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Capillary electrophoretic separations of peptides using micelle-forming compounds and cyclodextrins as additives[a]

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ABSTRACT

The value of electrokinetic capillary chromatography for separating structurally similar model peptides and tryptic digests is demonstrated. The behavior of model peptides in buffer systems containing dodecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, sodium dodecyl sulfate and two cyclodextrins as additives is described. These additives, under different analytical circumstances, exhibit certain beneficial effects for peptides with similar net charges but different hydrophobicities. Separations of underivatized peptides, utilizing UV detection, are presented. In addition, separation of fluorescent products of peptides derivatized with *o*-phthalaldehyde, fluorescamine, and a new reagent, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde, are demonstrated and discussed. Beneficial spectroscopic detection effects with cyclodextrin are also noted.

INTRODUCTION

Separation of complex peptide mixtures was one of the early demonstrations of the analytical power of capillary zone electrophoresis (CZE) [1,2]. With the rapidly growing capabilities of this analytical method and the recent emphasis on biotechnological products characterization, peptide separation will continue to be among the most common applications of CZE. In particular, CZE has received considerable attention as a complementary method to reversed-phase liquid chromatography in peptide mapping efforts [3,4]. While migration rates of various peptides can be optimized through an appropriate pH adjustment, the use of electrokinetic capillary chromatography (EKC) can be beneficial in separating substances with similar net charge values. As a modified version of CZE, EKC introduces additional separation mechanisms to supplement the differences in electrophoretic mobilities of analytes. Through the addition of micelle-forming surfactants or inclusion-forming compounds (such as cyclodextrins) to the buffer medium, a dynamic partition mechanism of solute separation is established. Thus, EKC incorporates aspects of both electrophoretic and chromatographic separations, so that subtle differences in the size, shape, hydrophobicity and charge of analytes can be explored. Although EKC was originally

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introduced to allow for the separation of neutral compounds [5], this technique has also been successfully applied to the separation of various charged species [6–9]. Most applications of CZE to peptide separations reported to date utilize UV detection at wavelengths close to 200 nm (absorbance of peptide bonds), which clearly has its limitations in high-sensitivity peptide mapping [10]. The recent emphasis on characterization of trace proteins and peptide hormones demands detection techniques of much higher sensitivity. Tagging peptides with fluorescent moieties thus becomes one of the logical strategies toward achieving such improvements.

The present study reports capillary electrophoretic separation of peptides, both in their native, underivatized form and as fluorescent derivatives formed using *o*-phthalaldehyde (OPA), fluorescamine and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [11]. The common anionic surfactant, sodium dodecyl sulfate (SDS) and cationic surfactants, dodecyltrimethylammonium bromide (DTAB) and hexadecyltrimethylammonium bromide (HTAB), are used in this study. In addition to these micelle-forming compounds, cyclodextrins are also utilized. Successful separations of structurally similar model peptides and peptides from the enzymatic digestion of proteins, incorporating either UV detection or fluorometric detection, are demonstrated.

EXPERIMENTAL

Apparatus

The instrumentation used in all experiments was an in-house built capillary electrophoresis system, as reported previously [8,11]. The high-voltage power supply, capable of delivering 0 to 30 kV, was purchased from Spellman High Voltage Electronics (Plainview, NY, U.S.A.). A Jasco UVIDEC-100-V UV detector (Tokyo, Japan), operated at 220 nm, was employed for detecting unmodified peptides, while the conventional fluorometric measurements utilized a Model FS 950 Fluoromat detector (Kratos, Ramsey, NJ, U.S.A.). The excitation wavelength was set at 365 nm, whereas a 418-nm cut-off filter was used to pass fluorescence emission for the OPA- and fluorescamine-tagged peptides. Ultrasensitive measurements with CBQCA-derivatized peptides [11] utilized a laser-induced fluorescence detector of a design similar to that described by this laboratory in connection with microcolumn liquid chromatography of fluorescent amino acid derivatives [12,13]. Since the CBQCA-derivatized fluorescent isoindoles exhibit excitation maxima near 450 nm, a helium/cadmium laser (Omnichrome, Chino, CA, U.S.A.) was employed as the excitation source (442 nm at 50 mW output power). On-column detection was facilitated by removing a section of the polyimide capillary coating to create an optical window.

