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Determination of peptides by capillary electrophoresis–electrochemical detection using on-column Cu(II) complexation

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ABSTRACT

A Cu(II)-coated capillary has been developed for the determination of peptides by capillary electrophoresis with electrochemical detection. Capillaries were prepared by forcing a solution 48 μM in $CuSO_4$, 120 μM in tartaric acid, 2.4 mM in NaOH and 120 μM in KI through them for 25 min; the resulting capillaries are stable for at least 12 h. Under alkaline conditions, peptides complex with Cu(II) present on the walls of the capillary to form Cu(II)–peptide complexes which can be detected oxidatively at a carbon fiber electrode. Di-, tri-, tetra- and pentaglycine were determined with a detection limit of $7 \cdot 10^{-7}$ M for triglycine. N-Terminal-blocked peptides can also be determined via this method. This system is more sensitive than direct detection of peptides by UV at 210 nm and exhibits higher selectivity than commonly employed derivatization procedures based on reactions with a primary amine functionality.

INTRODUCTION

High-performance liquid chromatography (HPLC) determination of peptides complexed with Cu(II) has been accomplished previously by two methods: postcolumn addition of Cu(II) with electrochemical detection (ED) at a glassy carbon electrode [1–5], or detection of eluting peptides at a solid copper electrode [6,7]. Detection at a copper electrode is best suited for determination of dipeptides since the sensitivity has been shown to decrease with increasing peptide length [6]. In 1989, Warner and Weber [1] reported using postcolumn addition of biuret for the selective detection of peptides by liquid chromatography–ED. The resulting Cu(II)–peptide complexes could be detected oxidatively at +0.70–0.90 V *vs.* Ag/AgCl. For many peptides, the oxidation was chemically reversible, and added selectivity could be obtained using dual electrode detection. Detection limits using this method were two orders of magnitude lower than those obtained with a copper electrode.

In this paper, the use of the biuret chemistry in the detection of peptides by capillary electrophoresis (CE)–ED is investigated. CE has been shown to be a powerful technique for the separation of charged compounds, in particular, peptides and amino acids. Because CE can be used for analysis of nanoliter sample volumes, it has been found to be useful in the fields of biotechnology and microanalysis of biological systems [8–10]. The fused-silica capillary used in CE, unlike bonded-phase HPLC columns, is

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stable at high pH, which means that postcolumn derivatization is no longer a necessity.

To date, UV, fluorescence and radiometric detection have been used for the detection of peptides by CE. Due to the pathlengths encountered in CE (25–100 μm), UV detection is very insensitive. In addition, the low wavelength commonly employed for the detection of peptides (210 nm) is not very selective. Fluorescence detection, while sensitive, is limited to naturally fluorescent molecules or fluorescently derivatized compounds. More derivatization reagents react only with peptides possessing a primary amine functionality; therefore, N-terminal-blocked peptides cannot be detected. There is also very little selectivity for peptides over amino acids. Radiometric detection necessitates the use of radioactively labeled species.

ED, unlike UV detection, does not suffer from a loss of sensitivity when the cell volume is decreased. The development of the porous glass coupler [11], the Nafion joint [12], and end-column detection [13] has enabled the use of ED for CE. To date, the investigation of the ED of peptides separated by CE has been limited to the use of a copper electrode for the detection of dipeptides [14] or selective determination of thiol-containing peptides such as glutathione [15]. In this paper, the on-column complexation of peptides with copper and subsequent determination by CE–ED is described.

EXPERIMENTAL

Reagents

All solutions were prepared in deionized water and filtered with a 0.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA). Biuret reagent (0.6 mol/l NaOH, 12 mmol/l $CuSO_4$, 32 mmol/l $C_4H_4KNaO_6 \cdot 4H_2O$, 30 mmol/l KI) was obtained from Sigma (St. Louis, MO, USA). Cupric sulfate, sodium hydroxide and sodium borate were supplied by Fisher Scientific (Pittsburgh, PA, USA). Potassium iodide and sodium potassium tartrate were obtained from Sigma. Di-, tri and tetraglycine, Arg–Gly–Asp–Ser, Arg–Gly–Glu–Ser and Pro–Leu–Gly–amide were obtained from Sigma; di-, tri- and tetraalanine and Ala–Gly–Gly were from Research Plus (Bayonne, NJ, USA). Ultrapure sodium hydroxide was obtained from Aldrich (Milwaukee, WI, USA). 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-amino-2-methyl-1-propanol and glycine were obtained from Sigma and were used to prepare buffer solutions. All chemicals were reagent grade and were used as received.

