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# Capillary electrophoresis separation of an asparagine containing hexapeptide and its deamidation products[1]

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**Abstract**

A capillary electrophoresis (CE) method was developed for the separation of a model hexapeptide (L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala) and its degradation products. Separations using CE had much shorter analysis times than the RP-HPLC assay used previously. CE was also evaluated for possible use in analyzing peptide samples containing polymers. With a thorough rinsing procedure between runs, the presence of a model polymer did not affect the separation of the peptides. The CE assay can be applied to the study of peptide stability in polymers. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Capillary electrophoresis; Peptide; Deamidation; Polyvinyl alcohol; RP-HPLC

## 1. Introduction

One of the major routes of degradation in proteins and peptides is deamidation at asparagine (Asn) and glutamine (Gln) residues [1,2]. In this work, an asparagine containing hexapeptide, shown in Fig. 1, was used as a model to study the kinetics of deamidation at alkaline pH. To accomplish this objective, capillary electrophoresis (CE) was applied to the separation of the Asn-hexapeptide and its degradation products. The overall goal was to develop a method to study the effect of polymeric additives on the degradation of the hexapeptide. Therefore, the developed analytical method should be rugged enough to withstand direct injection of samples containing a model polymeric additive, polyvinyl alcohol.

The degradation pathway shown for this model peptide in the solution state has been well characterized and is shown in Fig. 2[1]. In solution at slightly acidic to basic pH, the peptide undergoes deamidation via formation of a cyclic imide intermediate which hydrolyzes to form both the Asp and isoAsp containing products. Previous studies have used reverse phase HPLC assays to resolve

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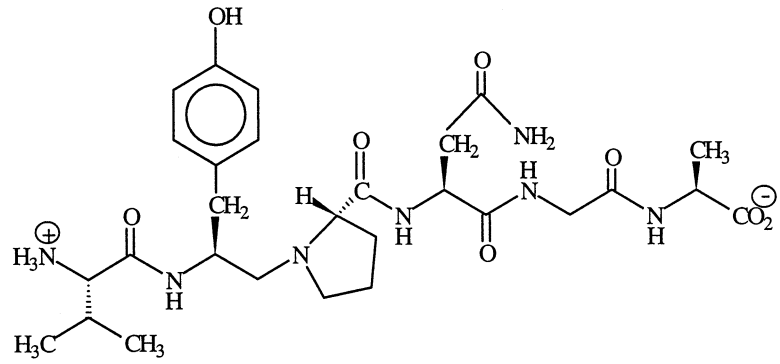

Fig. 1. Structure of the Asn-hexapeptide.

the deamidated degradation products [1,2]. The HPLC assay suffered from long analysis times, taking up to 50 min to elute all the peptides [1]. In addition, the separation could potentially deteriorate and column life could be shortened as a result of bonded phase modification by different polymers present in the samples.

Capillary electrophoresis (CE) has proven to be an excellent technique for the separation of closely related protein and peptide degradants [3–8]. Speed and efficiency have made CE a popular technique for the analysis of protein and peptide pharmaceuticals. CE is capable of separating closely related species that exhibit small differences in either mass, charge, or conformation. Several authors have demonstrated the ability of CE to separate the deamidated degradants of asparagine containing peptides [9–11]. Separations of recombinant insulin from its deamidated degradant, and recombinant hGH from both mono- and di-desamido forms by capillary electrophoresis have also been reported [9–11].

Thus, CE offered an excellent alternative to RP-HPLC for the separation of the Asn-hexapeptide and its degradation products. The lack of a bonded phase, and the ability to rinse the capillary between injections to regenerate the surface offered the possibility of a rapid, reproducible assay with direct injection of the polymer containing samples.

