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Analysis of endoproteinase Arg C action on adrenocorticotrophic hormone by capillary electrophoresis and reversed-phase high-performance liquid chromatography[a]

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ABSTRACT

The specificity and rate of cleavage of adrenocorticotrophic hormone (ACTH) peptide bonds by endoproteinase Arg C were analyzed using capillary electrophoresis (CE) and reversed-phase (C,,) high-performance liquid chromatography (HPLC). Acidic cleavage products were readily resolved by CE in uncoated capillaries using low ionic strength electrolytes. However, products predicted to have a net positive charge greater than 2 or more than 4 positively charged groups per peptide did not migrate out from the capillary at low ionic strength. Addition of salts and zwitterions to the electrolyte decreased capillary-peptide interactions such that all of the ACTH peptides examined were eluted with high efficiency separation by CE. Commercially obtained endoproteinase Arg C preparations exhibited peptidase activity at Lys^{15}–Lys^{16} and at Lys^{16}–Arg^{17} in addition to the expected cleavage at Arg-X bonds. ACTH peptide bond cleavage rates for Arg^{8}–Trp^{9}, Arg^{17}–Arg^{18}, Lys^{15}–Lys^{16}, and Lys^{16}–Arg^{17} were 1.46, 0.096, 0.057, and 0.029 $\mu mol\,min^{-1}\,mg^{-1}$ respectively. CE separations generally exhibited better resolution and were accomplished in shorter times than C_{18} HPLC separations. These properties make CE a particularly appropriate method for kinetic analysis of proteolytic enzyme action on peptide substrates.

INTRODUCTION

Development of high efficiency peptide separation methods has stimulated progress in areas as diverse as purification of prohormone processing proteinases [1]

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and the study of protein structure using tryptic digestion [2]. Peptide separations are often achieved with reversed-phase (C_{18}) high-performance liquid chromatographic (HPLC) methods. However, resolution of complex or structurally similar mixtures of peptides by C_{18} HPLC may require relatively long analysis times and elaborate gradient solvent programming.

Application of capillary electrophoresis (CE) to protein and peptide separations has demonstrated that extremely high separation efficiency and resolution can be obtained by this method [3–5]. A potential obstacle in CE separations is adsorption of peptides and proteins to the wall of the capillary, resulting in low separation efficiency or irreversible binding of analyte to the capillary. Such interactions are generally strongest for basic peptides and proteins [3].

Three approaches have been used to reduce these interactions. One strategy is to derivatize the inner wall of the capillary with functional groups that chemically block silanol groups or sterically shield them from interaction with analytes [6,7]. A second method is to alter the pH of the electrolyte such that silanol groups of the capillary are protonated [8] (low pH) or basic groups of the analyte are deprotonated [9] (high pH). These changes can reduce capillary-analyte interactions, but the pH extremes required may alter the structure of biological analytes. The third approach is to decrease binding interactions by increasing the ionic strength of the electrolyte. Jorgenson and co-workers [3,10] have employed this tactic to achieve high efficiency separation of relatively basic proteins at pH values near neutrality.

In this report we compare CE and C_{18} HPLC separation methods for kinetic analysis of the action of a proteolytic enzyme, endoproteinase Arg C, on a peptide substrate, adrenocorticotrophic hormone (ACTH). Endoproteinase Arg C hydrolyzes most Arg-X peptide bonds [11]. However, additional cleavage at Lys-Lys and Lys-Arg bonds was observed for ACTH (see Fig. 1). We also demonstrate the application of CE for measurement of rates of peptide bond cleavage. The influence of net charge and total positive charges on peptide adsorption to the capillary wall during CE separation is discussed.

EXPERIMENTAL

Materials

Tyr^3ACTH(3–9), ACTH(1–10), and ACTH(l-16) were obtained from Bachem Bioscience (Philadelphia, PA, U.S.A.). ACTH(7–24) was prepared by solid phase methods using *tert.*-butyloxycarbonyl protected amino acids. Porcine ACTH(l-39) was purchased from Sigma (St. Louis, MO, U.S.A.) and purified by chromatography on Whatman CM-52 cellulose (Hillsboro, OR, U.S.A.) and preparative C_{18} HPLC. Acetyl-L-methionine-1-naphthyl ester was from Serva (Garden City Park, NY, U.S.A.). CE electrolyte components were obtained from Sigma.

