# Fluorometric Determination of Amino Acids and Proteins Utilizing a Copper(II) Catalyzed Reaction

Hisakazu MORI,* Kazue SAKAI, Kyoko YAMASHINA, Sayuri HIRATA, and Kumiko HORIE
Kyoritsu College of Pharmacy, Shibakoen, Minato-ku, Tokyo 105
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Amino acids accelerated the copper(II)-catalyzed oxidation of di-2-pyridyl ketone hydrazone (DPKH) to form a fluorescent compound. With the use of this enhancement effect of catalysis, various amino acids (L-histidine, L-cysteine, L-glutamic acid, glycine, DL-serine, and L-arginine) could be determined by flow injection analysis. The detection limit of L-histidine was less than 2 pmol. On the contrary, proteins decreased the catalysis. Proteins were also determined through the utilization of this effect. In this determination, the addition of citric acid was found to be very effective. The detection limit of bovine serum albumin was less than 20 ng. The effect of protein upon the catalysis of copper(II) was considered to be ascribable to an inhibition of the coordination of DPKH to copper(II).

In trace analyses of amino acids they are usually transformed to derivatives which can be detected colorimetrically or fluorometrically at low level. One mole of the derivative corresponds to one mole of an amino acid in such reactions. Amino acids can also be determined by other methods than those with derivatisation. In these methods, two types of reactions seem to be utilized. One type is such a chemiluminescence reaction as that between luminol and hydrogen peroxide catalyzed by cobalt(II)[1] or copper(II).[2] The addition of an amino acid to this reaction system, forming a complex with a metal ion, reduces the chemiluminescence. Thus, amino acids are determined quantitatively. The other type is a fluorogenic oxidation reaction catalyzed by copper(II) in which amino acids accelerate the reaction rate. With the use of this enhancement effect of catalysis, amino acids were determined[3] by flow injection analysis (FIA). In this catalytic reaction, one mole of an amino acid may correspond to more than one mole of the fluorescent compound. Thus, a more sensitive detection would be achieved by optimizing the reaction conditions. This would also be done by the use of a reaction substrate which is oxidized to a compound having intense fluorescence.

In the present study regarding the determination of amino acids, di-2-pyridyl ketone hydrazone (DPKH) was adopted as a reaction substrate instead of phenyl 2-pyridyl ketone hydrazone (PPKH), which was used in the previous study.[3] It is known[4] that the oxidation product of DPKH shows an intense fluorescence in the acidic media. Thus, the FIA system was applied with a flow line of hydrochloric acid in order to detect the oxidation product in an acidic media. The effects of proteins on the copper(II)-catalyzed oxidation were also investigated, compared with those of amino acids. The necessary conditions for the sensitive detection of proteins were pursued.

## Experimental

**Chemicals.** DPKH was synthesized by the reaction of di-2-pyridyl ketone with hydrazine monohydrate according to a method described in the literature.[4] Amino acids and copper(II) salts were of analytical reagent grade. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. and human serum albumin (HSA), from Wako Pure Chemical Industries, LTD.

**Apparatus and Procedures.** Figure 1 shows a schematic diagram of the FIA system used in the present study. It contains four flow lines (Teflon, 0.5 mm i.d.). $C_1$ is a $5\times10^{-5}$ M( 1 M $\equiv$1 mol dm$^{-3}$) aqueous solution of DPKH. $C_2$ is 0.1 M NaOH. $C_3$ is water and $C_4$ is 0.4 M HCl. An alkaline solution of DPKH could not be used instead of the flow lines, $C_1$ and $C_2$, because of the gradual change of the base line on the recorder. All solutions were pumped by two double-plunger-type pumps (Nihonseimitsu NP-D-322U) at a flow rate of 1.2 cm$^3$ min$^{-1}$. Samples were injected to the flow system by an injector (Nihonseimitsu NVI-3002) with a 50 mm$^3$ sample loop. The aerial oxidation of DPKH, catalyzed by the amino acid–copper(II) complex, was carried out in a reaction coil (150 cm) immersed in a water bath thermostated at 25 °C. The fluorescence[4] ($\lambda_{ex}$ 349 nm, $\lambda_{em}$ 435 nm ) of the reaction

