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Detection of Copper Ions in Liquid Foods and Beverages Based on an Enzymatic Method

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ABSTRACT

An economic, simple and sensitive enzymatic method for the determination of copper in aqueous samples was proposed in this study. By removal of copper, superoxide dismutase (SOD) becomes apoenzyme SOD (apo-SOD) that has no SOD activity. The amount of copper added can be estimated by the recovery of SOD activity from reconstituted apo-SOD. The concentration of copper in an aqueous sample can thus be determined. A linear relationship (R^2 = 0.998) between the concentrations of copper, ranging from 20 to 80 ppb, and the recovered SOD activities was observed. The detection limit for copper in the proposed assay was 20 ppb. Moreover, interfering effects of some common metals on the recovery of SOD activity was evaluated. It showed that the concentrations of these metals less than 45 μg/mL exhibited no interference in the assay. In addition, data obtained from this assay were very close to those estimated by inductively-coupled plasma atomic emission spectrometry (ICP-AES). This enzymatic method can be adapted as a routine assay for the determination of copper in aqueous samples such as beverages, drinking water and liquid foods. The assay can be applied for large number of samples (about 96 samples per hour) on an ELISA microplate reader.

Key words: enzymatic method, superoxide dismutase, apoenzyme, copper ion, ELSIA microplate reader

INTRODUCTION

Copper (II) is one of the most important heavy metals, and a microelement in mammalian nutrition. In general, a daily copper intake of 1.5 - 2 mg is essential. However, copper becomes toxic to human if high amount is ingested and accumulated in tissue. For example, catalytic copper, because of its mobilization and redox activity, is believed to play an important role in the formation of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and OH radicals, that bind very fast to DNA and produce damage by breaking the DNA strands or modifying the bases and/or deoxyribose leading to carcinogenesis[(1)]. Overexposure to copper causes ptyalism, nausea, vomiting, epigastric burning and diarrhea. High doses of copper result in a series of systematic toxic effects such as hemolysis, hepatic neurosis, gastrointestinal bleeding, oliguria azotemia, hemoglobinuria, hematuria, proteinuria, hypertension, tachycardia, convulsions, and coma[(2)]. Therefore, the trace amount of copper in water must be controlled on a daily basis. In view of this, the separation and determination of copper from associated elements is indispensable. Moreover, the determination of copper levels both in serum and urine samples are of great importance in the early diagnosis of certain diseases[(3)].

The amount of copper ion could be assayed by employing many techniques. Among them, flame atomic absorption spectrometry (FAAS) is a convenient technique and available in most laboratories. FAAS has more advantages over other methods, including less subject to interferences and simplicity of instrumentations. However, direct determination of copper in liquid foods or biological samples by FAAS is difficult due to its low sensitivity[(4)]. In many instances, it needs a pre-concentration prior to analysis[(5,6)]. From the practical point of view, to be applied to

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the *in situ* analysis of aqueous samples, such as liquid foods and beverages, a method must be rapid, simple and without complicated pretreatments. In the case of copper, limit of detection of at least 0.5 ppm is required[7]. The methods of inductively-coupled plasma atomic emission spectrometry (ICP-AES)[8,9] or inductively coupled plasma mass spectrometry (ICP-MS)[10] provide sufficient detection limits (about 6 ppb for ICP-AES, 1 ppb for ICP-MS). However, these methods require expensive instrumentations. Therefore, they are not suitable to analyze large number of samples. Other methods including electrothermal/graphite furnace atomic absorption spectroscopy (ETV-AAS or GFAAS)[11], X-ray fluorescence[12], differential pulse anodic (or cathodic) stripping voltammetry[13], electrothermal atomic absorption spectrometry (ETAAS)[14], and evanescent wave absorption spectroscopy [15] have also been developed to measure copper content, but neither the instruments nor the techniques are familiar to the routine analysis.

