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Analysis of dilute peptide samples by capillary zone electrophoresis

RUEDI AEBERSOLD* and HAMISH D. MORRISON
Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, B.C. V6T 1W5 (Canada)

ABSTRACT

We report a method for the analysis of dilute peptide solutions by capillary zone electrophoresis. The procedure is based on an electrophoretic concentration step of the applied peptide solution in the capillary (stacking) prior to separation, thus allowing the application of increased sample volumes without a breakdown in resolution. Given a constant configuration of the hardware, the method permits the analysis of peptide solutions of an at least 5 times lower concentration than previously possible. The method was applied to the direct analysis of peptide samples separated by narrow-bore reversed-phase high-performance liquid chromatography for high-sensitivity peptide-sequence analysis.

INTRODUCTION

Capillary zone electrophoresis (CZE) is among the methods with the highest resolving power in a single dimension for charged molecules. Separations with several hundred thousand theoretical plates have already been achieved[1,2]. In addition, the basis of separation is different from popular chromatographic methods such as reversed-phase high-performance liquid chromatography (RP-HPLC). The concentration of the analyte in a small detection volume gives CZE a high intrinsic sensitivity. High resolution, high sensitivity and orthogonal selectivity make CZE an ideal method for the further characterization of HPLC fractions.

In spite of the high intrinsic detection sensitivity, the usefulness of CZE for the analysis of dilute samples is limited. Sample volumes bigger than the 5–10 nl typically applied to the capillary lead to a dramatic breakdown in resolution.

Currently, automated high-sensitivity methods allow the sequence determination of peptide samples at the 10–20 pmole level[3]. The preparation and isolation of such small amounts of peptides in a form compatible with sequence analysis represents a considerable technical challenge. The most successful methods rely on the separation of proteolytic cleavage fragments by narrow-bore RP-HPLC[4,5]. For a successful sequence determination it is of extreme importance to verify that the sample applied to the sequenator contains but a single peptide species. Multiple peptides simultaneously

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applied to the sequenator generally make the sequence uninterpretable. Furthermore, peptides applied to the sequenator cartridge cannot be recovered for further separation.

The analysis of HPLC fractions which contain peptides at a concentration of 0.5–5 ng/μl in an essentially non-destructive way is therefore a common problem in high-sensitivity protein-sequence analysis. To date, no satisfactory method has been available to perform this task.

We have evaluated the potential of CZE for the analysis of dilute peptide solutions. In agreement with other groups we found that even with high-quality equipment, the detection limit imposed by the application volume is reached at sample concentrations in the order of 5–20 ng/μl.

We have developed a simple and fast procedure for the analysis of peptide solutions too dilute to be analyzed in the standard CZE operating mode. The method is based on the electrophoretic concentration of peptides in the capillary before the separation is started. In the concentrating mode significantly higher sample volumes can be applied without breakdown in resolution. We demonstrate the successful use of the method for the analysis of peptide fractions collected from narrow-bore HPLC systems prior to sequence analysis and discuss implications of this method for other analytical problems as well as for further developments in CZE methodology.

MATERIALS AND METHODS

Capillary electrophoresis

In this study we used an ABI Model 270A (Applied Biosystems, Foster City, CA, U.S.A.) capillary electrophoresis instrument. Materials and reagent for CZE, including capillaries, electrophoresis buffers and a calibrated mixture of three synthetic peptides (performance evaluation standard) were purchased from the manufacturer of the instrument.

UV absorption was measured at 215 nm. In the standard and concentrating operating mode, samples were loaded to the capillary by applying a reduced pressure to the cathodic electrode reservoir for a specified time, while the anodic end of the capillary was immersed in the sample solution. After sample application, the anodic end of the capillary was placed back into the electrophoresis buffer, along with the anodic electrode and electrophoresis was started. The temperature was maintained at 30°C for all experiments and electrophoresis was performed at 30 kV.

In the concentrating operating mode, the pH of the sample solution was raised to pH >10 by the addition of small volumes of ammonia. The volume of ammonia required to raise the pH of a given sample volume was determined by direct measurement of the pH value of a scaled-up volume of sample buffer only.