Endoproteinase Arg C was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and Sigma. Similar results were obtained with both preparations. The heterogeneity of these enzyme preparations was examined by non-denaturing polyacrylamide gel electrophoresis (PAGE) [12]. Three major and six minor protein bands were observed when the gel was silver-stained. Four activity bands were observed when a comparable gel was stained for esterase activity using acetyl-L-methionine-1-naphthyl ester as the substrate [12], one corresponding to each of the

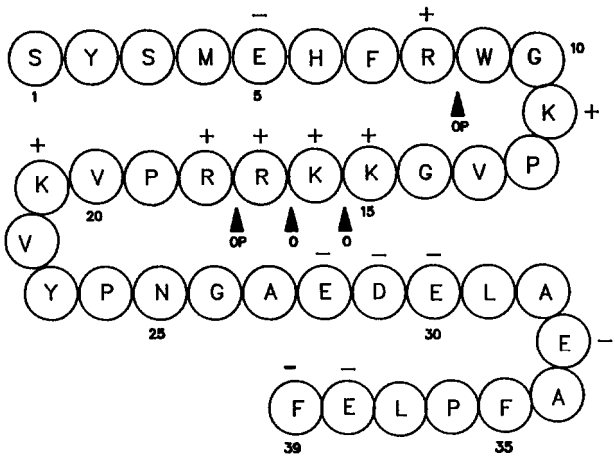

Fig. 1. ACTH sequence and points of cleavage by endoproteinase Arg C. The amino acid sequence of porcine ACTH and the sites of predicted (P) and observed (0) cleavage by endoproteinase Arg C preparations are shown. Residues denoted by + or – signs are predicted to be charged at pH 8.0, as calculated using the constants of Noszal and Osztas [18].

major silver-stained bands, and one to a minor silver-stained band. This indicates that these enzyme preparations probably contain more than one proteolytic activity. Non-endoproteinase Arg C activities may account for the cleavage observed at Lys-Lys and Lys-Arg bonds (see Discussion).

Peptide bond cleavage measurements

Enzymatic cleavages were carried out with endoproteinase Arg C in 0.12 *M* $NaHCO_3 \cdot NH_4OH$, pH 8.5 or 40 m*M* Tris–40 m*M* Tricine, pH 8.2 at 37°C. Peptide bond cleavage rates measured in these two buffers were the same. For HPLC analysis proteinase action was stopped by addition of 0.05 volumes of 6 *M* HCl. Samples were centrifuged at 14 000 g for 5 min and stored at -20°C until the supernatant was analyzed by C_{18} HPLC. Samples analyzed by CE were injected directly into the capillary without a quench step.

The identity of reaction products was established by amino acid analysis of fractions collected from HPLC separations. Peptides were hydrolyzed in 6 *M* HCl, converted to their phenylthiocarbamyl forms, and resolved by C_{18} HPLC [13].

High-performance liquid chromatography

Products from proteolytic cleavage reactions were resolved by C_{18} HPLC using a BakerBond wide pore (J. T. Baker) 5 μm, 250 x 4.6 mm I.D. column. Separations were achieved with linear gradients of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA, 50% aq. acetonitrile (solvent B); using 80% solvent A to 30% Solvent A over 25 min (except where noted), followed by a 2-min wash cycle with 100% solvent B, at a flow-rate of 1 ml/min. The absorbance of the eluate was monitored at 210 nm. Peak areas were calculated using a Waters Maxima 820 data analysis system.