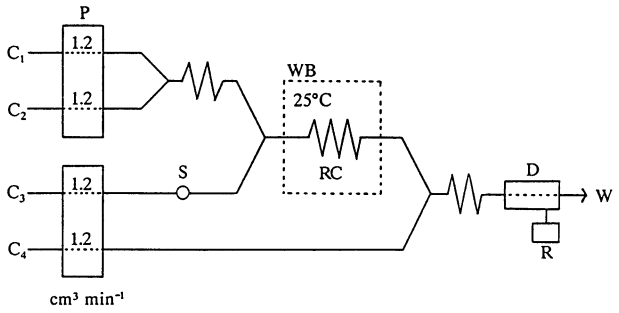


Fig. 1. Schematic diagram of the FIA system.
S: sample injector, $C_1$: $5\times10^{-5}$ M aqueous solution of DPKH, $C_2$: 0.1 M NaOH, $C_3$: water, $C_4$: 0.4 M HCl, P: pump, RC: reaction coil, WB: water bath, D: detector, R: recorder, W: waste.

product acidified by 0.4 M HCl was monitored by a spectrofluorometer (Hitachi F-1000) with a 12 $mm^3$ flow cell, and recorded on a recorder (data processor, Shimadzu C-R1A). The peak height of the signal was calculated using this recorder.

The samples of amino acids were prepared in a buffer solution as mixtures of $4\times10^{-7}$ M copper(II) and various amounts of each amino acid. After stirring for 10 min it was subjected to analysis. The samples of proteins were usually prepared in a similar manner as described above. Those in the Atkins–Pantin buffer (boric acid–potassium chloride–sodium carbonate) containing citrate were prepared as follows. A copper(II) solution in a citric acid–disodium hydrogenphosphate buffer (pH 3.0) was diluted with the Atkins–Pantin buffer. This solution was mixed with a Atkins–Pantin buffer solution of protein.

**Absorption Spectra Measurements.** The absorption spectra of the mixtures of copper(II), L-histidine (or BSA), and DPKH were measured at room temperature as follows. A mixture in a quartz cell of 0.5 $cm^3$ of $2\times10^{-3}$ M aqueous copper(II) solution, 1 $cm^3$ of aqueous L-histidine (or BSA) solution, and 0.5 $cm^3$ of 0.05 M Clark–Lubs buffer (boric acid–potassium chloride–sodium hydroxide, pH 9.0) was bubbled with $N_2$ gas for 5 min. Then, into this mixture was added 1 $cm^3$ of a $1\times10^{-3}$ M aqueous solution of DPKH through which $N_2$ gas had been previously passed. About 5 s after mixing, a measurement of the absorption spectrum was started with a spectrophotometer (Hitachi U-3210).

## Results and Discussion

**Determination of Amino Acids.** As reported in a previous paper,[3] the amino acid–copper(II) complex in the alkaline buffer solution had shown an enhanced catalytic activity in the aerial oxidation of PPKH. In the present work using DPKH, the catalytic activities of the amino acid–copper(II) (1 : 1) complex ($4\times10^{-7}$ M) in the buffer solutions of various pH values were compared with those of copper(II). The amino acid used was L-histidine, which had given the maximum activity in a previous study.[3] Phosphate buffer (0.005 M) was employed in the pH range of 5.0 to 7.0, and Clark–Lubs buffer (0.005 M) in the pH range of 8.0 to 10.0. As shown in Fig. 2, copper(II) and the L-histidine–copper(II) complex indicated comparable catalytic activities in weakly acidic media. In more basic media, the catalytic activities of both decreased, though that of the L-histidine–copper(II) complex became superior to that of copper(II), showing that the addition of L-histidine to copper(II) in a basic media enhances the catalytic activity of copper(II), as in the case of the aerial oxidation of PPKH.[3] It was suggested that the determination of this amino acid could be made most sensitively with the use of a buffer of pH 9.0 among those of various pH values. The decrease in the catalytic activity in more basic media would be due to the formation of phosphate(s) or hydroxide(s) of copper(II); this decrease could be partly prevented by the formation of an active L-histidine–copper(II) complex.

