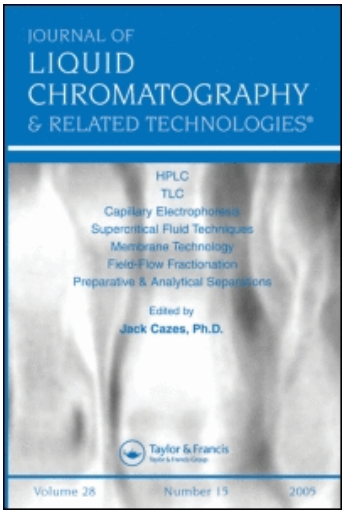

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Huimin Yuan[a]; George M. Janini[b]; Haleem J. Issaq[b]; Richard A. Thompson[a]; Dean K. Ellison[a]
[a] Analytical Research Department, Merck Research Laboratories, Merck & Co., Inc., Rahway, NJ, U.S.A. [b] NCI-Frederick Cancer Research and Development Center, Frederick, MD, U.S.A.

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SEPARATION OF CLOSELY RELATED HEPTADECAPEPTIDES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

Huimin Yuan,[1,*] George M. Janini,[2] Haleem J. Issaq,[2]
Richard A. Thompson,[1] Dean K. Ellison[1]

[1]Analytical Research Department
Merck Research Laboratories
Merck & Co., Inc.
Rahway, NJ 07065, USA

[2]SAIC Frederick
NCI-Frederick Cancer Research and Development Center
P.O. Box B
Frederick, MD 21702-1201, USA

ABSTRACT

Separations of five synthetic heptadecapeptides from the p-loop region of p21 Ras proteins, differing mostly in a single amino acid at the same position, were investigated. The studies were performed at low pHs in polyacryamide-coated capillaries, where the peptides were slightly positively charged. CZE, without surfactants, failed to separate all the peptides. Separations by MEKC employing an anionic surfactant sodium dodecyl sulfate (SDS), a neutral surfactant Tween 20 and a cationic surfactant cetyltrimethylammonium bromide (CTAB) provided results with varying degrees of success. Tween 20, slightly improved the separation while CTAB resulted in almost baseline separation of all the peptides.

The effects of applying acetonitrile to MEKC were also examined. The separation results are analyzed with regard to the properties of the peptides and the surfactants. Implications of the results on peptide-micelle interactions and general aspects of peptide separation by MEKC are discussed.

INTRODUCTION

Micellar electrokinetic chromatography (MEKC)[1] has rapidly developed in the last decade. As a modified capillary zone electrophoresis (CZE) technique, MEKC adds another dimension of selectivity based on hydrophobicity of analytes. CZE, although proven to be powerful in differentiating minute mass-to-charge ratio in peptide separation,[2-4] it fails in distinguishing small hydrophobicity changes in amino acid sidechains in peptides,[5,6] particularly when the size of peptides increases. However, in MEKC, a micelle-forming surfactant is added to the background electrolyte at concentrations above its critical micelle concentration (CMC). Differential distribution of analytes into the surfactant micelle leads to differences in apparent mobility and then consequent separation. Any factor that affects the partitioning of analytes into the micelle can be exploited in MEKC in a way similar to liquid chromatography. Therefore, MEKC in design is much more capable of distinguishing small differences in size, shape, charge distribution, and hydrophobicity.

Although developed initially to separate neutral analytes by employing charged surfactants, the scope of MEKC has been widened tremendously to separate both neutral and charged analytes by using various ionic or nonionic surfactants. MEKC not only has inherited CZE's advantages of short analysis time, small sample usage, and high efficiency, but also has added the much needed and almost infinite versatility in the optimization of a separation.

In recent years, MEKC peptide analysis has provided competitive and complementary information to reversed-phase HPLC, the conventional technique of choice for peptide analysis.[6-8] Studies of MEKC in peptide separation have encompassed the use of different types of surfactant, peptide-analyte interactions, analysis of a wide range of natural and synthetic peptides, and analysis of large and highly similar peptides. The power of MEKC has been explored and shown particularly well in separations of large closely-related peptides.