Capillary electrophoresis

An Isco (Lincoln, NE, U.S.A.) prototype electrophoresis system was used for these studies. The system includes an Isco CV [4] variable-wavelength UV monitor with cassette-mounted capillary cell, a Spellman Model EPM30PNX1696 ± 30 kV power supply, and a split flow sample injection system [14]. CE separations were carried out with 50 cm long uncoated capillaries, 50 μm I.D. (Polymicron, Phoenix, AZ, U.S.A.). Temperature was controlled by forced ambient air stabilization of the capillary compartment. Data were recorded and analyzed with an Isco ChemResearch data management system using an IBM PC computer. In addition to the electrolytes noted in figure legends, a range of electrolytes was used for CE analysis of ACTH peptides. The systems tested, with ACTH(l-39) theoretical plate values and migration times, respectively, shown in parentheses were: 40 m*M* Tris–40 m*M* Tricine–0.1 *M* NaCl, pH 8.3 (16 050, 9.2 min); 40 m*M* phosphate-2 *M* betaine pH 7.5 (44 000, 9.7 min); 40 m*M* phosphate–0.1 *M* K_2SO_4, pH 7.0 (107600, 19.7 min); 40 m*M* phosphate-2 *M* betaine–0. 1 *M* K_2SO_4, pH 7.6 (201 000, 28.2 min); 0.1 A4 2-(N-cyclohexylamino)ethanesulfonic acid (CHES)–0.25 *M* K_2SO_4–1 m*M* EDTA, pH 9.0 (84 500, 49.4 min); 20 m*M* 3-(cyclohexylamino)-1-propenesulfonic acid (CAPS)–10 m*M* KCl, pH 10.5 (7100, 4.9 min); 40 m*M* 2-(N-morpholino)ethanesulfonic acid (MES)–2 *M* betaine–40 m*M* K_2SO_4, pH 5.5 (221700, 48.5 min); and 0.1 *M* sodium phosphate, pH 2.5 (109000, 5.9 min).

Analysis of peptide bond cleavage rates

Peptide quantities in cleavage reactions were determined from peak areas, which were converted to molar quantities by reference to peak areas obtained from injections of standard peptide solutions. The peptide concentration of standard solutions was determined by ultraviolet absorbance measurements [15]. Rates of peptide cleavage were determined from plots of peptide concentration vs. reaction time. The slopes of linear portions of these plots gave the rate of peptide bond cleavage. Rates were usually determined with reactions that had gone to less than 10% of completion. However, no product inhibition was observed even in reactions carried to 50% of completion. For determination of K_M values, the rate of peptide bond cleavage was measured as a function of substrate concentration. K_M values were then obtained from plots of $1/V$ vs. 1/[s].

RESULTS

Identification of cleavage products resolved by C_{18} HPLC

The peptides derived from endoproteinase Arg C hydrolysis of porcine ACTH(l-39) were initially examined by monitoring the reaction products as a function of time using C_{18} HPLC (Fig. 2). Incubation for 0.5 h (upper trace, Fig. 2) resulted in two peptide products. Peaks 4 (retention time, t_R = 12.8 min) and 5 (t_R = 26.5 min) were identified as ACTH(l-8) and ACTH(9–39), respectively, by amino acid analysis, which indicated that the Arg^8–Trp^9 bond was the most rapidly cleaved by endoproteinase Arg C action. After a 3-h incubation, ACTH(9–39) was largely cleaved to other products (Fig. 2, lower trace). The peaks were identified as: peak 1, ACTH(9–16) (t_R = *5.6* min); peak 3, ACTH(9–15) (t_R = *7.4* min); peak 7, ACTH(17-39) (t_R = *27.4* min); and peak 8, ACTH(18-39) (t_R = *28.1* min). Peaks