Other reaction conditions were examined in order to detect lower amounts of L-histidine. Favorable peaks were obtained at a DPKH concentration of $5\times10^{-5}$ M in the series of $5\times10^{-4}$, $5\times10^{-5}$ and $5\times10^{-6}$ M. Among three concentrations (0.05, 0.005, and 0.0005 M) of the Clark–Lubs buffer (pH 9.0), that of 0.005 M gave the greatest difference between the signal due to aerial oxidation with and without L-histidine. Several copper(II) salts, i.e., nitrate, chloride, perchlorate, and sulfate were examined, and the use of copper(II) nitrate gave the maximum signal. The temperature of the water bath where the reaction coil was immersed was varied (15, 25, 35, and 45 °C). Since the signal did not increase very

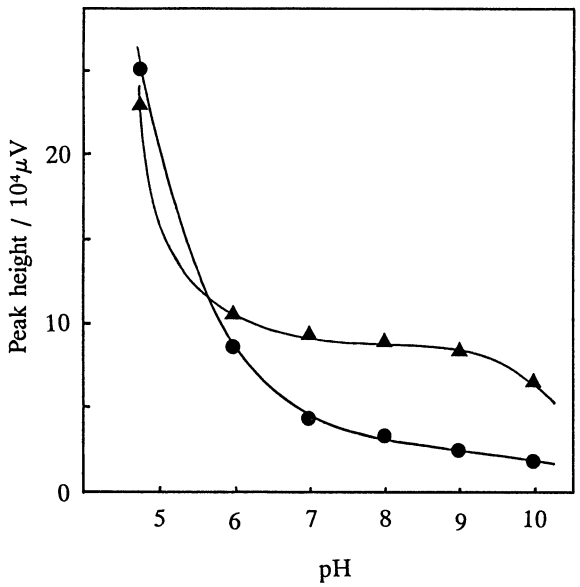


Fig. 2. Variation of the peak heights of signals with a change of the pH values of sample solutions.
●: copper(II), ▲: copper(II) + L-histidine(1 : 1).

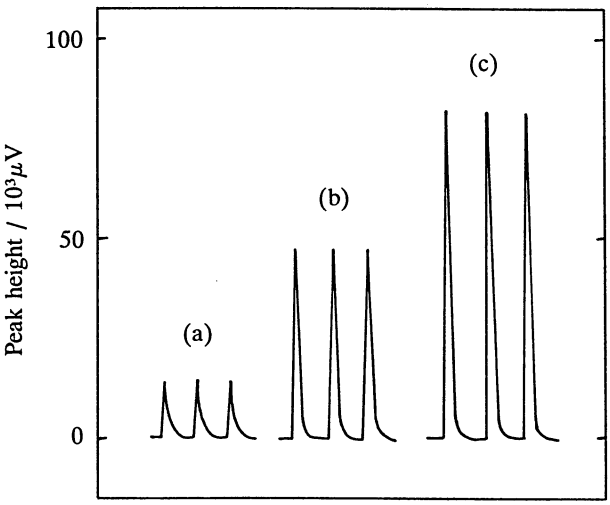


Fig. 3. Peaks with increased height by the addition of L-histidine.
(a): 0, (b): 0.4, (c): 0.8.
The amount of added L-histidine is represented by the molar ratio to copper(II).

much at temperatures greater than 25 °C, a temperature of 25 °C was adopted for the reaction. The variation of the concentration of hydrochloric acid did not change the peak height, provided that the concentration of hydrochloric acid is higher than that of the sodium hydroxide solution. Thus, concentrations of 0.4 and 0.1 M were respectively employed as those of the hydrochloric acid and sodium hydroxide solutions.