Capillary electrophoresis system

The CE-ED system used was described previously [12]. The detector cell was operated in a three-electrode configuration with a 33-μm carbon fiber working electrode (AVCO Specialty Products, Lowell, MA, USA), a platinum wire auxiliary and a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA). The detection cell contained 0.1 *M* NaCl as the electrolyte. An ISCO CV^4 absorbance detector (ISCO, Lincoln, NE, USA) was used for UV detection. Separations were performed on a fused-silica capillary, 1.2 m × 50 μm I.D. × 375 μm O.D. (Polymicro Technologies, Phoenix AZ, USA). Unless otherwise indicated, the separation voltage was 25 kV and the detector potential was +0.900 V *vs.* a Ag/AgCl reference electrode. All washings were accomplished using positive pressure.

Cyclic voltammetry

A solution of $1 \cdot 10^{-2}$ *M* Gly–Gly–Gly was prepared in 50 m*M* NaOH, 1 m*M* $CuSO_4$, 2.6 m*M* tartaric acid and 2.5 m*M* KI and allowed to react for 10 min. This solution was then diluted 1:10 in 10 m*M* NaOH, pH 9.5. Cyclic voltammetry experiments were conducted in a three-electrode configuration using a Model CySy-1 computerized electrochemical analyzer (Cypress Systems, Lawrence, KS, USA). The scan rate was 10 mV/s. A carbon fiber working electrode, a Ag/AgCl reference and a platinum auxiliary electrode were used in all studies.

Electrochemical pretreatment

Two different electrochemical pretreatments of the carbon fiber microelectrode were investigated.

(a) Application of a 50-Hz square-wave waveform of 2 V amplitude for 1 min. This pretreat-

ment was performed while the microelectrode was inserted in the capillary column and operating buffer was flowing past its surface.

(b) Anodization of the microelectrode in an electrochemical cell at +900 mV in a solution of 1 *M* NaOH. This pretreatment was carried out for 15 min.

Precolumn derivatization

Precolumn derivatization was first evaluated for the detection of peptides. In this case, the sample was dissolved in 10 m*M* borate buffer, pH 10.3. To 900 μl of sample was added 100 μl of a solution consisting of 120 μM $CuSO_4$, 319 μM $C_4H_4KNaO_6$, 301 μM KI and 6 m*M* NaOH. The reaction was allowed to proceed for a minimum of 10 min and injected by pressure injection. The run buffer consisted of 10 m*M* borate, pH 10.3.

On-column derivatization

In this case, the Cu(II) was added to the buffer system so that the complexation would occur during the separation. The run buffer consisted of 10 m*M* borate, pH 9.5, 1 m*M* $CuSO_4$ and 3 m*M* tartaric acid. Samples were injected by pressure injection and the separation voltage was then applied.

Derivatization using a Cu(II)-saturated capillary

The capillary was precoated with Cu(II) prior to the separation in this procedure. A solution of 1 *M* NaOH was flushed through the capillary for 5 min, 0.1 *M* NaOH for 5 min and then a solution of 2.4 m*M* NaOH, 48 μM $CuSO_4$, 120 μM potassium tartrate and 120 μM KI was flushed through the capillary for 10 min. Lastly, a 2.5 m*M* NaOH solution was pushed through for 15 min. Separations were performed in the 2.5 m*M* NaOH run buffer.

RESULTS AND DISCUSSION

Electrochemistry

Electrochemical pretreatment is well known to have a pronounced effect on the electron transfer properties of many solution species, in particular, enhancement of the electrochemical response [16,17]. In this study, two pretreatment regimes were investigated. The first was the application of a high-frequency 2 V potential window to the electrode; this was considered a severe pretreatment. A second, milder pretreatment was also investigated, which involved preanodization of the microelectrode in 1 *M* NaOH for 15 min at +900 mV. This second approach was utilized in subsequent studies as it was found to provide activation of the microelectrode comparable to that of the harsher pretreatment, but was simpler to apply. The pretreatment was found to be necessary only for new or unused microelectrodes. Once pretreated, the microelectrodes did not require further pretreatment for reactivation, *i.e.*, oxidation of the Cu(II)–peptide complexes did not foul the surface of the electrodes. It is most probable that the pretreatment removed an initial polymeric layer which inhibited electron transfer on the surface of the carbon fiber. Shown in Fig. 1 is the background response at an unpretreated microelectrode (A) and at a pretreated microelectrode (B). The response of Cu(II)–Gly–Gly–Gly at a pretreated microelectrode is illustrated in Fig. 1C.

