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Analysis of derivatized peptides by capillary electrophoresis

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ABSTRACT

Thirteen synthetic prothrombin leader peptides differing only in C terminus were analyzed by capillary electrophoresis. These peptides were derivatized using the novel, fluorescent derivatizing agent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Derivatized peptides were detected using fluorescence and ultraviolet absorption signals, and underivatized peptides were detected using ultraviolet absorbance at 185 nm. Data are presented which compare the analysis of the derivatized and underivatized peptides.

INTRODUCTION

The analysis of peptides is important for a wide variety of samples such as physiological fluids, foods, protein digests and synthetic mixtures. Peptide analysis is frequently performed using either high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Historically, high sensitivity peptide detection has been difficult because peptides lack a strong UV absorbing chromophore. Detection in the low UV range (215 nm and below) is often used, but interference from the solvents and other modifiers present can be prohibitive. In order to avoid the complications involved with low UV measurements, peptide derivatization techniques are often employed which allow mid UV and fluorescence detection [1]. In addition, fluorescence detection greatly enhances analytical selectivity and sensitivity.

There are a number of essential requirements for successful peptide derivatization. Ideally, the derivatizing agent should produce a single, stable derivative for each target analyte. The derivatization reaction should be rapid and simple, and quantitative yields should be obtained easily. The derivatizing agent should generate a strong signal especially for high-sensitivity analyses, and the reaction byproducts should not complicate the separation and analysis.

A number of peptide derivatizing agents have been recently reported including *ortho*-phthalaldehyde (OPA), ninhydrin, fluorescamine, naphthalene-2,3-dicarboxaldehyde (NDA/CN), 9-fluorenylmethyl chloroformate (FMOC), 1-dimethyl aminonaphthalenesulfonyl chloride (Dansyl), and phenylisothiocyanate (PITC) [2–5]. All of these tags are sensitive for the analysis of primary amines, but each has unique disadvantages. OPA, which is the most popular derivatizing agent, ninhydrin and fluorescamine produce relatively unstable derivatives. In addition, ninhydrin is not useful for CE since post capillary derivatization is not available. NDA/CN, the naphthalene analogue of OPA, produces

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stable derivatives, but the reaction byproducts produced when in the presence of an excess of reagent complicate the analysis [5]. Also, the pH conditions for derivatization are very specific and vary from peptide to peptide, rendering quantitative derivatization of peptide mixtures very difficult. Both FMOC and Dansyl react with water to produce large hydrolysis product peaks which interfere with the analysis. In the case of PITC, the derivatization is complicated by the fact that excess reagent must be removed prior to analysis.

The derivatization of amino acids using the highly fluorescent derivatizing agent, 6-aminoquinolyl - N - hydroxysuccinimidyl carbamate (AQC) has been demonstrated and has a number of advantages [6,7]. This derivatizing agent reacts quickly and easily with both primary and secondary amines, and the derivatives are highly stable (see Fig. 1). Due to the unique fluorescence characteristics of the derivatizing agent, the fluorescence signal produced by the hydrolysis byproducts is significantly reduced, eliminating the problem of interference with the analysis [6]. In addition, the reaction is not hindered by buffer salts such as sodium phosphate, sodium acetate, sodium citrate, sodium chloride and sodium dodecyl sulfate which may be present in peptide containing samples. In this research, the feasibility of peptide derivatization with AQC was evaluated.

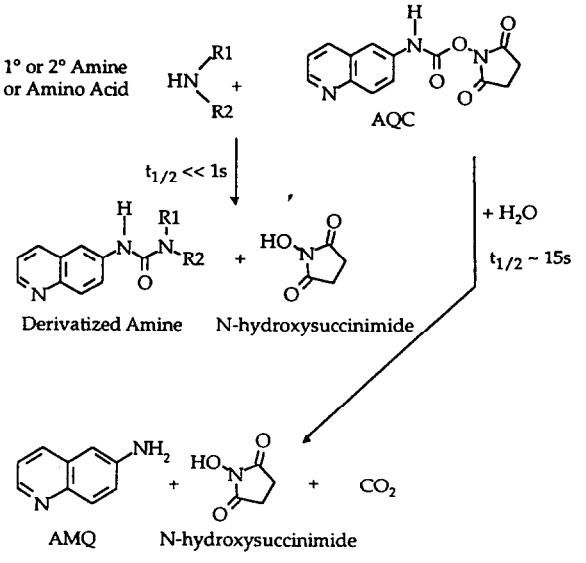

Fig. 1. AQC derivatization reaction [6,7].