Materials and reagents

Fused-silica capillaries with 50 μm I.D. and 187 μm O.D. were obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). All peptide standards, and cytochrome *c*, SDS, DTAB and HTAB detergents, α- and β-cyclodextrin, tris(hydroxymethyl)aminomethane (Tris) and fluorescamine were purchased from Sigma (St. Louis, MO, U.S.A.). *o*-Phthalaldehyde, ethanethiol and 2-mercaptoethanol were received from Fluka (Ronkonkoma, NY, U.S.A.). The CBQCA fluorogenic reagent was synthesized in our laboratory [11]. Methanol and tetrahydrofuran (THF) were

HPLC grade, from Mallinckrodt (Paris, KY, U.S.A.). Disodium hydrogenphosphate and boric acid were analytical reagent grade, from Fisher Scientific (Fairlawn, NJ, U.S.A.). EKC and CZE buffers were either Tris–phosphate (pH = 7.05) or sodium borate (pH = 9.50), with appropriate additions of surfactants, cyclodextrins and organic modifiers.

Preparation of fluorescent derivatives

Aliquots of peptide standard solutions or the protein samples after tryptic digestion [10] were mixed with 0.4 *M* borate buffer (pH = 9.50) and OPA–ethanethiol or OPA–mercaptoethanol solutions, as described previously [8] for the amino acids. Alternatively, a fluorescamine derivatizing solution [2] was mixed directly with these samples. The procedure for preparing CBQCA derivatives is described elsewhere [11].

A hydrodynamic sample introduction technique [14] was employed in all analyses.

RESULTS AND DISCUSSION

Separation of underivatized peptides in the presence of surfactants

Due to the increasing importance of CZE in protein characterization studies, it is essential to understand various parameters affecting separation of the protein fragments, *i.e.*, peptides with different amino acid compositions and molecular weights. Due to the high resolving power of CZE, it is feasible to separate peptides that differ only slightly in their net charge. Depending on a particular peptide mixture, buffer pH adjustment can be used to control the net charge, and thus, migration rates, appreciably [15,16]. However, in complex peptide mixtures, such as tryptic digests, a complementary use of chromatographic techniques (with the elution order being a sensitive function of hydrophobicity or selective interactions) appears desirable [4]. It also seems logical to explore EKC for the separation of peptides, particularly those having similar net charge values.

As seen from Table I, where structural sequences, calculated net charge values and isoelectric points of several angiotensin peptides are listed, minor structural

TABLE I

SEQUENCE OF ANGIOTENSIN (Ang) PEPTIDES, CALCULATED NET CHARGE AND ISOELECTRIC POINTS

Peak no.	Peptide	Sequence	Net charge		*pI*
			pH 7.05	pH 9.50	
1	[Ile^7]-Ang III	Arg-Val-Tyr-Ile-His-Pro-Ile	1.07	0.04	9.55
2	[Val^4]-Ang III	Arg-Val-Tyr-Val-His-Pro-Phe	1.07	0.04	9.55
3	[Asn^1,Val^5]-Ang II	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	1.06	−0.04	9.45
4	Ang III	Arg-Val-Tyr-Ile-His-Pro-Phe	1.07	0.04	9.55
5	[Val^5]-Ang II	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	0.08	−0.54	7.81
6	Ang II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0.08	−0.54	7.81
7	Des-Asp^1-Ang I	Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	1.15	0.04	9.55
8	Ang I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	0.16	−0.54	7.96

differences between the individual solutes are unlikely to be resolved by the CZE principle alone. Net charge values calculated according to a modified version of the method of Skoog and Wichman [17] indicate that at pH 7.05, each of these peptides possess a positive charge. Since our initial experiments with an anionic surfactant, SDS, did not result in adequate separations, additional cationic surfactants, HTAB and DTAB, were explored. A separation of eight model peptides, shown in Fig. 1, was obtained with the addition of HTAB at a level above its critical micelle concentration (CMC) into a Tris–phosphate buffer.

