ a simple method to identify and separate enzymatically generated fragments of WKpG-PKC as standards for future CE-LIF analyses in cell-based biochemical assays. A buffer commonly used to separate basic-to-neutral peptides (100 mM Tris, 100 mM Tricine, pH 8.1) by CE-LIF did not resolve WKpG-PKC and its fragments (Fig. 3a) [9]. Buffer optimization for separation of the trypsin-generated fragments was undertaken initially since fewer peptide fragments permitted the electropherograms to be more easily interpreted. Since fluorescein is only weakly fluorescent at low pH, neutral to basic buffers were assessed initially. The phosphate, tris/tricine, and borate buffers screened using the trypsin-generated fragments were selected due to their common usage in CE for peptide separations (ESM Table S2) [8, 9, 27]. These buffers did not resolve the 7 peaks nor did the buffer systems display reproducible peak migration times. Acidic CE buffers have been successfully used for separation of highly basic analytes including peptides [42–44]. Peptides created by tryptic digestion of human β globin chains have also been separated by CE using acidic buffers [45]. Consequently, buffers in the pH range of 1.5 to 4.8 were tested for separation of the WKpG-PKC fragments since the parent peptide is basic at most pH. Three different buffer systems (citrate, acetate and phosphate) were tested for separation of trypsin-formed fragments (ESM Table S2). The phosphate buffer system yielded seven identifiable peaks whereas the citrate and acetate buffers yielded few resolvable peaks (data not shown). Thus, the phosphate buffer was selected for further optimization.

To improve the separation of trypsin-generated fragments, the H_3PO_4 concentration was varied from 20 to 300 mM (pH 1.5). When a low H_3PO_4 concentration (100 mM) was used, seven peaks were not identifiable; however, greater concentrations (200–250 mM) did reveal 7 peaks on the electropherogram (ESM Figs. S3B, S5A, S5B). At concentrations of H_3PO_4 greater than 250 mM, multiple peaks were observed but poorly resolved. Based on these results, 250 mM was selected as the optimal H_3PO_4 concentration. The pH of the 250 mM H_3PO_4 buffer was also varied between 1 and 3 (ESM Table S2). When judged qualitatively, pH 1.5 yielded the best separation (Fig. 3b, c, ESM Figs. S5A, S5B); consequently, 250 mM H_3PO_4 pH, 1.5 buffer was used in all subsequent steps. The parent WKpG-PKC peptide also exhibited a sharp peak when electrophoresed in the optimized buffer (ESM Fig. S4). Under the optimal electrophoretic conditions, the 6 fragments and the parent peptide were separated in less than 16 min. WKpG-PKC possessed a migration time of 592 ± 5 s and separation efficiency (N) of $8.3 \pm 2.6 \times 10^4$ theoretical plates per meter. The resolution between the parent and the first eluting fragment was 0.76 ± 0.02 ($n=3$) (Fig. 3c, Table 1). The migration times of all 7 analytes were highly repeatable, and the resolution

between all other fragments was >2 . Separation of the 7 peptides was also achieved using different sample matrices (ESM Table S3).

The limit of detection (LOD), calculated as the amount of WKpG-PKC peptide required to yield a signal-to-noise ratio of 3 under the optimized experimental conditions, was determined to be $4.7 \pm 0.07 \times 10^{-13}$ moles. The poor LOD was likely due to the use of the fluorophore chosen to label the peptide (fluorescein) which is protonated and weakly fluorescent in acidic buffer. An improved LOD could readily be achieved by labeling the peptide with a pH-insensitive fluorophore, for example, BODIPY®FL dye.

Separation of Pronase E-generated fragments—The peptide mixture formed by incubation of the WKpG-PKC peptide with Pronase E was separated in 250 mM H_3PO_4 , pH 1.5. When the parent peptide was incubated with 0.3 $\mu\text{g/mL}$ Pronase E for 15 to 30 min, the fragments formed were derived from cleavage within the PKC substrate sequence (Fig. 2b, ESM Fig. S2). As discussed previously, 13 of the expected fragments were identified by MALDI-MS and the presence of 12 peaks was demonstrated by CE-LIF (Fig. 3d, ESM Figs. S5C, S5D). While more peaks were identified by using the Gaussian fit, only those that have a signal-to-noise ratio higher than 3 were selected. Moreover, all the peptide fragments were separated in less than 16 min. Since a future goal was to assay peptide fate in the presence of cellular constituents, a Ba/F3 cell lysate was used as a sample matrix for the Pronase E-formed peptide standards. The samples of the proteolytic standards were mixed into the cell lysate and electrophoresed (Fig. 4a, b). All 12 analytes were visualized within 16 min with migration times having standard deviations between ± 0.4 and ± 1.2 s ($n=5$ runs). The WKpG-PKC peak was only partially resolved from fragment α ; however, the separation appeared sufficient to determine whether fragment α was being formed when the parent peptide was placed into a complex cellular mixture with proteolytic capabilities. The detection of the Pronase E-generated fragments in a complex matrix with partial resolution was accomplished, demonstrating that a simple buffer system was suitable for the CE-based separation of this reporter and its proteolytic metabolites in a complex environment.