In the present study, an economic and convenient enzymatic method was developed to determine the concentration of copper in aqueous samples. The analysis is performed using an enzyme copper-zinc-superoxide dismutase (Cu_2Zn_2-SOD, EC 1.15.1.1) which is a metalloprotein that catalyzes the dismutation of superoxide anion (O_2^-) into hydrogen peroxide and oxygen molecule[16]. By removal of copper, Cu_2Zn_2-SOD becomes apo-SOD with no SOD activity. Therefore, the amount of copper in an aqueous sample can be estimated through the recovery of SOD activity by mixing with apo-SOD.

MATERIALS AND METHODS

I. *Materials*

Riboflavin, *o*-dianisidine, diethylenetriamine penta-acetic acid (DTPA), and purified Cu_2Zn_2-SOD (4,000 U/mg protein) from bovine erythrocytes were obtained from Sigma Chemical Company (St. Louis, MO, USA). 96-well microplates (Cell Wells, made of polystyrene, flat bottom with diameter of 6.4 mm) with lid cover were products of Corning Glass Works. A stock standard of copper, 1000 ppm (1000 mg/L) certified atomic absorption standard solutions, was purchased from Fisher Scientific (Dubuque, IA, USA). Chemical reagents were all prepared using Millipore milli-Q water pretreated with Chelex 100 (Bio-Rad, Hercules, CA, USA) to remove metal ions.

II. *Preparing the Apoenzyme of SOD (apo-SOD)*

The apo-SOD was prepared by dialysis of Cu_2Zn_2-SOD for 48 h against several changes of 50 mM sodium acetate (pH 3.8) buffer containing 10 mM disodium salt of ethylenediaminetetraacetic acid (EDTA). This treatment was followed by exhaustive dialysis against 0.1 M NaCl in 50 mM sodium acetate (pH 3.8) to remove protein-bound EDTA. The apoenzyme of SOD was then obtained by dialysis against 10 mM sodium acetate (pH 3.8) to remove salt[17]. The apo-SOD was also prepared by column chromatography[18]. Briefly, metal removal from Cu_2Zn_2-SOD was accomplished by chromatography on a Sephadex G-25 column (1.6 × 100 cm) eluted with 50 mM sodium acetate (pH 3.8) buffer containing 10 mM EDTA. The EDTA was removed from the sample by eluting 10 mM sodium acetate (pH 3.8) through a second Sephadex G-25 column. The resulting solution was then dialyzed against 10 mM sodium acetate (pH 3.8).

III. *Assay of SOD Activity*

SOD activity was determined according to Misra and Fridovich's procedure with some modification[19,20]. Samples (70 - 80 μL) were added into the microplate wells, together with 80 μL of freshly prepared potassium phosphate (50 mM, pH 7.5) containing 0.4 mM *o*-dianisidine, 0.8 mM diethylenetriamine penta-acetic acid and 20 μM riboflavin. Each combined solution was mixed vigorously in a well for two min on a mixer (Tuple mixer Twin 3-28, Iwaki Glass Co. Ltd., Japan). Reaction was initiated by illuminating the lid-covered microplate with an argon lamp (Philip, 13 W) positioned 15 cm above the microplate at room temperature for 10 to 20 min. The absorbance of reaction mixtures in the wells of plate were measured at 450 nm by an automatic ELISA microplate reader (MR5000, Dynatech). SOD activities either in aqueous samples or copper standard solutions were assayed in triplicate.

IV. *Copper Assay Using the Present Method*

Test aqueous samples (20 μL) or copper (II) standard solutions (10 μL) were added into the microplate wells, together with 10 μL of apo-SOD (original SOD activity was 20 U) and 50 μL potassium phosphate buffer (30 mM, pH 5.9). The reaction mixtures were shaken on a Tuple mixer (Twin 3-28, Iwaki Glass Co., Ltd., Japan) for two min and stood at room temperature (25 ± 2°C) for 15 min. The mixtures were then evaluated for SOD activity as mentioned above, and the amount of copper was calculated from the standard curve.