Synthesis and purification of model decapeptide

The peptide NH_2–Val–Gln–Ala–Ala–Ile–Asn–Tyr–Ile–Asn–Gly–COOH was chemically synthesized using an Applied Biosystems Model 430 A peptide synthesizer. The crude peptide (50–100 mg) was dissolved in dilute acetic acid containing 6 *M* guanidine hydrochloride and injected into an Altex HPLC system equipped with a Waters 440A detector and a Vydac C4 column (250 × 4.6 mm I.D., 5 μm). Fractions containing the pure peptide were collected, pooled and lyophilyzed. Chromatographic

conditions were the following: buffer A: 0.1% trifluoroacetic acid (TFA) in water; buffer B: 0.1% TFA in acetonitrile–water (60:40, v/v); column temperature: 50°C; flow-rate: 1.2 ml/min. A linear gradient of 0–100% buffer B over 90 min was applied.

Proteolytic cleavage and peptide isolation

The 85000-dalton protein used for the generation of tryptic cleavage fragments was isolated and processed as described before[4]. Approximately 5 μg of protein was separated from contaminating bands by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose and cleaved on the matrix by trypsin. Resulting cleavage fragments were released into the supernatant and separated on a Vydac C4 narrow-bore (250 × 2.1 mm I.D.) reversed-phase column on a Waters peptide analyzer (Waters Associates, Milford, MA, U.S.A.). The system was equipped with a 200-μl sample-injector loop. Digestion mixture was acidified with 10 μl of 10% TFA, vortexed quickly and centrifuged for 1 min in a microfuge at high speed. The supernatant was removed and immediately injected into the HPLC unit. The following buffer system was used: buffer A: 0.1% TFA (Sequenal grade, Pierce, Rockford, IL, U.S.A.) in water; buffer B: 0.08–0.095% TFA in acetonitrile–water (70:30, v/v). The optical densities of buffers A and B were matched at 215 nm by titrating the TFA concentration in buffer B. Both buffers were continuously degassed with a stream of helium. All experiments were carried out with the column at 50°C at a flow-rate of 100 μl/min. Following sample injection, buffer A was pumped through the column for 5 min at a flow-rate of 200 μl/min before the gradient was started.

Peptides were detected with a Waters Model 900E photodiode array detector. The leading wavelength was 215 nm. Peptide-containing fractions were collected manually into microfuge tubes.

Buffers, HPLC solvents and other reagents were of analytical or HPLC grade. All aqueous solutions were prepared with deionized ultrafiltered water.

RESULTS AND DISCUSSION

Sensitivity limit of peptide analysis by CZE in the standard operating mode

We first determined the lower concentration limit for peptide analysis by CZE in the standard operating mode in the given hardware configuration of an Applied Biosystems Model 270A Instrument. The sample was the performance evaluation standard supplied by the manufacturer of the instrument. The three synthetic peptides were dissolved at an initial concentration of 100 ng/μl and then serially diluted two-fold in 10 m*M* citrate buffer, pH 2.5. Electrophoresis was performed in the same buffer and the peptides were detected at 215 nm. Samples were loaded to the capillary by applying reduced pressure at the cathodic end of the capillary for 5 s. To minimize the reduction of the apparent detection sensitivity by peak broadening, the loading time was kept constant and the sample concentration was varied.

The results are shown in Fig. 1. The lower concentration limit with this sample was reached at a sample concentration of 5–10 ng/μl. The application of larger sample volumes lead to significant peak broadening and did not improve on the detection limit as judged by the signal to noise ratio. The required minimal sample concentration for operation in the standard operating mode is about 10 ng/μl. This value is ap-

