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Utility of high resolution capillary electrophoresis for monitoring peptide homo- and hetero-dimer formation

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ABSTRACT

The monomer and disufide-linked homo-dimer of two different peptides, one with an amino-terminal cysteine, the other with a cysteine at the carboxy-terminal, are shown to be baseline resolved by capillary electrophoresis in less than 15 min. Time-course for homo-dimer formation with both peptides, either under mild (air) or stronger (hydrogen peroxide oxidizing conditions, was easily monitored. Confirmation that the second peak appearing under oxidizing conditions was indeed the homo-dimer was obtained with mass spectrometry. The possibility that stronger oxidizing conditions led to the production of the sulfonic acid derivative of the monomeric peptide, was ruled out through generation of the derivative by performic acid oxidation. As expected, the negative charge of the sulfonic acid moiety gives the peptide a slower electrophoretic mobility than both the monomer and the dimer. Moreover, as would be expected with a sulfonic acid derivative, oxidation to the dimeric form was not possible. This was consistent with the observation that the homo-dimer peak could be reduced to monomeric form in the presence of dithiotreitol. Co-oxidization of the amino- and carboxy-terminal peptides led to the expected production of both homo-dimers and the hetero-dimer, all of which were resolved.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) is an analytical technique applicable to both small and macromolecular components of biological interest. The capability of attaining separation efficiencies superior to those with high-performance liquid chromatography (several hundred thousand to one million theoretical plates) in a rapid, automated and reproducible manner has set the stage for HPCE to become a premier method for the separation of complex mixtures of molecules having a diverse size and nature [1–4]. Electrophoretic separation is carried out in a capillary having an internal diameter of less than 100 μm and a total volume of no greater than several microliters. The simplicity of using a polyimide-coated fused-silica capillary allows for the efficient dissipation of Joule heat and, hence, for electrophoretic separations to be carried out in free-solution under high potentials (up to 30 000 V).

The development of conditions for reproducible peptide analysis by CE was one of the first successes for the technique. Low pH buffer systems reduce the negative character of the inner capillary wall, thus minimizing interaction with peptides. Under these conditions, CE has been shown to be an excellent technique for separating acidic peptides which are typically difficult to resolve by reversed-phase HPLC methods. The selectivity of CE has ben highlighted by the ability to resolve peptides with small structural differences such as deamidation [5], single amino acid substitutions which do not change the calculated peptide net charge [6], and geometrical isomers which vary only in the surface of the peptide exposed to the run buffer matrix [7]. Collectively, these studies suggest that the isoelectric point (p*I*) should be used

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with caution as a predictive method for peptide migration in CE, since it does not account for variable surface presentation and amino acid sequence which induce "nearest neighbor" effects. This has been demonstrated in an elegant study by Field *et al.* [8] who used strategically located histidine and arginine residues in "shuffled" peptide sequences (same total composition, shuffled amino acid order). These peptides, all theoretically possessing the same charge-to-mass (q/m) ratio were found to have dramatically different mobilities. While there have been attempts to model peptide mobility on altered p*I* predictions which take the nearest neighbor phenomenon into consideration [9], larger peptides, which are non-linear molecules, still do not behave in a theoretically predictable manner.

With the high selectivity inherent in CE, it seems probable that this technique could be exploited for the analysis of disulfide-mediated peptide dimerization. A single illustration in the review by Grossman *et al.* [1] showing the reduction of STP-3 peptide by dithiothreitol provided the first intimation that such analyses could be done with CE. In the present work, we demonstrate this to be the case. The rapid analysis times associated with CE make it ideal for monitoring disulfide-mediated dimerization/reduction processes quantitatively and qualitatively, as well as providing information with respect to homo- and hetero-dimer formation.

EXPERIMENTAL

Materials

Citric acid was a Gold Label reagent purchased from Aldrich. Sodium hydroxide, phosphoric acid (85%) and hydrogen peroxide (30%) were purchased from Fisher Scientific. All chemicals for peptide synthesis were purchased from Applied Biosystems (ABI, Foster City, CA, USA). Dithiothreitol (DTT) was purchased from Sigma (St. Louis, MO, USA).