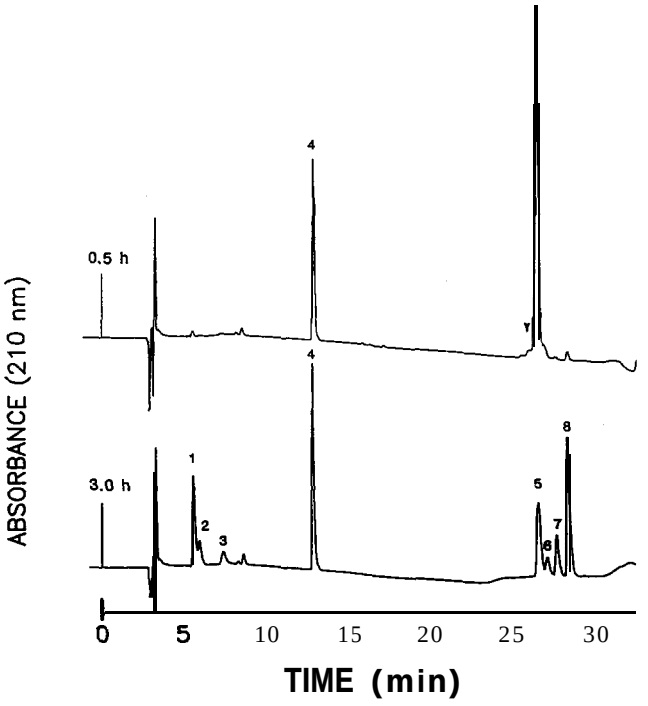

Fig. 2. Resolution of ACTH(l-39) cleavage products by C_{18} HPLC. ACTH(l-39) (80 μM) and endoproteinase Arg C (14 μg/ml) were incubated in a volume of 33 μl at 37°C as described under Experimental. The reactions were quenched after 0.5 (upper) and 3.0 h (lower), and the products resolved by C_{18} HPLC. The elution time of the substrate, ACTH(1–39), is indicated by the arrow (t_R = 26.0 mm). The vertical bar at time $t \simeq 0$ represents 0.05 absorbance units. Peaks were identified as ACTH peptides: 1 = 9-16; 3 = 9–15; 4 = l-8; 5 = 9-39; 7 = 17-39; and 8 = 18-39. Peaks 2 and 6 appeared transiently and were not obtained in sufficient quantity to permit identification.

2 and 6 appeared transiently, and were not obtained in sufficient quantity to permit identification. These results indicated that cleavage occurred at Lys^{15} and Lys^{16}, in addition to the expected cleavage at Arg^{17}. ACTH peptides (1–8), (9–15), (17–39), and (18-39) were resistant to further cleavage when incubated with endoproteinasc Arg C for up to 24 h.

CE separation of peptides

CE separations of endoproteinase Arg C treated ACTH were initially performed in 80 m*M* Tris, 80 m*M* Tricine, pH 8.2, and a typical electropherogram is shown in Fig. 3. The electrolyte employed for this separation was similar in pH and ionic strength to that previously reported for analysis of tryptic digests of proteins [16]. Under these conditions, neither the starting material, ACTH(1–39), nor the cleavage products (9-39) and (9-16) were observed in the electropherogram. Injection of standard solutions of these three peptides, as well as ACTH(1–16), confirmed that these peptides did not elute from the capillary. However, ACTH fragments (9–15), (1–8), (17-39).

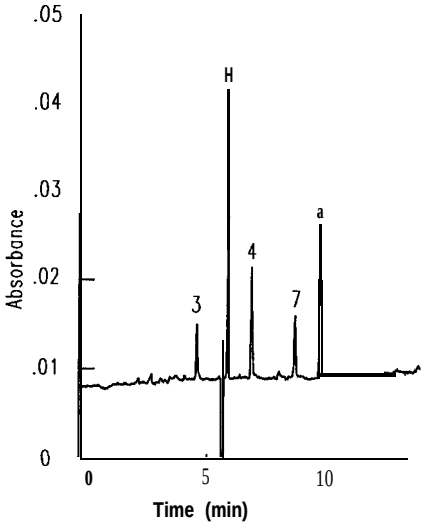

Fig. 3. Analysis of ACTH(1–39) cleavage products in low ionic strength electrolyte. ACTH(1–39) (6 nmol, 27.4 μg) was incubated with endoproteinase Arg C (1 μg) as indicated in the Experimental section. The reaction products were then subjected to CE, which was carried out at 25 kV, 12 μA in 80 m*M* Tris-80 m*M* Tricine, pH 8.2. The elution profile was monitored at 200 nm (0.10 AUFS). Peaks were identified as: 3 =9-15; 4 = l-8; 7 = 17-39; 8 = 18-39; H = histidine standard.

and (18-39) did elute and were well resolved in a relatively short analysis time under these conditions.

Effect of pH and ionic strength on CE separation of ACTH peptides

The peptides that did not elute from the capillary were those predicted to have a higher number of positively charged groups, and/or net charge (Table I). It seemed likely that interactions between these positively charged groups of the peptide and

TABLE I

PREDICTED CHARGE PROPERTIES FOR ACTH DERIVED PEPTIDES AT pH 8.0

Predicted charges present at pH 8.0 on the residue side chains and a-amino and a-carboxyl groups were calculated using the microconstants determined by Noszal and Osztas [18].