Figure 3 shows the peaks obtained when the samples containing various amounts of L-histidine were subjected to analysis. As the molar ratio of L-histidine to copper(II) was increased, the fluorescence intensity increased linearly in the molar ratio range from 0 to 1. The correlation coefficient ($r$) was 0.996 ($n$=7) and the relative standard deviation at a molar ratio of 0.4 was 2.3% ($n$=5). All of the amino acids investigated increased the catalysis of copper(II). In the cases of L-histidine and L-cysteine, the peak height of the signal increased linearly over a molar ratio range of about 1, but deviated from a linear relationship at a molar ratio greater than 1. The maximum molar ratio below which a linear relationship was observed was 3 for L-glutamic acid, 4 for glycine and DL-serine, and 5 for L-arginine. The calibration curves for all amino acids included the point of molar ratio 0 in the linear range. Table 1 gives the upper limits of the linear range for each amino acid. In Table 1 are also shown the slopes of the calibration curves which were defined as the difference between the peak height at a molar ratio of 1 and that at a molar ratio of 0. The slopes for L-histidine and L-cysteine are larger than those for other amino acids. A similar tendency had been observed in a determination using PPKH,[3] though this tendency was more marked in the present study.

In the determination of L-histidine, the content of this amino acid was 2 pmol in a sample with a molar ratio of 0.1. This value was less than one third of that in the case of PPKH. This difference would come from the character of the oxidation product of DPKH, which shows intense fluorescence in an acidic media.

Table 2 shows the effects of foreign metal ions in the determination of L-histidine. The relative peak height of the signal in the case of the mixture to that in the case of copper(II) was used as an indication of the effect of a foreign metal ion. Zinc(II), manganese(II), silver(I), mercury(II), and cobalt(II) increased the peak height in this order. On the other hand, nickel(II) and iron(III) decreased the peak height. EDTA at the same level as copper(II) interfered with the determination. This would be ascribed to the formation of a copper(II)–EDTA complex with a large formation constant, to which DPKH cannot coordinate.

**Determination of Proteins.** We further applied this method to the determination of protein which is practically important in biological studies. Preliminary

Table 1. Upper Limits of the Linear Ranges and Slopes of Calibration Curves for Various Amino Acids

| Amino acid | Upper limit/$10^{-7}$ M | Slope/$10^3$ μV |
|---|---|---|
| L-Histidine | 4 | 69.0 |
| L-Cysteine | 3.2 | 73.7 |
| L-Glutamic acid | 12 | 15.4 |
| Glycine | 16 | 11.6 |
| DL-Serine | 16 | 6.3 |
| L-Arginine | 20 | 2.1 |

Table 2. Relative Peak Heights in the Presence of Foreign Metal Ions

| Metal ion | Added Amount/[Metal ion][Cu(II)]$^{-1}$ | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1 | 10 | 100 | 500 |
| None | | 1 | | | |
| Co(II) | | 1.7 | 4.3 | | |
| Hg(II) | | 1.3 | 2.8 | | |
| Ag(I) | | 1.1 | 1.8 | | |
| Mn(II) | | 1.1 | 1.4 | | |
| Zn(II) | | 1.0 | 1.1 | 1.4 | 1.7 |
| Al(III) | | 1.0 | 1.0 | 1.0 | 1.0 |
| Ni(II) | | 0.9 | 0.2 | | |
| Fe(III) | | 0.9 | 0.7 | | |
| EDTA | 0.5 | 0.1 | ≈0 | | |

