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Analysis of underivatized amino acids by capillary electrophoresis using constant potential amperometric detection

A mixture of native (underivatized) amino acids is separated by capillary electrophoresis under alkaline conditions (pH ≈ 12) and amperometrically detected with a copper-disk microelectrode. A simple design facilitates capillary-electrode alignment without the need for micropositioning equipment. The limits of detection for the amino acids are in the low μM concentration range, and the signal response is linear over 2–3 orders of magnitude. This procedure is applied to analyze the amino acid hydrolysis products from cytochrome c.

1 Introduction

The determination of amino acids by capillary electrophoresis (CE) has received considerable attention in recent years [1–8] because of the high separation efficiencies achieved with CE. In most cases, the amino acids have been labeled with appropriate probes to enhance detectability (*e.g.*, by photometric means). Although effective in enhancing sensitivity, these labeling procedures are troublesome and time-consuming. In many situations, a rapid analysis accompanied with high separation efficiencies is required (*e.g.*, monitoring of a particular process). In such cases, a simple method for the determination of underivatized amino acids with relatively fast analysis time is warranted. Indirect detection methods have been implemented to detect underivatized amino acids [6–8]. These methods are based, however, on a displacement mechanism that strongly depends on the concentration of the background (separation) electrolyte that can compromise separation performance and sensitivity.

Underivatized amino acids are generally considered nonelectroactive species at modest potentials on conventional carbon electrodes (with the exception of tyrosine, tryptophan, methionine, and cysteine) [9–12]. This problem has motivated many researchers to focus their attention on metallic electrodes [12–16]. Because of its characteristics, copper has received attention in recent years, especially by Baldwin and co-workers [15, 16]. This attention is in part because of the possibility of performing amperometric detection for a variety of compounds at a constant applied potential. It has been proposed that detection of amino acids at copper electrodes is possible by two different mechanisms [14, 16]. One proposed mechanism is based on copper-analyte complexation at moderate pH conditions, and the other is based on electrocatalytic oxidation under strong alkaline solutions. The conditions that facilitate each proposed mechanism have been coupled with HPLC for the determination of native amino acids. Operation of the copper electrode under conditions that favor the catalytic oxidation of amino acids (*i.e.*, alkaline conditions) has been reported to produce better results than those under conditions of the analyte-complexation mechanism (detection limits of 1–50 pmol in comparison with 10–100 pmol) [16].

Ewing and co-workers [17] reported the use of CE with amperometric detection using a copper-wire microelectrode to detect some amino acids; their operating conditions favored the copper (II)-analyte complexation mechanism at the surface of the electrode. Under such conditions, some peak tailing was observed in the electropherograms that can be detrimental in resolving multiple components. The tailing was associated with a slow adsorption step in the analyte-copper (II) complexation process. Lunte and co-workers [18] used another copper-complexation approach to detect peptides amperometrically, after separation by CE. Under alkaline conditions, the peptides were complexed with copper (II) that was previously introduced and adsorbed on the wall of the separation capillary. The copper-peptide complexes were detected with a carbon fiber electrode.

In a recent publication [19], we reported the use of a copper microelectrode in combination with CE for the amperometric detection of carbohydrates. Separation was achieved in a strong alkaline electrolyte solution, and detection was performed with a copper microelectrode operated at a constant applied potential (0.6 V *vs.* Ag/AgCl). The detection mechanism for carbohydrates has been attributed to electrocatalytic oxidation of the sugars at the surface of the electrode [15]. More recently, Baldwin and co-workers [20] used a similar method to determine amino acids and peptides. The alkaline conditions favor the proposed electrocatalytic oxidation mechanism that has been reported to produce better performance than the one involving complexation of the analyte [16]. Furthermore, these conditions improve the separation, and the peak tailing associated with the complexation mechanism is not observed.