Thirteen synthetic prothrombin leader sequences, ANKGFLEEX (where X is valine in the unmodified sequence), differing only in C terminus were chosen as the model. The different C termini were aspartic acid (PT-Asp), serine (PT-Ser), glutamic acid (PT-Glu), glycine (PT-Gly), threonine (PT-Thr), alanine (PT-Ala), valine (PT-Val), methionine (PT-Met), lysine (PT-Lys), isoleucine (PT-Ile), leucine (PT-Leu), phenylalanine (PT-Phe), and tryptophan (PT-Trp). AQC reacts with the N terminal amine group as well as the amine groups of all lysine residues. As a result, all of the peptides except PT-Lys have two possible derivatization sites, and PT-Lys three potential sites. The effect of differing numbers of tags will be discussed.

EXPERIMENTAL

Materials, chemicals and reagents

Acetonitrile (ACN) and boric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA), and ethylenediaminetetraacetic acid (calcium disodium salt, EDTA) was purchased from EM Science (Gibbstown, NJ, USA). AQC (Waters AccQ · Fluor reagent, Millipore, Milford, MA, USA) was synthesized according to ref. 6. Deionized water, which was used in all eluents, was purified on site using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Glass tubes (50 × 6 mm) were purchased from Corning Glassworks (Corning, NY, USA), and were pyrolyzed at 500°C for 6 h prior to use.

Peptide synthesis

Solid-phase peptide synthesis was performed using FMOC methodology on a 9050 Plus flow peptide synthesizer (Millipore) [8]. Preactivated pentafluorophenyl esters were used for peptide assembly on a solid polyethylene glycol polystyrene support (Millipore). After synthesis, the peptide was cleaved from the solid support and deprotected using a solution of trifluoroacetic acid–triethylsilane (95:5).

Sample preparation

A 10-μl aliquot of a peptide containing solution was delivered to a pyrolyzed 50 × 6 mm glass tube. A 70-μl volume of borate buffer (200 m*M* boric acid and 5.0 m*M* EDTA, pH 8.8) was added and the mixture was vortexed. A 20-μl volume of a solution containing the AQC reagent (3 mg/ml dissolved in acetonitrile) was added and the solution was vortexed quickly to insure complete derivatization of all amines. The samples were then heated for 10 min at 50°C and injected. The underivatized peptide samples were prepared in the same fashion using ACN instead of AQC reagent.

Apparatus

The derivatized and underivatized peptides samples were analyzed under identical conditions using two separate CE systems (described below as systems 1 and 2). The eluent was an aqueous borate buffer (50 m*M*, pH 8.5). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 69 cm to the window × 75 mm I.D. The applied voltage was 14 kV and the resulting current was 11–12 μA. The samples were injected hydrostatically for 6 s unless otherwise noted.

CE System 1 was a Waters Quanta 4000 (Millipore). The UV absorbance signal was measured at 254, 214 and 185 nm. Electrophoretic data were collected using Expert Ease software (Millipore).

CE System 2 was a homemade system consisting of a DA-30 high-voltage power supply (Spectrovision, Chelmsford, MA, USA) and a Waters M470 scanning fluorescence detector (Millipore) with a modified flow cell for CE. The excitation wavelength was 250 nm and the emission wavelength was 395 nm. Hydrostatic injections were made using a pneumatic piston (Bimba, Monee, IL, USA) which drove a timed lift. Electrophoretic data were collected using Maxima software (Millipore).