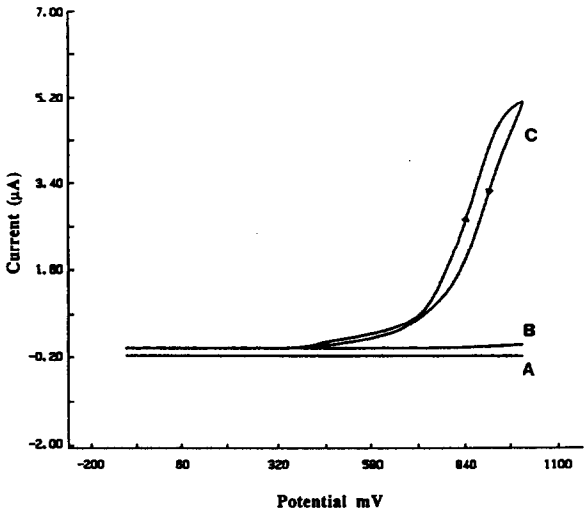

Fig. 1. Cyclic voltammetric behavior of 1 m*M* Cu(II)–Gly–Gly–Gly in 10 m*M* NaOH at a carbon fiber microelectrode. A = Initial response to complex; B = background electrolyte response after pretreatment of the electrode for 15 min in 1 *M* NaOH with stirring; C = response to complex following electrochemical pretreatment. Scan rate 10 mV/s.

Precolumn complexation of peptides with copper

Initial studies focused on precolumn complexation of compounds with the biuret reagent. Samples were derivatized with diluted biuret, allowed to react 10 min, and then injected into the CE system. Using this method, a detection limit of $4 \cdot 10^{-5}$ *M* was achieved (signal-to-noise ratio = 3) for the peptide Ala–Gly–Gly. The high ionic strength of the standard biuret reagent caused band-broadening, so it was diluted prior to derivatization. The run buffer consisted of the same solution. In an attempt to reduce the amount of current generated in the separation capillary, the use of several zwitterionic buffers, including CAPS and 2-amino-2-methyl-1-propanol, was investigated. However, it was found that when a buffer consisting of 10 m*M* CAPS (pH 10.3) was employed, the response for Cu(II)–Ala–Gly–Gly was reduced by 70%. 2-Amino-2-methyl-1-propanol buffers produced high background after electrode pretreatment, which took >30 min to decay to baseline.

On-column complexation of peptides with copper

Derivatization on-column by employing a buffer containing the biuret reagent was investigated. Initially, a buffer consisting of 10 m*M* borate, 1 m*M* $CuSO_4$ and 3 m*M* potassium tartrate was employed, but this resulted in a noisy baseline. It was then found that if the Cu(II) concentration were reduced by a factor of 42, the noise was substantially decreased. Detection limits of $1 \cdot 10^{-4}$ *M* for Ala–Gly–Gly (signal-to-noise ratio = 3) were obtained using a buffer consisting of 1 m*M* borate buffer, pH 10, 24 μM $CuSO_4$, 64 μM $C_4H_4KNaO_6 \cdot 4H_2O$, 60 μM KI, and 1.2 m*M* NaOH. However, even with this buffer system there was a gradual increase in noise over time that was not eliminated by replacing the electrode with a new carbon fiber. Based on this observation, it was concluded that the large amounts of Cu(II) present were interfering with the functioning of the Nafion joint. The high negative potential at the anode caused the Cu(II) to accumulate at the Nafion joint, hindering the flow of ions across the joint and causing a gradual increase in noise at the electrode.

Derivatization using a Cu(II)-saturated capillary

The formation of a "Cu(II)-coated" capillary was investigated. Above pH 2, fused-silica capillaries contain negatively caused silanol groups that can retain metal cations [18]. If a Cu(II)-coated capillary could be produced, Cu(II) would be available for complexation with peptides without the need for it in the running buffer. This would also eliminate the need for prederivatization. Coating the capillary was achieved using a pressure injection system to push a solution containing copper through the column. While copper still comes into contact with the Nafion joint, in this case it is for a minimal period of time, without the presence of the applied voltage. Copper is thus available for complexation with the peptides, but does not accumulate at the Nafion joint.