In the present report, we demonstrate further the capabilities of the CE-amperometric system to the separation and detection of underivatized amino acids. Specifically, we analyze the hydrolysis products from cytochrome c. In addition, we also report a simple design of the electrochemical cell which facilitates electrode-column align-

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ment, thus eliminating the need for micropositioners and microscopes in the experimental process. As with the method developed for the carbohydrates, this procedure is simple, sensitive, offers high separation efficiencies, and is relatively easy to implement.

2 Materials and methods

2.1 Apparatus

All separation experiments were performed with a home-built CE system. The high-voltage power supply (Glassman High Voltage, Inc., Whitehouse Station, NJ) was capable of delivering 0–30 kV. Separations were performed in fused silica capillaries (Polymicro Technologies, Phoenix, AZ), 20 μm ID and 360 μm OD. New capillaries were treated by flushing with 0.5 M NaOH solution, distilled water, and the separation electrolyte before use. Prior to each injection, the capillary column was flushed with the separation electrolyte and allowed to equilibrate under the electric field for 2–3 min. The capillaries were filled either with the separation electrolyte or water for overnight storage. Sample injection was performed by electromigration. The copper disk working electrodes were constructed by carefully inserting a piece of copper wire (~ 5 cm long, 90–100 μm diameter) through a small piece of fused silica capillary (360 μm OD, 100 μm ID, ~ 2 cm long). The copper wire was sealed with epoxy at both ends of the capillary. After the epoxy had dried, the copper wire protruding from one end of the capillary was cut off with a stainless steel scalpel, which left a shiny copper disk surface with a diameter of about 100 μm. Amperometric detection was performed using the end-column approach at the grounded end of the capillary [21]. The separation electrolyte also served as the supporting electrolyte in the electrochemical cell. Our previous electrochemical cell [19] was modified, so alignment with micromanipulators was not required. Three pieces of a glass slide were assembled as illustrated in Fig. 1, and kept in place with epoxy. The electrode and separation capillary were placed against the edge of the larger piece of glass and allowed to gently touch each other, and then were glued in place with epoxy. Thus, alignment was automatically achieved because of the same outer diameter of the separation capillary (20 μm ID) and the capillary containing the disk working electrode (~ 100 μm ID). The actual size of the assembly shown in Fig. 1 is approximately 1 in^2. After the electrode-capillary holder was dried, it was placed into the reservoir designated for the outlet separation electrolyte. A silver-silver chloride (Ag/AgCl, in 3 M KCl) reference electrode and a platinum wire counter electrode were also immersed in the outlet reservoir. The detection end of the system was housed in a Faraday cage to minimize external noise. The detection potential was controlled through a three-electrode potentiostat (CV-37) from Bioanalytical Systems, Inc. (West Lafayette, IN). The signal output from the current amplifier of the CV-37 was fed into an A/D converter board mounted on an IBM PC. Data acquisition was controlled by means of the GRAMS 386 for Chromatography software (Galactic Industries, NH). The electrode was turned off at the end of each working day (typically

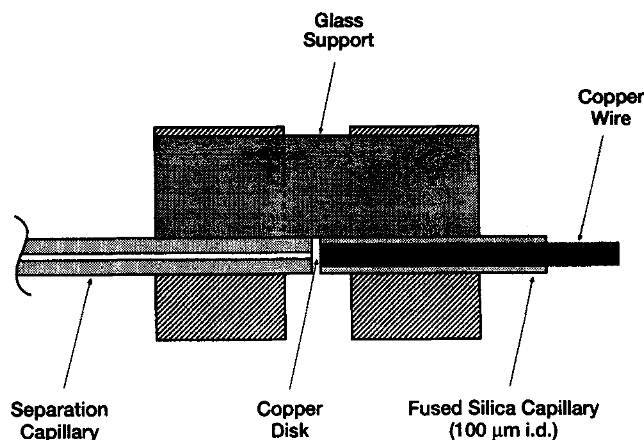

Figure 1. Schematic of the electrode assembly.

after 10–12 h of constant operation) to ensure similar conditions day after day. At the beginning of the day the electrode was exposed to −1.0 V for ~ 5 min and reset to the detection potential (0.7 V) until a stable background current was observed (~ 30 min). Cyclic voltametry (CV) was performed in unstirred solutions on a CS-1090 computer-controlled electroanalytical system from Cypress Inc. (Lawrence, KS).