Peptide synthesis

The synthetic peptide, N-terminal cysteine (Ntc), CFLGIPFAEPPVGSRRFMPPEPKRPW-SGVL contained residues 32–60 from mouse RNA acetylcholinesterase with the addition of an amino-terminal cysteine. A second synthetic peptide contained a 13 amino acid sequence from a proprietary protein, with the addition of a carboxy-terminal cysteine. This peptide, TFQTNPDGTIQFRC, is denoted as the carboxy-terminus cysteine peptide or Ctc. Both were synthesized by 9-fluorenylmethoxycarbonyl (FMOC) amino acid strategy on an ABI 431A peptide synthesizer using the protocols and reagents provided by the manufacturer. Each peptide was purified by reversed phase HPLC using a Vydac C_{18} column (25 × 2.2 cm) using a trifluoroacetic acid–acetonitrile buffer system. Peptide integrity was monitored by either amino acid analysis or positive ion mass spectrometry.

Peptide dimerization

Dimerization was carried out under mild conditions through air oxidation. The peptide was dissolved in water to a final concentration of 0.1 mg/ml. The pH was adjusted to 8.0 with NH_4OH and the solution was stirred at room temperature for up to 18 h. Under these conditions, oxidation was monitored through the concentration of free cysteine using Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid)] to a zero absorbance at 412 nm [10]. For HPCE time course analysis of the dimerization process, the sample was allowed to stand in a microvial (suspended in the sample vial containing 1 ml of water and sealed with a rubber cap) in the HPCE vial tray which maintained a temperature of *ca.* 27°C or in a refrigerator at 4°C. Dimerization was reversed by the addition of DTT at a final concentration of 1 m*M* (addition from a 1 *M* stock solution). Homo-dimer formation was enhanced by the addition of H_2O_2 at a final concentration of 0.015% added to the sample from a 0.3% stock solution.

Formation of the peptide sulfonic acid derivative

Performic acid oxidation was carried out by the method described by Hirs [11]. Briefly, 50 μl of 30% H_2O_2 was added to 950 μl of 88% formic acid and reacted for 2 h at room temperature. A 50-μg amount of peptide was dissolved in 2.5 μl

formic acid and 0.5 μl methanol and cooled at −20°C. A 5-μl volume of performic acid was added, and the reaction allowed to proceed for 2 h at −20°C. The reaction was stopped by dilution with 250 μl of water and the solution speed vacuumed to dryness. The residue was dissolved in 100 μl of water and stored at −20°C until analysis.

Buffer and sample preparation

Citrate buffer (20 m*M*) was made by solubilizing citric acid and adjusting the pH using either NaOH or HCl. Phosphate buffer was made by diluting a 0.1 *M* phosphoric acid stock solution and adjusting the pH with NaOH. All buffers were made with Milli-Q (Millipore) water, and filtered through an 0.2-μm filter (Gelman) before use. The monomeric and dimeric forms of the peptides were solubilized in Milli-Q purified water at a final concentration of 1 mg/ml.

Instrumentation

HPCE separation was carried out on a Beckman P/ACE System 2050 interfaced with an IBM 55SX computer utilizing System Gold software (version 7.1) for control and data collection. All peak information (migration time) was obtained through the System Gold software.

Capillary electrophoresis separation conditions

For peptide analyses, running buffer was either 20 m*M* citrate or 50 m*M* phosphate buffer, pH 2.5, and the following method was typically used: a three-column-volumes rinse with running buffer, 3 s pressure injection of peptide, separation at 25 kV (constant voltage with the inlet as the anode and the outlet as the cathode), a five-column-volumes wash with 0.1 *M* NaOH followed by a five-column-volumes rinse with running buffer. Capillaries were polyimide-coated fused silica with a 57 cm in length (50 cm to the detector) × 50 μm I.D. All electrophoretic separations were carried out at 25 kV constant voltage and capillary temperature was maintained at 28°C. Detection was by absorbance was at 200 nm.

RESULTS AND DISCUSSION

Oxidation of the peptide monomer results in homo-dimer formation

Two unrelated peptides were used to evaluate the utility of HPCE for separation of peptide monomers from their disulfide-linked dimers. The N-terminal cysteine peptide was a 30-mer containing residues 32–60 of the mouse mRNA acetylcholinesterase, has a molecular mass of 3369 and is termed the N-terminal cysteine or Ntc peptide. The second peptide used in this study was a 14-mer containing 13 residues from a proprietary protein and a C-terminal cysteine. It has a molecular mass of 1629 and is referred to as the Ctc peptide.