The coating procedure described in the Experimental section produced a capillary which could be run in 2.5 m*M* NaOH and was stable for >12 h of continuous analysis. The column was coated on a daily basis and the electrode was left in the column during the coating procedure. Increasing the concentration of the Cu(II) solution used for washing did not result in an increase in response for the peptides tested. No response was obtained if the capillaries were not initially coated with Cu(II).

The exact mechanism by which Cu(II) is associated with silica is not known. However, the interaction of metal cations such as Cu(II) with silica is well documented. It is believed that this interaction is not just due to ionic attraction forces but may also involve the formation of covalent bonds between the metal ion and the silanol group. The silica surface may be viewed as a polydentate ligand if one considers the close spacing of the SiOH groups on the surface [19]. In this application, the use of high pH and the presence of an additional complexing agent (tartaric acid) make the exact prediction of the mechanism difficult. However, it has been shown that the SiO^- ion can penetrate and displace a ligand from the coordination sphere of the metal atom, thus forming a covalent bond between the complex and the silica surface [20]. Whether the Cu(II) is bound to the silica is association with tartaric acid is not known. However, it is clear that Cu(II) does become associated with the

capillary wall and is available for peptide complexation.

Increasing the concentration of NaOH used to run the system to 10 m*M* decreased the migration time for the peptides, but also decreased the signal by 66%. However, NaOH was necessary to obtain the best signal. The use of other more conventional buffer systems such as borate, 2-amino-2-methyl-1-propanol, CAPS and glycine caused a decrease in response. Addition of 2.5% methanol or acetonitrile caused a reduction in sensitivity.

Separation of peptides

The separation of di-, tri-, tetra- and pentaglycine is seen in Fig. 2. A detection potential of +900 mV was chosen based on previous reports [1–4] and cyclic voltammograms obtained in our laboratory. The best response was obtained for triglycine with a detection limit of $7 \cdot 10^{-7}$ *M*. The response decreased with increasing size of the peptide. Detection limits for di-, tetra- and pentaglycine were $9.5 \cdot 10^{-7}$ *M*, $1.6 \cdot 10^{-6}$ *M*, and $5.5 \cdot 10^{-6}$ *M*, respectively. For Gly–Gly–Gly, response with linear from $1 \cdot 10^{-4}$ to $5 \cdot 10^{-6}$ *M* with a correlation coefficient of 0.998. The slope was 9 nA/m*M*. The responses for homogeneous peptides of alanine were less than those for glycine. When a 50 μM solution of di-, tri-, and tetraalanine was injected, only the trialanine was detectable. This system can be employed for the detection of heterogeneous peptides as well. Fig. 3 shows a separation of two peptides differing by a single amino acid, Arg–Gly–Asp–Ser (which supports fibroblast attachment and inhibits fibronectin binding to platelets) and Arg–Gly–Glu–Ser (an inhibitor of platelet aggregation).

Most methods for peptide analysis by HPLC or CE rely on the presence of a nucleophilic primary amine for derivative formation [21–23]. The biuret reaction has an advantage over this method, since it is selective for the peptide bond. Thus, the determination of the amide-protected Pro–Leu–Gly–amide was investigated. The detection limit for this compound was $2 \cdot 10^{-6}$ *M* (signal-to-noise ratio = 3). The relative standard deviations for the reproducibility of the detector response and migration time for Pro–Leu–Gly-amide were 6.8 and 3.6%, respectively ($n = 9$). A comparison of electrochemical and UV detection for the determination of Pro–Leu–Gly is shown in Fig. 4. It can be seen that the sensitivity of the Cu(II) complexation method is much

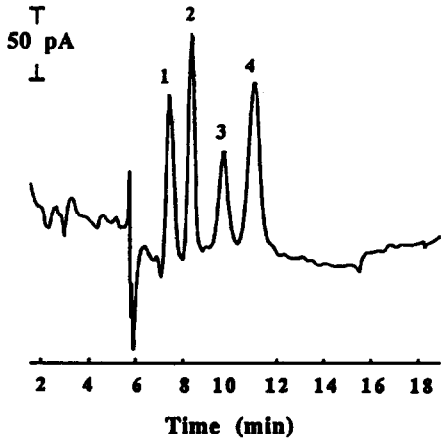

Fig. 2. Separation of $9 \cdot 10^{-6}$ *M* (1) di-, (2) tri-, (3) tetra- and (4) $5 \cdot 10^{-5}$ *M* pentaglycine in 2.5 m*M* NaOH. Capillary 1.2 m, coated at 10 p.s.i. (1 p.s.i. = 6894.76 Pa) for 5 min in 1 *M* NaOH, 5 min in 0.1 *M* NaOH, 10 min in a solution 2.4 m*M* in NaOH, 48 μM in $CuSO_4$, 120 μM in tartaric acid, 120 μM in KI, and finally 15 min in 2.5 m*M* NaOH. Applied potential 25 kV. Detection potential +900 mV.