Peptide	Positive	Negative	Net
(l-39)	7.1	7	+0.1
(l-8)	1.1	2	-0.9
(1-16)	4.1	2	+2.1
(9-16)	3.1	1	+2.1
(9-15)	2.1	1	+1.1
(17-39)	3.1	6	-2.9
(18-39)	2.1	6	-3.9

silanol groups of the capillary were responsible for adsorption of peptides to the capillary. Therefore the influence of ionic strength on CE separation was examined for two of the peptides that were predicted to have a large number of positively charged groups, i.e., ACTH(l-39) and (I-16).

Addition of NaCl to the electrolyte (80 m*M* Tris-80 m*M* Tricine, pH 8.2) decreased adsorption and increased the separation efficiency for both ACTH(l-16) and (l-39) (Fig. 4). The theoretical plate values observed for ACTH(l-16) at 20, 40, and 60 m*M* NaCl were 570, 6160, and 9190, respectively. ACTH(l-39) appeared to interact most strongly with the capillary and was not observed until the NaCl concentration in the electrolyte was raised to 60 m*M*. Increasing the NaCl concentration to 90 m*M* improved theoretical plate values from 5060 (60 m*M*) to 17020. These results strongly suggest that the cause of low efficiency in these separations was electrostatic in nature.

The efficiency of ACTH(l-39) separation was examined in a number of electrolyte systems (see Methods). In general, increased electrolyte ionic strength or relatively acidic conditions resulted in higher theoretical plate values. The combined phosphate–betaine–K_2SO_4, pH 7.6 electrolyte was among the most efficient, yielding 201000 theoretical plates. Electrophoresis conducted at pH 10.5 resulted in relatively

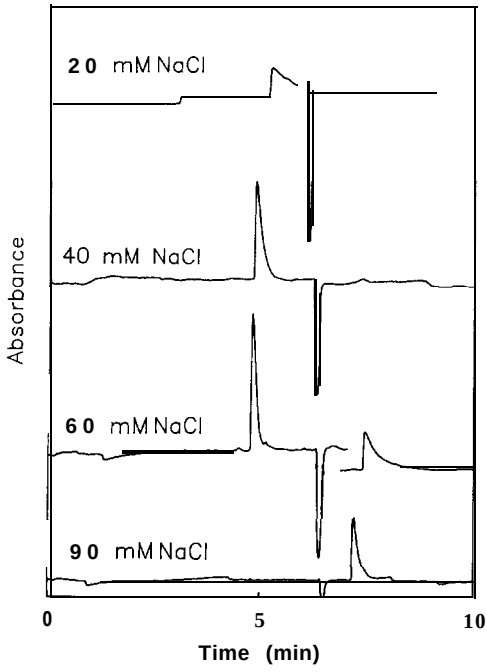

Fig. 4. Effect of NaCl concentration on CE mobility of ACTH(l-39) and (1-16). ACTH(1–39) or ACTH(I-16) was introduced into the capillary, and peptides were analyzed using 80 m*M* Tris–80 m*M* Tricine, pH 8.2 as the electrolyte, to which NaCl was added: 20 m*M*, (1-16) only; 40 m*M*, (1-16) only; 60 m*M* (1–16) and (l-39); 90 m*M* (1–39) only. The separation was carried out at 25 kV, and the elution profile was monitored at 200 nm. The negative pen deflection corresponds to the migration of the neutral zone.

low separation efficiencies. However, migration times were extremely short, and this can be advantageous for kinetic measurements (see Discussion). Conversely, the highest separation efficiency was obtained at pH 5.5 in the presence of betaine and K_2SO_4.

Comparison of CE and C_{18} HPLC separation of peptides derived from endoproteinase Arg C cleavage of ACTH(l-39)

Comparison of CE and C_{18} HPLC separation of ACTH(l-39) cleavage products resulting from endoproteinase Arg C action is shown in Fig. 5. CE separation was performed using a Tris-Tricine electrolyte system that provided moderate resolution and relatively short analysis times. Under these conditions, CE generally provided better separation of peptide pairs [except ACTH(l-39) and (9–39)]. The total time required for analysis is substantially less with CE, particularly as a wash cycle and column re-equilibration time were not required, as they were for the C_{18} separation.