For CE analysis of the monomers and homodimer of each of the respective peptide systems, the purified homo-dimer was generated via air oxidation. Oxidation was found to be complete with stirring at room temperature overnight (≈18 h) at which point no free cysteine could be detected using Ellman's reagent [10]. Positive ion mass spectrometric analysis of the peptide before and after oxidation yielded molecular masses consistent with the monomer and the dimer. CE analysis of the freshly dissolved, HPLC-purified Ntc peptide monomer in low-pH buffer (pH 2.5) showed that the peptide ran as a single discrete peak at *ca.* 8.2 min, indicating that there was minimal contamination from either incomplete synthesis/deprotection or side reactions (Fig. 1A). CE analysis of the oxidized product shows that the disulfide-linked homodimer had a markedly faster electrophoretic mobility (migration time of 7.2 min) and appeared to be baseline resolved from the monomer (Fig. 1B). Similar profiles were seen in both 50 m*M* phosphate and 20 m*M* citrate. While the peaks were somewhat sharper in the phosphate buffer, the migration times were longer.

Two lines of evidence supported the postulate that the identity of the oxidized product was indeed the disulfide-linked dimer. First, the purified homo-dimer co-migrates with the faster migrating peak which appears in time-dependent manner under oxidizing conditions. Second, a mixture containing the purified homo-dimer and monomer could be reversibly converted to either

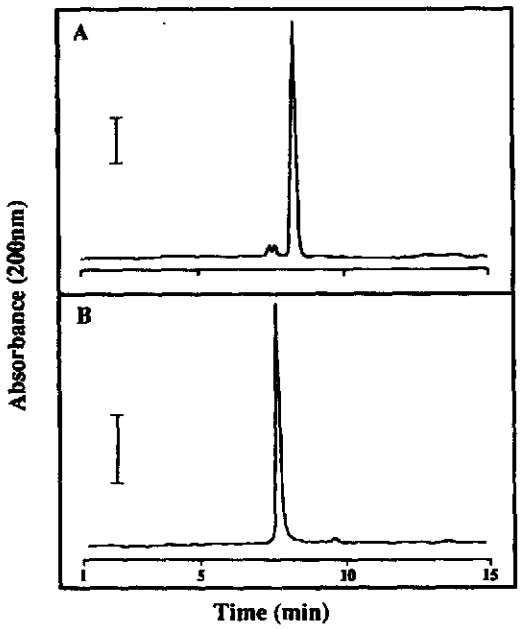

Fig. 1. CE separation of Ntc monomer and the disulfide-linked dimer. Purified Ntc monomer (A) and dimer (B) were dissolved in water at 0.5 mg/ml. Separation was carried out in 20 m*M* citrate buffer, pH 2.5. Bar represents 0.005 AU.

the monomer under reducing conditions (DTT) or the homo-dimer under oxidizing conditions (H_2O_2). This is shown in Fig. 2 (left panels) where, upon addition of DTT at a final concentration of 1 mM, an initial 1:2 mixture of Ntc monomer–dimer is immediately and almost completely converted to the monomeric form (96% as determined by peak areas). In contrast, a 2:1 monomer–dimer mixture is rapidly converted to the dimeric form (90% as determined by peak areas) with the addition H_2O_2 at a final concentration of 0.015% (v/v) (Fig. 2, right panels). In both of these cases, injection and subsequent electrophoretic separation of the oxidized/reduced mixture was carried out within 2–3 min of addition of the reagent. This highlights the rapid rate of conversion between the Ntc monomeric and dimeric forms. In both cases conversion was 100% complete after 30 min.

The possibility that the H_2O_2 had oxidized the sulfhydryl group of the monomer to sulfonic acid (as opposed to generating the disulfide-linked dimer) was thought to be unlikely for two reasons. First, analysis of both preparations on positive ion mass spectrometry gave molecular masses expected for the monomer and dimer. Second, it is unlikely that a sulfonic acid moiety on the peptide would lead to a more rapid electrophoretic mobility than the monomer. In fact, the additional negative charge on the peptide, due to the ionized sulfonic acid at pH 2.5, would presumably lead to a slower electrophoretic mobility (*i.e.* a longer migration time). This was confirmed by generating the sulfonic acid derivative of the Ntc peptide by performic acid oxidation [11]. As predicted, the presence of a sulfonic acid moiety markedly slows the electrophoretic mobility of the Ntc peptide (Fig. 3). Injection of a mixture containing the Ntc monomer, homo-dimer and monomeric sulfonic acid derivative shows that the order in which they migrate is as would be predicted. However, the migration times for all components have been increased as result of residual formic acid in the sample matrix. Addition of DTT to untreated monomer and the sulfonic acid derivative showed that only the former could be oxidized to the dimer. These results indicate that the peak signified with an arrow in Fig. 2 (lower right panel) is most likely the sulfonic acid derivative. Under the strong oxidizing conditions afforded by H_2O_2, some sulfonic acid derivative is formed as a minor side reaction during the dimerization process. The oxidation of the Ntc monomer to the disulfide-linked homo-dimer was substantially rapid at room temperature. Fig. 4 shows approximately 32% conversion of the Ntc monomer (as determined by peak areas) within 2 h at 27°C. The temperature-dependent nature of this process is indicated by the slower loss of monomer at 4°C. It is clear that the loss of the monomer (peak at 8.2 min) and corresponding appearance of the peak at 7.2 min does not represent some type of degradation process, since the purified homo-dimer is apparently unaffected within the same time-frame under identical conditions.

