Poster Presentation

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Capillary Electrophoresis for Monitoring Deblocking and Assembly of Bioactive Peptides

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Abstract

Modern peptide synthesizers routinely produce high yields of the intended sequence. However, the product, as cleaved from the resin, requires further processing before it can be used experimentally. In particular, the side chain protecting groups must be quantitatively removed while minimizing extraneous side reactions. Further manipulation may be required to induce the synthetic peptide to assume a biologically active configuration. While the progress of these steps can be successfully monitored with reversed phase HPLC, such assays take too long to be used for real-time control of these reactions. High performance capillary electrophoresis (CE) provides an alternative assay technique. The speed and intrinsically high resolving power of CE should be suitable for monitoring reaction kinetics. This approach will be used to assess the time course of release of synthetic peptides from the resin as well as for the subsequent removal of stable blocking groups from peptides that also contain labile side chains. The kinetics of reduction of sulfhydryl side chains will also be demonstrated. Capillary electrophoresis should prove a useful tool for improving the yield of post-synthetic processing steps in producing biologically active peptides.

Introduction

Synthetic peptides are widely used in a variety of biological experiments as well as for fundamental physical and chemical studies. With modern automated peptide synthesizers, the intended sequence is usually obtained in good yield with minimal failure and deletion sequences. Such contaminants can be easily resolved by reversed phase HPLC. However, additional heterogeneity, often with appreciable reduction in yield, results from both incomplete reactions and side reactions during release from the resin and removal of protecting groups. For example, a peptide that contains tryptophan may reattach to the resin through the indole if the time allowed for release is longer than necessary. The released peptide should be separated from the resin as soon as possible, even if longer periods are required for deblocking. Arginine, for example, requires hours for deblocking, particularly if MTR is used as the side chain protecting group. Since such a time course can result in significant deamidation or other side chain cleavage, deblocking should be terminated at the point of maximum yield. Since these effects are sequence dependent, it is desirable to control the compromise between insufficient and excessive deblocking for each peptide. Finally, the synthetic peptide may require additional processing before experimental use. For example a terminal cysteine is often included for use in conjugation. This side chain must be completely reduced. The same chromatographic techniques can be used to judge these reactions, but the time required for a series of HPLC analyses is relatively long compared to the various reaction kinetics. Since HPLC cannot, therefore, contribute to real time control, it is often desirable to optimize conditions on one or more small aliquots of the resin-bound product. A faster analytical technique would facilitate such pilot experiments. A relatively new technique, capillary electrophoresis could meet this need.

Electrophoretic separations of peptides derive selectivity from net charge, mass, Stoke's radius, and intrinsic viscosity. Capillary electrophoresis uses high voltages to yield good resolution, with short analysis times. These properties should be suitable for real time monitoring of the post-synthesis processing of peptides. This was tested in a release experiment, in an extended de-blocking , and in a thiol reduction

Materials and Methods

Test Peptides and Protecting Groups

Ram8: $S^1VR^2AS^1Y^1I$-$CONH_2$

Ram8-Cys: $C^3S^1VR^2AS^1Y^1I$-$CONH_2$

1 tertiary butyl ether (tBu)
2 methoxytrimethylbenzene sulfonyl (Mtr)
3 S-triphenylmethyl (Trt)

Synthesis

Both peptides were synthesized at the 0.1 nmole scale on a polystyrene-based support PAL™ using an Excell™ Peptide Synthesizer (MilliGen/Biosearch). Peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) alpha-amino protected amino acids and benzotriazolyloxy-trisdimethylaminophosphonium hexafluorophosphate (BOP) / 1-hydroxybenzotriazole (HOBt) activation. The acylation times used for each amino acid addition were determined by an expert system. All peptide synthesis chemicals were supplied by Milligen/Biosearch. The completed resins was dried under vacuum and stored at -20° until use.

Release and Deblocking

Dry resin (25mg) was suspended in reagent R (9ml trifluoracetic acid, 0.2ml anisole, 0.5ml thioanisole, 0.3mlethanedithiol)(500µl) and incubated at room temperature. For monitoring, the suspension was briefly centrifuged and a 5µl sample of supernatant was removed. The resin was resuspended by vortexing. When release was complete, the resin was separated from the soluble peptide with a glass wool column. The column was washed with an additional 500µl of Reagent R and deblocking continued at room temperature with continued sampling (10µl). Each aliquot was immediately dried with a stream of nitrogen and redissolved in 50µl 2% acetic acid for electrophoresis.

Reduction

Peptide Ram8-Cys was dried from Reagent R under a stream of nitrogen and then lyophilized from 500µl 2% acetic acid. It was resuspended in 1ml of 0.04M Tris-Cl, 1mM EDTA, pH8. Dithiothreitol (75mg;50-fold molar excess)was added. Timed samples (10µl) were diluted in 100mN Phosphoric acid (40µl)for electrophoresis. An additional 75mg of DTT was added at 101min to ensure complete reduction.

Capillary Electrophoresis

Capillary electrophoresis was performed using a Waters Quanta™4000 with a 50µ X 60cm capillary. All analyses were in 100mN phosphoric acid at 25kV with 10sec hydrostatic injection. The separations were monitored at 185nm.

Figure 1: Monitoring Cleavage from Resin

The supernatant above the synthetic resin was sampled at intervals after adding Reagent R. Each sample was assayed by capillary electrophoresis. The first material released migrates near 5min. As the reaction progresses, more released material is apparent. Further, the number of peaks first increases and then decreases. This is consistent with the expected simultaneous release and deprotection. After 75min, no further cleavage from the resin is detected. The reaction can then be terminated by separating the soluble peptide from the resin before extraneous side reaction products begin to appear. The CE assay provides rapid monitoring consistent with real time control of cleavage.