2.2 Reagents

Amino acids and cytochrome c (from bovine heart) were purchased from Sigma (St. Louis, MO) and used as received. All other chemicals were obtained from Fisher (Pittsburgh, PA). Stock solutions of amino acids and separation electrolytes were prepared in water purified with an Ultra-Pure water system (Millipore, Bedford, MA), and filtered with 0.2 μm filters prior to use. The standard solutions of amino acids were prepared daily by serial dilution in the separation electrolyte. A 1 mg quantity of cytochrome c was hydrolyzed by dissolving it in 1 mL of 6 M HCl contained in a hydrolysis tube, and heating at 110°C for 24 h in vacuum [22]. After hydrolysis, a portion of the solution was dried and reconstituted in the separation electrolyte prior to injection into the CE system.

3 Results and discussion

3.1 Signal enhancement

Anodic current enhancements for carbohydrates and amino acids on copper electrodes have been observed in strong alkaline solutions and have been attributed to electrocatalytic oxidation of the analyte at the surface of the electrode [15, 16]. Cyclic voltammograms revealing the anodic current signals at copper electrodes (in 50 mM NaOH solutions) for several native amino acids are shown in Fig. 2. The anodic signal observed (starting at *ca.* 0.50 V) showed peak potentials ranging between 0.60 V and 0.70 V for the amino acids studied. This signal was characteristic in all CV experiments. A small anodic current was also observed around 50 mV during the first CV scan, but disappeared after the first scan. This small response has been attributed to a Cu(II)-amino acid complexation mechanism, and disappears after genera-

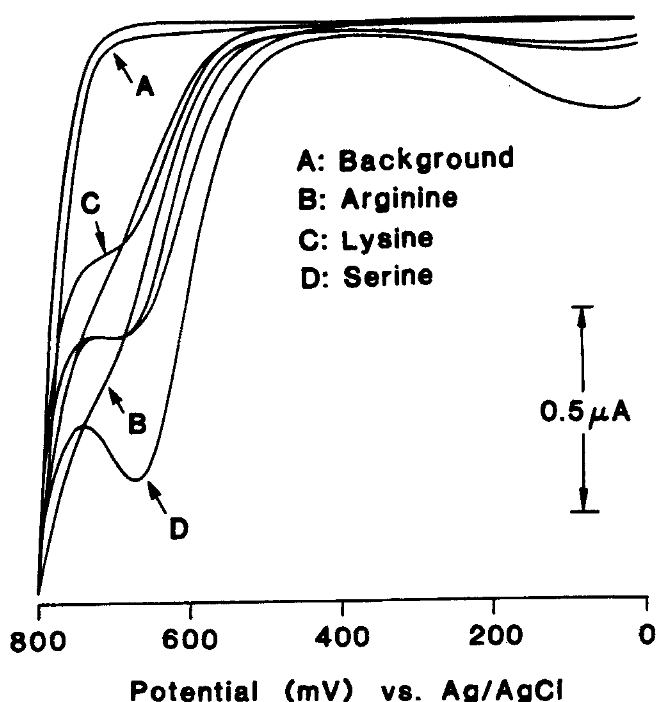

Figure 2. Cyclic voltammograms of three probe amino acids (1 mM) in 50 mM NaOH (scan rate 50 mV/s).

tion of a copper oxide layer as the potential is increased (which is believed to passivate the electrode surface) [16]. The signal enhancements were fully in accord with those previously reported by Baldwin and co-workers [16]. The peak current increased upon changing the NaOH concentration from 10 to 100 mM, and decreased upon switching the electrolyte solution from 50 mM NaOH to 50 mM Na_2CO_3 or 50 mM Na_3PO_4, which indicates agreement with a proposed reaction mechanism involving hydroxide ions in the catalytic reaction [23]. Hydrodynamic voltammograms under CE flowing conditions showed a similar trend of response for the amino acids studied. An operating potential of 0.70 V (*vs.* Ag/AgCl) gave the best compromise between the signal and the background current. Therefore, the potential of 0.70 V was chosen for the amperometric detection of amino acids after separation by CE.