Similar studies were carried out with a second peptide having a carboxy-terminal cysteine and these studies clearly show the utility of CE for time course analysis if peptide dimerization (Fig. 5). Analysis of the purified Ctc monomer and homo-dimer showed the Ctc monomer to have a

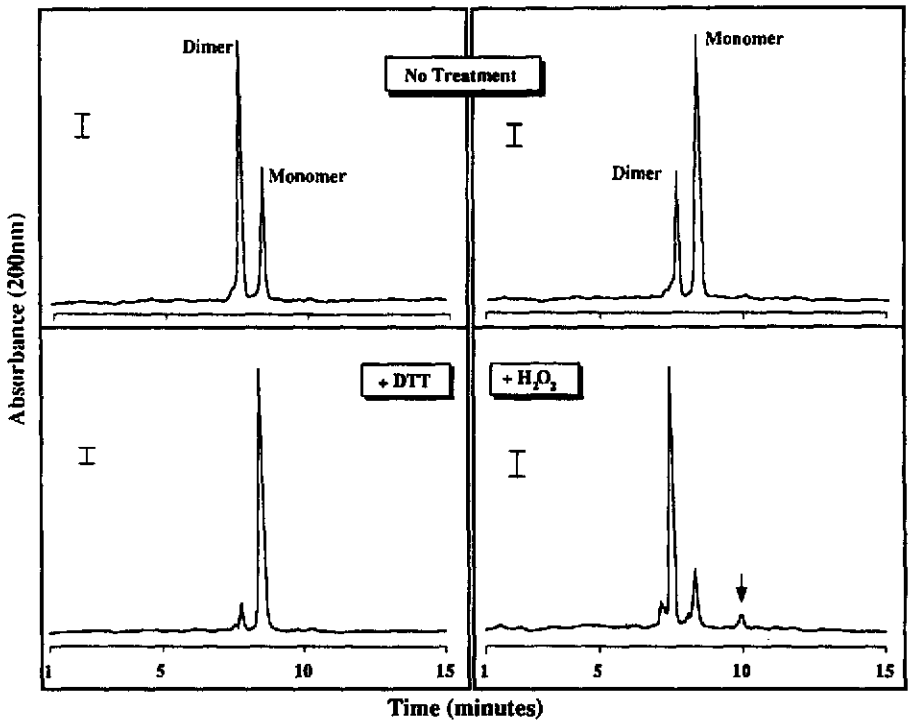

Fig. 2. Oxidization and reduction of an Ntc monomer–dimer mixture. A 1:2 mixture of monomer–dimer is reduced in the presence of 1 m*M* DTT (left panels) while a 2:1 mixture monomer–dimer is oxidized in the presence of 0.015% H_2O_2 (right panels). Separation was carried out in 20 m*M* citrate buffer, pH 2.5. Bar represents 0.002 AU.

migration time of 11.3 min while the dimer, formed more rapidly under the strongly oxidizing conditions, has a migration time of 9.6 min. Consistent with the Ntc system, the Ctc homo-dimer had a more rapid electrophoretic mobility than the Ctc monomer and, thus, they are baseline resolved. Oxidation of the Ctc monomeric peptide was markedly slower at 27°C than that observed with the Ntc peptide (compare with Fig. 4C), with only negligible conversion to homo-dimer observed after 8 h. In order to induce substantial conversion of the monomer to the dimer, H_2O_2 was added at a final concentration of 0.015% (v/v) at which point a more accelerated conversion to the homo-dimer occurred. Where similar conditions (H_2O_2) led to complete monomer-to-dimer conversion of the Ntc system within minutes, Ctc dimerization was only 59% complete after 8 h. This suggests that either the nature of the peptide itself (size, amino acid composition, primary structure), the different location of the cysteine (N- *vs.* C-terminal) or both are playing a role in the rate of dimerization.