An interesting feature of cationic surfactants is their electrostatic attraction to the negatively charged capillary wall, resulting in a dynamic modification of the double layer and the possible reversal in direction of the electroosmotic flow (depending on surfactant concentration). In our experiments, the bulk of the micellar solution is electroosmotically driven toward the positive (ground) electrode. Peptides possessing a negative charge will thus be accelerated in the direction of the electroosmotic flow, while the positively charged species tend to migrate in the opposite direction but are eventually overcome by the stronger electroosmotic flow. The distribution of solutes between the buffer medium and the pseudostationary micellar phase provides the needed separation selectivity. The extent of interaction will largely depend on the charge, size and hydrophobicity of the peptides. While this separation principle was originally developed for the analysis of neutral molecules [5], it has also been found successful for various charged species [6–9]. Under the given conditions, all positively charged peptides shall migrate in the same direction as HTAB and DTAB micelles.

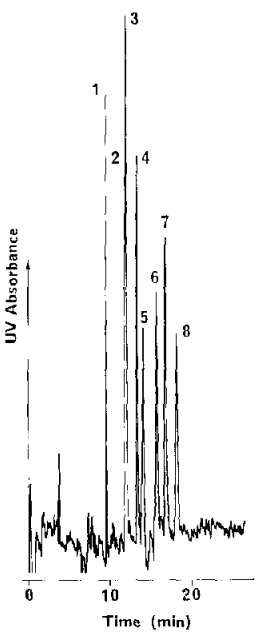

Fig. 1. Separation of underivatized angiotensin analogues using a cationic detergent as a buffer additive. Peak assignment corresponds to the numbers in Table I. Capillary: 80 cm in length (50 cm to detector), 50 μm I.D. Separation buffer: 25 m*M* Tris–25 m*M* Na_2HPO_4 (pH 7.05) 0.05 *M* HTAB. Operating voltage: −20 kV. UV detection at 220 nm.

The separation mechanisms in the micellar system appear more complex compared with CZE of the same compounds. As seen in Table I, the pair of angiotensin II Val^5-angiotensin II and angiotensin III–Val^4-angiotensin III possess the same charge, differing only in the neutral amino acid between Tyr and His. A clear separation of these peptides, as seen in Fig. 1, is likely due to their different hydrophobicities and partition rate into the micelles. Angiotensin I and Des-Asp^1-angiotensin I can be separated completely due to their difference in size, charge or hydrophobicity. On the other hand, Val^4-angiotensin III and Asn^1-Val^5-angiotensin II, differing in a single amino acid, have been only partially resolved.

It should be pointed out that a surfactant concentration above the CMC is essential to achieving good separations. Fig. 2 shows a comparison of analyses carried out at different concentrations of DTAB. A concentration of $2 \cdot 10^{-3}$ *M*, which is below the CMC ($1.4 \cdot 10^{-2}$ *M* [18]) gives poor resolution (Fig. 2A), although it is sufficient to reverse the direction of electroosmotic flow. In contrast, with a 0.05