3.2 Electrode configuration

Recently, it was reported that the use of disk electrodes in a wall-jet configuration and with sizes as large as 10 times the ID of the separation capillary does not have a major impact on separation efficiency [24]. Such a design showed an improved reproducibility when compared with the cylindrical microelectrodes. The same configuration has also been employed with commercially available electrodes [25]. Despite the improvements and modifications, a typical arrangement using a micropositioner and a microscope was still required for proper manipulation of the electrode at the end of the separation capillary. Furthermore, the alignment procedure has to be performed in the electrochemical cell, limiting the capillary-electrode assembly setup. Once in place, the setup is extremely difficult to move from place to place without disturbing the capillary-electrode alignment. The simple arrangement that we have configured (as shown in Fig. 1) overcomes the above-mentioned problems.

The separation capillary and the electrode (having similar OD) are guided by the edge of a piece of glass, and the centers of the two tubes become aligned with each other. Using a disk electrode with a diameter larger than the capillary ID in the wall-jet configuration [22] further facilitates the alignment. After fixing the electrode and the capillary in place, the assembly is immersed in the electrolyte reservoir, and, if desired, it can be moved from one location to another without problems associated with misalignment. We have constructed many assemblies (> 15) without the use of micropositioners or microscopes. All have shown similar performance characteristics (in terms of limit of detection and band broadening, factors that may indicate misalignment). A drawback of the present assembly is the use of epoxy to fix the capillary and the electrode, which does not allow exchange of the electrode.

3.3 Separation of amino acids

The separation of the amino acids was performed in a strong alkaline solution (pH ~ 12), that also favors the conditions for detection at the copper electrode. All amino acids are negatively charged at such a pH. Sodium hydroxide solutions modified with sodium acetate (see

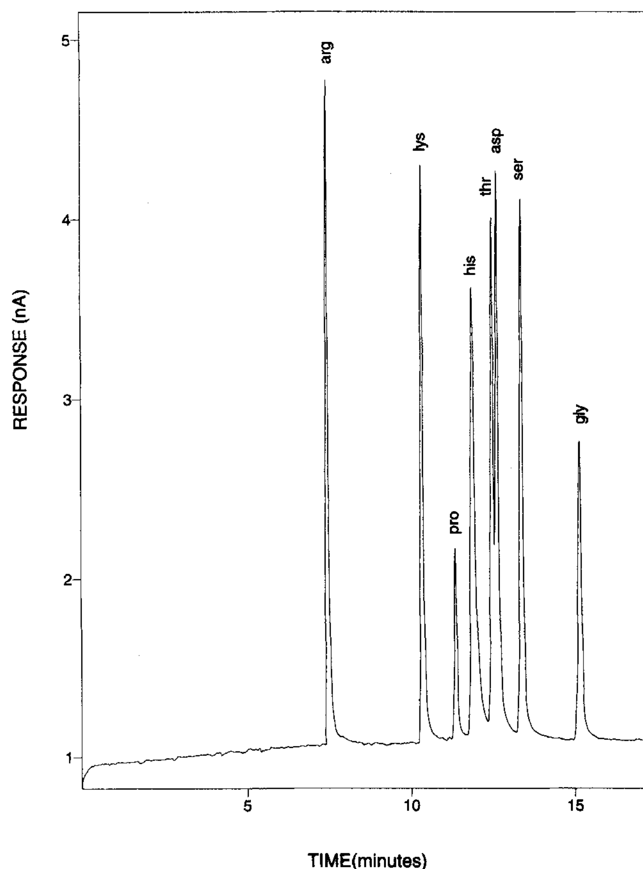

Figure 3. Separation of eight amino acids (~ 100 µM). Separation conditions: fused silica capillary, 48 cm long (20 µm ID × 360 µm OD); electrolyte, 10 mM NaOH-10 mM sodium acetate; separation voltage, 8 kV; injection, 5 s at 8 kV; detection at 0.7 V (*vs.* Ag/AgCl).