RESULTS

Peptide detection by UV and fluorescence

Both the derivatized and underivatized PT-Ser peptide were analyzed on both systems using four different detection conditions. The peptide was analyzed on system 1 using 254, 214 and 185 nm detection, and on system 2 using fluorescence detection. The optimal fluorescence excitation and emission wavelengths for the aminoquinolyl tag are 250 and 395 nm, respectively. The absorbance signal generated by the underivatized peptide was strongest at 185 nm, and it was not detected at all at 254 nm. Apparently, the presence of phenylalanine in the peptide was insufficient to produce a peak. The derivatized peptide was detected strongly at all UV and fluorescence wavelengths. See Fig. 2 for the electropherograms of derivatized and underivatized PT-Ser.

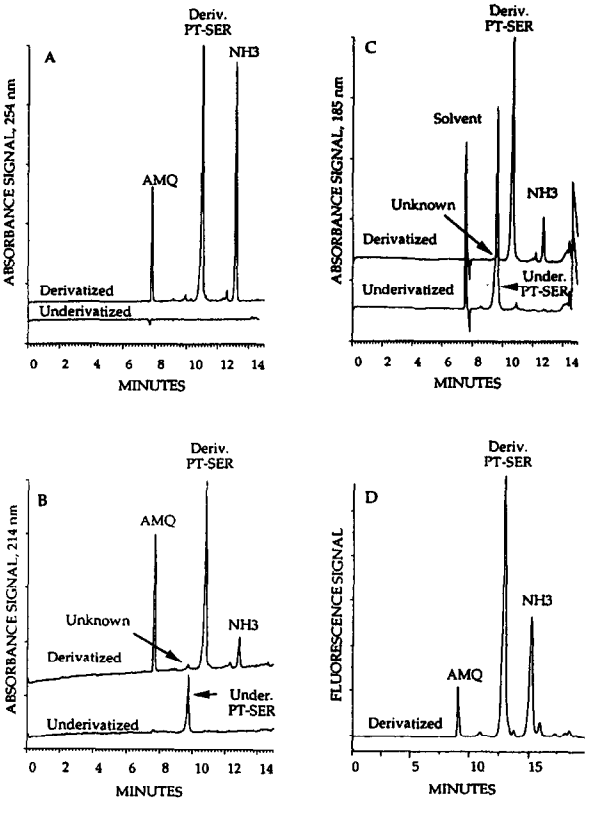

Fig. 2. (A) Derivatized and underivatized PT-Ser detected on system 1 at 254 nm. (B) Derivatized and underivatized PT-Ser detected on system 1 at 214 nm. (C) Derivatized and underivatized PT-Ser detected on system 1 at 185 nm. (D) Derivatized PT-Ser detected on system 2 using fluorescence detection. The excitation wavelength is 250 nm, and the emission wavelength is 395 nm.

Derivatization

We have recently demonstrated the rapid, quantitative formation of stable urea derivatives via reaction of AQC with amine compounds such as amino acids. Thus, the derivatization is expected to label the peptide at the free amino terminus, and on the amino-containing side chain of any lysine residues in the peptide. Excess reagent is hydrolyzed to AMQ, N-hydroxysuccinimide and carbon dioxide.

Derivatization yields of better than 95%, as determined by a loss of starting material, were observed for all peptides studied. Fig. 2 shows overlayed electropherograms of underivatized and derivatized PT-Ser peptides. At 214 nm, where both the underivatized and derivatized peptides generate a strong signal, a small unknown peak in the electropherogram of the derivatized peptide appears to be coeluted with the underivatized peptide. This peak, which correlates to 3% of underivatized peptide, is not the underivatized peptide because it is also visible at 254 nm where the underivatized peptide is not detected.

The addition of insufficient reagent will result in incomplete derivatization [6]. Fig. 3 contains overlays of two derivatized PT-Lys samples. In the upper electropherogram, there are two peaks corresponding to derivatized PT-Lys. The later peak is the completely derivatized peptide, and the earlier peak is mono- and/or di-derivatized PT-Lys. As has been shown for amino acids, addition of a second aliquot of reagent to the sample eliminated the first peak indicating that the derivatization was completed.