The octapeptide angiotensin II analogs, differing in a single amino acid, have been separated using nonionic surfactant Tween 20[9,10] and sucrose monododecanoate[11] at low pHs, with cationic surfactant CTAB or DTAB at a neutral pH and with anionic surfactant SDS at a high pH of 9.5.[12] The separation of eight neurohypophyseal nonapeptide analogs was studied using cationic surfactant CHAPS, anionic surfactant SDS, and neutral surfactant Triton X-100, where CHAPS gave the best results.[5] Zwitterionic surfactant PAPS has been reported effective in the

separation of a few polymyxin decapeptide analogs.[13] Recently, three closely related variants of 13-mer neurotensin analogs were separated using a nonionic surfactant sucrose monododecanoate.[11] Longer peptides of 16- to 18-mers of synthetic basic neuropeptide Y analogs were studied using cationic surfactant CTAC.[5]

In the study, a maximum of four of fourteen peptides were separated at a time. MEKC was shown to separate two 22-mer motilin variants using neutral surfactant Tween 20 or CTAB with 5% acetonitrile.[14] In the latter case, four variants of insulin from different species were also separated. Finally, two 70-mer insulin-like growth factor I variants differing in a single amino acid were separated using a zwitterionic surfactant DAPS with 15% acetonitrile.[15] These studies not only have shown the capability and versatility of MEKC in peptide analysis, but also have enriched our knowledge of the nature of interactions between surfactant micelles and peptides.

In this study, a test set of synthetic heptadecapeptides (Table 1), differing mostly in a single amino acid, was employed to study the separation through MEKC mechanism. Very few studies have been reported using a set of highly related peptides of such a length. The capillary column used in the study was coated with a polyacrylamide to eliminate electroosmotic flow (EOF),[16,17] decrease adsorption of analyte to the capillary surface,[18] and increase separation reproducibility. MEKC under zero EOF infinitely expands the migration window of analytes interacting with the micelle and increases peak capacity.[17] Elimination of EOF eases the optimization of the separation.[17]

In this study, the five highly-related heptadecapeptides were separated using either Tween 20 or cetyltrimethylammonium bromide (CTAB) under the MEKC mechanism; some commonly employed variables, such as pH, type of surfactant and addition of organic solvent, were examined in an effort to obtain the best separation.

EXPERIMENTAL

Reagents

The peptides (Table I) were custom-synthesized by commercial laboratories for Dr. J. A. Berzofsky (NCI, NIH, USA). Peptide mixtures were prepared by mixing aliquots of pure standard solutions of each peptide to a final concentration of ~50 μM each. All chemicals, including buffers, buffer additives, and surfactants, were purchased from Sigma (St. Louis, MO, USA), except for SDS, which was purchased from Fluka (Fluka Chemika-BioChemika, Buchs, Switzerland).

Table 1

Sequences and Physicochemical Properties of the Peptides

Code	Sequence	pH 2.47 Charge[a]	pH 2.47 $q/M^{2/3}$	pH 3.07 Charge	pH 3.07 $q/M^{2/3}$	Hydrophobicity Coefficient[b]
1	YKLVVVGA**A**GVGKSALT	2.83	0.0204	2.574	0.0186	42.3
2	YKLVVVGA**C**GVGKSALT	2.83	0.0201	2.574	0.0183	42.9
3	YKLVVVGA**V**GVGKSALT	2.83	0.0202	2.574	0.0184	45.3
4	KLVVVGA**GD**VGKALTI	2.74	0.0198	2.303	0.0166	43.2
5	YKLVVVGA**D**GVGKSALT	2.74	0.0194	2.303	0.0163	40.4

[a] Calculated using Henderson-Hasselbalch equation (ref. 27). [b] Sum of retention coefficients of comprised amino acids from ref. 23.