below) were employed as the separation electrolyte in most cases. Figure 3 illustrates a separation of a standard mixture containing eight different amino acids. The peaks were identified by spiking each individual solute and matching their migration times. The time of analysis is relatively fast with the separation completed in less than 20 min. Separation efficiencies ranged from 70 000 to 100 000 theoretical plates for the solutes studied. Resolution was best when using NaOH concentrations above 20 mM. At NaOH concentrations greater than 40 mM, however, the noise level also increased considerably. Variations in migration time during the course of one day was less than 1% RSD ($n = 8$). At the lower NaOH concentrations the migration time varied between 1.5–3% (RSD) for some amino acids.

Alternatively, resolution can be improved by adding another electrolyte to the separation medium (changing the ionic strength). Sodium phosphate (Na_3PO_4) and sodium acetate were tested. Sodium acetate was not detrimental to the electrode response, while Na_3PO_4 caused a decrease in signal by about 45%, although the resolution was improved. The effect of sodium acetate on the resolution of eight amino acids is illustrated in Fig. 4. Proline was not resolved from histidine in 10 mM NaOH as the separation electrolyte. The addition of 10 mM sodium acetate, however, improved the separation of proline and histidine ($R \approx 3$); the resolution between threonine and asparagine was also improved (from $R \approx 0.4$ to $R \approx 0.8$). Representative calibration plots demon- strated linearity over 2–3 orders of magnitude (µM-mM) with correlation coefficients of 0.998–0.999. The limit of detection (S/N = 3) for the amino acids studied were between 1 and 3 fmol (1–2 µM) under our experimental conditions.

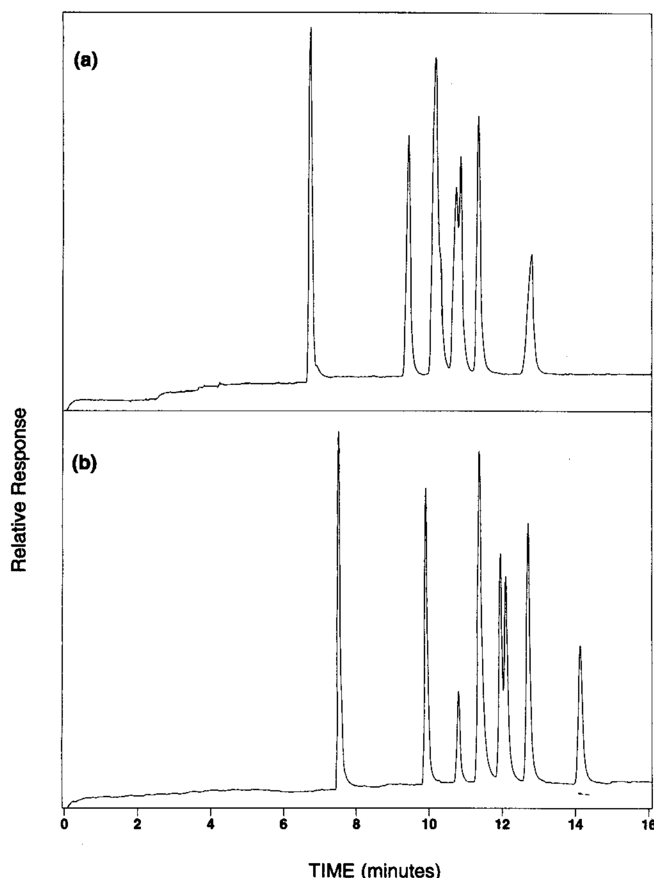

Figure 4. Effect of acetate concentration on the resolution of eight amino acids. The separation electrolyte contained (a) 0 and (b) 10 mM sodium acetate and 10 mM NaOH. See Fig. 3 for other experimental conditions and identification of peaks.