V. *Tolerance Limits of Some Common Metal Ions in the Current Method*

The following metal ions were used for the tolerance experiments: Na (I), K (I), Zn (II), Mn (II), Ni (II), Pb (II), Co (II), Cd (III), Fe (III), and Cr (III). These metals were 1000 μg/mL (1000 ppm) certified atomic absorption standard solutions from Fisher Scientific (Dubuque, IA, USA). To test if these metals were able to compete with the copper (II), 10 mL of apo-SOD (original SOD activity was 20 U) was mixed with 10 μL copper standard solution (1000 ppm) and 50 μL of these metal ions in phosphate buffer (30 mM, pH 5.9) for 20 min, and followed by SOD activity assay. In addition, these *metal* solutions were prepared at concentrations ranging from 10 to 500 ppm.

Journal of Food and Drug Analysis, Vol. 20, No. 1, 2012

VI. *Determination of Copper in Aqueous Samples Using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)*

Samples were submitted to Regional Instruments Center of National Science Council for copper ion concentration analyses. Measurements were made on inductively coupled plasma-atomic emission spectrometer (ICP-AES, Jarrell-Ash, ICAP 9000). The analyses were carried out by ICP-AES at 324.754 nm[9] using a 1000 ppm certified atomic absorption standard solution (Fisher Scientific, Dubuque, IA, USA) for calibration.

RESULTS AND DISCUSSION

I. *Preparing Apoenzyme of SOD*

The principle of copper estimation is demonstrated in Figure 1. The apo-SOD was prepared by the dialysis of Cu_2Zn_2-SOD against EDTA. The apo-SOD could recover its SOD activity by the addition of copper from aqueous samples or standard solutions[17,18]. Thus, the concentrations of copper were determined by evaluating the degree of the recovered SOD from of apo-SOD.

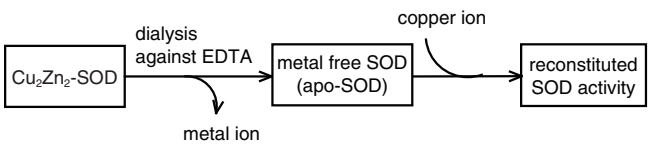

Figure 1. The principal mechanism of current assay for the determination of copper ion in aqueous solution.

Initially, apo-SOD was also prepared from column chromatography[18] as described in Materials and Methods. However, the apo-SOD prepared from column chromatography did not perform well. Therefore, the dialysis method was chosen for the preparation of apo-SOD in this study.

II. *Optimal Conditions for Reconstitution of Apo-SOD*

The apo-SOD could recover its SOD enzyme activity by adding copper ions at various pH (Figure 2). The maximal activity recovered was found at pH 5.9. It has been shown that at pH 5.9, copper binds efficiently to the appropriate SOD sites[17,21]. At pH below 5, apo-SOD retrieved low enzyme activity possibly due to poor interaction of copper with apo-SOD[17]. Time course of the reconstitution of apo-SOD was also investigated. The recovered SOD activity was found to be proportional to the reaction time (Figure 3). The restored SOD activity reached a plateau (about 72% original activity) after 10 min. When the reaction was extended to 50 min, SOD activity did not increase further. It was assumed that 28% of the original activity was not recovered due to slight denaturation during preparing apo-SOD.

III. *Calibration Curve and Sensitivity*

The SOD activity regenerated by adding various amounts of copper to apo-SOD was traced by the absorbance at 450 nm as shown in Figure 4. It indicated that the apo-SOD recovers the enzyme activity in accordance with the amount of copper added. Thus, the recovered SOD activity corresponds to the amount of copper added, and the concentration of copper is thus determined. A linear relationship (R^2 = 0.998) between the concentrations of copper, ranging from 20 to 80 ppb, and the activities of recovered