Linearity and reproducibility

Reproducibility of migration time (MT) and peak height was determined for six consecutive injections of derivatized PT-Ser using system 1 at 254 nm and system 2. Reproducibility of migration time increases significantly when the relative migration time (RMT) is calculated with respect to the AMQ peak. The relative standard deviation of the RMT is 0.3% for system 1 and 0.2% for system 2. These numbers are significantly improved from the R.S.D. of the MT which were 3 and 2% for systems 1 and 2, respectively (Table I).

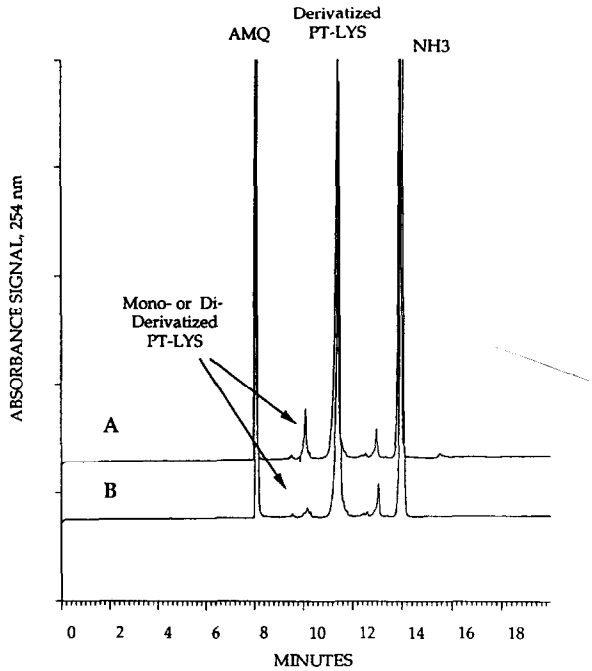

Fig. 3. (A) Incomplete derivatization of PT-Lys. (B) PT-Lys sample after the addition of another aliquot of reagent.

Linearity and the lower detection limits (LDL) were determined for derivatized and underivatized PT-Ser (Table II). Derivatized and underivatized peptide samples were prepared at the concentration levels of 660, 2700, 11 000, 42 000 and 170 000 n*M*. The derivatized samples were analyzed on system 1 at 254 nm and system 2, and the underivatized samples were analyzed on system 1 at 185 nm. Good linearity was achieved for all three systems with correlation coefficients better than 0.997 in the cases. The LDL ($S/N = 3$) was determined for each of the three condi-

TABLE I

REPRODUCIBILITY OF SYSTEM 1 AND SYSTEM 2

$n = 6$.

System	R.S.D. (%)		
	MT	RMT	Height
1 (254 nm)	3	0.3	2
2 (Fluorescence)	2	0.6	8

TABLE II

LINEARITY AND LOWER DETECTION LIMITS

System	Peptide	Correlation coefficient	LDL (nm)	LDL (fmol)
1 (185 nm)	Underivatized PT-Ser	0.997	300	11
1 (254 nm)	Derivatized PT-Ser	0.999	400	15
2 (Fluorescence)	Derivatized PT-Ser	0.999	300	11

tions. The LDL for both the underivatized peptide at 185 nm and the derivatized peptide on system 2 with fluorescence detection was 300 n*M* which corresponds to a mass detection limit of approximately 11 fmol of peptide on capillary. The LDL for the derivatized peptide on system 1 at 254 nm was slightly higher than these at 400 n*M* which corresponds to a mass detection limit of approximately 15 fmol of peptide on capillary.

Peptide resolution

Each of the thirteen peptides was analyzed in the underivatized state on system 1 at 185 nm and derivatized state on system 1 with UV detection at 254 nm. The underivatized peptides had positively charged lysine residues and, most likely, neutral N termini under the analysis conditions used (pH 8.5). In addition, the free carboxyl terminus and both of the glutamic acid residues were in the carboxylate form. Thus, the overall net charge of the underivatized peptides was −1 for PT-Lys, −3 for PT-Asp and PT-Glu, and −2 for the others except for PT-Ala. The net charge on PT-Ala was −1 because a glutamic acid was accidentally deleted from the sequence (as determined by amino acid analysis). Table III lists the RMT values for the underivatized peptides. As expected, PT-Asp and PT-Glu have the largest RMT values (1.44) because they have the largest negative charge. PT-Ala and PT-Lys have the smallest RMT values (1.14 and 1.12, respectively) because they had the smallest negative charge.

