



Peptide mapping and analysis using capillary electrophoresis

Application Note

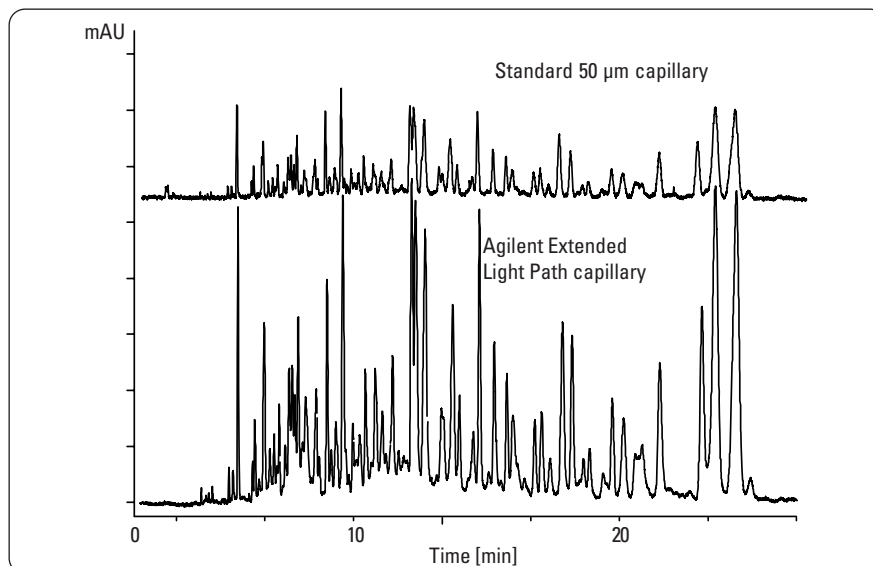
Life Sciences

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Acknowledgments

The authors wish to thank Prof. K. Kirschner for supplying aspartate aminotransferase, and W. Schlamann and Prof. S. Braslavsky for providing phytochrome.



Abstract

Capillary electrophoresis (CE) has proven to be a powerful tool in peptide analysis especially for:

- protein identification by peptide mapping;
- re-analysis of peptide fractions collected by HPLC prior to sequencing;
- purity checking of synthetic peptides.

This Application Note focuses on the first two areas. The methodological aspects of peptide analysis are described, including the optimization of protein digestion buffers, electrophoretic conditions, reproducibility, detection sensitivity and spectral analysis.



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Introduction

Peptide mapping, a widely used tool for identifying proteins and determining protein modification, is routinely performed with reversed phase liquid chromatography (RP-HPLC). Recently, CE has been applied to peptide analysis and many users have found it to be an excellent complementary tool to HPLC.¹ This is mainly because the separation mechanisms in CE differ from HPLC.

Capillary zone electrophoresis (CZE) is the CE mode that is most commonly used for peptide analysis. CZE is also the simplest mode because the analysis is performed in free solution (that is, the capillary is filled only with buffer). With both CE and HPLC, successful peptide mapping requires high resolution, elution time reproducibility and sensitive detection.

The benefits that make CE an attractive technique include the speed of analysis, minimal sample consumption, lack of organic waste and its general versatility. The versatility results from the ease of changing the separation mode and affecting selectivity simply by altering buffer composition.

Method development in CZE is primarily focused on buffer composition: pH, ionic strength, the physical properties of the buffering ions, and addition of additives. Since peptides are amphoteric they are ideally suited for electrophoretic analysis. In CZE, separation of neutral species is not possible, therefore it is important to maintain a charge on the peptides. Best results are generally obtained at one or two pH units above or below the pI value of the peptide, which varies from pI 3 to pI 10. Method development therefore usually entails a wide range of buffer pH values. Since many peptides are uncharged at approximately pH 4 to pH 6, this range is generally not ideal. The inert nature of the fused-silica capillary enables the running

buffer to be varied from pH 2 to pH 12. Alteration of buffer pH not only affects peptide charge but also the magnitude of the electro-osmotic flow (EOF). At low pH values, the silanol groups of the capillary wall are essentially protonated, uncharged, and the EOF is extremely low. In this case the peptides are also protonated, positively charged and migrate toward the cathode (negative electrode). At moderate pH, the peptides may be cationic, neutral, or anionic, but nonetheless elute at the cathodic end because the magnitude of the EOF is greater than peptide mobility. At high pH, both the capillary wall and the peptides are deprotonated and negatively charged. Again, all are swept toward the cathode by the EOF.