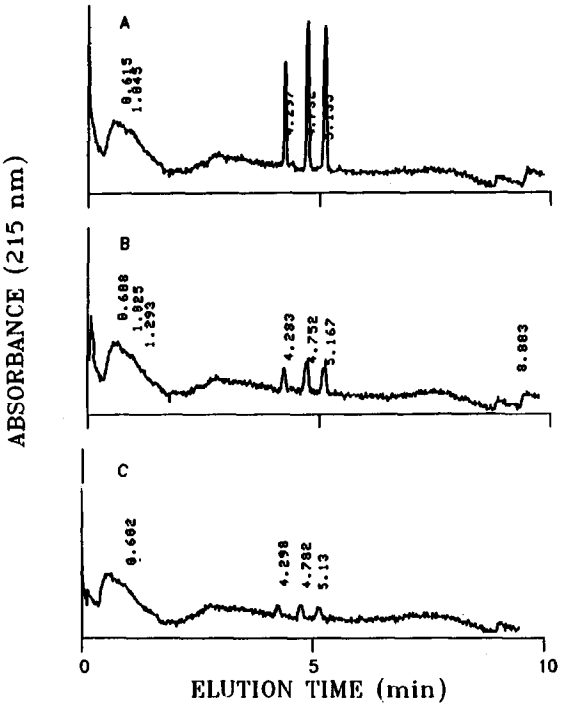

Fig. 1. Sensitivity of CZE in standard operating mode. A calibrated mixture of three synthetic peptides (performance evaluation standard) was dissolved in 10 m*M* citrate buffer, pH 2.5. Samples were loaded to the capillary by applying reduced pressure to the cathodic end of the capillary for 5 s. Electrophoresis carried out at 30 kV. Detector setting 0.005 a.u.f.s. at 215 nm. Sample concentration: (A) 25 ng/μl; (B) 12.5 ng/μl; (C) 6.25 ng/μl.

proximately 5–10-fold higher than actual peptide concentrations in typical peptide fractions collected for high-sensitivity sequence analysis from a narrow-bore HPLC system.

Electrophoretic concentration of dilute peptide samples in the capillary

The sensitivity of CZE in the standard operating mode is not sufficient for the analysis of peptide samples collected from narrow-bore HPLC columns for high-sensitivity sequence analysis. One way to overcome this limitation is to apply higher sample volumes to the capillary and to avoid the breakdown in resolution.

We attempted to concentrate the applied peptide solution electrophoretically in the capillary into a narrow zone before the separation was started. This was achieved by electrophoretic stacking of the peptides at the interface between the sample application solution and the electrophoresis buffer. The procedure is schematically illustrated in Fig. 2. The pH of the sample solution was raised to a value well above the isoelectric point (p*I*) of the peptides by the addition of base. The basic sample solution was then applied to the capillary previously filled with low-pH electrophoresis buffer (citrate buffer, pH 2.5). Immediately after sample application, the anodic end of the

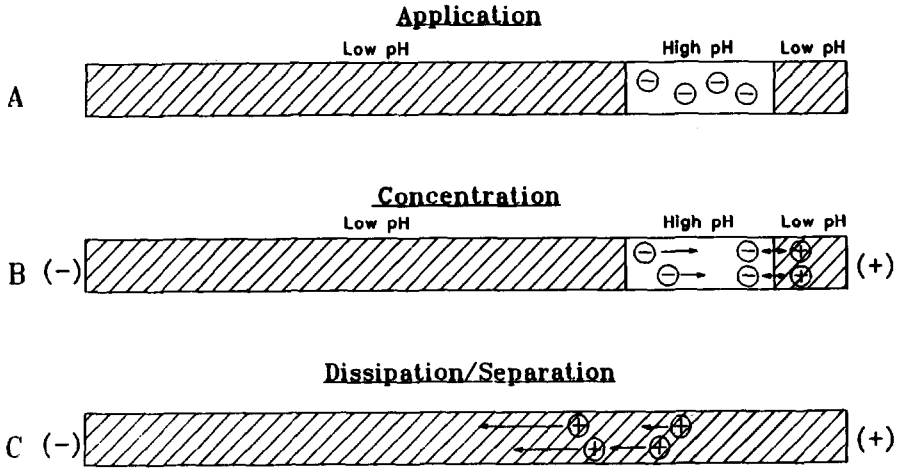

Fig. 2. Schematic illustration of electrophoretic stacking. (A) Samples are applied to the capillary in a solution with a pH value higher than the p*I* of the peptides, resulting in deprotonated, negatively charged peptides. (B) Application of a potential gradient over the capillary leads to concentration of the peptides at the electrophoresis buffer–sample interface. (C) After dissipation of the pH-step gradient, peptides resume mobility towards the cathode and are separated in the electric field.

capillary was immersed into low-pH electrophoresis buffer. This procedure resulted in a pH-step gradient at the interface of the electrophoresis buffer and the applied sample.

Application of a potential gradient over the capillary initiated the movement of the peptides towards the anode (Fig. 2B). The direction of electrophoretic mobility was reversed, as soon as the peptides migrated into the acidic milieu of the electrophoresis buffer, leading to the concentration of the applied peptides at the sample solution–electrophoresis buffer interface.

If the potential gradient was maintained over the capillary, the pH step rapidly dissipated and peptides started to move from the concentrating zone towards the cathode.