The aminoquinolyl tag is uncharged pH 8.5. As a result, derivatization at the positive lysine residues reduces the overall positive charge on the peptide causing it to be eluted later on the CE system (positive power supply). Since PT-

TABLE III

PEPTIDE MIGRATION

Peptide	MT		RMT		ΔRMT
	Underivatized	Derivatized	Underivatized	Derivatized	
PT-Asp	12.136	12.675	1443	1.586	0.143
PT-Ser	10.642	11.369	1.283	1.429	0.146
PT-Glu	11.917	12.617	1.442	1.584	0.142
PT-Gly	10.675	11.421	1.291	1.437	0.146
PT-Thr	10.500	11.242	1.287	1.418	0.140
PT-Ala	9.367	10.146	1.139	1.284	0.145
PT-Val	10.225	11.242	1.278	1.421	0.143
PT-Met	10.188	11.533	1.279	1.427	0.148
PT-Lys	8.291	11.454	1.124	1.415	0.291
PT-Ile	10.129	11.575	1.278	1.425	0.147
PT-Leu	10.158	11.613	1.278	1.425	0.146
PT-Phe	10.161	11.767	1.279	1.430	0.151
PT-Trp	10.138	11.800	1.275	1.427	0.152

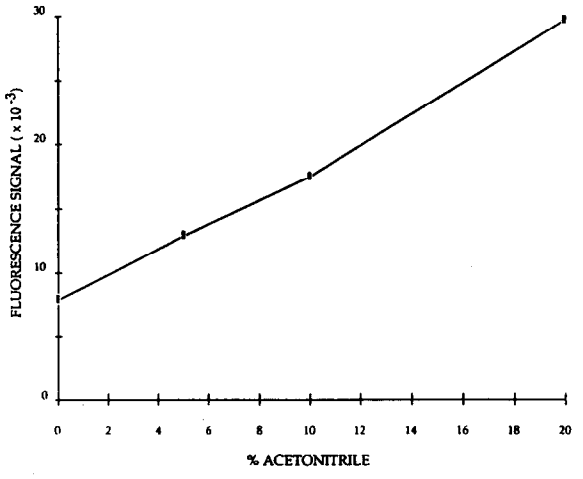

Fig. 4. Fluorescence signal enhancement in the presence of ACN. Signal intensity was measured for derivatized PT-Ile in the presence of 0, 5, 10 and 20% ACN.

Lys is doubly positive in the underivatized state, derivatization has a larger relative effect on its migration time. Table III lists the differences in RMT between the derivatized and underivatized peptides (ΔRMT). The values for all of the peptides except PT-Lys are approximately the same (0.14 to 0.15). PT-Lys has an RMT difference value that is twice the other values (0.29). This is to be expected because two positive charges are eliminated from the PT-Lys upon derivatization, but only one positive charge is eliminated from the others.

Fluorescence signal enhancement

Derivatized PT-Ile was analyzed using four different eluents containing varying percentages of ACN. The eluents were the borate buffer (50 m*M*, pH 8.5) with 0, 5, 10 and 20% ACN. The eluent containing 20% ACN was found to enhance the fluorescence signal by a factor of 3 to 4 over the buffer without ACN. These results agree with data previously generated for derivatized alanine [6] (Fig. 4).

DISCUSSION

AQC was shown to be an effective reagent for the derivatization of peptides. Complete derivatization took less than one minute, and the derivatives were stable for at least several days. It has been demonstrated for amino acids that the derivatives are stable for a minimum of one week at room temperature [6]. Unreacted AQC was hydrolyzed, and the byproducts of derivatization and hydrolysis did not interfere with the CE analysis. Derivatization yields of close to 100% were routinely achieved. It has been previously reported for amino acids that a 4–5-fold excess of AQC to amine is sufficient to generate quantitative yields of derivatized product [6]. However, when insufficient reagent was added to the PT-Lys peptide sample, quantitative conversion was achieved simply by adding a second aliquot of reagent.