CE analysis of homo- and hetero-dimer formation

It is clear from a comparison of Figs. 4C and 5 that the Ntc dimerization is markedly faster than that observed with the Ctc system (under mild or strong oxidizing conditions). To determine whether CE could resolve both homo-dimers, as well as the heterodimer, a solution containing both the purified Ntc and Ctc monomer was incubated at 27°C in the absence of H_2O_2 due to the comparatively rapid oxidation of the Ntc monomer (Fig. 2). Beginning initially with pure Ntc and Ctc monomer (Fig. 6A), incubation for 2 h and 46 min resulted in the formation of substantial Ntc homo-dimer (migration time 8.0 min) representing *ca.* 16% conversion (Fig. 6B). In contrast, a negligible amount of Ctc monomer had been oxidized to the homo-dimeric state (signified by the arrow at 9.4 min). In addition,

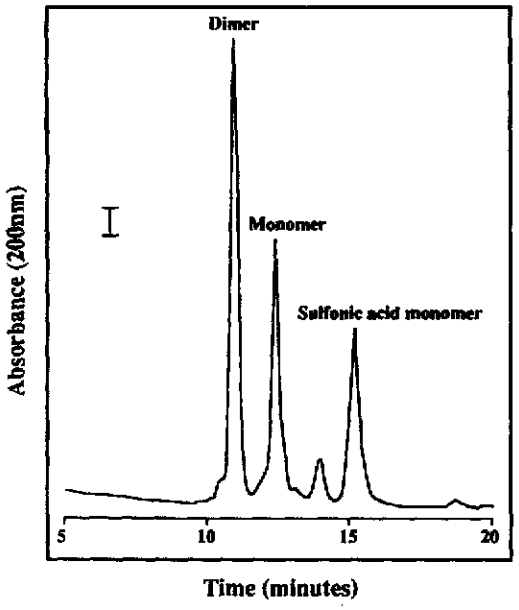

Fig. 3. CE analysis of the Ntc monomer, homo-dimer and sulfonic acid derivative. A mixture of purified monomer (1.0 mg/ml), dimer (0.5 mg/ml) and sulfonic acid derivative (0.5 mg/ml) obtained as described in the Experimental section was injected into the capillary which contained 50 m*M* phosphate buffer, pH 2.5. Bar represents 0.005 AU.

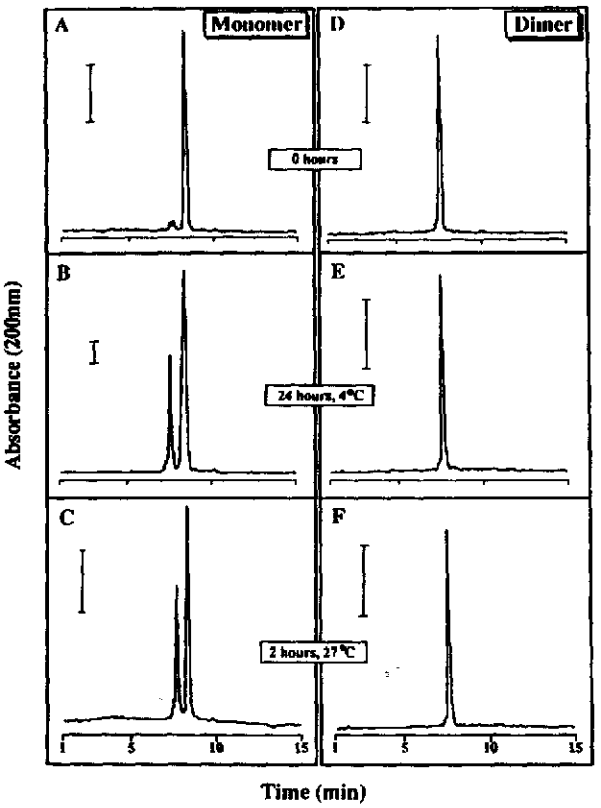

Fig. 4. Oxidization of Ntc peptide. Purified Ntc monomer and dimer were dissolved in water (1 mg/ml), analysed immediately (0 h) and after incubation at 27°C for 2 h and 4°C for 24 h. Bar represents 0.005 AU.

there is the appearance of a peak between the Ntc monomer and homo-dimer that was not previously observed. This represents the first intimation of heterodimer formation between the Ntc and Ctc peptide monomers (8.6 min). This peak appears to increase in concert with the Ntc homo-dimer peak (Fig. 6B and C). Only after *ca.* 11 h is there a clearly definable amount of Ctc homo-dimer (Fig. 6D). As would be predicted from the comparatively slow rate of dimerization (relative to the Ntc peptide), the magnitude of the Ctc homo-dimer peak is small in comparison with those of the Ntc homo-dimer and Ntc-Ctc heterodimer.