Apparatus and Methods

Separations were performed using a Beckman CZE system 2000 (model P/ACE), equipped with an autosampler, a liquid-cooled column cartridge, a UV detector and a System Gold data collection system. Fused-silica capillaries (50 μm i.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were custom coated with polyacrylamide as described before.[16] The coated capillary displayed negligible electroosmotic flow (EOF) and well-controlled migration reproducibility.[17] The capillary was purged 3 minutes each time before injection. Injections were performed under pressure mode for 3~5 seconds at 20 psi. All buffer solutions were degassed and filtered through 0.22 μm filters. A Fisher Accumet (model 750) Selective ion analyzer was used for pH measurement. The wavelength for UV detection was set at 200 nm for studies with Tween 20 and at 214 nm for studies with CTAB. Identifications of peaks in an electropherogram of a mixture were made by spiking each of the peptides in the mixture.

RESULTS AND DISCUSSION

The sequences of the five peptides used in this study and their relevant physicochemical properties are listed in Table 1. The charges of the peptides were calculated using the Henderson-Hasselbalch equation. Since electrophoretic mobility is best correlated with $q/M^{2/3}$, where q is the net charge and M is the mass of analyte,[4] the values of $q/M^{2/3}$ were also listed in Table 1 to compare the mobility of each peptide. The 17-mer peptides encompass the p-loop of Ras p21 protein, whose mutation of the 2nd G in the sequence (G12 in the protein) is known to be carcinogenic in mammalians.[19] Four of the five peptides differ only at the ninth position, while the last one has a "shift" and differs from the others at both

termini. All of the peptides are slightly basic due to the presence of two lysine residues in the sequences. In p21 Ras the sequence is a part of the structure, assuming a β strand-turn-α helix motif with the center part of the peptide in the "turn" portion.[20]

Peptides **1** to **3** have amino acids with nonionizable sidechains at the ninth position, while peptides **4** and **5** have aspartic acids at the corresponding positions. Therefore, the isoelectric points for the last two peptides are slightly lower than those of the other three. Due to the small mass differences of these peptides, the calculated mobilities are very close at both pHs of 2.47 and 3.07, where separations were performed.

Separation by CZE

The pKa values of the C-terminal -COOH group and the N-terminal -NH_2 group of a peptide are usually close to 3.2 and 8.2, respectively.[21] The pKa values for aspartic acid and lysine residues present in the current 17-mer peptides are 3.5 and 10.3, respectively.[21] Therefore, the pKa values for ionizable groups in the present peptides are centered at two regions, one around 3 and the other around 8~10. Separation at either of these pHs is expected to give the largest charge differences among the peptides. Since the polyacrylamide coating of the capillary is sensitive to alkaline pHs, separations at acidic conditions were performed. Acidic pHs also ensure that all the peptides assume net positive charges and migrate towards the same direction for easy detection in the absence of EOF.[3-4]

Figure 1 shows the separation of the peptide mixture under different pHs without addition of any surfactant. At pH 2 no separation can be seen; all five peptides migrated as a single broad peak. Because all the acidic groups are protonated and do not contribute to the net charge, all the peptides assume an almost identical positive charge from their basic groups. Due to higher conductivity at this pH, the voltage applied was lower at the constant power mode, which resulted in longer migration time. Raising pH to 2.47 greatly differentiated the mobilities of the peptides; the five peptides were separated into three peaks. Further increasing pH to 3.07 separated all the peptides but **1** and **2**.

The migration order at this pH corresponds well to the calculated mobility, except for peptide **2**. The first three peptides, having similar mobilities, migrated as a group, and were baseline separated from the last two peptides, which have lower mobilities. The first three peptides have exactly the same charge (Table 1) and differ slightly by a single amino acid. Compared to peptide **1** with an alanine at the ninth position, peptide **2** has an extra sulfhydryl group due to the substitution of alanine by cystein; peptide **3** has an extra methyl group due to the presence of valine.