3.4 Electrode stability

The stability of the copper disk electrode under CE conditions was investigated by analyzing a standard sample mixture. The relative standard deviation for the current response of five consecutive injections in a period of 3 h for a typical electrode assembly was about 5%. No apparent deterioration in performance was observed in more than 10 h of constant operation. Furthermore, under our experimental conditions we have used a single electrode with similar performance for more than a week.

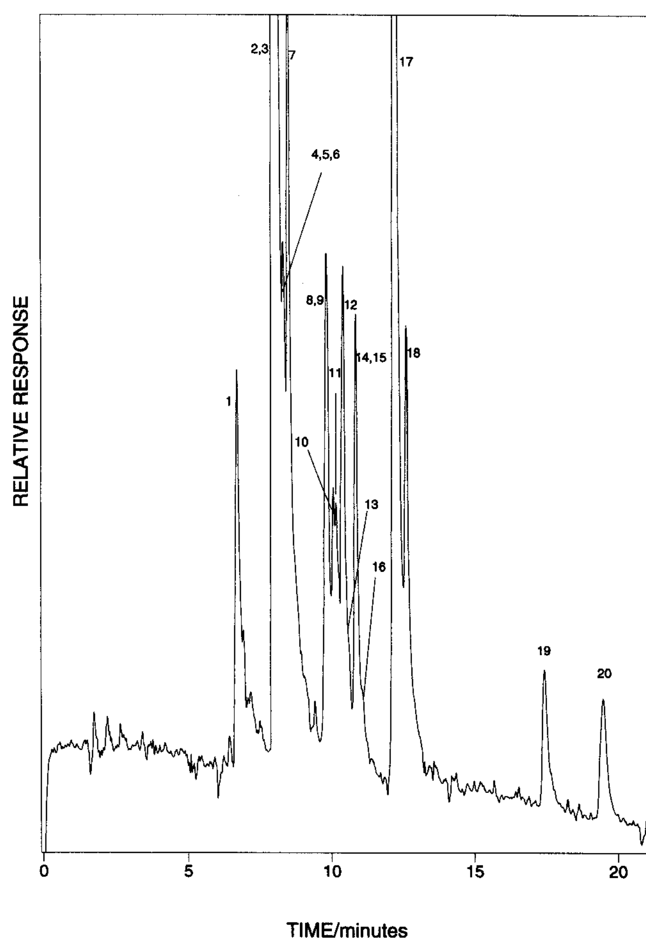

Figure 5. Electropherogram of a sample containing the hydrolysis products of cytochrome c (1- arginine; 2,3- tryptophan, lysine; 4,5,6-valine, phenylalanine, alanine; 7- histidine; 8.9-leucine, isoleusine; 10-glutamine; 11- methionine; 12,13- threonine, proline; 14,15- asparagine, serine; 16- cysteine; 17- glycine; 18- tyrosine; 19- glutamic acid; 20- aspartic acid). Separation conditions: fused silica capillary, 43 cm long (20 µm ID × 360 µm OD); electrolyte, 20 mM NaOH – 23 mM acetate; separation voltage, 8 kV; injection, 3 s at 8 kV; detection at 0.7 V (*vs.* Ag/AgCl).

3.5 Protein hydrolyzate

We also used the procedure to separate the hydrolysis products of cytochrome c. This protein was hydrolyzed following commonly used methods [22]. An electropherogram is shown in Fig. 5. The electrode response was not affected by the type of sample, giving reproducible peak heights for the five injections made (RSD < 5%). The sample components were identified by migration time matching and spiking the amino acids expected in the sample. Our approach, however, does not involve complicated derivatization procedures.

4 Concluding remarks

In summary, the analytical procedure shown above for the determination of native amino acids by CE with constant potential amperometric detection is simple, does not involve derivatization procedures, and offers detectabilities in the low μM range. We demonstrated the potential of the method by analyzing the hydrolysis products of a protein. This procedure should also be applicable to the analysis of samples containing small peptides.

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