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# Electrochemical detection of oligopeptides through the precolumn formation of biuret complexes

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## ABSTRACT

The relatively slow kinetics of formation of the electroactive Cu(II)–peptide complexes from larger (>6 amino acids) peptides requires relatively high temperature and long reaction times for a postcolumn reactor. The precolumn incubation of bradykinin, $Tyr^8$-bradykinin and insulin A chain with biuret reagent for 20 min at 60°C leads to the formation of biuret complexes which can be subjected to chromatography in acidic or basic eluents. These complexes are detected electrochemically with a sensitivity similar to the Cu(II)–$(ala)_3$ complex (1 nC/pmol at 1.0 ml/min). The influence of the column-packing material on the electrochemical detector response of the Cu–peptide complexes has also been studied.

## INTRODUCTION

Reaction detection in liquid chromatography [1] has been used to increase detection sensitivity by the introduction of suitable chromophores or fluorophores, and to increase detection selectivity by using specific reactions. While certainly less convenient than detection by means of the native property of a compound, the gain in detection limit, sensitivity or selectivity is often substantial.

Recently, we [2,3] have introduced a method for the detection of peptides following their liquid chromatographic separation. The postcolumn reaction is the biuret reaction [4] which has been employed in an absorbance scheme for detection [5]. The poor detection limits in the latter technique do not recommend it for trace determinations. However, the electrochemical approach yields detection limits for small peptides (3–6 amino acids) of around 0.2 pmol in a 20-$\mu$l injection into a 15 cm $\times$ 4.6 mm I.D. reversed-phase column [2] (10 n*M*).

The detection system is based on the formation of Cu(II)–peptide complexes in the classical biuret reaction. The chemistry of Cu(II)–tripeptide complexes has recently been studied in great detail in a series of papers by Margerum [6] who showed in particular that these coordination compounds are easily oxidized to the Cu(III) form. Dual-electrode electrochemical detection can be used because the biuret electrochemistry is reversible. The first electrode in the series of two electrodes acts as an anode, or generator, by oxidizing the solutes passing by it in the flowing stream. The second electrode acts as a cathode or collector, reducing the products of the first electrode that are carried across its surface by the flow stream.

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We reported previously [2] that a tetradecapeptide, fibrinopeptide A, was about 100-fold less sensitively detected than a model tripeptide, $A_3$. This note demonstrates that precolumn formation of the complex leads to sensitivity equal to or better than the model compound for three larger peptides.

## EXPERIMENTAL

The measurements were carried out on an apparatus consisting of two Waters M45 pumps, a Rheodyne 7125 injection valve with a 20-$\mu$l sample loop, a BAS detection cell with a dual glassy carbon electrode, a BAS LC-4B amperometric detector and a BBC Goerz dual channel recorder. A stainless-steel auxiliary electrode and a silver–silver chloride reference electrode (purchased from BAS) were used. With no chromatographic column, this is a flow-injection system. $C_{18}$ columns (15 cm long, Waters Nova-Pak) and $ZrO_2$-polybutadiene (PBD) columns (a kind gift from Professor P. W. Carr, University of Minnesota) were also used. Control over postcolumn reactor temperature was by placement of knotted PTFE tubing into a BAS column heater.

Optical absorbance measurements were carried out with an IBM Model 9420 UV–visible spectrophotometer equipped with a cell holder, the temperature of which could be controlled with a recirculating bath (Haake). Reagents were brought to the appropriate temperature before mixing and spectrophotometric observation.

The following reagents were used without further purification: potassium phosphate monobasic and dibasic GR crystals, acetonitrile (HPLC grade), and phosphoric acid (E.M. Science, Cherry Hill, NJ, U.S.A.), sodium carbonate anhydrous and sodium bicarbonate (Fisher Scientific, Pittsburgh, PA, U.S.A.), sodium hydroxide pellets (J.T. Baker, Phillipsburgh, NJ, U.S.A.). All peptides were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium sodium tartrate (Aldrich, Milwaukee, WI, U.S.A.) was recrystallized from water before use. Water was doubly deionized and passed through an activated carbon column before distillation in a Corning system. All the solutions were filtered through nylon-66 filters (pore size 0.2 $\mu$m, purchased from Rainin, Woburn, MA, U.S.A.) before use.

Aqueous peptide solutions containing 4 nmol in a 20-$\mu$l injection were injected for the postcolumn experiment; 0.2–0.4 nmol of the Cu–peptide complexes were injected in the precolumn reaction system.