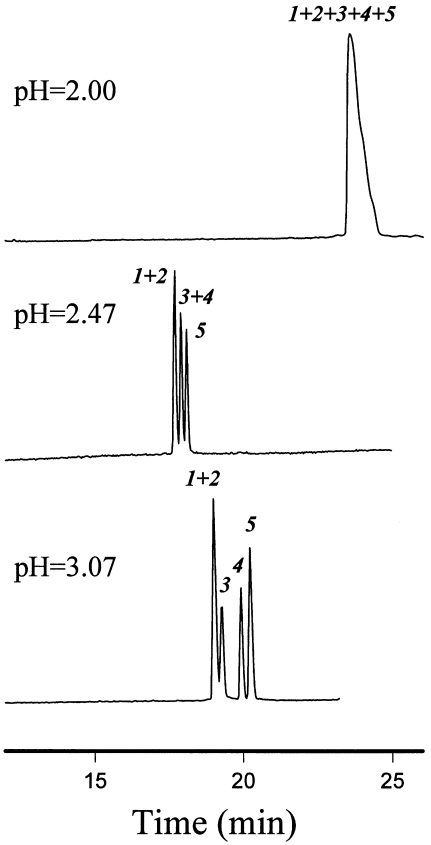

Figure 1. CZE separation of a mixture of five heptadecapeptides at different pHs. Instrument: Beckman model P/ACE, System 2000. Buffer: 50 mM phosphoric acid (pH adjusted using triethyl amine). Column: 10% polyacrylamide-coated fused-silica 47 cm (effective length of 40 cm) x 50 μm I.D. Power: 0.2 W (12 kV; 16-18 μA). The pH for each run and the peak identities are indicated on the graph. The sequences for the peptides are shown in Table 1.

It is challenging for CZE to completely differentiate the small mass difference between these three 17-mers. According to calculations shown in Table 1, peptide **2** has a slightly lower mobility than peptide **3**, and both peptide **2** and **3** are expected to migrate more slowly than peptide **1**. However, as observed in Figure 1 (pH 3.07), peptide **2** migrated slightly faster than peptide **3** and was inseparable from peptide **1**. This was probably caused by the conformational characteristics associated with each key (the ninth) amino acid, which overwhelms the charge-to-mass determinant.

It has been proposed that secondary conformation should be considered for peptides longer than 12~14 residues.[22] The conformations, in terms of helicity, of 17-mer peptides have been shown to change by single amino acid substitutions. It was suggested that single amino acid substitution may alter the folding, charge distribution or hydrophobic sites, which, in turn, influences the mobility under CZE and MEKC modes.

The last two peptides have an aspartic acid in the center of the sequence. Increasing the pH to approach the pKa of aspartic acid (~3.5) apparently differentiated the mobility of peptide **4** and **5** from others. The migration order of **4** and **5** is in accordance with the calculated mobility, although their separation was still minimal. For the present 17-mer peptides, better separations have to rely on differences other than that of charge-to-mass ratio. In this work different modes of MEKC were tested to exploit hydrophobicity differences.

MEKC Using the Anionic Surfactant SDS

SDS was the first surfactant to be tested. Adding up to 50 mM SDS to the background electrolyte at pH 3.07 did not improve the separation at all; instead, it heavily broadened individual peaks. In fact, this was not unexpected. We believe that the reason for this outcome is that, at this pH, the peptides and SDS are oppositely charged, which probably led to significant nonspecific ionic interactions.

MEKC Using the Neutral Surfactant, Tween 20

The use of Tween 20 was considered because neutral surfactants usually provide advantages, such as minimized nonspecific charge-charge interactions with the analytes, and allows the use of high concentrations without increasing the current. Strictly speaking, under only one kind of MEKC is the migration order solely determined by the interactions between the analytes and the micelle; that is, using a charged surfactant and neutral analytes, where the electrophoretic mobilities of the analytes are zero in the absence of EOF. When charged analytes are used, the electrophoretic mobilities of the analytes are not zero and are usually unequal. Whether the migration order of the analytes can be dominated by the analytes-micelle interactions is determined by how strong the interactions are and how closely the electrophoretic mobilities of the analytes are. For analytes that cannot be well separated through CZE, the migration order is more likely a measurement of analytes-micelle interactions in MEKC.