## 2. Materials and methods

### 2.1. Materials

L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala (Asn-hexapeptide) and L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala (Asp-hexapeptide) were synthesized by Dr Madhup Dhaon (Abbott Laboratories, North Chicago, IL). The cyclic imide (L-Val-L-Tyr-L-Pro-L-Asu-Gly-L-Ala) was collected from the degradation of the Asn-hexapeptide via HPLC. HPLC-grade trifluoroacetic acid (TFA) and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). The buffer salts were also purchased from Fisher Scientific. Polyvinyl alcohol (Mw = 50000) was purchased from Aldrich (Milwaukee, WI). Distilled and deionized water was used in all studies (Millipore MILLI-Q water system).

### 2.2. Instrumentation

The HPLC analytical system consisted of a Shimadzu LC-6A pump, an SPD-6A variable-wavelength UV detector, a CR601 Chromatopac integrator, and a Rheodyne 7125 manual injector outfitted with a 20 μl injection loop. The Asn-hexapeptide and its degradation products were separated on an Alltech Econosphere C-18 reversed-phase column (5 μm resin, 4.6 × 250 mm) at room temperature, using an isocratic system

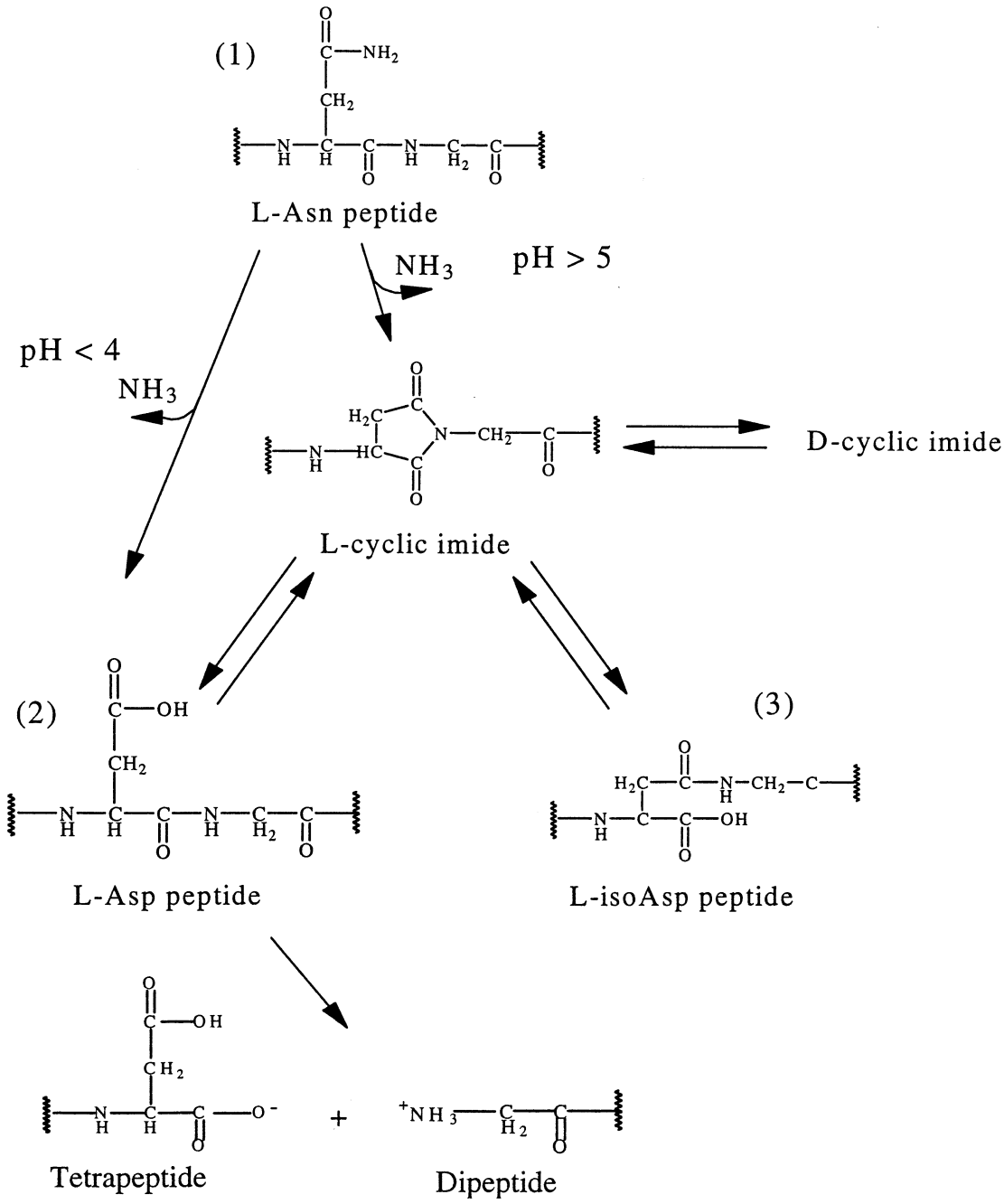


Fig. 2. Degradation pathway of the Asn-hexapeptide showing the cyclic imide intermediate and the Asp- and isoAsp-hexapeptide products.

with a mobile phase of 7% (v/v) acetonitrile, 0.1% (v/v) TFA in water. The flow rate was 1.2 ml $min^{-1}$. The detection wavelength was 214 nm.