The use of extreme pH values (< 3 and > 9) can have numerous other effects. High or low pH values can decrease resolution of some peptides by inducing the same charge on all peptides, thereby minimizing mobility differences. A potential benefit, however, is the reduction of peptide adsorption to the capillary walls. Adsorption via coulombic interaction is minimized by protonation of the wall at low pH (making it essentially neutral). At high pH, where both the peptides and surface are anionic, coulombic repulsion is induced.

Also important in method optimization are the type of buffer ion and its concentration. These factors can influence EOF, Joule heating, peak shape, adsorption, and sensitivity. Further details are discussed in the "results and discussion" section of this note.

Experimental

All CE experiments were performed using an Agilent Capillary Electrophoresis system from Agilent Technologies. The system comprises a CE unit with built-in diode array detector and an Agilent ChemStation for system control, data collection and data analysis. RP-HPLC was performed with an Agilent LC system.

Bovine serum albumin (BSA) lysozyme, and beta lactalbumin were purchased from Sigma (St. Louis, Mo., U.S.A.). Phytochrome protein was a generous gift from the Max Planck Institute (Mülheim, Germany). Aspartate aminotransferase (AAT) was a generous gift from the Biocenter of the University of Basel, (Switzerland). Acetonitrile and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Deventer, The Netherlands) and were of HPLC grade. Tryptic digestions of the proteins were performed similarly to the method described by Stone and coworkers.² Detection in CE was performed at 200 nm with a spectral bandwidth of 16 nm, unless otherwise specified.

Results and discussion

Digestion matrix

Complete enzymatic digestion is usually desired for peptide mapping, although limited digestion can be used to obtain information on protein structure and conformation.³ To facilitate digestion, proteins are typically denatured by unfolding and by reduction of disulfide bridges. The resulting matrices typically include more than 50 mM buffer, up to 1 M guanidinium chloride, or up to 1 M urea, together with other additives. These high salt concentrations, even after dilution or dialysis must be taken into consideration prior to analysis.

In RP-HPLC, the salts and other additives are unretained and thus do not interfere with the separation. In CE, these species often migrate with the peptides and can cause difficulties for both resolution and sensitivity.

Examples of BSA digestions in buffers of various composition are shown in figure 1. Each BSA digest was prepared at the same protein concentration and electrophoresed under identical conditions. A satisfactory separation was obtained when the digestion and separation were both phosphate buffer (figure 1a).

Note that the addition of urea to the digestion matrix caused a large peak between 9 and 10 minutes (figure 1b). This peak was due to elution of urea with the EOF and absorption at the detection wavelength of 200 nm. (Detection at 200 nm often gives better sensitivity than at 214 nm or higher.) The urea peak can be useful for measuring EOF because it is a neutral species; however, if the concentration is too high the zone may be broad and overlap with neighboring peptide zones.

Spectral analysis

Diode-array detection can have significant advantages in peptide mapping. Although peptide spectra are very similar, aromatic peptides can easily be identified at 280 nm. Identification of tryptophan and tyrosine-containing peptides using 200- and 280-nm detection wavelengths is illustrated for a digestion of carbonic anhydrase in figure 2. Although the absorbance at 280 nm was significantly lower than that of the peptide bond at 200 nm, the diode array detector was sufficiently sensitive for this application.

Spectral information can also be invaluable for the identification of peptides and proteins containing chromophoric groups such as a bound co-enzyme. Figure 3 shows a peptide map of phytochrome, a 125 kDa plant photoreceptor protein that contains a covalently bound linear tetrapyrrole chromophore having a characteristic absorbance at 380 nm. Although the separation of this complex digest was not ideal, multiwavelength diode array detection clearly identified the chromophore-containing peptides.