After incubation of this same sample for 24 h at 27°C, the profile given in Fig. 7A was observed. From the rapid rate of dimerization of the Ntc peptide and the comparatively slow rate of Ctc dimerization, it is predicted that after this period of time, there should be little or no monomeric Ntc but detectable levels of Ctc monomer. This appears to be the case. Co-injection of pure Ctc (Fig. 7B) and Ntc (Fig. 7C) monomer with the mixture clearly shows that, as would be predicted from migration times, peaks 6 and 3 represents residual monomeric Ctc and Ntc respectively, while peaks 4 and 1 respectively represent the Ctc and Ntc dimers. It follows that peak 2 represents the Ctc:Ntc hetero-dimer and that peak 5 represents the sulfonic acid derivative of the Ntc peptide. As would be expected from the mobility of the Ntc and Ctc monomers, the hetero-dimer has a mobility that is median to both. It is interesting that the heterodimer appears to be in a slightly higher concentration (assuming equal molar absorptivity) than both of the homo-dimers. This may indicate that both monomers have a slightly higher affinity for hetero- rather than homo-dimerization. This is particularly the case for the Ctc monomer and raises some interesting questions regarding the physical characteristics of

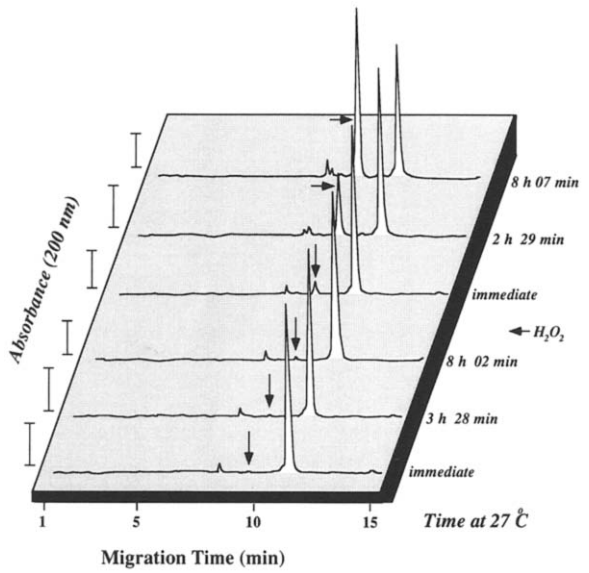

Fig. 5. CE time-course analysis of the Ctc dimerization process. Ctc peptide (1 mg/ml in water) was incubated at 27°C and analysed at 0 min (immediately), 3 h 28 min, 8 h 2 min. Hydrogen peroxide was added and analysis carried out at 0 min (immediately), 2 h 29 min, 8 h 7 min. Separation was carried out in 20 m*M* citrate buffer, pH 2.5. Bar represents 0.005 AU.

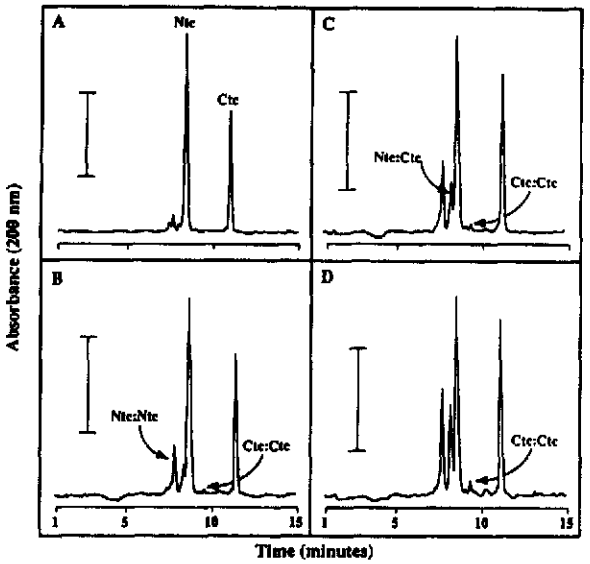

Fig. 6. Co-oxidization of the Ctc and Ntc peptides. A mixture of the purified Ntc and Ctc monomers was incubated at 27°C and analysed at 0 min (A), 2 h 46 min (B), 5 h 31 min (C) and 11 h 1 min (D). Separation was carried out in 20 m*M* citrate buffer, pH 2.5. Bar represents 0.005 AU.

each of the peptides that result in the observed dimerization properties.