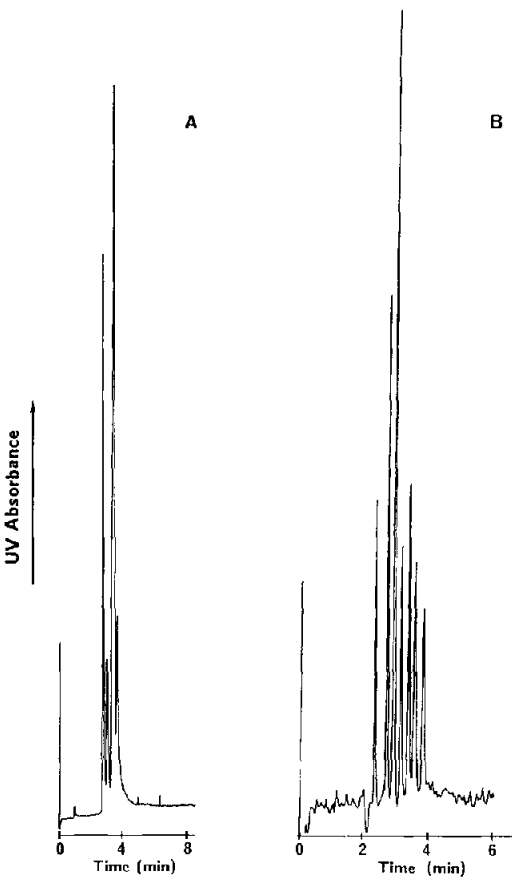

Fig. 2. Comparison of separations of angiotensin analogues using cationic detergent above and below the critical micelle concentration. Capillary: 45 cm in length (25 cm to detector), 50 μm I.D. Separation buffer: 10 m*M* Tris–10 m*M* Na_2HPO_4 (pH 7.05) with DTAB of (A) $2.0 \cdot 10^{-3}$ *M*, and (B) 0.05 *M*. Operating voltage: −15 kV. UV detection at 220 nm.

M DTAB solution, excellent resolution of seven peptides is achieved (Fig. 2B), emphasizing the importance of the micellar state.

Separation of fluorescent peptide derivatives in the presence of surfactants and cyclodextrins

For high-sensitivity determinations of peptides, fluorescence measurements become feasible after derivatization with a suitable fluorogenic reagent. Among numerous reagents, OPA and fluorescamine have found widespread use in chromatographic analyses of amino acids and peptides. Recently, we have reported successful separations of OPA-derivatized amino acids [8] by capillary electrophoresis, and it thus appeared logical to extend this derivatization method to electrokinetic capillary chromatography of peptides.

Separation of various OPA-derivatized peptides, using SDS as an additive, is shown in Fig. 3. At pH 9.50, the small peptides (Fig. 3A) are all negatively charged, migrating electrophoretically against the electroosmotic flow in the expected order. The situation with the four angiotension peptides, separated as OPA-derivatives, is slightly more complex. Angiotension III, with a positive charge of 0.04, migrates in the direction of electroosmotic flow and is detected first. At pH 9.50, angiotension I, angiotensin II and Val^5-angiotensin II possess the same negative charge (-0.54), and their retention in the system is determined primarily by the extent of partitioning

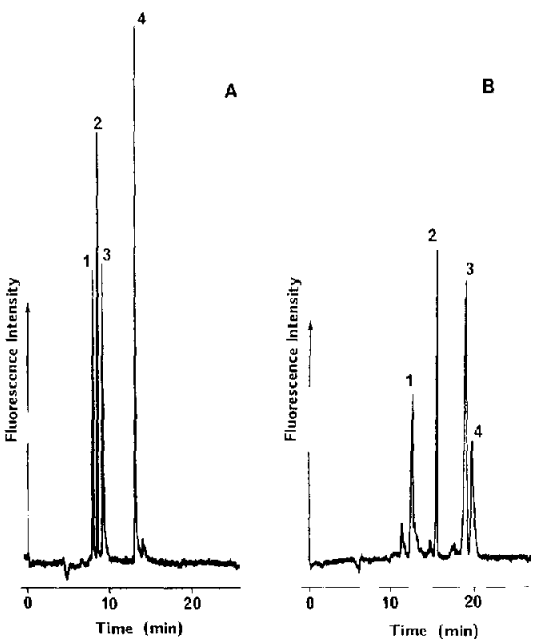

Fig. 3. Electropherograms of OPA-derivatized peptides. Capillary: 80 cm in length (50 cm to detector), 50 μm I.D. Operating voltage: 23 kV. (A) Separation buffer: 0.05 *M* borate buffer (pH 9.50)–1% THF–50 m*M* SDS. Peaks: 1 = Ala-Gly-Ser-Glu; 2 = Val-Gly-Ser-Glu; 3 = Val-Gly-Asp-Glu; 4 = 2-aminoethanol (internal standard). (B) Separation buffer: 0.05 *M* borate buffer (pH 9.50)–1% THF–15% methanol–50 m*M* SDS. Peaks: 1 = angiotensin III; 2 = angiotensin I; 3 = [Val^5]-angiotensin II; 4 = angiotensin II.

between the micellar and bulk aqueous phases (size and hydrophobicity are important).