RMT is a useful indicator for peptide charge characteristics. The underivatized peptides with the smallest RMT values were the peptides with the smallest negative charge (PT-Lys and PT-Ala which had a glutamic acid deletion), and the peptides with the largest RMT were those with the largest negative charge (PT-Asp and PT-Glu). The remaining underivatized peptides have a median RMT of approximately 1.28. In the case of the derivatized peptides, PT-Ala (net charge −2) is the only peptide with a low RMT value (1.28), and PT-Glu and PT-Asp still have the highest RMT values (1.58). The RMT value for derivatized PT-Lys is in line with the median peptides (1.43) since both of its positive charges have been eliminated. Note that derivatized PT-Ala has an RMT which is approximately equal to those underivatized peptides with net −2 charge, and that underivatized PT-Asp and PT-Glu have similar RMT values to the majority of the derivatized peptides, all of which have a net charge of −3.

The RMT value for derivatized PT-Lys is 1.42, and the RMT value for the incompletely derivatized peptide peak was 1.25. The ΔRMT value for these peaks were 0.29 and 0.13, respectively. Since 0.13 is close to the ΔRMT value generated for the peptides which have a net charge change of one, it is likely that the small peak is the result of incomplete derivatization at one of the lysine residues, rather than the N terminus. This is consistent with the RMT data and with data reported previously which indicated that the ε amine on lysine is less reactive than the α amino

group, and when reagent is limited, lysine is incompletely derivatized [6].

Selectivity of derivatized peptides is influenced by the number of derivatizable lysine residues. The ΔRMT was greater for PT-Lys because it had one more derivatization site than the other peptides, and thus the change in net charge was larger. Electrolyte pH will influence the effect that the number of derivatizable lysine groups will have on migration time. For instance, if the pH of the buffer is below the pK_a of the lysine residues, the N terminus, and the AMQ tag, or above the pK_a of all three, the effect will not be as dramatic. In both situations, the net peptide charge will not be changed upon derivatization. In the pH range used in this study, the addition of the derivatizing agent changed the overall charge of the peptide. In addition, though it was not demonstrated here, the sulfhydryl group of the amino acid cysteine is also derivatizable and could potentially be used to influence resolution.

A potential problem with fluorescence detection in aqueous environments is fluorescence quenching. The use of cyclodextrins in the eluent has been reported to be useful for fluorescence signal enhancement of derivatized peptides [9,10]. In this paper, a non-polar solvent was used to enhance the fluorescence signal by a factor of 3 to 4. As a result, the LDL was reduced to below 100 n*M* (mass detection limit less than 4 fmol) which is significantly better than the LDL for the underivatized peptides under their optimal detection conditions at 185 nm.

REFERENCES

1 M.C. Polo, D. Gonzalez de Llano and M. Ramos, in L.M.L. Nollet (Editor), *Food Analysis by HPLC*, Marcel Dekker, New York, 1992, pp. 117–140.
2 H. Konig, H. Wolf, K. Venema and J. Korf, *J. Chromatogr.*, 533 (1990) 171.
3 S. Lunte and O.S. Wong, *Curr. Sep.*, 10 (1990) 19.
4 R.G. Carlson, K. Srinivasachar, R.S. Givens and B.K. Matuszewski, *J. Org. Chem.*, 51 (1986) 3978.
5 S.M. Lunte and O.S. Wong, *LC·GC*, 7, 11 (1989) 908.
6 S.A. Cohen and D.P. Michaud, *Anal. Biochem.*, 211 (1993) 279.
7 S.A. Cohen, K.M. De Antonis and D.P. Michaud, in R.H. Angelletti (Editor), *Techniques in Protein Chemistry IV*, Academic Press, New York, 1993, pp. 289–298.
8 A. Rustici, M. Velucchi, R. Faggioni, M. Sironi, P. Ghezzi, S. Quataert, B. Green and M. Porro, *Science*, 259 (1993) 361.
9 J. Liu, K.A. Cobb and M. Novotny, *J. Chromatogr.*, 519 (1990) 189.
10 J. Liu, Y.-Z. Hsieh, D. Wiesler and M. Novotny, *Anal. Chem.*, 63 (1991) 408.