The CE system consisted of a manual pressure injector (sustained positive pressure of 5 psi for 1 s, injection volume of 10 nl), a Spellman high

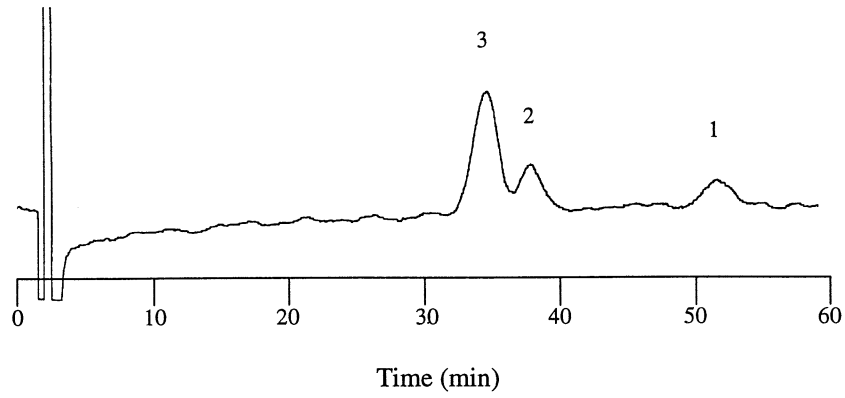


Fig. 3. HPLC chromatogram of the Asn-hexapeptide after degradation at pH 10.0 for 210 min. Conditions: C-18 reversed-phase column (5 μm resin, 4.6 × 250 mm), with a mobile phase of 7% (v/v) acetonitrile, 0.1% (v/v) TFA in water. The flow rate was 1.2 ml $min^{-1}$ with UV detection at 214 nm. Sample volume: 20 μl. Peaks: (1) Asn-hexapeptide, (2) Asp-hexapeptide, (3) isoAsp hexapeptide.

voltage DC supply, and an ISCO $CV^4$ UV detector (Lincoln, NE). Data were collected and analyzed by an Isco ChemResearch data analysis system 150 program on a 486 SX computer. The 50 μm i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ) had a total length of 75 cm and a length of 40 cm to the detector. The detection wavelength was 214 nm. The applied voltage was 25 kV, with a current of 25 μA.

The peptide separations were performed in a pH 3.0, 40 mM phosphate running buffer. The capillary was rinsed with the phosphate buffer for 3 min between runs. Before the first use and after the last run (about 20 runs), the capillary was flushed for 15 min with 0.1 N NaOH. The capillary was equilibrated for at least 30 min with run buffer prior to use.