In the presence of negligible EOF, neutral micelles do not migrate under an electric field. Thus, analytes which are distributed more greatly in the micelle are more significantly slowed down. Figure 2 shows the separation of the peptides using 0, 100 mM, and 150 mM Tween 20 at pH 3.07. As a neutral surfactant, Tween 20 is expected to have mostly hydrophobic interactions with

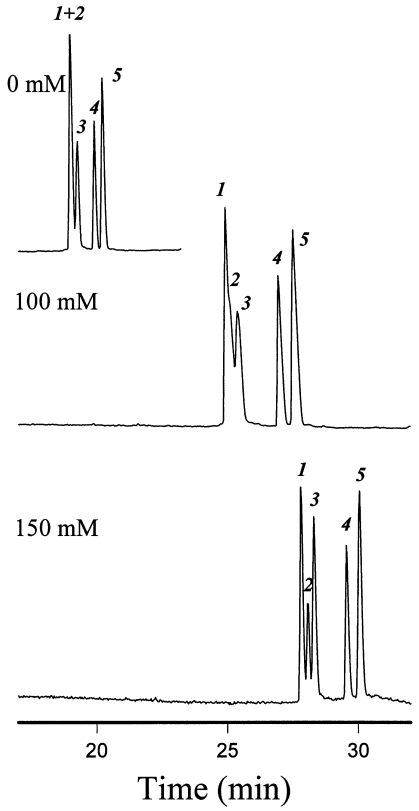

Figure 2. Separations of the peptide mixture at pH 3.07 with different Tween 20 concentrations. The Tween 20 concentrations and the peak identities are indicated on the graph. Other experimental conditions were the same as in Figure 1.

the peptides. The hydrophobicity coefficient for each peptide, estimated by the sum of that for each amino acid by Guo *et al*. at pH 2, is listed in Table 1.[23] In the first group (peptides **1** to **3**), the hydrophobicity increases in the order of peptides **1**, **2** and **3**. For the effect of Tween 20 *per se,* they should be slowed down to a greater degree in the said order. Addition of Tween 20 to 150 mM clearly resolved peptides **1** and **2**, which co-migrated as a single peak in the CZE mode in Figure 1. Further increasing the Tween 20 concentration showed no more improvements. The observed migration order after the resolution of peptides **1** and **2** is in agreement with the hydrophobic prediction. Apparently, the slight hydrophobicity difference is responsible for the separation of peptides **1** and **2**. However, the separations among the peptides **1** to **3** were still minimal, indicating the peptide-micelle interactions were not strong.

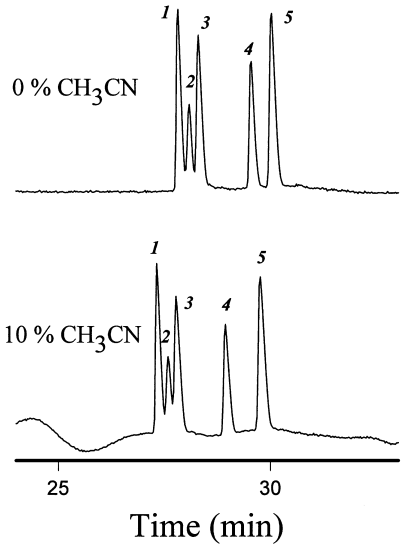

Figure 3. Effect of acetonitrile addition (10% v/v) on the resolution of the peptides at pH 3.07. The Tween 20 concentration was 150 mM. The peak identities are indicated on the graph. Voltage and current vary to keep a constant power of 0.2 W. Other experimental conditions were the same as in Figure 1.