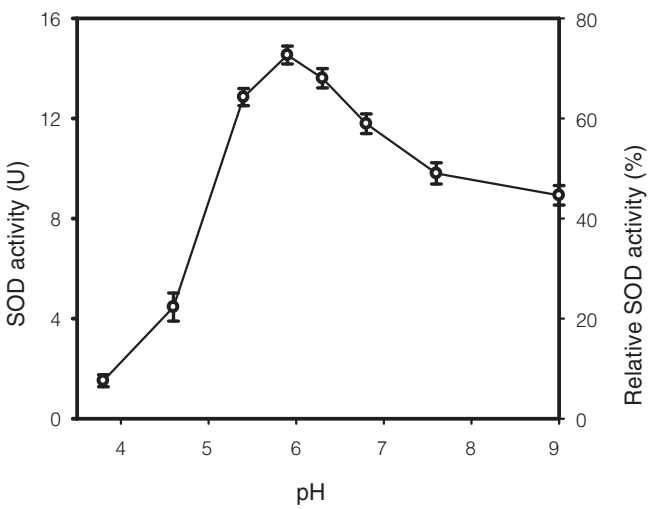

Figure 2. Reconstitution of apo-SOD at different pH. Ten microliter of apo-SOD (original SOD activity was 20 U as labeled by vendor) was mixed with 20 μL Cu ion (II) and 50 μL of buffers (30 mM) with different pH for 20 min, followed by determination of SOD activity.

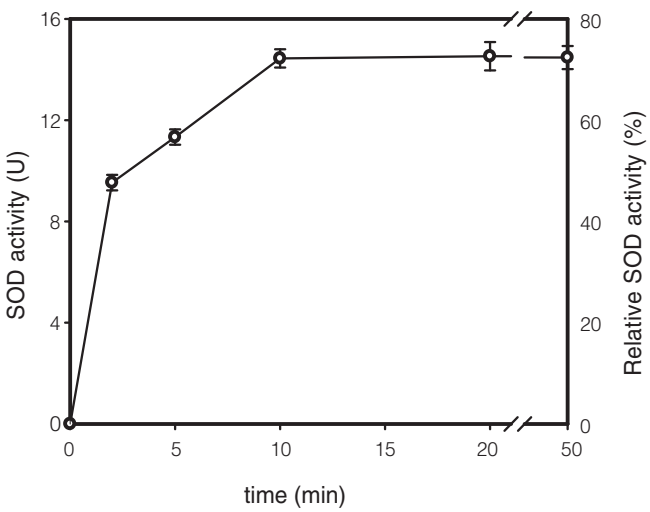

Figure 3. Time course of the reconstituted apo-SOD at pH 5.9. 10 microliter of apo-SOD (original SOD activity was 20 U) were added into the microplate wells, together with 50 μL of potassium phosphate buffer (30 mM, pH 5.9) and 20 μL of Cu ion (II) followed by determination of SOD activity.

SOD (absorance at 450 nm) was observed. In addition, the detection limit of the present assay for aqueous copper is 20 ppb (Figure 4).

IV. *Tolerance Limits of Some Common Metal and Sample Analysis*

When other metal ions were added alone without copper, the apo-SOD could not recover its enzyme activity in the assay. However, the recovery of apo-SOD by copper addition could be interfered by some common metals present in the test solution. Therefore, the interfering effects of these metal ions on apo-SOD activity recovery were examined and their tolerance limits were determined. In this experiment, the tolerance limit was set as the maximal concentration (μg/mL) of a certain metal ion causing no interference to the SOD activity recovery from apo-SOD on the basis of 10 μg of copper (10 μL of 1000 mg/L standard solution). Among the metals examined, sodium (Na^+) and potassium (K^+) showed very little influence (150 and 120 μg/mL, respectively). The tolerance limit range of divalent and trivalant metal ions were from 90 to 45 μg/mL in this assay (Table 1). However, interference from these metals could be reduced or even eliminated by diluting test samples to suitable concentrations.

Zinc involves in the active site of Cu_2Zn_2-SOD. However, the SOD activity in this assay was not recovered when only zinc was added to apo-SOD solution. Liu *et al.*[22] as well as Pelmenschikov and Siegbahn[23] demonstrated similar findings. That is, the current assay is very specific. Only copper could restore SOD activity. However, zinc might interfere the reconstitution of apo-SOD with copper when its concentration was more than 45 ppm (Table 1). Therefore, combination of this method with pre-concentration techniques[5,6] or proper serial dilution will increase the detection limit.

The assay was applied for the determination of the amount of copper in drinking water (mineral water), alcoholic beverages (beer and wine), and tomato juice (Table 2). The results of the assay were compared with the measurements by ICP-AES. It showed that data obtained from the two methods were not significantly different at $p > 0.05$ (Table 2).