### 2.3. Kinetic measurements

The degradation of the Asn-hexapeptide was studied in aqueous buffer at 37°C. The following buffer solutions were used: pH 7.5, 0.1 M phosphate; pH 10.0, 0.05 M bicarbonate; pH 12.0, 0.01 M NaOH. A constant ionic strength of 0.5 M was maintained for each buffer by adding the appropriate amount of NaCl. The buffers were prepared at 37°C. The pH was measured with an Orion Research (701A) digital ionalyzer equipped with a Ross combination electrode. The peptide and buffer were combined to yield a concentration of $1.6 \times 10^{-3}$ M. At appropriate time points, 10 μl aliquots were removed from the reaction vial and quenched with 90 μl of the CE running buffer (pH 3.0, 0.04 M phosphate). Samples for the HPLC were diluted 10 fold with mobile phase prior to analysis. Samples were analyzed immediately using either HPLC or CE. The pH of the reaction solutions was measured at the end of each study to verify that no change in pH had occurred. The peak areas from the CE analyses were normalized by dividing the observed peak area by the peak retention time. The observed rate constants for the loss of the Asn-hexapeptide ($k_{obs}$) were determined from the slope of the plot of ln (amount of peptide remaining) versus time. This calculation was based on the pseudo-first-order kinetics previously observed for this peptide [1], according to the relationship:

$$\ln A = \ln A_0 - k_{obs}\, t$$

where $A$ is the amount of peptide at the sampling time ($t$), and $A_0$ is the initial peptide concentration.

## 3. Results and discussion

Although HPLC is the most popular analytical method for the separation of polypeptides, some

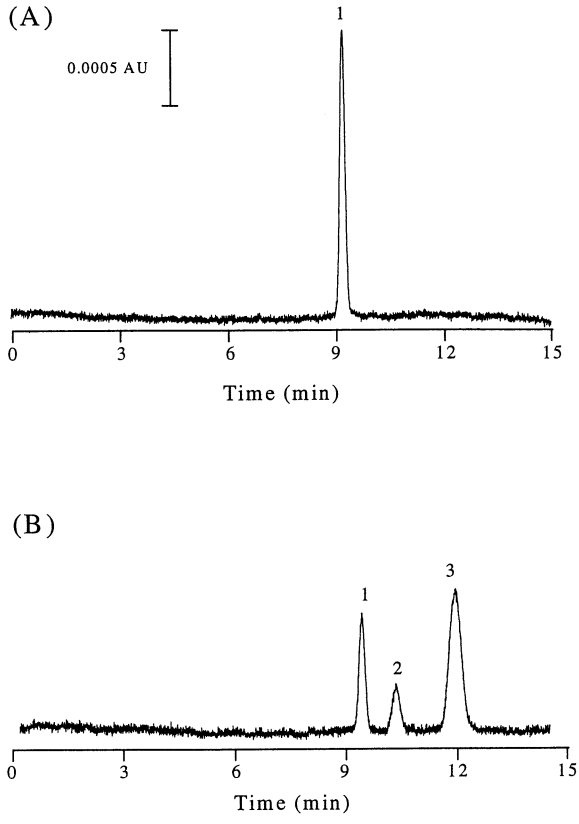


Fig. 4. CE separation of the native Asn-hexapeptide and its degradation products after degradation at pH 10.0 for (a) $t = 0$ min and (b) $t = 120$ min. Conditions: 75 cm × 50 μm id. fused-silica capillary with an effective length of 40 cm; 40 mM acetate buffer, pH 3.0; 25 kV applied voltage, 25 μA current; UV detection at 214 nm. Sample: 5 psi positive pressure for 1 s (injection volume of ~10 nl). Peaks: (1) Asn-hexapeptide, (2) Asp-hexapeptide, (3) isoAsp hexapeptide.