For the second group of peptides (**4** and **5**), peptide **4** has significantly higher hydrophobicity than peptide **5**, but it migrated ahead of **5**, maintaining the order as seen under CZE, confirming that the MEKC selectivity using Tween 20 was only a small modulation of peptide electrophoretic mobility due to their weak interactions. It is necessary to mention that the foregoing discussions have not taken into considerations any potential conformational changes associated with sequence alteration, which may influence the partitioning of analytes into the micelle.

Adding a small percentage of organic solvent to MEKC sometimes increases the resolution and expands the peak capacities by modifying the analyte-micelle interactions.[24] Such an effect can be critical in improving separation when the interactions between the two are too strong, which usually occurs on highly hydrophobic molecules or long peptide. In this study, acetonitrile was used for testing potential further improvement of the resolution. As shown in Figure 3, the addition of 10% acetonitrile to the 150 mM Tween 20 added running buffer was not significant on peptides **1** to **3**, due to probably too small differences between the three peptides. However, improvement of the resolution between peptides **4** and **5** was clearly seen. These two peptides differ more in amino acid composition due to the differences at terminal residues.

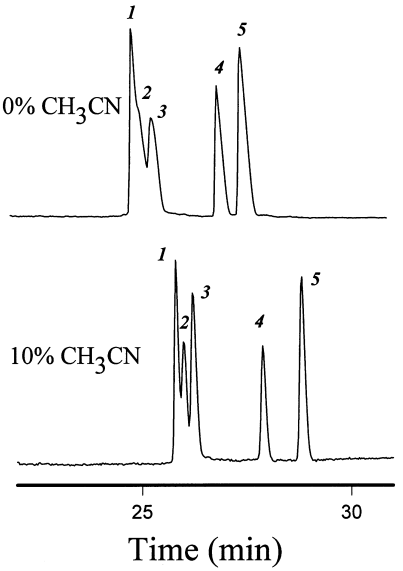

Figure 4. Effect of acetonitrile addition (10% v/v) on the resolution of the peptides at pH 3.07. The Tween 20 concentration was 100 mM. The peak identities are indicated on the graph. Other experimental conditions were the same as in Figure 3.

As shown in Figure 4, the improvement resulting from the addition of acetonitrile was more visible at 100 mM Tween 20, where the separation was not optimized and peptides **1** and **2** were indistinguishable. Adding 10% acetonitrile not only improved the resolution between **4** and **5** as seen at 150 mM Tween 20, but also improved resolution among peptides **1**, **2,** and **3**.

At pH 2.47, the effect of Tween 20 on the separation was slightly different from that at pH 3.07. Compared to the separation by CZE without buffer additives, adding 100 mM Tween 20 separated peptides **1** and **2** and peptides **3** and **4**, separating three zones into five distinctive peaks (Figure 5). Increasing Tween 20 concentration to 150 mM further improved the resolution among peptides **1** to **3**, but deteriorated **4** and **5**. At pH 2.47 the charge difference between the first group of peptides (**1** to **3**) and the second group of peptides (**4** and **5**) is smaller (Table 1).

The separation between the two groups was much less. Compared to pH 3.07, pH 2.47 better protonated the carboxyl group at the C-terminus, making the peptides slightly more hydrophobic. This may, in fact, increase the interaction between the peptides and the neutral surfactant Tween 20.

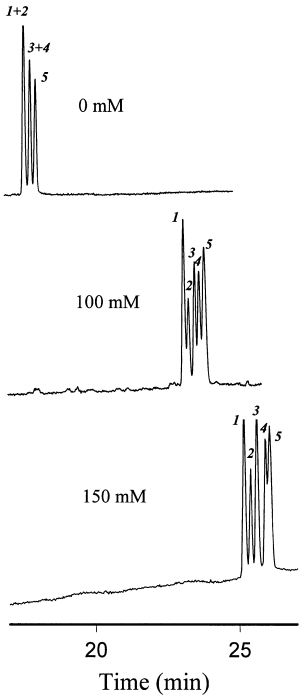

Figure 5. Separations of the peptide mixture at pH 2.47 but with different Tween 20 concentrations. The Tween 20 concentrations and the peak identities are indicated on the graph. Other experimental conditions were the same as in Figure 1.