Another chromophore-containing protein is native AAT. This protein has a characteristic absorbance at 340 nm from the co-enzyme, pyridoxyl-5'-phosphate (vitamin B6). The complex, bound via a Schiff base, is intact at neutral or basic pH but not when acidic. This is illustrated in figure 4, which shows the spectra obtained from CZE analyses of the protein at

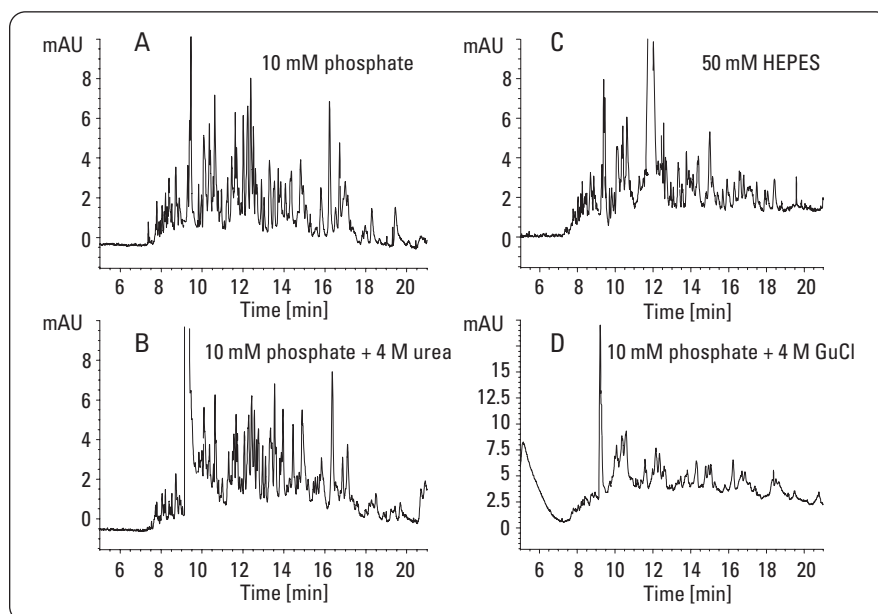


Figure 1
Effect of buffer additives on separation of a tryptic digest of bovine serum albumin.

Chromatographic conditions

Running buffer:	30 mM phosphate, pH 7
Effective capillary length:	70 cm
Internal diameter:	50 μ m
Electric field:	314 V/cm
Current:	19 μ A
Temperature:	25 $^{\circ}$ C
Injection:	200 mbar x s
Detection:	200 nm/16 nm bandwidth

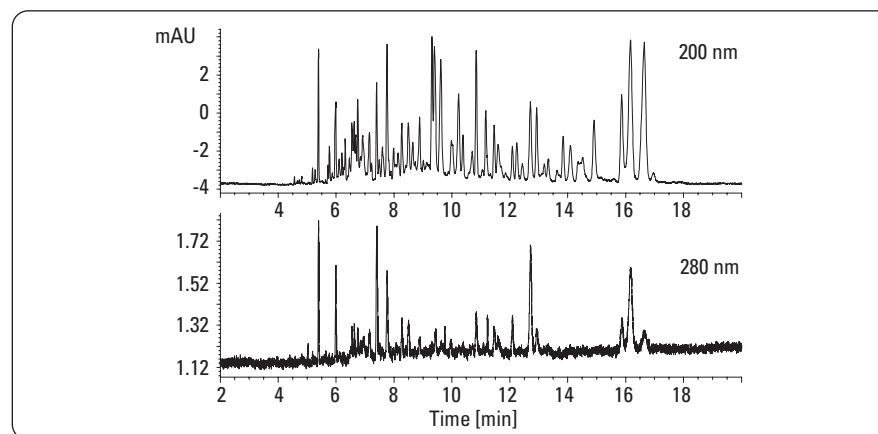


Figure 2
Multi-wavelength detection of carbonic anhydrase digestion at 200 and 280 nm to detect peptides containing aromatic amino acids.

Chromatographic conditions

Running buffer:	20 mM phosphate, pH 3.0
Effective capillary length:	50 cm
Internal diameter:	50 μ m with extended light path
Electric field:	314 V/cm
Current:	22 μ A
Temperature:	25 $^{\circ}$ C
Injection:	100 mbar x s

pH 2.5, pH 7, and pH 9. CZE enables the analysis to be performed at any pH, unlike RP-HPLC where, in most cases, acidic pH values must be used.

Conclusion

Capillary electrophoresis is being used increasingly together with HPLC for protein characterization by peptide mapping and for general peptide analyses. CE both complements and expands the characterization of peptides because the separation mechanism is different from HPLC while its capabilities for automation and on-line quantification are similar.