Resolution of the monomer and dimer

An interesting question arises from the observation that the monomer and dimer in both systems are baseline resolved by CE. Can the baseline resolution of the monomer and dimer be explained by differences in mass/charge properties? Covalent dimerization through oxidation of the terminal cysteines involves only the loss of two hydrogen atoms with no obvious change in net charge. Hence, from the perspective of simple mass-to-charge ratio (m/q), which is the basis for separation in free solution CE, the difference between the monomer and the homodimer appears negligible (0.034%). However, the 1966 study by Offord [12] demonstrated that the relationship between the mass/charge properties of a peptide and its electrophoretic mobility was linear when expressed as a quotient of mass$^{2/3}$/charge ($m^{2/3}/q$). This was shown to be a reasonable postulate with a mixture of peptides which included bradykinin, angiotensin, leucine enkephalin, methionine enkephalin, and oxytocin (Fig. 8). The charge on each peptide at pH 2.5 was calculated with a program that compensated for the presence of amidated or acetylated residues[a] and the $m^{2/3}/q$ ratios calculated accordingly. A plot of the $m^{2/3}/q$ *vs.* migration time shows a reasonably linear relationship between these two parameters with a correlation coefficient (r) of 0.971. Co-injection of Ntc and Ctc monomer/dimer with the standard peptides allowed for the determination of their respective mobilities relative to the standards. Determining the net charge at pH 2.5 for the Ntc monomer (+4.88), Ntc dimer (+8.85), Ctc monomer (+1.969) and Ctc dimer (+3.390), $m^{2/3}/q$ values of 46.32, 37.51, 70.32 and 55.90 result respectively. The differences in the calculated $m^{2/3}/q$ for the monomer and dimer in both peptide systems suggest that, indeed, they should resolve

[a] The net charge on a peptide was calculated using the NCHARGE software written by Dr. L. Holladay of Alza Corporation. Parameters for the calculation including temperature of 28°C (capillary temperature), ionic strength of the buffer and a C-terminal pK_a of 2.90.

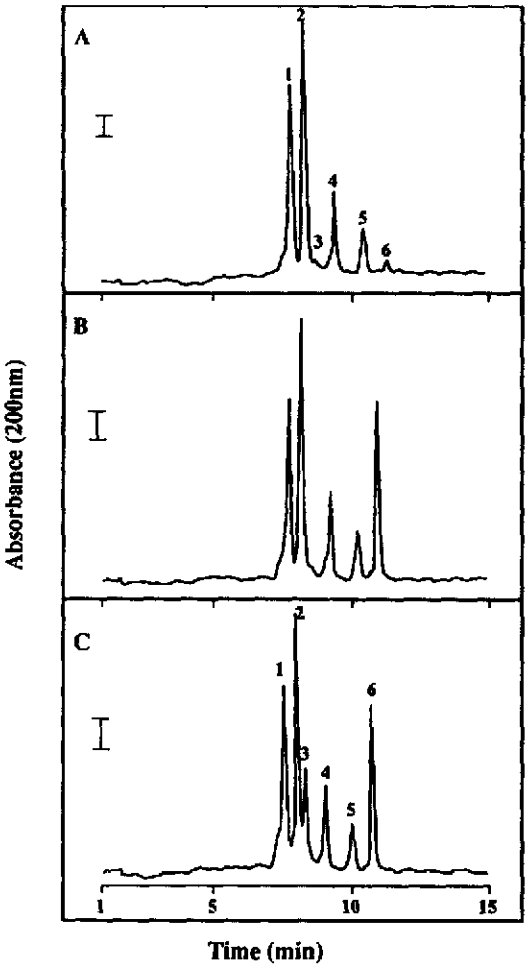

Fig. 7. Confirmation of peak identity through co-injection of peptide monomers. (A) The oxidized mixture described in Fig. 6 except after incubation at 27°C for 28.5 h. By co-injection of purified components with the mixture, peaks 6 and 3 are shown to represent the Ctc (B) and Ntc (C) monomers respectively. As indicated by migration times, peaks 1 and 4 represent the Ntc and Ctc dimer while peak 2 represents the Ntc:Ctc hetero-dimer and peak 5 the sulfonic acid derivative of the Ntc monomer. Separation was carried out in 20 mM citrate buffer, pH 2.5. Bar represents 0.001 AU.