The effect of the electrophoretic concentration procedure is shown in Fig. 3. Aliquots of the performance evaluation standard were dissolved at 10 ng/μl in either 10 m*M* citrate buffer (pH 2.5) or in 20 m*M* NH_4OH. Both samples were applied to the capillary by applying negative pressure to the cathodic end of the capillary for 10, 20 or 30 s (Fig. 3). In the normal operating mode, severe peak broadening and a concurrent breakdown in resolution was observed at an application time as short as 10 s (Fig. 3A–C). In the concentrating mode, loading times in excess of 30 s were easily tolerated with minimal peak broadening and loss of resolution (Fig. 3D–F).

We next attempted to determine the minimal sample concentration required for peptide analysis by CZE in the concentrating mode. Aliquots of the performance evaluation standard were dissolved in 20 m*M* citrate buffer (pH 2.5) at concentrations ranging from 1–50 ng/μl. The pH of the sample solutions was raised by the addition of concentrated ammonia and the samples were loaded into the capillary by applying negative pressure at the cathodic end of the capillary for 60 s before electrophoresis was started (data not shown).

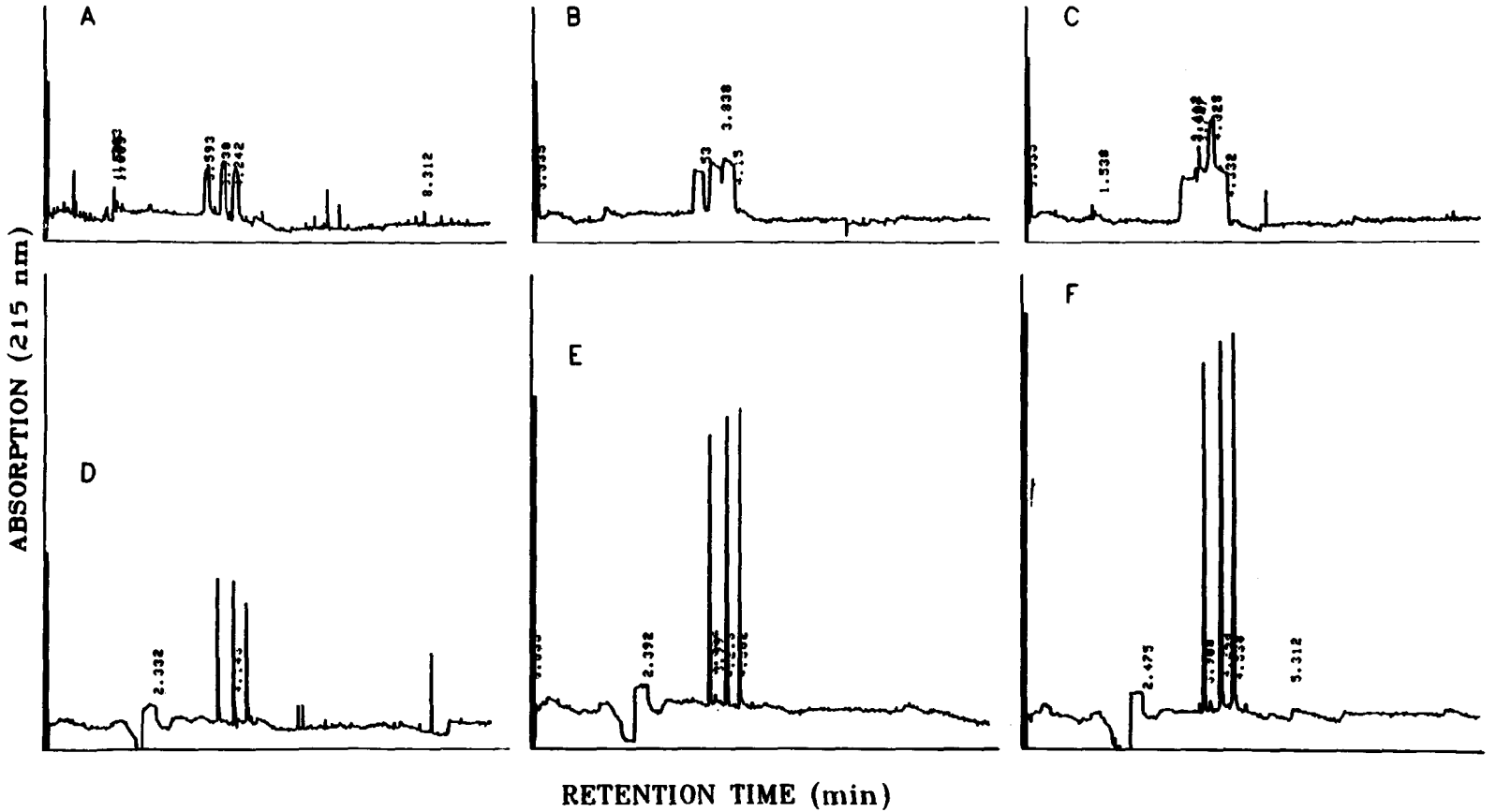

Fig. 3. Effect of the concentrating procedure. Performance evaluation standard was dissolved at 10 ng/μl in either 10 m*M* citrate buffer, pH 2.5 (A, B, C) or in 10 m*M* NH_4OH (D, E, F). Samples were loaded to the capillary by applying reduced pressure at the cathodic end of the capillary for 10 s (A, D) 20 s (B, E) or 30 s (C, F). Electrophoresis was performed at 30 kV. Peptides were detected at 215 nm at a detector setting of 0.005 a.u.f.s.

These results, together with the results shown in Fig. 3D–F and Fig. 4, showed that the minimal concentration required for analysis of performance evaluation standard by CZE in the concentrating mode was below 1 ng/ml. This represented a significant improvement over the limits observed in the same instrument in the same configuration with the same sample using the standard operating mode. Furthermore, the ability to successfully analyze samples at the 1–2-ng/μl level raised the possibility to directly analyze peptide fractions collected from narrow-bore HPLC prior to high-sensitivity sequence analysis.

CZE of peptide fractions separated by narrow-bore HPLC

We next determined whether the conditions for the electrophoretic concentration of dilute peptide samples in the capillary were applicable to the analysis of peptide fractions collected from a narrow-bore HPLC system.

Aliquots of 60–500 ng of the synthetic decapeptide NH_2–Val–Gln–Ala–Ala–Ile–Asn–Tyr–Ile–Asn–Gly–COOH were applied to a C_4 HPLC column. The column was developed with an acetonitrile gradient in 0.1% TFA and the peptides were detected by UV absorbance at 215 nm. Peptides were collected manually into a microfuge tube. The fraction volume was 80 μl. Two idential samples were collected at each concentration. One sample was directly applied to the capillary of the CZE