Separation of the WKpG-PKC peptide substrate and product after incubation with PKC α

To confirm that the WKpG-PKC peptide remained a substrate for PKC and the two forms could be identified under the CE conditions optimized for separating the proteolytic products, the peptide was incubated in the presence of purified PKC α . Near complete phosphorylation of the peptide was achieved in 3 h, as confirmed by CE-LIF (ESM Figs. S6A, S6B). Analysis by MALDI mass spectrometry demonstrated the presence of an analyte of the same molecular mass as the phosphopeptide. A negative control omitting ATP from the incubation mixture showed no phosphorylation of the peptide under the same conditions. Non-phosphorylated (1.5 μM) and phosphorylated (1.5 μM) WKpG-PKC was co-mixed, diluted in the sample matrix buffer, and electrophoresed using the optimized buffer conditions. The two components were separated with migration times of 599 ± 1.8 s for non-phosphorylated and 659 ± 6 s for phosphorylated WKpG-PKC, respectively (ESM Fig. S6C). The resolution between the two peaks was 2.5 ± 0.4 while the number of theoretical plates was $1.5 \pm 0.8 \times 10^4$ for non-phosphorylated WKpG-PKC peptide and $1.1 \pm 0.2 \times 10^4$ for the phosphorylated peptide (Table 1).

Proteolysis of the WKpG-PKC peptide in a Ba/F3 cell lysate

To evaluate generation of proteolytic fragments of the WKpG-PKC peptide in a cell lysate, the conjugated peptide was incubated with a Ba/F3 cell lysate for 60 min. The electropherogram of this sample revealed only two peaks supporting little degradation of the peptide with only 6.1 % of the peptide appearing in a single fragment peak (Fig. 5a, b). For comparison purposes, control experiments using the unconjugated peptide (controlPKC) were incubated with cell lysate. ControlPKC was almost completely degraded within 60 min (Fig. 5c, d). While the identity of the fragment formed from WKpG-PKC was not known, the fragment co-migrated with fragment θ formed in the Pronase E reaction mixture (when the reaction mixture alone was electrophoresed in the same capillary and under the same experimental conditions). The cell lysate-WKpG-PKC reaction mixture was co-mixed with phosphorylated WKpG-PKC and electrophoresed to assay separation of WKpG-PKC, phosphorylated WKpG-PKC, and fragment θ (ESM Fig. S7). The intact non-phosphorylated and phosphorylated WKpG-PKC peptides possessed distinct and repeatable migration times (631.3 ± 3.3 and 681.8 ± 1.4 s, respectively) from that of the degradation fragment (774.1 ± 4.5 s), demonstrating that the optimized separation conditions enabled separation of the 3 species from within the complex milieu of the lysate. Furthermore, the optimized separation conditions permitted identification of the number of fragments formed within the lysate, an attribute critical to understanding the extent of degradation of the WKpG-PKC by cytosolic peptidases.

Conclusion

In the current work, a simple buffer system was developed for reproducible separation of multiple peptide fragments generated by enzymatic metabolism of a peptide-based kinase substrate possessing a β -hairpin structure. The separations were routinely reproducible with only a simple wash step between analytical runs. Two enzymatic digestions using trypsin and Pronase E generated 7 and 12 peptide fragments, respectively. Mass spectrometry revealed that under the conditions used, the fluorescently labeled β -hairpin domain and PEG₂ linker remained intact after incubation with these degradative enzymes, cleavage occurring only within the substrate in the C-terminal half of the construct. The optimized buffer method demonstrated CE-based separation of the majority of the fragments generated under both conditions in a simple buffer system in less than 16 min. Moreover, all of the fragments were identifiable when the sample matrix was comprised of a concentrated cellular lysate. The non-phosphorylated and phosphorylated forms of the β -hairpin peptide were also separated in the same buffer optimized for separation of the proteolytic fragments. However, under the acidic separation conditions, fluorescein was protonated, resulting in greatly decreased detection limits for the peptides. While the method is expected to be a valuable tool for quantification of PKC activity in small biological samples, such as single cells, the substrate peptide properties must be enhanced by addition of a pH-insensitive fluorophore in order to achieve the needed limits of detection for ultra-small samples such as single cells. Additionally, if desirable, the electrophoretic separation can be directly coupled to electrospray mass spectrometry to definitively identify all peptide fragments. As expected, the degradation profile of the WKpG-PKC peptide in a Ba/F3 cell lysate demonstrated little proteolysis by virtue of the β -hairpin domain. This was in contrast to that

of the peptide lacking the β -hairpin, controlPKC, which showed almost complete degradation. The separation method has potential application for the measurement of PKC activity and metabolic processes in complex biological systems where knowledge of fragment migration times is important for quantification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