MEKC Using the Cationic Surfactant, CTAB

The effectiveness of surfactant in separating highly related peptides is still difficult to predict, since the structures of both peptide and surfactant contribute to specific interactions. Use of surfactants and peptides of the same type of net charge (either positive or negative) is more likely to minimize nonspecific ionic interactions. In this study, a cationic surfactant CTAB was tested at pH 3.07. Since both the peptide and CTAB are positively charged at pH 3.07, they migrated toward the same electrode. However, since the mobility of CTAB (and probably CTAB micelles) is larger than any of the peptides (due to a larger charge-to-mass ratio), peptides which distribute more strongly into the micelle gain more mobility.

Adding CTAB to the running buffer greatly improved the separation as shown in Figure 6. With 20 mM CTAB, all five peaks were distinguished; at 50 mM CTAB, all the peptides were separated to almost baseline resolution.

However, the migration order with 50 mM CTAB was different from that in unmodified CZE or CZE with Tween 20. In contrast to the migration order of **1, 2**, **3**, **4,** and **5** shown in Figure 1, the peptides migrated now in the sequence of **4**, **2**, **3**, **1,** and **5**. If the migration were totally controlled by hydrophobicity, the migration order would be in the order of decreasing hydrophobicity, which is **3**, **4**, **2**, **1,** and **5**.

In MEKC using charged analytes and charged micelles, the determinants of migration order are more complicated in two aspects than when neutral analytes are used. Firstly, the interactions could be a combination of hydrophobic and ionic.[25] Secondly, the migration order is not straightforwardly hydrophobic dependent, since the analytes have electrophoretic mobilities when free in solution. CTAB forms micelles with its positively charged quaternary amine moiety pointing outside the hydrophobic core. Analytes which have stronger ionic interactions with the micelle or have higher hydrophobicities exhibit higher affinity for the micelle.

Peptide **4**, having the second highest hydrophobicity, migrated by far the fastest with the CTAB addition. The "abnormally" strong interaction of peptide **4** with the micelle could be related to the presence of a partially charged aspartic acid side chain in the center of the peptide, which provides ionic interaction with the micelle. In other words, each individual charge plays a more important role than the overall net charge in peptide-micelle interaction. Although peptide **5** also has an aspartic acid, it migrated the slowest. This was probably because peptide **5** has the lowest electrophoretic mobility among the five peptides and also has the lowest hydrophobicity of all, which may stabilize its interaction with CTAB micelle to the least degree among the five. Peptides **1**, **2,** and **3** have the same charge distribution, but increasing hydrophobicity in the said order. Peptides **2** and **3** migrated faster than **1**, indicating stronger interactions with the micelle. However, peptide **2** had a higher apparent mobility than **3**, in contrast to the hydrophobicity order.

The positively charged surfactant CTAB has been known to form a dynamic coating on the unmodified fused silica capillary wall, due to ionic interactions with the silanol group. Such dynamic coating results in a reversal of EOF at CTAB concentrations of millimolar or micromolar levels.[16] Since the peptides were detected at the cathode side in a reasonable time, the polyacryamide-modified capillary used in the current study did not seem to bind CTAB extensively; otherwise, a strong EOF would have reversed the apparent migration direction of the peptide. Increasing CTAB concentration is expected to accelerate the migration of the peptide due to peptide-micelle interactions, but, in reality, the migration velocity decreased with the increase in CTAB concentration (Figure 6). Considering that the voltage decrease from zero CTAB to 50 mM CTAB was small under the constant power mode (11.06 kV to 9.03 kV) and that the viscosity increase should be negligible, the increase in migration time in general may indicate a slight adsorption of CTAB to the capillary wall causing a slight reversed-flow EOF.