Kinetic analysis of endoproteinase Arg C hydrolysis of ACTH peptide bonds

Kinetic measurements of peptide bond hydrolysis can be performed if either product can be resolved from the substrate peptide. We therefore used CE separations for rate measurements that provided moderate separation efficiency and relatively short migration times.

ACTH(l-39) was incubated with endoproteinase Arg C and samples analyzed at various times to determine the rates of hydrolysis of the Arg^8–Trp^9, Lys^{16}–Arg^{17}, and Arg^{17}–Arg^{18} bonds. These values determined by C_{18} HPLC separations were 1.46, 0.029. and 0.096 μmol min^{-1} mg^{-1}. The hydrolysis rate of the Lys^{15}–Lys^{16} bond of

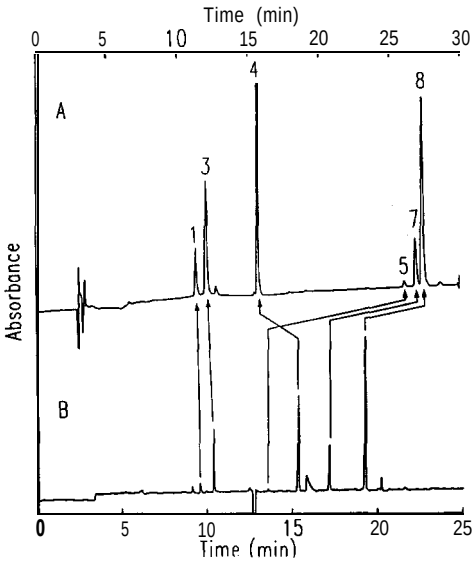

Fig. 5. Separation of cleavage products derived from endoproteinase Arg C treatment of ACTH(1–39). ACTH(1–39) was subjected to proteolytic cleavage as described in the Experimental section. The components of the reaction mixture were then analyzed by C_{18} HPLC (upper panel) using a linear gradient from 10 to 80% solvent B over 28 min or CE (lower panel) using 200 m*M* Tris–200 m*M* Tricine, pH 8.2 as the electrolyte at 25 kV. Peak identities are as noted in Fig. 2.

TABLE II

RATES OF Arg-Trp BOND CLEAVAGE OF ACTH PEPTIDES BY ENDOPROTEINASE Arg C

Substrate	Separation method[a]	
	C_{18} HPLC	CE
ACTH(1-39)	1.46[b]	1.49
ACTH(1–16)	1.28[b]	1.25
ACTH(1–10)	0.895	0.136
Tyr^3ACTH(3–9)	0.007[b]	N.D.[c]
ACTH(7–24)	0.55	N.D.

[a] Units are μmol min^{-1} mg^{-1} endoproteinase Arp C.

[b] V_{max} determined from plot of l/V *vs.* 1/[s]. All other values were initial rates determined at 100 μM peptide substrate.

[c] N.D. = Not determined.

ACTH(11–16), measured by C_{18} HPLC analysis of an ACTH(l-16) cleavage reaction, was 0.057 μmol min^{-1} mg^{-1}.

The hydrolysis observed at the Lys^{15}–Lys^{16} and Lys^{16}–Arg^{17} bonds was unexpected both because of the known specificity of endoproteinase Arg C action on the carboxyl side of arginine residues and for the Lys^{15}–Lys^{16} bond because of the exoproteolytic nature of the cleavage. The rate of Arg^8–Trp^9 bond cleavage was therefore examined in a number of truncated ACTH peptides to determine the influence of the proximity of the amino and carboxyl termini on peptide bond hydrolysis rate. The results in Table II show that relatively rapid cleavage rates are maintained when the Arg^8–Trp^9 bond is only one residue away from the amino terminus, 0.55 μmol min^{-1} mg^{-1} in ACTH(7–24) vs. 1.46 μmol min^{-1} mg^{-1} in ACTH(1–39), a factor of 2.7 difference. Close proximity to the carboxyl terminus has a larger effect, as the cleavage rate decreased by a factor of 15 [1.46 μmol min^{-1} mg^{-1} vs. 0.095 μmol min^{-1} mg^{-1} for ACTH(1–10)] when Trp was one residue removed from the carboxyl terminus. The hydrolytic rate was decreased by a factor of 200 when Trp was the C terminal residue [0.007 μmol min^{-1} mg^{-1} for Tyr^3ACTH(3–9)]. These effects are due to alterations in k_{cat}, not K_M, because the K_M values for cleavage of the Arg^8–Trp^9 bond are quite similar for ACTH(1–39) (6.9 μM), ACTH(l-16) (6.2 μM), and Tyr^3ACTH(3–9) (6.1 μM). Peptide bond cleavage rates measured by CE analysis were quite similar to those measured by C_{18} HPLC. K_M values could not be determined by CE analysis however, because of detector sensitivity limitations.