6-FAM	6-Carboxyfluorescein
Ac	Acetyl
CE-LIF	Capillary electrophoresis with laser-induced fluorescence
DBU	1,8-Diazobicyclo[5.4.0]undec-7-ene
DIPEA	<i>N,N'</i> -Diisopropylethylamine
DMF	<i>N,N</i> -Dimethylformamide
HBTU	<i>O</i> -(Benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HOBt	<i>N</i> -Hydroxybenzotriazole
MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometry time-of-flight
MM	Multiple myeloma
NMP	<i>N</i> -Methylmorpholine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PKC	Protein kinase C
PyBOP	Bromo-tris-pyrrolidono phosphonium hexafluorophosphate
PyClock	6-Chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane

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Fig. 1.
Chemical structure of WKpG-PKC

Fig. 2.

MALDI-MS of enzymatically generated fragments of WKpG-PKC. **A** MALDI-MS spectrum of the mixed aliquots obtained after incubation of WKpG-PKC with trypsin (2.6, 13.2, and 26.3 ng/mL) for 5, 10, 15, 20, and 30 min. **B** MALDI-MS spectrum of the mixed aliquots obtained after incubation of WKpG-PKC with Pronase E (0.3 $\mu\text{g/mL}$) for 15, 20, and 30 min. The letters designate the parent (*a*) and fragments (*b–o*) (ESM Table S1) and the peaks marked *asterisk* co-migrate with impurities in the synthesized WKpG-PKC

Fig. 3.

CE-LIF-based separation of trypsin and Pronase E-generated WKpG-PKC fragments. **A** Separation of WKpG-PKC parent and the 6 trypsin-generated fragments in 100 mM Tris, 100 mM Tricine, pH 8.1. **B** Separation of WKpG-PKC trypsin-formed peptides using 250 mM H₃PO₄, pH 1.5 as electrophoretic buffer. **C** Separation of WKpG-PKC fragments formed by trypsin after co-mixing with the parent peptide. The electrophoretic buffer was 250 mM H₃PO₄, pH 1.5. **D** Separation of the Pronase E-generated fragments using 250 mM H₃PO₄, pH 1.5 as electrophoretic buffer. Since the correspondence between the MALDI-MS peaks and the electrophoretic peaks was unknown except for the parent peptide, the electropherogram peaks are labeled with numbers and Greek letters. The peaks marked *asterisk* co-migrate with impurities in the synthesized WKpG-PKC

Fig. 4.

Separation of Pronase E-generated fragments in the optimized buffer using a cellular lysate as a sample matrix. WKpG-PKC (1.7 μ M panel **A** or 3.0 μ M panel **B**) was incubated with Pronase E for 15, 20, and 30 min and the aliquots combined. The resulting reaction mixture was added to a Ba/F3 cell lysate (3 mg/mL) and then electrophoresed. The *Greek letters* represent the fragments while the peaks marked *asterisk* co-migrate with impurities in the synthetic WKpG-PKC

Fig. 5.

Degradation of WKpG-PKC and controlPKC in a Ba/F3 cellular lysate. WKpG-PKC was incubated in the cytosolic lysate for 0 (A) or 60 min (B) and then electrophoresed. The peaks marked “ θ ” and “*” co-migrate with fragment θ or impurities in the synthetic WKpG-PKC. ControlPKC was incubated in the cytosolic lysate for 0 (C) or 60 min (D) and then electrophoresed. “G1,” “G2,” “G3” label fragments formed from the peptide, controlPKC

Migration time, peak area, separation efficiency and resolution of WKpG-PKC trypsin-generated fragments and phosphorylated peptide (*P*) separated in 250 mM H₃PO₄, pH 1.5 using 10 mM Na₂HPO₄, 140 mM NaCl, pH 7.6 as a matrix; *n*=3

Table 1

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	<i>P</i>
Migration time (s)	592±5	604±5	627±5	651±6	707±7	748±8	903±11	659±6.0
Peak area (×10 ⁴)	1.6±1.3	74.6±3.3	3.79±0.4	77.9±5.4	48.5±4.4	3.04±0.5	13.2±1.7	27.12±3.0
Peak efficiency (N×10 ⁴)	8.3±2.6	6.2±0.2	8.1±0.5	5.9±0.2	5.7±0.2	5.3±0.2	4.0±0.2	1.1±0.2
Resolution	Peaks 1,2 ^a 0.76±0.02	Peaks 2,3 2.38±0.04	Peaks 3,4 2.54±0.02	Peaks 4,5 4.93±0.08	Peaks 5,6 3.30±0.03	Peaks 6,7 9.99±0.05	Peaks 1,P 2.5±0.4	

^aThe resolution between peak 1 and peak 2 was calculated based on the electropherogram corresponding to the trypsin fragments co-mixed with the parent