by CE and that the homo-dimer should have a faster electrophoretic migration than the monomer. When these values are plotted on the curve obtained with standard peptides, it becomes clear that the Ctc monomer and homo-dimer both behave ideally, *i.e.* do not deviate from the linear relationship between charge/mass and

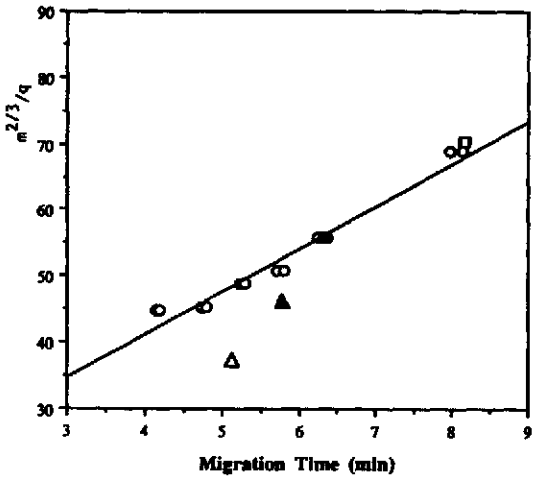

Fig. 8. Plot of mass-to-charge ratio ($m^{2/3}/q$) *vs.* migration time. Calculated values for $m^{2/3}/q$ were plotted against the migration times for two analyses (hollow circles) and linear regression analysis done on the combined data set (standard peptides include bradykinin, angiotensin, leucine enkephalin, methionine enkephalin, and oxytocin). Calculated values plotted for comparison: ○ = standard peptides ($r = 0.971$); □ = Ctc monomer; ■ = Ctc dimer; ▲ = Ntc monomer; △ = Ntc dimer.

electrophoretic mobility. In contrast, neither the Ntc monomer nor homo-dimer appear to behave ideally. Previous studies have shown that larger peptides are typically not linear and, hence, are less likely to behave ideally. Therefore, whether this observed deviation from linearity is a function a peptide size, character or both, is not clear.

It is reasonable to suggest that the differences in the $m^{2/3}/q$ values for the monomers and dimers reflect some type of secondary structure in the dimeric form, which ultimately results in the dimer having more charge per unit mass than the monomer. This could simply be due to "nearest neighbor" effects induced by the secondary structure of the dimer. The study by Field *et al.* [8] showed the dramatic outcome of the "nearest neighbor effect" on the mobility of peptides. They found that a series of peptides containing the identical amino acid composition (and little or no secondary structure) but differing in their primary structure (*i.e.* amino acid sequence) were separable by HPCE under the appropriate conditions. They argued that the

residues flanking charged amino acids (*i.e.* their environment) can alter the pK_a of the charged amino acid. Their data were also consistent with the concept that varying the primary sequence may lead to subtle changes in secondary structure, which ultimately may affect electrophoretic mobility. While there is no evidence that any type of secondary structure exist in the Ntc and Ctc systems (as predicted using the Chow–Fasman or Garnier–Osguthorpe–Robson methods[a]), we can not rule out the possibility that random secondary structure of the dimer is affecting the pK_a values of charged residues when in proximity to certain residues of the covalently-bound complimentary peptide. This most certainly is a possibility with the larger Ntc system which, as per Fig. 8, coincidently does not behave ideally from an electrophoretic mobility point of view.

CONCLUSIONS

This study demonstrates the utility of CE for the analysis of peptide dimerization through disulfide linkages. Selectivity is highlighted by the ability to resolve, not only monomers and dimers, but disulfide-linked homo- and hetero-dimers. The advantages of CE for this type of analysis include all the characteristics that make it useful as a microanalytical technique for biomolecules [13], the most important of which are selectivity, rapid analysis time and automation. The time-course analysis of the oxidization of peptide monomer to the dimeric form is easily automated, the results revealing qualitative and quantitative information about monomeric, homo-dimeric and hetero-dimeric components. This presents the possibility that CE could be used for measuring the kinetics of dimerization.

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[a] Obtained through the "Peptide Structure Program" contained within the GCG (Genetics Computer Group) sequence analysis software (version 7.0). Idem., Ibid.