The use of fluorescamine in CZE separations of tryptic peptides was first reported by Jorgenson and Lukacs [2]. Derivatization with this reagent is both effective and convenient. While searching for a suitable additive to optimize resolution of fluorescamine-labeled peptides, we explored certain cyclodextrin substances that were previously found effective in isotachophoretic separations of small molecules [19,20]. Two beneficial effects were found with the use of such additives: (1) appearance of very narrow peaks; and (2) enhancement of fluorescence intensity. Fig. 4 demonstrates highly efficient separations of (A) nine standard peptides, and (B) peptides obtained through tryptic digestion of cytochrome *c*. While additional research is needed to elucidate the factors controlling both the efficiency and separation selectivity due to solute–cyclodextrin interactions, a favorable match between the solute's size or shape and the cyclodextrin hydrophobic cavity is strongly suggested. The excellent peak shapes and high efficiencies could possibly be caused by decreased diffusion coefficients due to the formation of aggregates, or by protection from adsorption to the capillary wall.

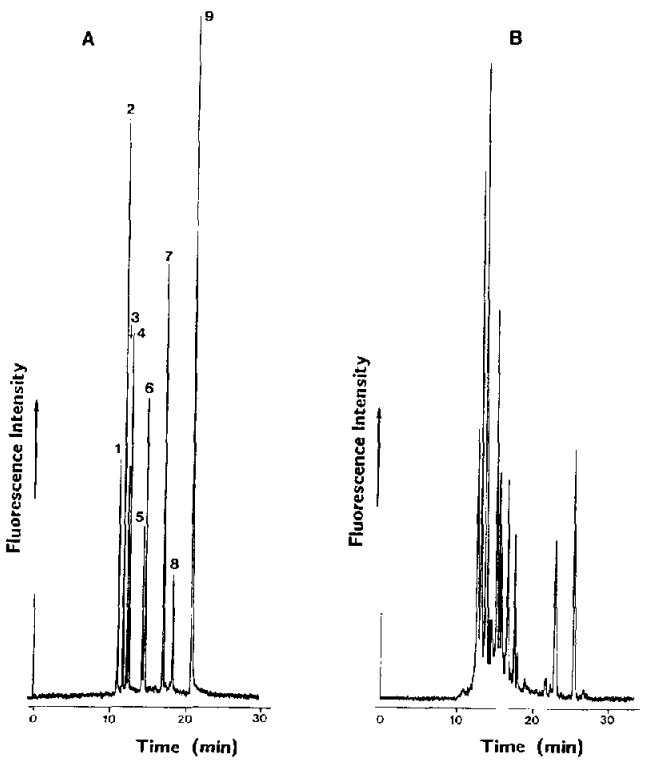

Fig. 4. Electropherograms of fluorescamine-derivatized peptides, using β-cyclodextrin as a buffer additive. (A) Model mixture of 9 peptides. Peaks: 1 = angiotension III; 2 = Gly-Gly-Tyr-Arg; 3 = angiotensin I; 4 = [Val^5]-angiotensin II; 5 = Met-Leu-Phe; 6 = Gly-Leu-Tyr; 7 = Val-Gly-Ser-Glu; 8 = Ala-Gly-Ser-Glu; 9 = Val-Gly-Asp-Glu. Capillary: 80 cm in length (50 cm to detector), 50 μm I.D. Separation buffer: 0.05 *M* borate buffer (pH 9.50)–20 m*M* β-cyclodextrin–15% methanol–1% THF. Operating voltage: 20 kV. (B) Tryptic digest of cytochrome *c*. Operating voltage: 15 kV. All other conditions are the same as in (A).