Once they have been optimized, peptide analysis results obtained by CE are indispensable for general peptide or protein characterization. The separation mechanism for CE, however, requires different sample pretreatment relative to LC. The availability of diode array detection greatly enhances the results by adding a qualitative dimension often essential when working with biomacromolecules.

References

1. Rush, R. S.; Derby, P. L.; Strickland, T. W.; Rohde, M. F. "Peptide Mapping and Evaluation of Glycopeptide Microheterogeneity Derived from Endoproteinase Digestion of Erythropoietin by Affinity Highperformance Capillary Electrophoresis", *Anal. Chem.*, **65**, 1834-1842, **1993**.
2. Stone, K.L.; Elliott, J.I.; Peterson, G.; McMurray, W.; Williams, K.R. "Reversed-phase high-performance liquid chromatography for fractionation of enzymatic digests and chemical cleavage products of proteins", *Methods in Enzymology*, **193**, 389-412, **1990**.
3. Herold, M.; Heiger, D. N.; Grimm, R. "Peptide Mapping by Reversedphase HPLC using Narrow-bore and Microbore Columns", *Amer. Lab*, **20J-20U**, **1993**.

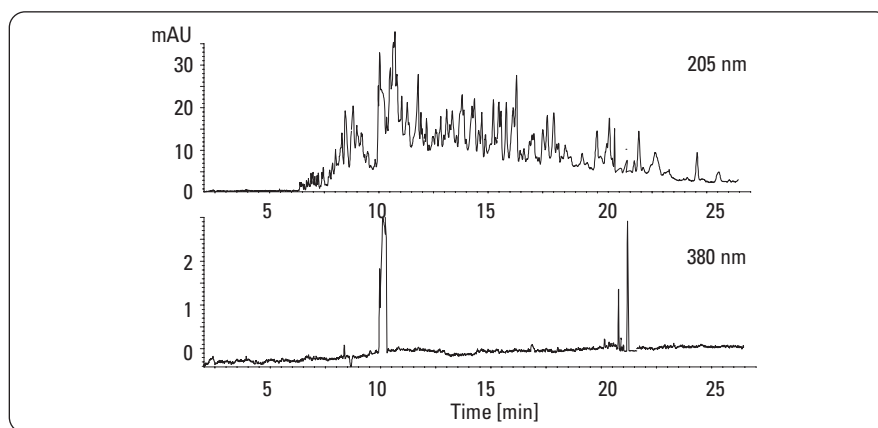


Figure 3
Multiple wavelength detection of phytochrome digestion at 205 and 380 nm to detect chromophore-containing peptides.

Chromatographic conditions

Running buffer:	50 mM phosphate, pH 7
Effective capillary length:	70 cm
Internal diameter:	50 μ m
Electric field:	360 V/cm
Current:	62 μ A
Temperature:	25 $^{\circ}$ C
Injection:	100 mbar x s

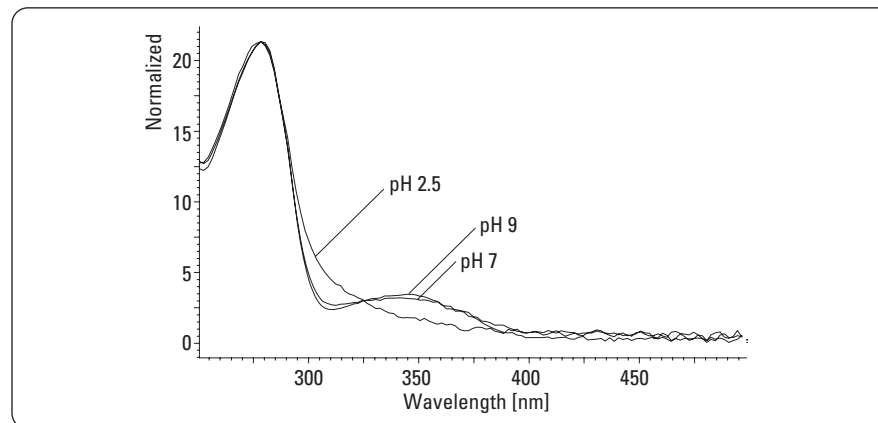


Figure 4
Electrophoresis and spectral analysis of native aspartate aminotransferase-pyridoxyl-5'-phosphate; CE was performed with pH 2.5, 7 and 9 (20 mM phosphate) (electropherograms not shown).

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Published November 1, 2008
Publication Number 5989-9807EN



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