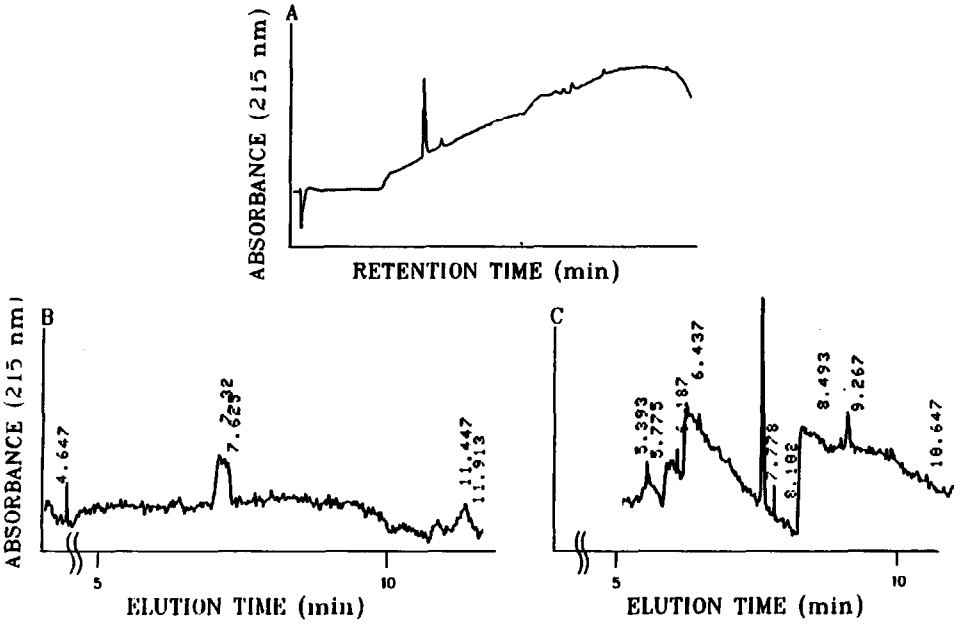

Fig. 4. Analysis of HPLC peptide fractions by CZE. Aliquots of 60 ng of the decapeptide NH_2–Val–Gln–Ala–Ala–Ile–Asn–Tyr–Ile–Asn–Gly–COOH were applied to a narrow-bore RP-HPLC column. Eluting peptides, as detected by UV absorption at 215 nm (A), were manually collected in a fraction volume of 80 μl. Collected peptide samples were either directly applied to the capillary of a CZE instrument for separation in the standard operating mode (B) or added with 2 μl of concentrated NH_4OH for separation in the concentrating mode (C). Application time: 30 s. Electrophoresis was carried out at 30 kV at 30°C, CZE detector setting 0.005 a.u.f.s. at 215 nm.

system for analysis in the standard operating mode. The second sample was added with 2 μl of concentrated NH_4OH to raise the pH of the sample solution well above the p*I* of the peptide. Aliquots of the basic peptide solution were then applied to the capillary for analysis in the concentrating mode. Results shown in Fig. 4 demonstrated that the concentrating mode was applicable to the analysis of peptides collected directly from narrow-bore HPLC systems. As little as 60 ng of the decapeptide applied to a HPLC column could be successfully analyzed by CZE in the concentrating mode (Fig. 4). Assuming a typical recovery of 70–80% of the peptide applied to the HPLC system and a fraction volume of 80 μl, the analyte concentration was in the range of 0.52–0.6 ng/μl. These results also indicated that the presence of small amounts of TFA and variable concentrations of acetonitrile in the peptide fractions collected from the HPLC system did not interfere with the concentrating process. These factors might however contribute to baseline noise in the CZE separation.

We then applied CZE in the concentrating mode to the analysis of selected peptides derived from the tryptic digestion of an estimated 5-μg aliquot of a 85000-dalton protein. Fractions were collected from a narrow-bore HPLC system. We were able to resolve peptides coeluting from the HPLC system into several peaks by CZE analysis in the concentrating operating mode but not in the standard operating mode (Fig. 5). Subsequent sequence analysis of the peptide fraction analyzed in Fig. 5 revealed the presence of three different peptide species. Once detected, fractions containing more than one peptide species can be further separated under chromatographic conditions with different selectivity before their application to the sequenator[5].

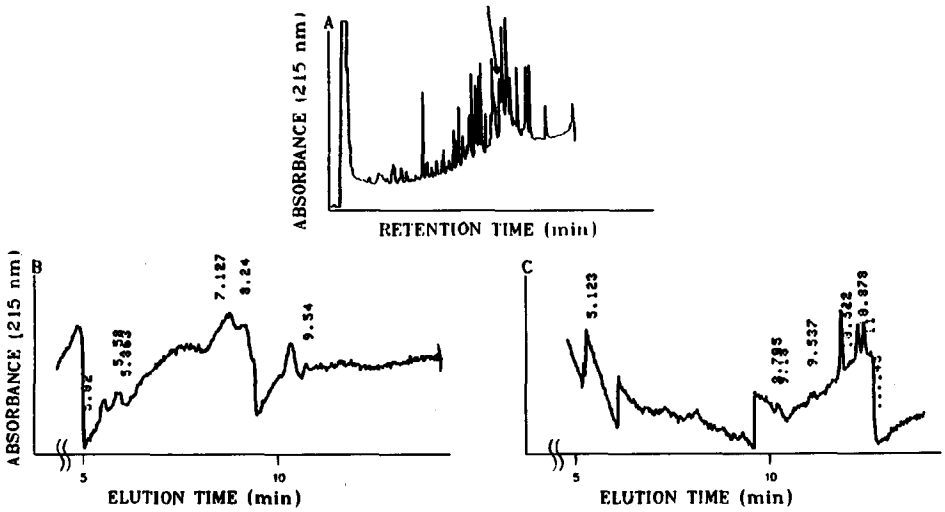

Fig. 5. Analysis of HPLC peptide fractions by CZE. An estimated 5 μg of a 85000-dalton protein were electroblotted from a polyacrylamide gel onto nitrocellulose and cleaved with trypsin on the matrix. Resulting peptides were released and separated by RP-HPLC (A). Fractions were collected manually. The marked peak (↓) was subjected to CZE analysis in the standard operating mode (B) or, after the addition of 2 μl of concentrated NH_4OH, in the concentrating mode (C). Electrophoresis was carried out at 30 kV at 30°C. Sample was applied for 30 s. Detector settings: HPLC: 0.1 a.u.f.s. at 215 nm; CZE: 0.005 a.u.f.s. at 215 nm.