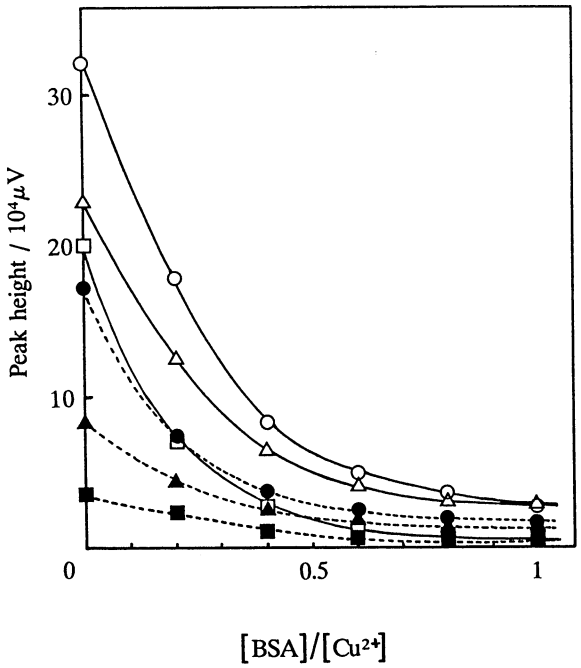


Fig. 4. Calibration curves for BSA samples in phosphate buffers (0.005 M) and citric acid–disodium hydrogenphosphate buffers of various pH values.
phosphate buffer, ●: pH 5.0, ▲: pH 6.0, ■: pH 8.0.
citric acid–disodium hydrogenphosphate buffer, ○: pH 5.0, △: pH 6.0, □: pH 8.0.
BSA samples were prepared on the assumption that the molecular weight of BSA was 69000. Thus, in the case that the molar ratio of BSA to copper(II) is unity, this sample contains 1380 ng of BSA. The samples in a citric acid–disodium hydrogenphosphate buffer contain 0.005 M citric acid.

experiments concerning BSA showed that this protein decreased the catalysis of copper(II), in contrast with amino acids. Figure 4 indicates the effects of the kinds of buffers and their pH values of BSA samples. With respect to the samples in a phosphate buffer, the catalysis of free copper(II) (that is, when no protein was added) was markedly lowered in the alkaline pH region. On the other hand, the samples in citric acid–disodium hydrogenphosphate buffer showed higher catalytic activities than those in phosphate buffer and the catalysis was not so much decreased, even in the alkaline pH region. This effect would be due to the formation of a soluble citric acid–copper(II) complex toward which DPKH can coordinate to be oxidated. Another oxyacid, tartaric acid, also showed a similar effect in the alkaline pH region, which was fairly smaller than that due to citric acid.

By experiments involving spectrum measurements (see below), it was previously confirmed that BSA reacts with greater amounts of copper(II) in more basic solutions. Thus, in order to detect lower amounts of protein, we exclusively examined samples in strongly alkaline buffers containing citrate. The Clark–Lubs buffer (pH 8.0—10.0) and the Atkins–Pantin buffer (pH 9.0—11.0) were employed for sample preparation. In the case of the Clark–Lubs buffer, that of pH 10.0 afforded the most acute decrease in signal over the range of small amounts of added protein. However, plotting of data over this range gave a calibration curve with comparatively large scattering. When the Atkins–Pantin buffer was applied, a more basic buffer reduced more extensively the signal due to free copper(II). Thus, a buffer of pH 9.0 afforded the best calibration curve, containing a linear line ($r$=−0.997) over the range of less than 200 ng of added protein (Fig. 5). The relative standard deviation upon the addition of 80 ng of BSA was 1.50% ($n$=5). Figure 5 also shows the calibration curve for HSA; it contains a linear line($r$=−1.000) over the range of less than 200 ng. The relative standard deviation upon the addition of 80 ng of HSA was 1.69%, ($n$=5). The detection limits were less than 20 ng in the respective calibration curves. At present, the sensitivity using the present method for protein determination does not attain to that in the method using the copper(II) catalyzed chemiluminescence reaction between 1,10-phenanthroline and hydrogen peroxide.[5]