Effects of cyclodextrin concentration on fluorescence intensity

The role of the cyclodextrin cavity in peptide separations was further investigated with the CBQCA-based fluorescent derivatives obtained recently in this laboratory [11] for ultrasensitive laser-induced fluorescence measurements. Using α-cyclodextrin (which has a smaller cavity size than β-cyclodextrin [21]) as a buffer additive, good resolution and peak shapes were achieved for model peptides derivatized with CBQCA (see Fig. 5).

While utilizing the cyclodextrins as buffer additives, we have noticed significant increases of the measured fluorescence intensity for CBQCA-tagged peptides with an increasing concentration of cyclodextrin. The effects of α-cyclodextrin and β-cyclodextrin on the fluorescence intensity of a small peptide (Gly-Gly-Tyr-Arg) and a larger peptide (Des-Asp^1-angiotensin I) are compared in Fig. 6. Relative fluorescence intensity of CBQCA–Des-Asp^1-Angiotensin I increased nearly 10 times with an increase of β-cyclodextrin concentration from 0 to 20 m*M*, while a much smaller increase of fluorescence intensity was observed for CBQCA–Gly-Gly-Tyr-Arg (as indicated by the solid lines in Fig. 6). In contrast, α-cyclodextrin seems to favor small peptides for enhancement of fluorescence intensity, as demonstrated by the dashed lines in Fig. 6. The cavity size of cyclodextrins and host-guest interactions between solutes and these additives appear to play a role. While this fluorescence enhancement is not surprising in view of previous spectroscopic research with organized chemical

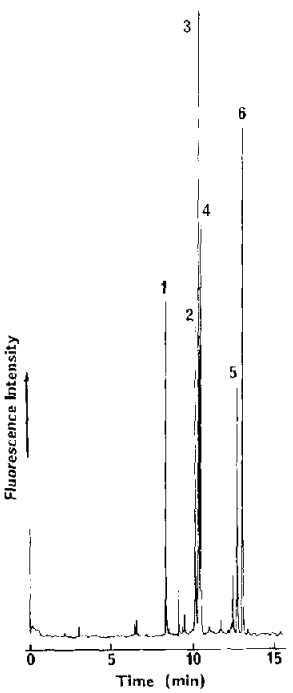

Fig. 5. Electropherogram of CBQCA-derivatized peptides, using α-cyclodextrin as a buffer additive. Capillary: 90 cm in length (60 cm to detector), 50 μm I.D. Separation buffer: 0.05 *M* borate buffer (pH 9.50)–20 m*M* α-cyclodextrin. Operating voltage: 20 kV. Peaks: 1 = Gly-Gly-Tyr-Arg; 2 = Gly-Leu-Tyr; 3 = Met-Leu-Tyr; 4 = Val-Ala-Ala-Phe; 5 = Glu-Gly-Phe; 6 = Glu-Val-Phe.

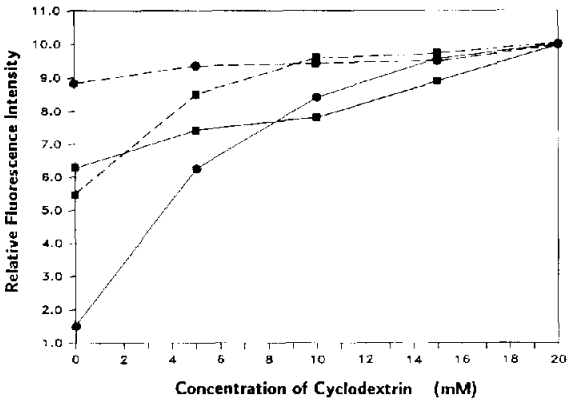

Fig. 6. Plots of relative fluorescence intensity *vs.* concentration (m*M*) of cyclodextrin. Solid lines correspond to β-cyclodextrin, dashed lines correspond to α-cyclodextrin. ■ = Gly-Gly-Tyr-Arg; ● = des-Asp[1]-Angiotensin I. Buffer: 50 m*M* borate buffer (pH 9.50) with addition of cyclodextrin.

media [22,23], the coincidence of desirable spectral properties and separation characteristics should have practical significance for the high-sensitivity determinations of complex peptide mixtures.

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