DISCUSSION

Kinetic analysis of ACTH cleavage by endoproteinase Arg C was conducted using CE and C_{18} HPLC to resolve peptide products. Resolution and measurement of the more basic ACTH peptides by CE required the presence of moderate levels of ionic or zwitterionic components in the electrolyte. Commercially obtained preparations of endoproteinase Arg C were found to cleave ACTH at some Arg-X bonds, as expected, and also at Lys-Lys and Lys–Arg bonds, but not at Arg-Pro, Lys-Pro, or Lys-Val.

The resistance of the Arg-Pro bond to cleavage is consistent with the observation that a mammalian endopeptidase activity that cleaves X-Pro bonds has not been observed to date [17].

Our initial attempts to resolve peptides from proteolytic reaction mixtures using CE showed that some ACTH peptides were adsorbed to the capillary. ACTH(1–39), (1–16), and (9-16) were not eluted from the capillary in CE separations carried out with low ionic strength electrolytes (Fig. 3). Similar low ionic strength CE electrolytes (pH 8) provided excellent separation of peptides derived from complete tryptic digests [19]. However, these systems are not useful for analysis of peptides containing a higher content of basic residues. Using similar electrolytes, we observed substantial adsorption of peptides containing as few as three basic residues. Higher concentrations of NaCl were required to achieve efficient elution for ACTH(l-39) than for ACTH(l-16) (Fig. 4), and this suggests that the number of positively charged groups may be as important as net charge in determining the strength of peptide–capillary interactions. Note that ACTH(l-16) is predicted to have a more positive net charge than ACTH(l-39) at pH 7-9. Net charge also appears to be a factor however, as ACTH(17-39) and ACTH(9–16) are predicted to have the same number of positively charged groups, yet ACTH(17–39), which has a negative net charge, showed no indication of adsorption, even at low ionic strength, whereas ACTH(9–16) did. These results are in agreement with previous studies of basic proteins that also demonstrated low separation efficiencies caused by adsorption to the capillary wall, which can be overcome by addition of salt or zwitterions to the electrolyte [3,10]. Our examination of the electrophoretic properties of ACTH(1–39) using the electrolyte systems developed by Bushey and Jorgenson [3] showed that the behavior of this peptide was generally similar to that of lysozyme and a-chymotrypsinogen with respect to theoretical plate values obtained in these systems. Their high efficiency phosphate–betaine–K_2SO_4 system also gave high (200 000) theoretical plate values with ACTH-(l-39).

CE resolved ACTH cleavage products with different net charge efficiently, and generally in shorter analysis times than C_{18} HPLC. CE separations also have the distinct advantage with respect to kinetic measurements of proteolytic enzyme action that analyses can be made in relatively short times. With some electrolytes thesetimes may be 5 min or less in some analyte combinations (see Methods), which is less than the time required to wash the HPLC column with 100% solvent B and re-establish starting solvent conditions in our C_{18} analyses. Separations by C_{18} HPLC, however, are capable of analyzing much larger sample volumes. This is particularly useful for analysis of low concentrations of peptides, which is often required when making K_M measurements.

Endoproteinase Arg C preparations obtained from two commercial sources gave essentially identical ACTH cleavage patterns. The relative bond cleavage rates were Arg^8–Trp^9, 50; Arg^{17}–Arg^{18}, 3.3; Lys^{15}–Lys^{16} [measured in hydrolysis of ACTH(1–16)], 2.0; and Lys^{16}–Arg^{17}, 1.0. Cleavage at Arg^8–Trp^9 and Arg^{17}–Arg^{18} was expected from previous work of Schenkein et al. [11]. It is likely that the unexpected hydrolyses occurring at Lys^{15}–Lys^{16} and Lys^{16}–Arg^{17} were catalyzed by another proteolytic enzyme present in the endoproteinase Arg C preparations. This putative non-endoproteinase Arg C activity appears to display significant cleavage specificity, as the Lys^{21}–Val^{22} bond was not hydrolyzed at a measurable rate.