We presently have interest in the different effects of proteins from amino acids on the catalysis of copper(II). In order to elucidate this difference, the effect of L-histidine or BSA on the coordination of DPKH to copper(II) was investigated. As the absorption spectrum (a) in Fig. 6 shows, the mixing of a solution of DPKH with a solution of copper(II) in alkaline buffer (Clark–Lubs, pH 9.0) afforded a new absorption maximum at 426 nm, indicating the coordination of DPKH to copper(II). When the solution of DPKH was mixed with a copper(II) solution containing L-histidine, the spectrum was little changed in the region of a molar ratio of L-histidine to copper(II) less than 1. On the contrary, when the DPKH solution was mixed with a copper(II) solution containing BSA, the absorbance was remarkably reduced at a molar ratio less than 1 (spectrum (b)—(e) in Fig. 6). In a more basic buffer, this tendency was much more marked. These facts would

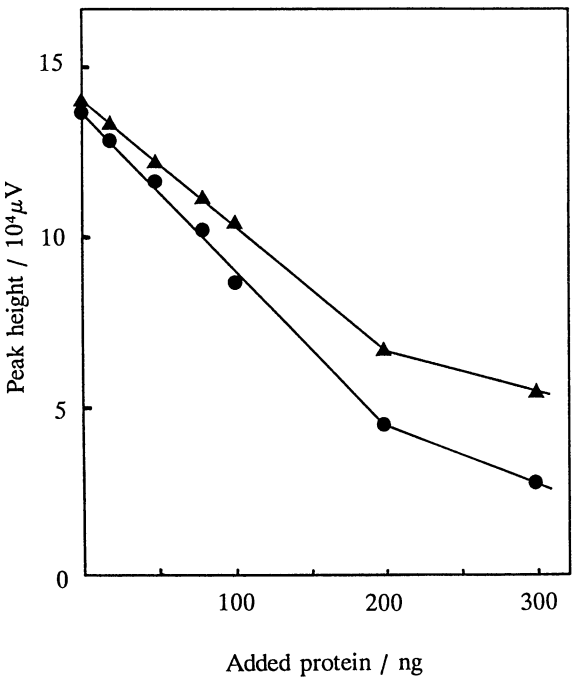


Fig. 5. Calibration curves for BSA and HSA samples in an Atkins–Pantin buffer containing citrate (pH 9.0).
●: BSA, ▲: HSA.
The sample solutions prepared contain $2\times10^{-5}$ M citric acid, 0.05 M boric acid, 0.05 M potassium chloride, and 0.029 M sodium carbonate.

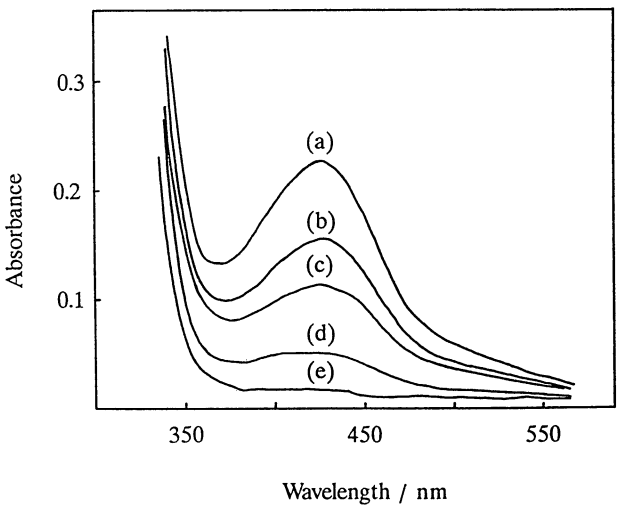


Fig. 6. Absorption spectra of the mixtures of copper(II), BSA, and DPKH with various molar ratios of BSA to copper(II).
(a): 0, (b): 0.047, (c): 0.094, (d): 0.19, (e): 0.47.

indicate that BSA firmly coordinated to copper(II) and inhibited the coordination of DPKH to copper(II), while L-histidine scarcely did so.

The determination of amino acids or proteins by the present method can be performed in 1 min. The sensitive detection of amino acids was achieved by the utilization of DPKH, owing to the intense fluorescence of the reaction product in an acidic media. The use of the compounds whose oxidation product have more intense fluorescence and/or which are oxidized with high velocity would increase the sensitivity in the detection of amino acids or proteins. The study in this direction is in progress.

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