## RESULTS AND DISCUSSION

### *Temperature and biuret reaction time effects*

We reason that the lower sensitivity to the larger peptide is kinetic in origin. The visible absorbance from the biuret complex measured *vs.* time at 20.8°C shows that bradykinin (nonapeptide) requires at least 2 min for complete reaction (Fig. 1). Fig. 2 shows that increasing the postcolumn reaction time from 0.1 to 0.7 min and increasing the temperature of the reactor up to 70°C increases the sensitivity of the system to bradykinin, but only by a factor of at most five (up to 0.1 nC/pmol). This still leaves one order of magnitude to be gained compared to smaller peptides like $A_3$ (typical sensitivity 1.0 nC/pmol). The reaction time was increased by using longer knotted PTFE tubing (0.01 in. I.D.) after the mixing "T". It is unlikely that the

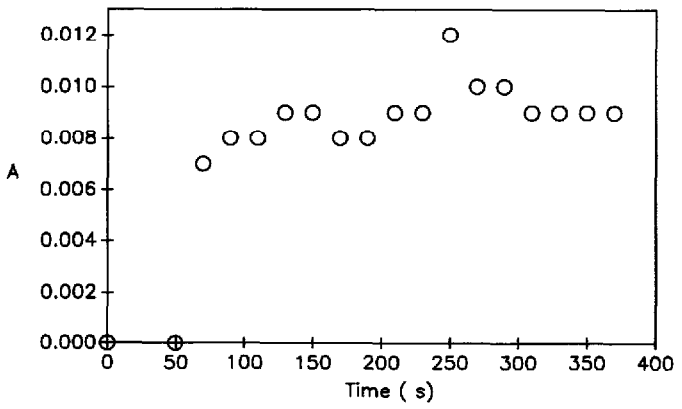


Fig. 1. Color development of bradykinin–Cu reaction *vs.* time. Concentrations are 0.1 m*M* bradykinin, 0.7 m*M* $Cu^{2+}$, 4.2 m*M* tartrate, pH 10.0 ± 0.1, wavelength set at 530 nm.

reaction solution heated up from room temperature to 70°C in 0.1 min, so the temperatures shown here must be regarded as those of the column heater, not measured reaction temperatures. Even so, it is evident that dramatic increases in reaction time would be required to obtain a sensitivity equivalent to $A_3$ for the larger oligopeptides.

An alternate approach is to use precolumn reaction. The incubation of peptides (bradykinin, $Tyr^8$-bradykinin, insulin A chain) with biuret reagent for 20 min at 60°C leads to the formation of the complex. These complexes yield good sensitivity in a flow-injection experiment (Table I). The eluent contained 1.0 m*M* $Cu^{2+}$, 3.0 m*M*

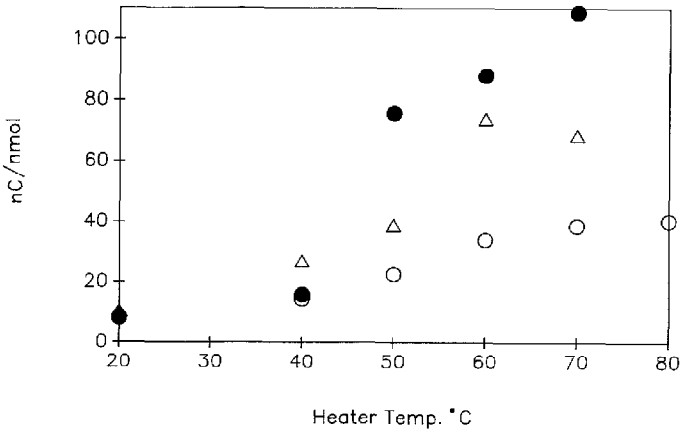


Fig. 2. Temperature and biuret reaction time effects on the electrochemical detector response of bradykinin–Cu complex. Postcolumn biuret reaction time: 0.1 min (○), 0.6 min (△), 0.7 min (●). Chromatographic conditions: column, Waters Nova-Pak $C_{18}$; mobile phase, 0.1% trifluoroacetic acid in acetonitrile–$H_2O$ (3:7); postcolumn reagent, 0.25 *M* $NaHCO_3$, 0.25 *M* $Na_2CO_3$, 0.1 m*M* $Cu^{2+}$, 2.0 m*M* tartrate. Mobile phase: postcolumn reagent ratio is 60:40, flow-rate is 2.0 ml/min. Anodic potential is 0.80 V *vs.* Ag/AgCl in 3 *M* NaCl reference electrode.