Figure 1A: 2min Cleavage

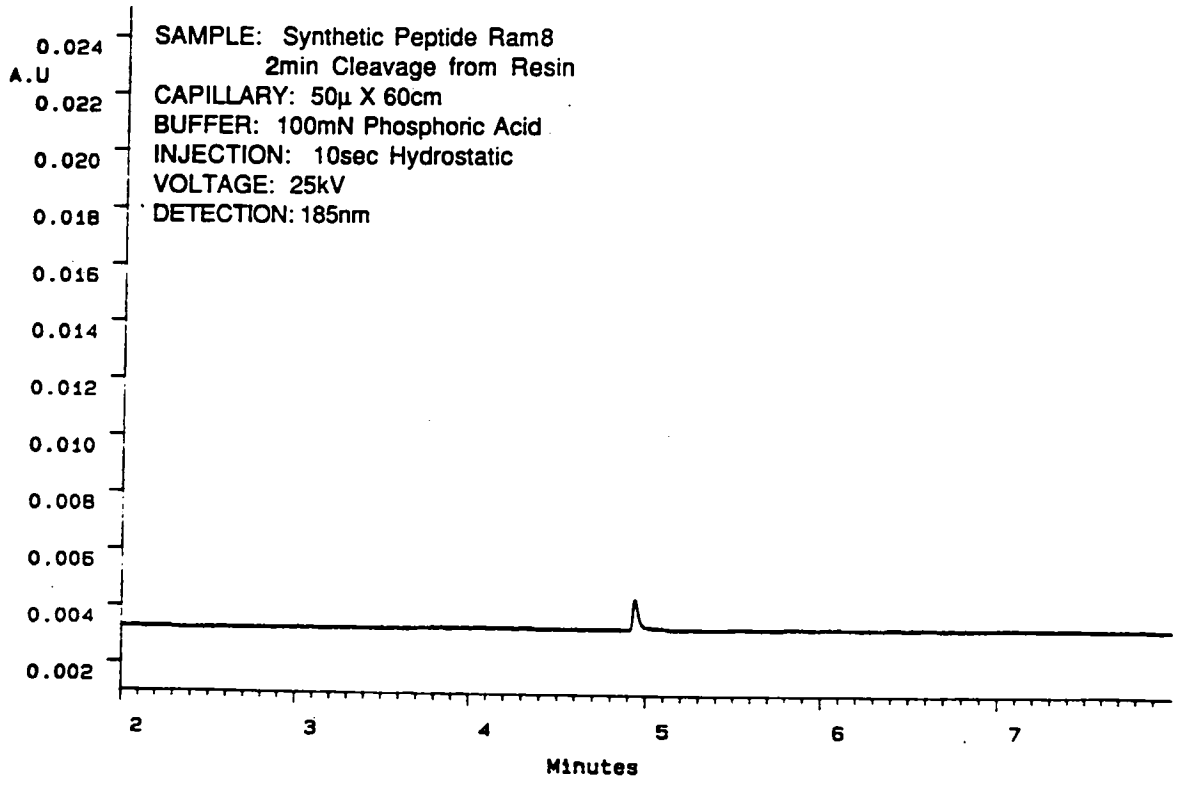


Figure 1B: 15min Cleavage

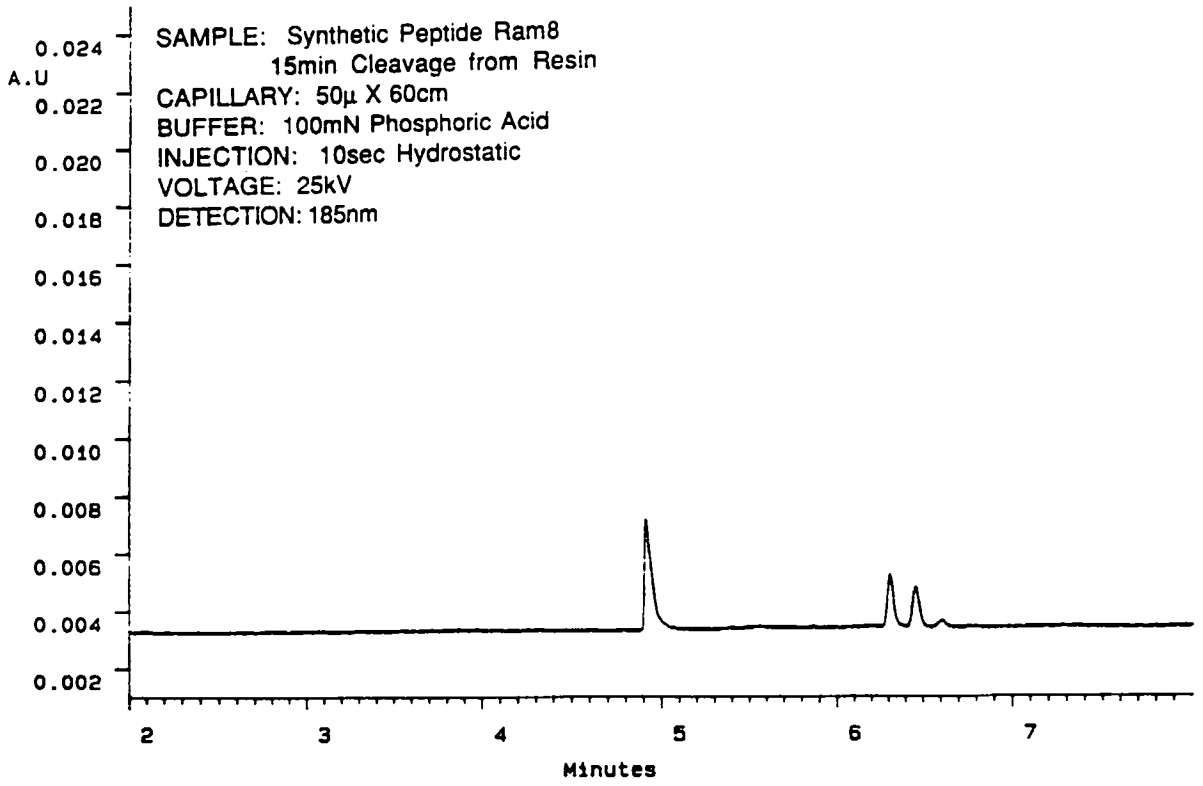


Figure 1C: 30min Cleavage

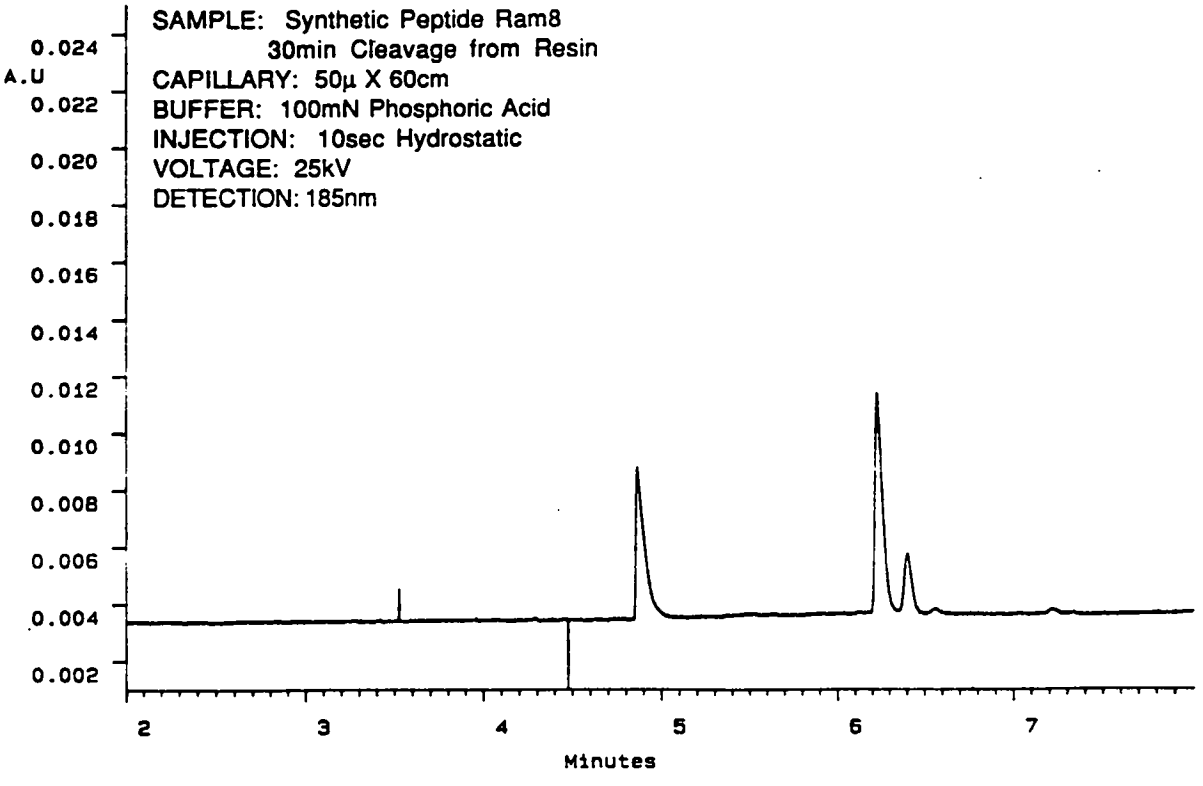


Figure 1D: 60min Cleavage

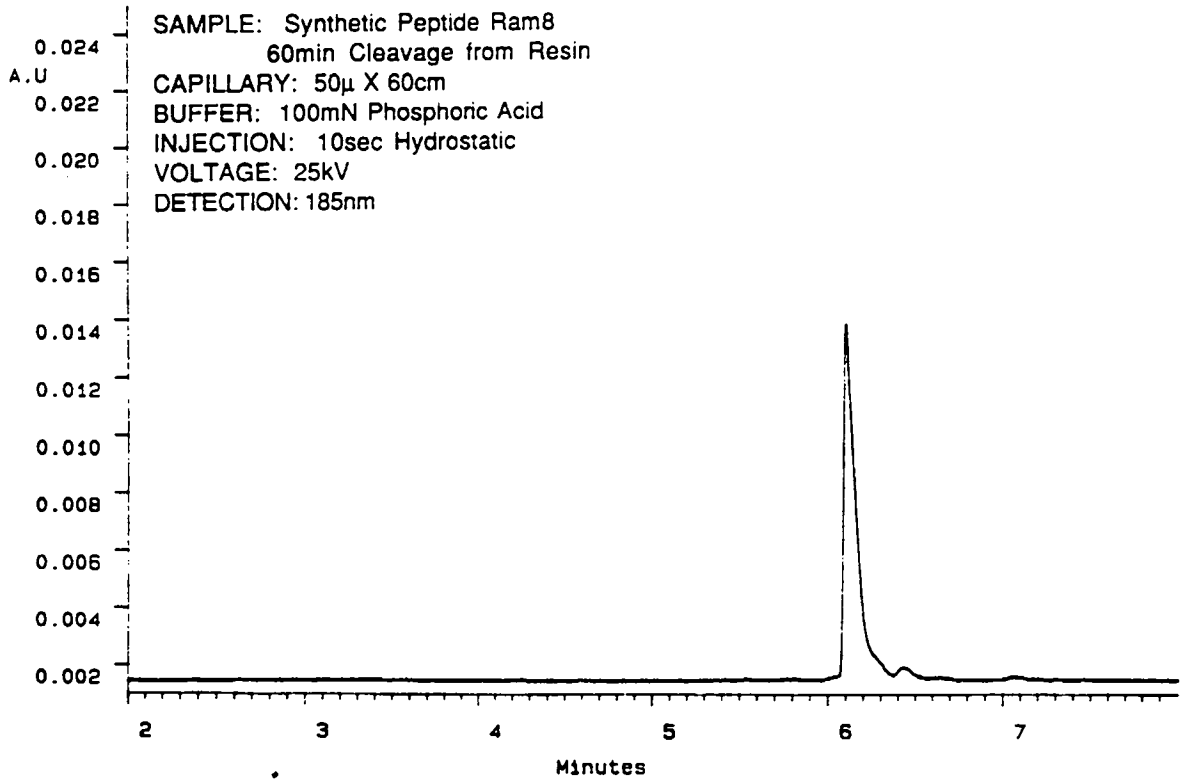


Figure 1E: 75min Cleavage

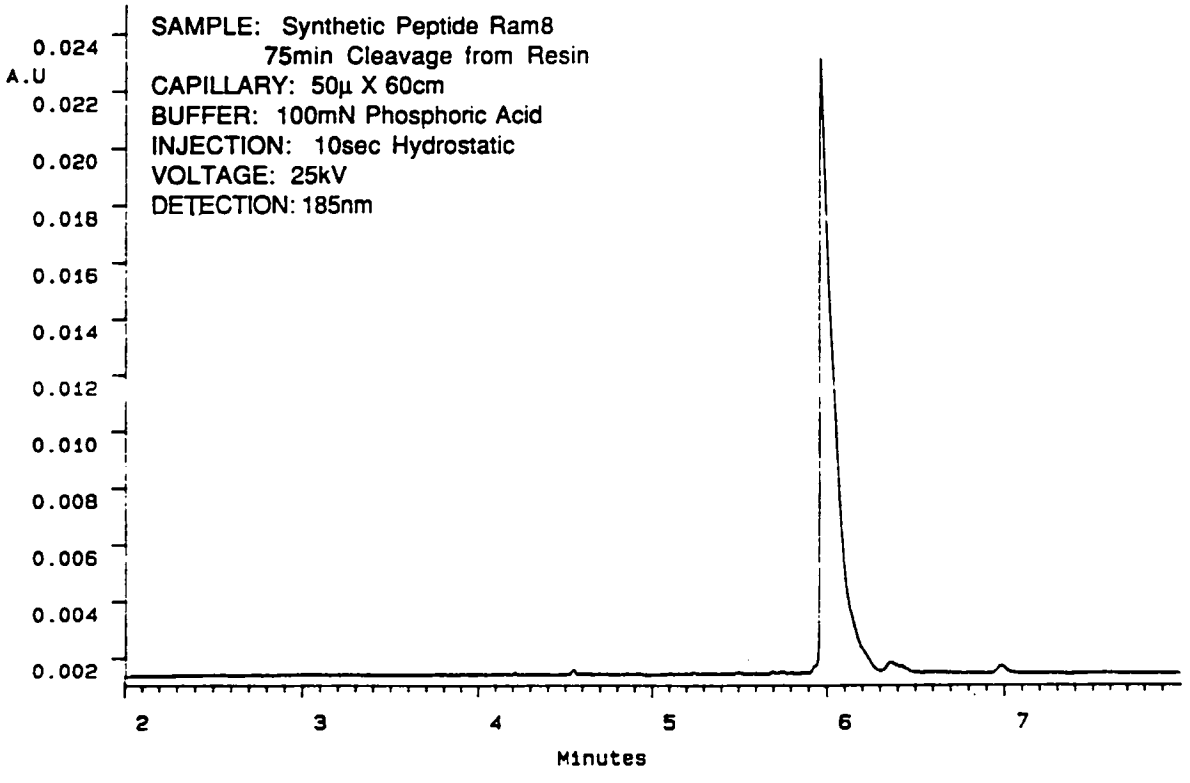


Figure 2: Monitoring Deprotection

The soluble peptide was sampled at intervals during incubation with Reagent R at room temperature. Initially, peaks near 6min and 10min are observed. As the reaction proceeds, the more slowly migrating peak decreases while the more cationic material increases. This is consistent with the removal of the Mtr group from the arginine side chain. By 300min, the conversion is essentially complete, and extension of the reaction to 780min yields additional side reaction products. Again, the assay times associated with capillary electrophoresis are short enought to control the deprotection reaction for maximum yield.