DISCUSSION

We describe a simple method for the analysis by CZE of peptide samples too dilute to be detected after separation by CZE in the standard operating mode. It consists of an electrophoretic concentration (stacking) of the applied sample at the sample–electrophoresis buffer interface prior to the separation and therefore permits the application of increased sample volumes without a breakdown in resolution. This procedure allowed the analysis of peptide samples at lower concentrations compared to standard operating conditions in the same hardware configuration. With this improvement, peptide fractions separated by narrow-bore HPLC for high-sensitivity peptide-sequence analysis could be monitored without additional manipulations. As very small sample volumes are applied ($\ll 1$ μl), this procedure is essentially non-destructive.

This method currently represents the most effective way to monitor the purity of peptide fractions collected from HPLC columns at the submicrogram level. Alternative methods include spectral analysis of the HPLC effluent using a photodiode-array detector[6,7], the systematic rechromatography of peptides under conditions of different selectivity[5] and the concentration of peptide solutions followed by mass-spectrometric analysis[8] or separation by CZE in the standard operating mode.

The assessment of the peak purity by spectral analysis of HPLC effluents using a photodiode-array detector is based on the assumption that different peptides vary in the compositon of aromatic amino acids and therefore show differences in their UV-absorption spectra in the range of 250–280 nm. The UV spectra of the ascending and descending slopes as well as at the apex of peptide peaks eluting from HPLC columns are compared with the expectation that peaks containing a single peptide would show identical spectra irrespective of the point of measurement whereas peaks containing more than one peptide slightly differing in chromatographic mobility and aromatic amino acids composition would show differences in the spectra as a function of the retention time. This method has been sucessfully used with microgram amounts of peptides[6,7]. Relatively weak UV absorption of aromatic amino acids in the 250–280 nm range prevents the successful use of the method for the analysis of submicrogram amounts of peptide samples. Furthermore, peptides containing equal amounts and compositons of aromatic amino acids or no aromatic amino acids at all cannot be discriminated.

The systematic rechromatography of peptide fractions under conditions of different selectivity is cumbersome, time consuming and associated with a systematic loss of approximately 20–40% of the sample per round of rechromatography (chromatography conditions reviewed in ref. 5). Mass spectrometry as a tool for the analysis of HPLC fractions holds tremendous promise for the future. Currently, the usefulness of mass spectrometry for this application is still restricted by limited sensitivity. Furthermore, for most laboratories the cost of mass spectrometric equipment is prohibitively high.

Concentration of HPLC effluents prior to analysis by CZE is associated with significant loss of samples and therefore no viable alternative to the electrophoretic concentration in the capillary. Peptides are eluted from the HPLC column under conditions of good solubility in a mixture of aqueous phase and acetonitrile. If the peptide solution is concentrated under reduced pressure or by lyophilization, an

over-proportional fraction of acetonitrile will evaporate, making the solvent more polar. As a consequence, initially dissolved more hydrophilic peptides frequently precipitate during concentration and are lost for further analysis.

Recently an alternative procedure for the electrophoretic stacking of samples in capillaries was described. Samples were applied in a buffer of lower conductivity than the electrophoresis buffer. The higher resistance in the dilute sample buffer led to a disproportionate voltage drop over the applied sample. The resulting higher electric field induced analytes to move faster and to stack up at the buffer interface[9]. This procedure requires the dilution and/or solvent change of the sample to be analyzed and is therefore less suitable for the separation of HPLC fractions.

The method has some limitations. Even with electrophoretic concentration, the sample volume which can be loaded into the capillary is limited. We found that the upper loading limit was reached after approximately 1 min of sample application under reduced pressure. For even more extensive sample loads, the concentration effect was still very efficient, but strong UV-absorption discontinuities lead to a very noisy baseline and made interpretation of the results difficult. For situations requiring the application of even larger volumes, alternative approaches for the sample concentration need to be developed.

The use of the described electrophoretic stacking procedure is not limited to the separation of dilute samples. Even if small sample volumes are applied, the peaks are sharpened and therefore the resolving power of the separation is dramatically increased (Fig. 3D).

Furthermore the described method is not limited to the separation of peptide solutions, but should be generally applicable for the analysis of dilute samples by CZE and is therefore an important contribution to the analysis of dilute solutions of ionic compounds.

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