TABLE I

ANODIC SENSITIVITIES (nC/pmol) *VS.* POTENTIAL

Data from flow injection. The eluent contains 1.0 m*M* $Cu^{2+}$, 3.0 m*M* KNa tartrate, 0.10 *M* $Na_2CO_3$ and 0.02 *M* $NaHCO_3$. The pH is 10.5 ± 0.1 and the flow-rate is 1.0 ml/min. The peptides were prepared in the eluent and incubated for 20 min at 60°C.

| Peptide | Potential (V *vs.* Ag/AgCl in 3 *M* NaCl) | | | |
|---|---|---|---|---|
| | 0.5 | 0.6 | 0.7 | 0.8 |
| Bradykinin | 0.17 | 0.66 | 3.70 | 6.14 |
| $Tyr^8$-bradykinin | 0.10 | 1.08 | 1.82 | 2.28 |
| Insulin A chain | 0.08 | 0.21 | 0.41 | 0.54 |

KNa tartrate, 0.10 *M* $Na_2CO_3$ and 0.02 *M* $NaHCO_3$. The pH was 10.5 ± 0.1 and the flow-rate was 1.0 ml/min. Note that the sensitivities of bradykinin and $Tyr^8$-bradykinin are greater than for the $A_3$ (*ca.* 1.0 nC/pmol at 0.80 V) implying that more than one copper binds to each molecule of these peptides. Signals at the cathode for bradykinin and insulin A chain are in the range of 20% of those at the anode just as for the smaller peptides [2]. It is somewhat lower, *ca.* 10%, for $Tyr^8$-bradykinin.

*Column packing material effects*

Fig. 3 shows sensitivity for bradykinin in three systems. Two of the sets of data are from injection of the biuret complex of bradykinin into a flowing stream of basic copper tartrate; one is flow injection while the other is chromatography on the PBD

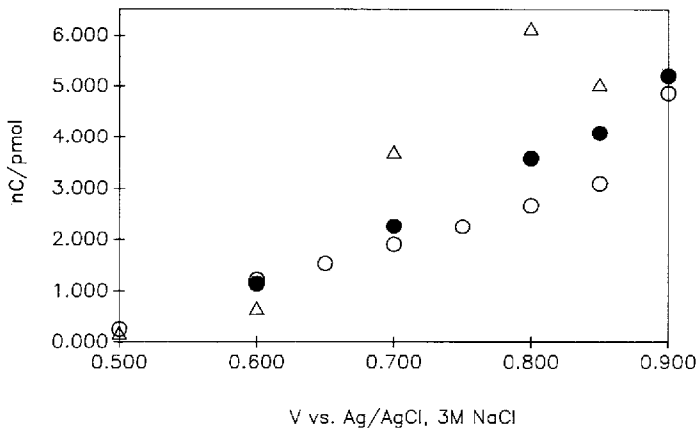


Fig. 3. Hydrodynamic voltammograms for bradykinin. Chromatographic conditions: (○) PBD column, eluent containing 1.0 m*M* $Cu^{2+}$, 3.0 m*M* KNa tartrate, 50 m*M* $Na_2HPO_4$, 0.1 *M* NaOH is added for adjusting the pH to 10.5 ± 0.1. Flow-rate is 1.0 ml/min. (△) Flow injection (*i.e.*, no column), eluent containing 1.0 m*M* $Cu^{2+}$, 3.0 m*M* tartrate, 0.10 *M* $Na_2CO_3$ and 0.02 *M* $NaHCO_3$, pH is 10.5 ± 0.1, flow-rate is 1.0 ml/min. (●) Waters Nova-Pak $C_{18}$ column; mobile phase is 25 m*M* $KH_2PO_4$, 2 g/l $H_3PO_4$ in acetonitrile–water (6:94); postcolumn reagent is the same as the eluent for the flow injection. Flow-rate is 2.0 ml/min; mobile phase, postcolumn reagent ratio is 1:1. Note: the sensitivities have been corrected for the postcolumn reagent dilution factor.

column which is stable to base [7]. The third set of data is for chromatography on a $C_{18}$ phase with an acidic mobile phase (pH 2.5 phosphate buffer–acetonitrile, 94:6) followed by the introduction of basic copper tartrate after the column as before. There is no electrochemical response if the basic copper tartrate is not introduced after the column. Signals at the cathode resulting from upstream oxidation at 0.6–0.7 V are in the range of 20–30% of those at the anode, just as for the smaller peptides.