As anticipated from the studies of Schenkein *et al.* [11], proximity of the cleavage site to the peptide termini influenced the rate of bond cleavage. Kinetic analyses indicated that this was a result of lower k_{cat} values, and not due to changes in K_M. Cleavage of the Arg^8–Trp^9 bond was much more rapid when Trp^9 was more than two residues removed from the C terminal, but still occurred at measurable rates even when Trp was the C terminal residue. Close proximity of Arg^8 to the N terminal had a smaller effect on the rate of bond hydrolysis. The rate of Arg^{17}–Arg^{18} bond cleavage was also influenced by the position of the bond relative to the N terminal. This bond was hydrolysed fairly rapidly when ACTH(1–39) was the substrate. The same bond appeared to be completely resistant to hydrolysis in ACTH(17–39).

Our results demonstrate that CE separations employing low ionic strength electrolytes that provide excellent separation of peptides derived from tryptic digests can result in significant adsorption of peptides by the capillary for molecules containing as few as three positively charged groups. However, extremely high efficiency separations can be achieved using high ionic strength electrolytes such as those developed by Jorgenson and co-workers or relatively acidic low ionic strength electrolytes. In addition, very short analysis times of peptide mixtures make CE a particularly attractive method for kinetic analysis of proteinase action on peptide substrates.

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REFERENCES

1 G. E. Maret and J.-L. Fauchere, *Anal.* Biochem., 172 (1988) 248.
2 J. H. Frenz, S. L. Wu and W. S. Hancock, *J.* Chromarogr., 480 (1989) 379.
3 M. M. Bushey and J. W. Jorgenson, *J.* Chromatogr., 480 (1989) 301.
4 F. Kilar and S. Hjertén, *J.* Chromatogr., 480 (1989) 351.
5 Z. Deyl, V. Rohlicek and R. Struzinsky, *J. Liq.* Chromatogr., 12 (1989) 2515.
6 S. Hjertin, *J. Chromatogr.,* 347 (1985) 191.
7 G. J. M. Bruin, R. Huisden, J. C. Kraak and H. Poppe, *J. Chromatogr., 480 (1989) 339.*
8 R. M. McCormick, *Anal. Chem.,* 60 (1988) 2322.
9 H. H. Lauer and D. McManigill, *Anal. Chem.,* 58 (1986) 166.
10 J. S. Green and J. W. Jorgenson, *J. Chromatogr., 478 (1989) 63.*
11 I. Schenkein, M. Levy, E. C. Franklin and B. Frangione, *Arch. Biochem. Biophys., 182 (1977) 64.*
12 E. Schaller and 0. von Deimling, *Anal. Biochem., 93 (1979) 251.*
13 R. A. Ebert, *Anal. Biochem., 154 (1986) 431.*
14 J. Tehrani and L. Day, *Am. Biofech. Lab., 7 (1989) 32.*
15 G. Allen, *Sequencing of Proteins and Peptides,* Elsevier, Amsterdam, 1981, pp. 2627.
16 R. G. Nielsen, R. M. Riggin and E. C. Rickard, *J. Chromatogr., 480 (1989) 393.*
17 *N.* A. Roberts, J. A. Martin, D. Kinchington, A. V. Broadhurst, J. C. Craig, I. B. Duncan, S. A. Galpin, B. K. Handa, J. Kay, A. Krohn, R. W. Lambert, J. H. Merrit, J. S. Mills. K. E. B. Parks, S. Redshaw, A. J. Ritchie, D. L. Taylor, G. J. Thomas and P. J. Machin, *Science (Washington D.C.), 248 (1990) 358.*
18 B. Noszal and E. Osztas, *Int. J. Peptide Protein Res., 33 (1989) 162.*
19 R. G. Nielsen and E. C. Rickard, *J. Chromatogr., 516 (1990) 99.*