difficulties still exist in achieving good, rapid separations. Capillary electrophoresis offers an alternative separation method. Fig. 3 shows the HPLC chromatogram of the Asn-hexapeptide and its degradation products based on a methodology described by Patel et al. [1]. Due to the similar selectivity of the Asn- and the isoAsp-hexapeptides, it was difficult to achieve a good separation using HPLC. Although the peaks for the Asn-hexapeptide and the isoAsp-hexapeptide separated, the long retention times resulted in peak broadening which decreased resolution. Increasing the organic modifiers caused the peaks to overlap. Furthermore, column variability made reproducibility of separation difficult to achieve from column to column. Finally, the analysis time of over 50 min on the HPLC is long enough that a more rapid separation would be desirable.

In contrast, a rapid separation of the peptides was achieved using CE, with baseline resolution of all three peptides. Peak assignments were made by the injection of peptide standards. Fig. 4 (a) shows an electropherogram of the Asn-hexapeptide before degradation. The CE elution time of 9 min for the Asn-hexapeptide was much shorter than that of over 40 min given by HPLC. Fig. 4 (b) shows the CE separation of the native peptide and its Asp and isoAsp containing degradation products which was accomplished with good resolution in $< 15$ min. An analysis time of $< 6$ min was achieved with an automated system which was equipped with a shorter capillary (data not shown).

The difference in retention order observed for the HPLC and CE assays was presumed to be due to a change in the separation mechanism. The CE separation of the Asn-hexapeptide from the Asp containing degradants is thought to be caused by the new negative charge formed as the amide converts to a carboxylic acid. The separation of

Table 1
Observed degradation rate constants ($k_{obs}$) for Asn-hexapeptide

| pH (Buffer) | Buffer concentration (M) | $k_{obs}$ [a] ($h^{-1}$) CE | $k_{obs}$ [a,b] ($h^{-1}$) HPLC |
|---|---|---|---|
| 7.7 phosphate | 0.10 | 0.028 | 0.0348 (0.001) |
| 10.0 (bicarbonate) | 0.05 | 0.571 | 0.617 (0.019) |
| 12.0 (NaOH) | 0.01 | 3.710 (0.06) | 1.85 (0.09) |

[a] Standard deviations given in parentheses ($n = 3$); $n = 1$ for values without SD.
[b] Values taken from [7].

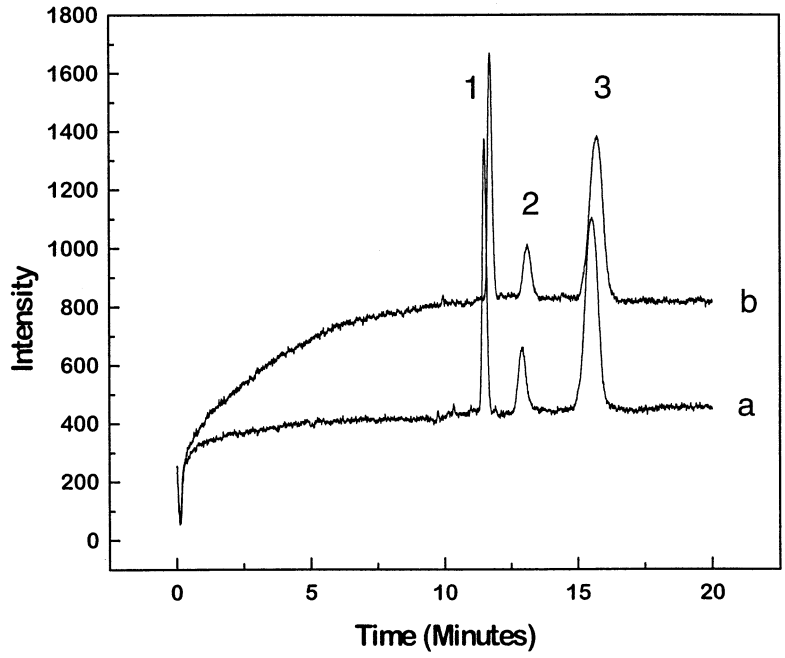


Fig. 5. CE separation of (1) Asn-hexapeptide, (2) Asp-hexapeptide, (3) isoAsp hexapeptide in samples (a) without PVA and (b) with PVA.