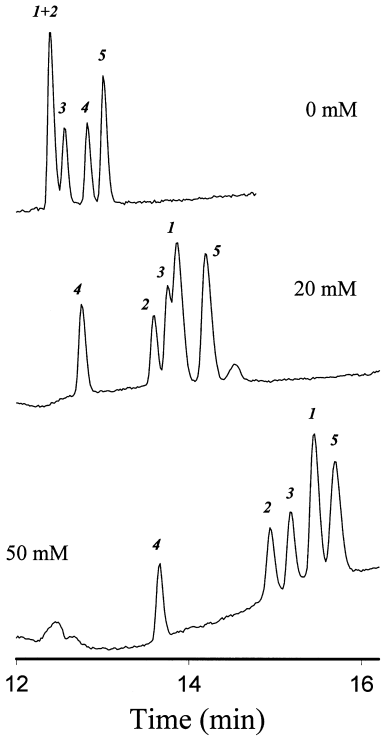

Figure 6. Separations of the peptide mixture at pH 3.07 but with different CTAB concentrations. The CTAB concentrations and the peak identities are indicated on the graph. The length of coated capillary was 37 cm (effective length of 30 cm) and the power was constant at 0.15 W. Other experimental conditions were the same as in Figure 1.

Addition of acetonitrile into the buffer containing 50 mM CTAB (data not shown) did not improve the separation of the peptides, but instead worsened the resolution, indicating, probably, there were no strong hydrophobic interactions between the peptides and the CTAB micelles.

MEKC Using Mixed Surfactants

Although MEKC introduces additional separation selectivity to CZE, the capability of MEKC is limited by the migration window imposed by the minimal and the maximal inclusion of analytes in the micelle. The window in principle can be expanded by combining two or more types of surfactants together. This has been shown on the separation of seventeen corticosteroids,

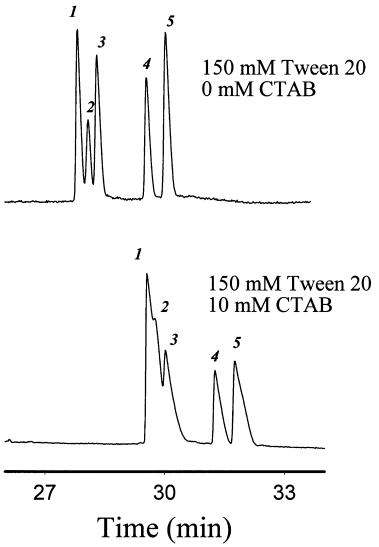

Figure 7. Separations of the peptide mixture at pH 3.07 and 150 mM Tween 20 with different CTAB concentrations. The CTAB concentrations and the peak identities are indicated on the graph. Other experimental conditions were the same as in Figure 6.

where a combination of two bile surfactants and an alkyl surfactant gave better separation than any other combinations using less types of surfactants.[26] In the present case the effect of combining Tween 20 and CTAB was investigated. Figure 7 shows the separation of the five peptides using 150 mM Tween 20 in combination with 10 mM CTAB. Inspection of Figure 7 shows that this effort resulted in deterioration, rather than improvement in the separation, compared to Figure 6.

CONCLUSIONS

In this study, five highly related heptadecapeptides, which were inadequately separated by CZE, were separated at low pHs through the MEKC mechanism, using either a nonionic surfactant Tween 20 or a cationic, surfactant CTAB, with CTAB providing better results. Coated capillaries highly suppressed the EOF, allowing a wider time frame for the separation, which proved to be critical in separating current peptides with subtle differences. The 17-mer peptides, which are relatively long and are positively charged at the pHs used in this study, interacted weakly with Tween 20 micelles but strongly with CTAB so as to alter their normal migration order based on charge-to-size ratio.

The interactions with CTAB appeared to involve electrostatic forces in addition to hydrophobic forces, although the peptides and CTAB all had net positive charges. Therefore, the conformation and the charge distribution of peptides play important roles in determining affinity for surfactant micelles.

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