The sensitivities for the two chromatographic experiments are equivalent. This indicates that the complex is kinetically stable in acid, and it can survive the chromatographic step. Thus, although the chromatography will be different than the chromatography of native peptides, the practitioner has the choice of whether to use $C_{18}$ columns with an acidic eluent, or a base-stable column with a basic eluent. A chromatogram of bradykinin and Tyr$^8$-bradykinin copper complexes on the $C_{18}$ column is shown in Fig. 4; the top trace is the response of the cathode and the bottom one is the response of the anode.

Two other points have been noted. The PBD column caused fouling. Table II shows the effect of the PBD column on the response of the electrochemical detector to the Cu–peptide complexes. Column A in Table II has the data from flow injection; the eluent was 1.0 m*M* $Cu^{2+}$, 3.0 m*M* KNa tartrate, 50 m*M* $Na_2HPO_4$, and NaOH was added for adjusting the pH to 10.5 $\pm$ 0.1; the flow-rate was 1.0 ml/min. Column B has the data from flow injection with the PBD column before the injector. The last column (C) has the data from chromatography with the PBD column. The sensitivity was lower even when the column was not used for chromatography (B). Also, the

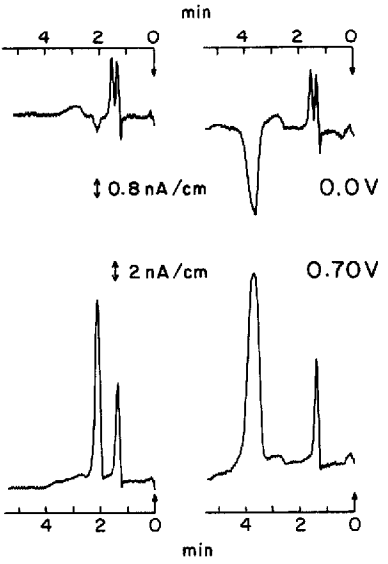


Fig. 4. Chromatogram of bradykinin– and Tyr$^8$-bradykinin–copper complexes. To the right: 0.48 nmol bradykinin–copper complex; to the left: 0.42 nmol Tyr$^8$-bradykinin–copper complex. Chromatographic conditions: mobile phase, 25 m*M* $KH_2PO_4$, 2 g/l $H_3PO_4$ in acetonitrile–water (6:94, v/v), postcolumn reagent, 1.0 m*M* $Cu^{2+}$, 3.0 m*M* tartrate, 0.10 *M* $Na_2CO_3$ and 0.02 *M* $NaHCO_3$. Flow-rate is 2.0 ml/min; mobile phase, post column reagent ratio is 1:1. The peptides were incubated in the post column reagent for 20 min at 60°C before being injected into the chromatography system.

TABLE II

| Cu–peptide complex | Anodic sensitivity at 0.8 V (nC/pmol) | | |
|---|---|---|---|
| | A | B | C |
| Bradykinin | 6.3 | 4.7 | 2.9 |
| $Tyr^8$-bradykinin | 1.4 | 1.1 | 0.53 |
| Insulin A chain | 0.53 | 0.21 | 0.12 |

drawn-out shape of the voltammetry with the PBD column should be compared to the voltammetry in flow-injection analysis (Fig. 3). This indicates electrode fouling by the column. The electrode fouling problem has been noted before when using $C_{18}$ columns [2], and it is lessened by operating at pH $\gtrsim$ 10. However, it is even less of a problem in flow injection than in chromatography, so we speculate that the columns are responsible for this. The other point to note is that the tyrosine-containing peptide, $Tyr^8$-bradykinin, has a lower collection efficiency than bradykinin, and insulin A chain has a lower sensitivity. Recently, we have begun to study the influence of an electroactive functional group on the electrochemical detector response to the Cu–peptide complexes. These results will be reported in due course.

It is thermodynamically feasible that the Cu(II)–peptide complexes can be oxidized by oxygen. The following casual observations suggest that the oxidation is slow, and therefore not a problem. The Cu(II)–tripeptide complexes are stable for at least several months. The Cu(II)–bradykinin complex in the biuret reagent containing $Na_2HPO_4$ buffer is still purple after 7 months. On the other hand, the purple color of a Cu(II)–polylysine complex disappeared after 20 days.

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