the isomeric Asp and isoAsp containing peptides was achieved by capitalizing on the slight differences in the p$K_a$'s of the carboxylic groups on the peptides, which result in small charge differences. At a pH near the p$K_a$ of these carboxylic acids (pH 3.0), the charge differences were enough to result in baseline resolution. At higher pH, the Asp and isoAsp peaks co-eluted since both peptides have the same charge. The peak retention times were quite reproducible from day to day and when the capillary was replaced. A calibration curve of absorbance as a function of Asn-hexapeptide concentration was linear from 0.01 mg ml$^{-1}$ to 1.0 mg ml$^{-1}$ ($R$ = 0.999).

The CE assay was used to follow the degradation of the Asn-hexapeptide and the formation of the products, producing a smooth and continuous profile. The observed rate constant ($k_{obs}$)of Asn-hexapeptide degradation was calculated at three alkaline pH values (Table 1). The rate constants measured using CE were comparable to those determined by HPLC at pH 7.5 and 10.0. However, at pH 12.0, the CE rate constant was greater by a factor of 2 than the HPLC determined rate constant. This discrepancy could be due to slight differences in methodology in the two stability studies. The standard error for the values determined using CE were comparable to those determined with HPLC.

In order to confirm that this CE method is rugged enough to handle planned studies in which large molecular weight polymers will be present in the analyte, we injected samples containing up to 5% PVA without adversely affecting the separation (Fig. 5). A prior sample preparation step would be necessary in HPLC to first remove the polymer in order to avoid column fouling. Since CE does not utilize a bonded phase, flushing the capillary with 0.01 N NaOH between subsequent runs was adequate to prevent the polymer from adhering to the silica wall. Fig. 6 shows that the degradation of the Asn-hexapeptide decreases as the percentage of PVA increases. Factors responsible for this effect are under investigation.

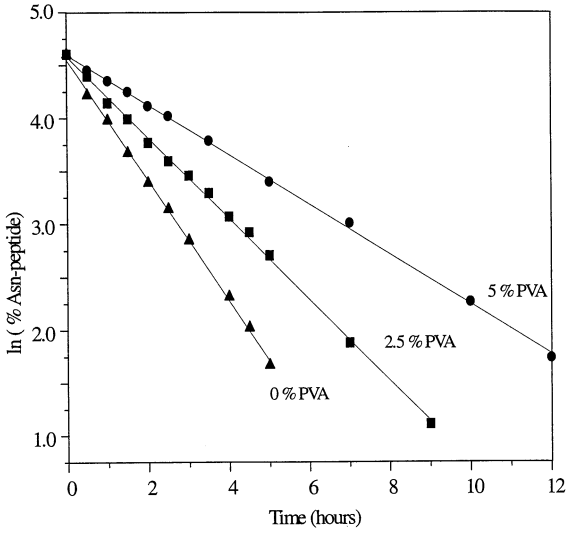


Fig. 6. Plots of Asn-hexapeptide degradation kinetics at pH 10.0 as determined by CE as a function of the % of PVA present in solution. The experimental $k_{obs}$ ($h^{-1}$) are 0.568 ($\pm$0.036) for 0% PVA, 0.382 ($\pm$0.038) for 2.5% PVA and 0.234 ($\pm$0.028) for 5.0% PVA.

## 4. Conclusion

The CE assay presented here is comparable to a previously reported HPLC assay in its ability to separate this group of peptides [1]. Furthermore, the CE assay offered better resolution in a much shorter analysis time. Capillary electrophoresis also proved to be a rugged analytical method which could be effectively used for direct analysis of polymer containing samples. Future studies will utilize the CE assay to monitor the degradation of this peptide in different polymer systems.

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