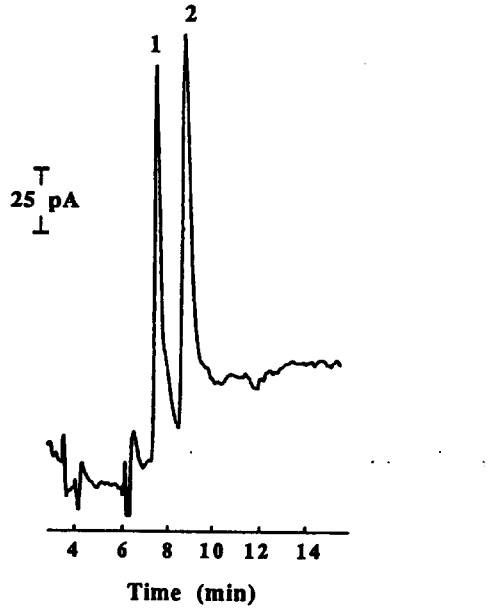

Fig. 3. Separation of $1 \cdot 10^{-5}$ *M* (1) Arg–Gly–Asp–Ser and (2) Arg–Gly–Glu–Ser in 2.5 m*M* NaOH. Run conditions as in Fig. 2.

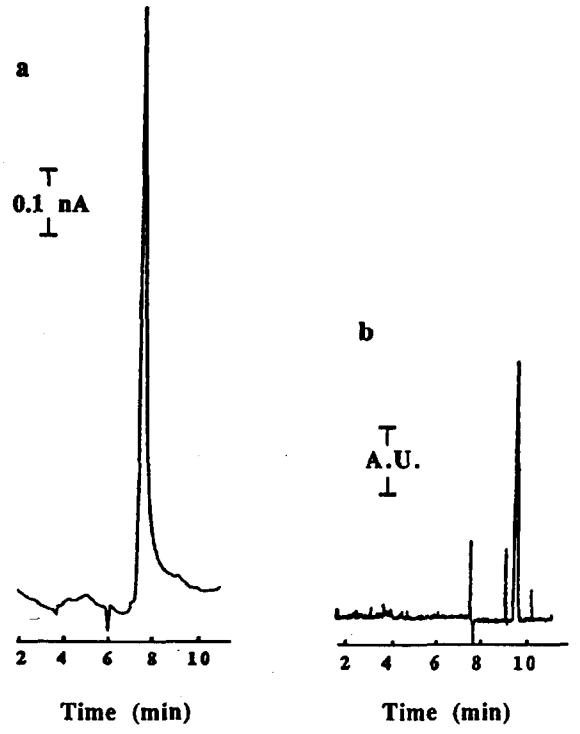

Fig. 4. A comparison of UV detection and ED: $5 \cdot 10^{-5}$ *M* Pro–Leu–Gly–amide. (a) ED, +900 mV; (b) UV detection, 210 nm.

better than with UV detection at 210 nm. In addition, this method affords greater selectivity than other commonly employed derivatization methods where there can be interference from amino acids. Warner and Weber [1] have shown than the selectivity for tripeptides using this method is on the order of 1000 times better than for amino acids.

The use of this method with capillary electrophoresis rather than liquid chromatography makes possible the analysis of submicroliter sample quantities. Since the Cu(II) complexation is accomplished on-column, there is no dilution of sample prior to injection as there is with other commonly employed derivatization reagents. At this time the analysis system is limited to the use of NaOH as the run buffer; however, a search for other compatible buffers is underway.

CONCLUSIONS

A very simple procedure for the determination of peptides has been developed, which allows direct injection of the peptide onto the column without the need for pre- or postcolumn derivatization. Investigations showed that the method can be used for the determination of selected peptides, including N-terminal-blocked peptides. The greater sensitivity and selectivity which can be achieved by the use of electrochemical rather than UV detection has been demonstrated. Future work will investigate the applicability of this method to the detection of larger peptides and the analysis of biological samples.

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