Figure 2A: 75min Deprotection

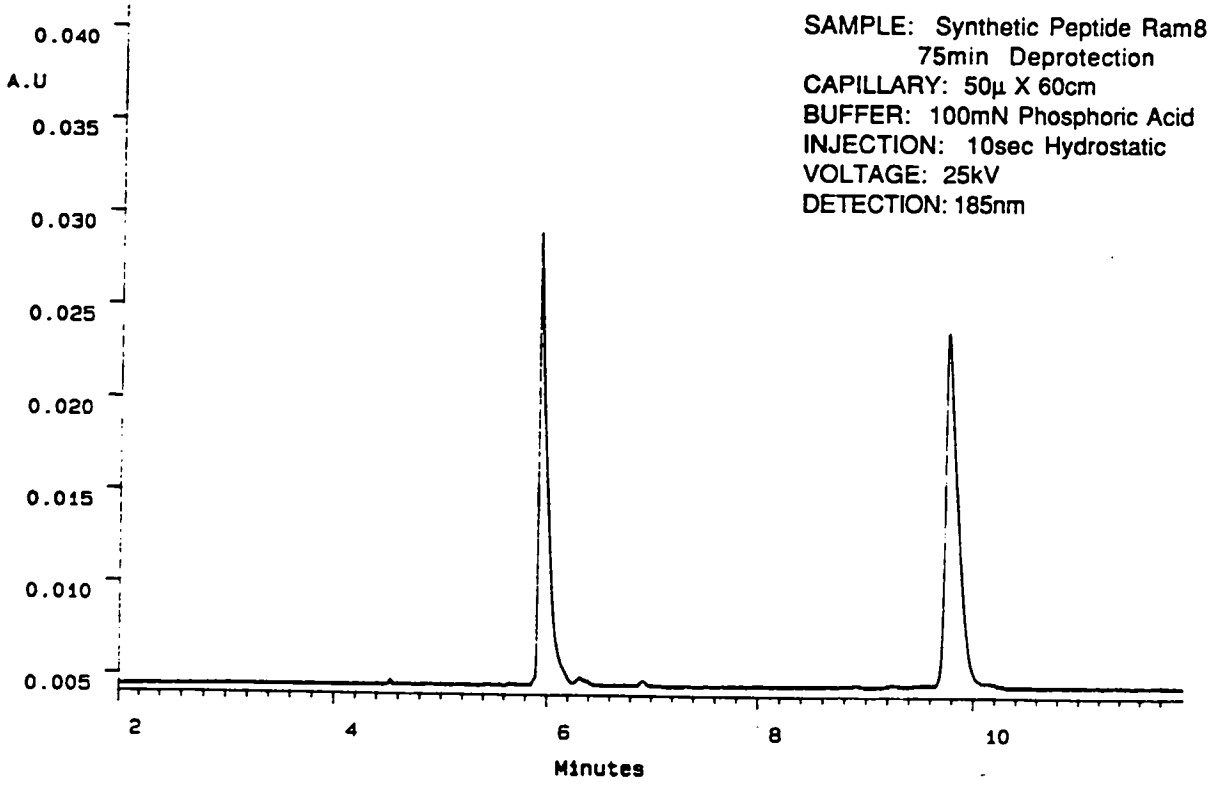


Figure 2B: 120min Deprotection

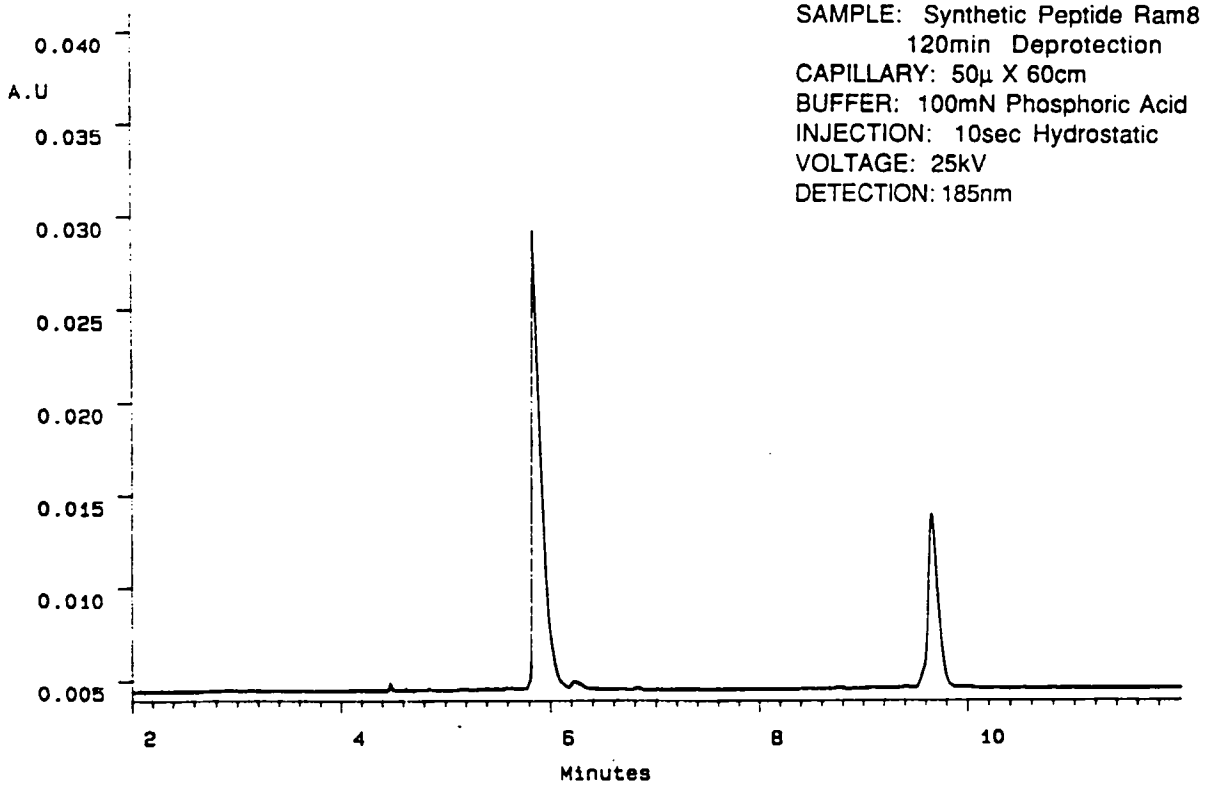


Figure 2C: 180min Deprotection

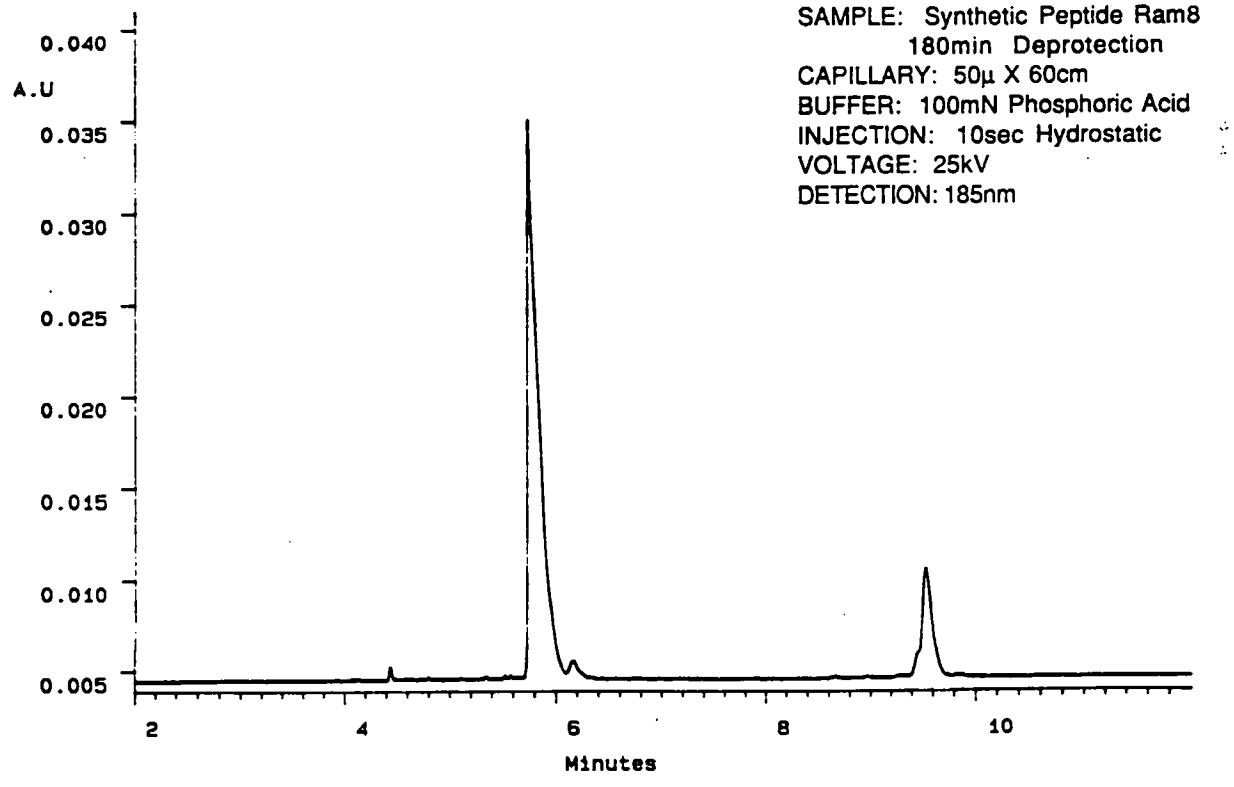
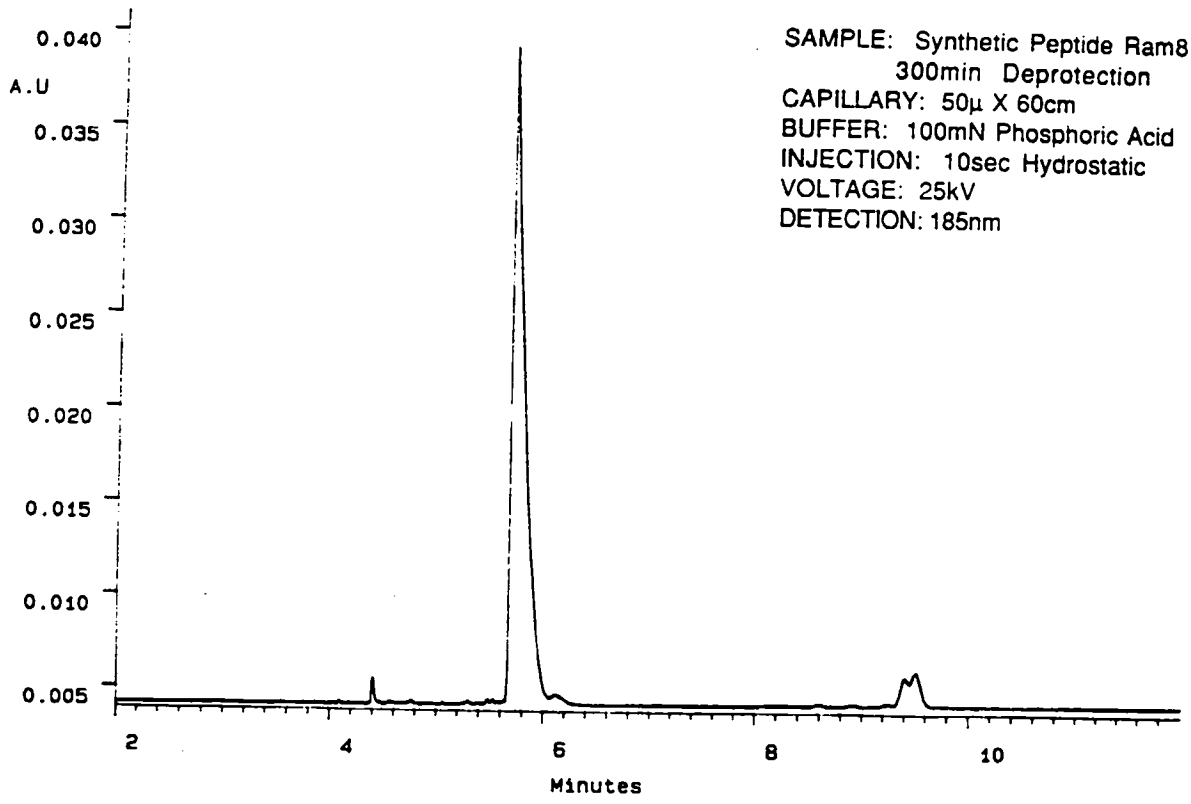
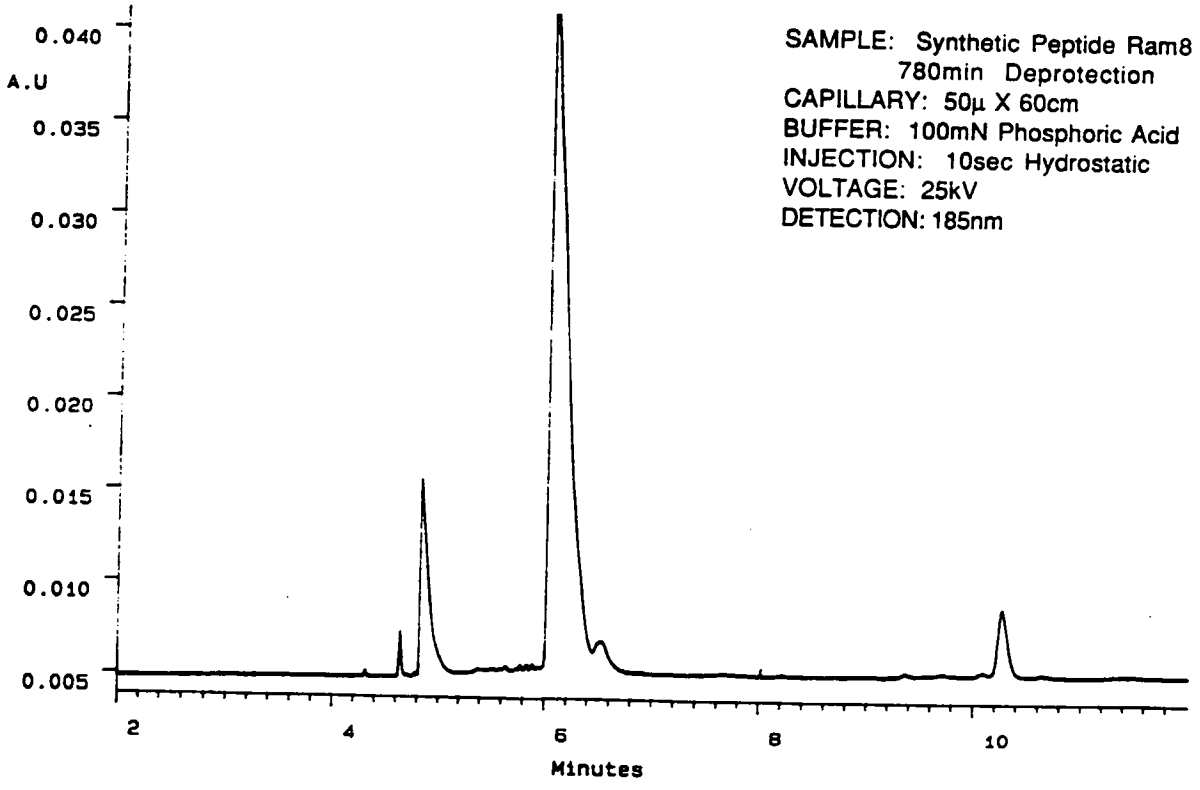


Figure 2D: 300min Deprotection



SAMPLE: Synthetic Peptide Ram8
300min Deprotection
CAPILLARY: 50µ X 60cm
BUFFER: 100mN Phosphoric Acid
INJECTION: 10sec Hydrostatic
VOLTAGE: 25kV
DETECTION: 185nm

Figure 2E: 780min Deprotection



SAMPLE: Synthetic Peptide Ram8
780min Deprotection
CAPILLARY: 50µ X 60cm
BUFFER: 100mN Phosphoric Acid
INJECTION: 10sec Hydrostatic
VOLTAGE: 25kV
DETECTION: 185nm

Figure 3: Monitoring Reduction

The deprotected peptide was dissolved in aqueous buffer and assayed. The sample contained several prominent peaks before reduction. One minute after the addition of a 50-fold excess of dithiothreitol, the mixture had been converted to a single peak. No further changes were observed after 100min of reduction. An additional 50-fold excess of DTT was added without producing any change in the electrophoretic pattern, suggesting that the peptide was completely reduced after 1min. The value of capillary electrophoresis in assessing the completeness of the reaction and suggesting the nedd for additional reductant is apparent.