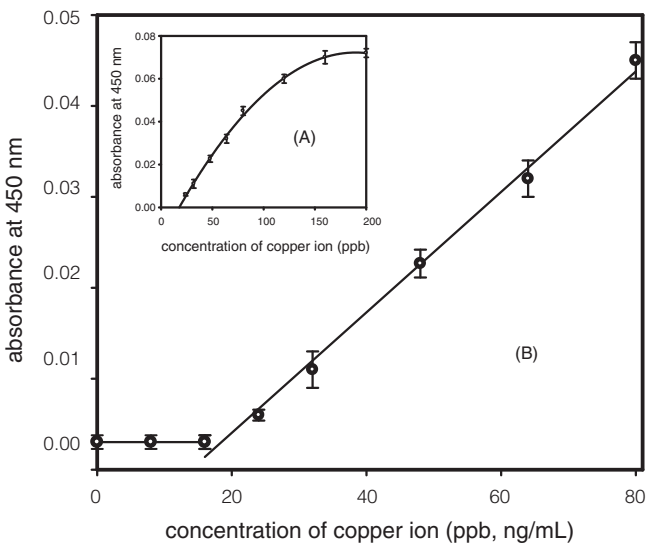

Figure 4. Calibration curve for Cu ion (II) determination by the enzymatic method. 10 microliter of apo-SOD (original SOD activity was 20 U) were added into the microplate wells, together with 50 μL of potassium phosphate buffer (30 mM, pH 5.9) and 20 μL of Cu (II) at concentrations ranges of 20 - 80 (A) and 20 - 200 ppb (ng/mL) (B) for 20 min, followed by the determination of SOD activity.

CONCLUSIONS

In present study, an enzymatic method was developed for the estimation of copper ions in aqueous samples. Advantages of this method include simplicity of instrumentation (a spectrophotometer), small volume of sample (20 - 10 μL),

Table 1. Tolerance limits[1] (μg/mL) of common metal ion solution (50 μL) in the current assay, on the basis of a 10 μL copper ion standard solution (1000 μg/mL)

ion	tolerance limit (μg/mL)	ion	tolerance limit (μg/mL)
Na (I)	150	Pb (II)	80
K (I)	120	Co (II)	50
Zn (II)	45	Cd (II)	50
Mn (II)	90	Fe (III)	50
Ni (II)	90	Cr (III)	45

[1] The metal ion solutions were prepared at concentrations ranging from 10 to 500 μg/mL. 50 μL of each metal ion solution was mixed with 10 μL of apo-SOD (original SOD activity was 20 U) and 10 μL copper ion (1000 μg/mL) for 20 min, and followed by SOD activity determination.

Table 2. Aqueous samples containing copper (II) assayed by the proposed method ($n = 3$) and ICP-AES ($n = 3$)

Method \ Sample[1]	Copper (II) concentration (ng/mL)				
	Mineral water	Beer	Tomato juice	Wine 1	Wine 2
Current assay[2]	26.7 ± 1.2	41.7 ± 0.7	65.4 ± 1.5	79.5 ± 2.5	107.1 ± 2.3
ICP-AES	24.9 ± 1.6	42.2 ± 0.8	63.2 ± 0.9	82.2 ± 1.7	110 ± 1.2

[1] The aqueous samples were filtered through a Millipore filtration membrane (0.2 μm) prior to analysis.
[2] 20 μL of aqueous samples mixed with 10 μL apo-SOD (original SOD activity was 20 U) and 50 μL potassium phosphate buffer (30 mM, pH 5.9) for 20 min, followed by assaying SOD activity. Concentration of Cu (II) was calculated from calibration curve.

short assay time (15 min), and applicability for large number of samples (about 96 samples per hour) on an ELISA microplate reader. Moreover, the cost of the assay is about ten-fold cheaper than that of ICP-AES. Five hundred samples could be measured in half a day. It can thus be developed a routine assay for the determination of copper ions in aqueous sample such as liquid foods and beverages.

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