Figure 3A: 0min Reduction

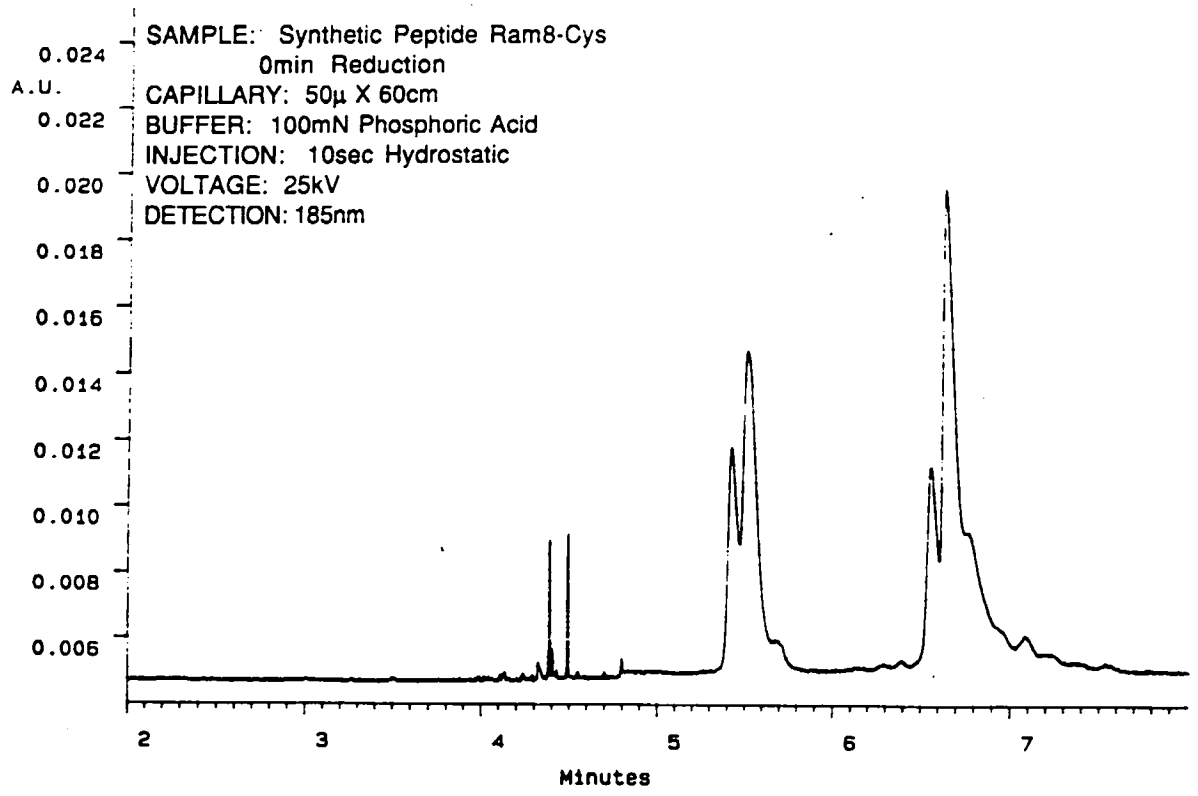


Figure 3B: 1min Reduction

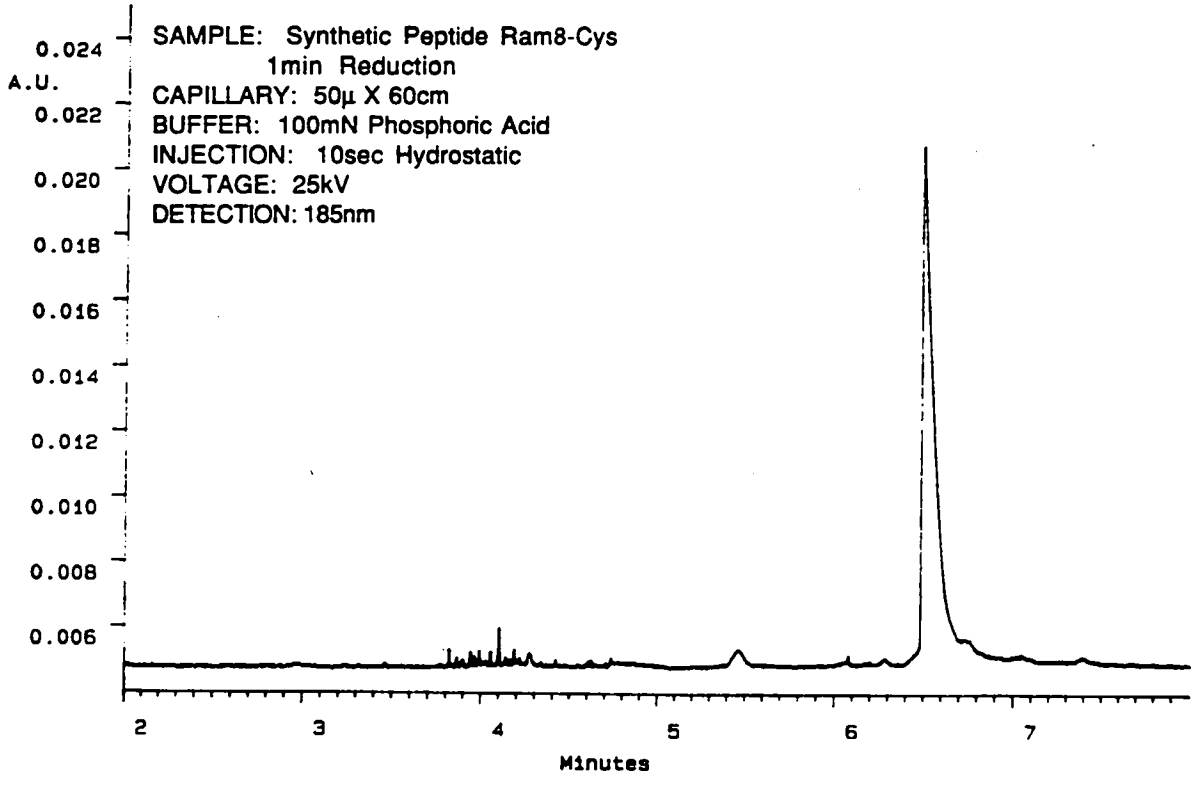


Figure 3C: 100min Reduction

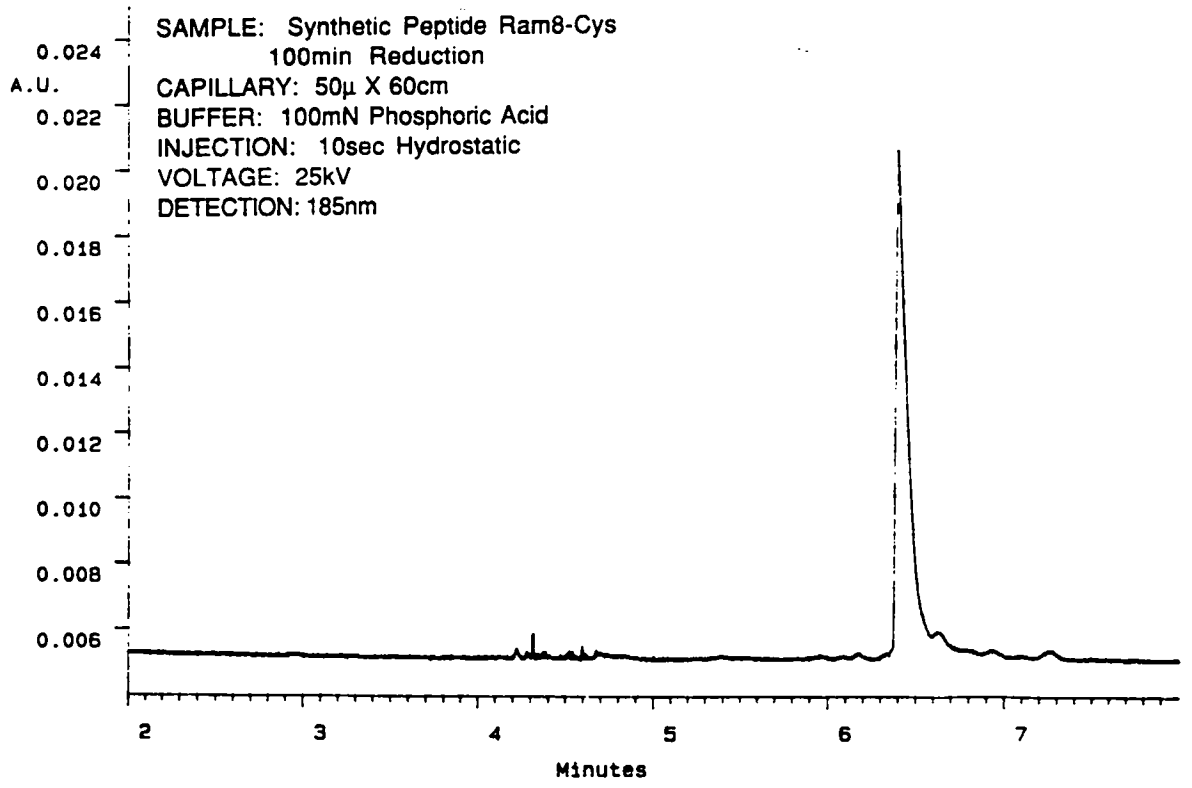
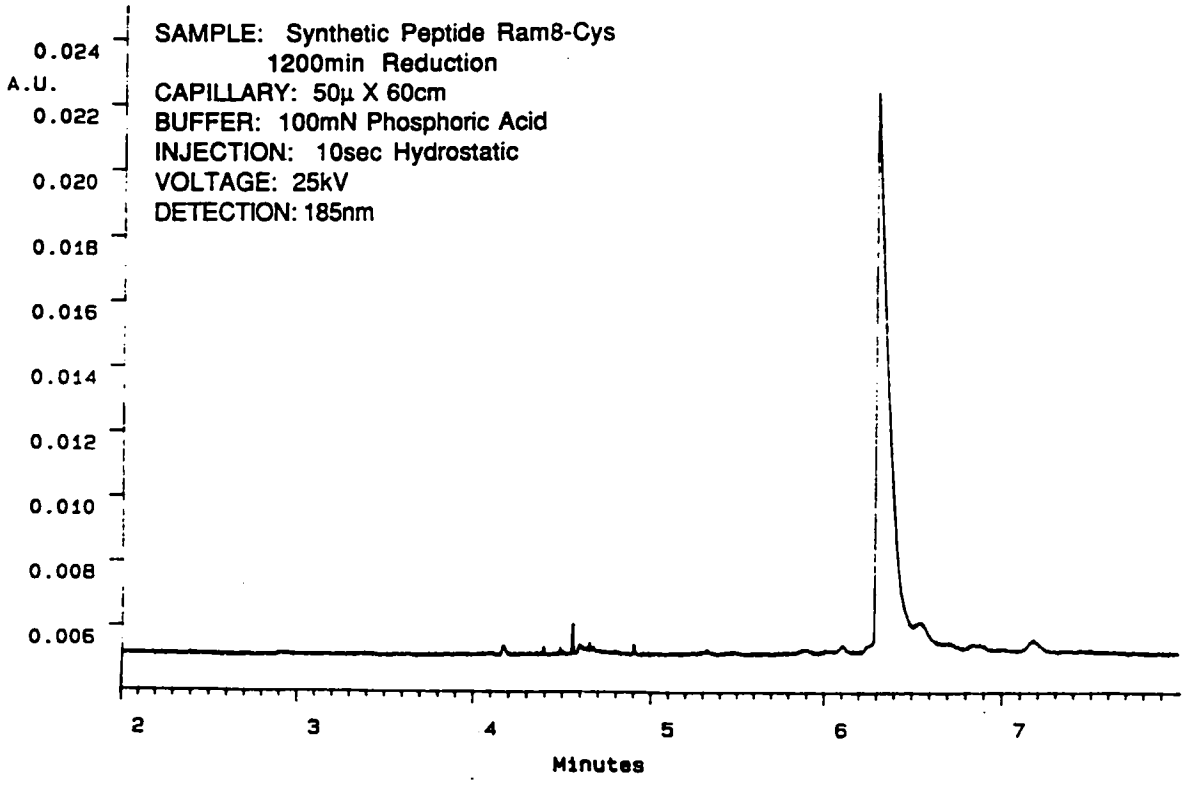


Figure 3D: 120min Reduction



Conclusions

1. Capillary electrophoresis is compatible with the reagents and concentrations encountered in post-synthetic processing of peptides.

2. The run time and cycle time of capillary electrophoretic assays are compatible with the reaction kinetics of cleavage, deprotection, and reduction.

3. Cleavage from the resin can be followed to an end point so that the released peptide can be isolated from any side reactions catalyzed by the support as well as from slowly released side products.

4. Removal of side chain protecting groups can be followed electrophoretically to ensure termination of the reaction at the maximum yield of peptide before degradation side reactions begin to appear in significant quantities.

5. The reduction of thiol-containing peptides can be reflected in electrophoretic mobility. It is, therefore, possible to optimize time, reagent amount, and so on for maximum yield of reduced peptide.

6. Capillary electrophoresis should be a valuable tool for the synthetic peptide chemist. It can provide information about the progress of post-synthetic processing reactions rapidly enough to control those processes for maximum yield of the intended product.