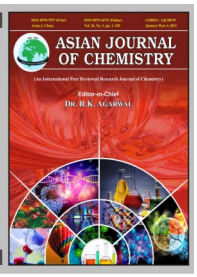
Asian Journal of Chemistry; Vol. 26, No. 1 (2014), 132-136

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.15349



Study of Allicin Extract Chelated with Some Heavy Metals (Cu^{2+}, Co^{2+} and Pb^{2+}) by Fluorescence Quenching Method and its Antioxidant Activity

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Received: 18 February 2013; *Accepted*: 9 July 2013; *Published online*: 26 December 2013; AJC-14488

Organosulfurs, mainly allicin, generally found in natural garlic clove are known responsible for its antioxidant property. Extraction with 20 % (v/v) ethanol/deionized water followed by clean-up with C_{18}-SPE of the allicin extract from fresh garlic clove was carried out and determined its antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 1,10-phenanthroline (Phen) methods. The complex formation between allicin and some heavy metal ions (Cu^{2+}, Co^{2+} and Pb^{2+}) was investigated by fluorescence quenching method. The experimental parameters were optimized including pH value, amount of the allicin extract, reaction time and concentration of nitric acid. The antioxidant activity of the allicin-metal complex was studied according to its labile chelating ability. It was found that their antioxidant activities of the allicin-metal complexes decreased (45 %) as compared with those of the allicin itself, considerably due to loss of free allicin in the extract, resulting in stability of the bound allicin complex under the optimum conditions. It is, therefore, implied that the organosulfurs play synergic functions of both as an antioxidant and a metal chelate.

Keywords: Organosulfurs, Allicin, Garlic clove, Antioxidant activity, Fluorescence quenching.

INTRODUCTION

Garlic (*Allium sativum* Linn.) is one of the oldest vegetables, which has long been used as a spice, food and medicine. In particular, it has been known to possess various biological functions, including antioxidant and antimicrobial activities[1]. The biological functions may be due to the presence of organosulfur compounds. Their antioxidant activity was widely interested to apply in pharmaceuticals, foods, cosmetics and agricultures[2-4]. Thus, scientists have been trying to validate their properties of the garlic, especially in terms of an identification of the active components, their mechanisms of action and explore the potential benefits as food supplements[5]. The nature of the volatile compounds in garlic has been proved to possess many beneficial activities for human health. The importance of thiosulfinates in the flavor of garlic distillates has been known[6]. Diallyl thiosulfinate (allicin) was discovered as the responsible of fresh garlic flavor[7,8]. In addition, the organosulfurs are identified for their unique pharmacological tasks associated with redox processes, metal binding and catalytic activities[9]. These are the basis of the antibiotic and anticancer activities. Medicinal properties of *Allium* species, such as garlic, onion, or shallot, including their role as natural cures for various human viral, bacterial and fungal infections have been mainly attributed to their content of the organosulfurs[10].

Furthermore, garlic is known to reduce metals toxicity in humans and animals. The removal of heavy metal ions in human body is an essential process. However, it has been proven that large amounts of many heavy metals, such as cobalt, copper, chromium, cadmium, mercury or lead seriously affect human health. The human body cannot process and dispose of the metal ions, therefore the metal ions would deposit in various internal organs. Heavy metals form compounds in the body that can be carcinogenic and mutagenic even at very low levels. It is, therefore, essential for people not expose excessive levels of heavy metals. Chelation of the heavy metal ions by natural ligands like allicin and/or organosulfurs is thus a choice of removal of the trace metals.

The aim of this study was to identify and characterize the allicin extract from fresh garlic clove by high-performance liquid chromatography (HPLC) and Fourier Transform infrared (FTIR) spectrometry, respectively. The optimum conditions for complex formation between the allicin extract and some heavy metal ions were investigated in details using spectrofluorophotometry. The antioxidant activities of the allicin extract and the allicin extract in the presence of the metal ions were comparatively determined by both 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and 1,10-phenanthroline (Phen) methods.

EXPERIMENTAL

The analytical reagent (AR) grade of chemicals and reagents were used. De-ionized water used for the preparation of all the solutions was purified by Milli-Q purification system (Millipore) (Massachusetts, USA). Butylated hydroxytoluene (BHT) was used as standard reference for antioxidant activity, which obtained from Sigma-Aldrich (USA). Ferric chloride ($FeCl_3$), ferrous sulfate heptahydrates ($FeSO_4.7H_2O$) was purchased from Carlo Erba (Italy). 1,1-Diphenyl-2-picry-hydrazyl radical (DPPH) was obtained from Fluka (Switzerland). Ethanol was purchased from Lab Scan (Ireland). Methanol was of HPLC grade also obtained from Lab Scan (Ireland).

A stock solution of Pb^{2+} (500 mgL^{-1}) was prepared by dissolving 0.1998 g of $Pb(NO_3)_2.5H_2O$ in 250 mL of 0.01 % HNO_3 solution. Also, 500 mgL^{-1} of stock solutions of Co^{2+} and Cu^{2+} were prepared by dissolving 0.6173 g of $Co(NO_3)_2{\cdot}2H_2O$ and 0.4911 g of $Cu(NO_3)_2$ in 250 mL, respectively. Working standard solutions of Co^{2+}, Cu^{2+} and Pb^{2+} were daily prepared by appropriate dilution of the stock solution with a 0.01 % HNO_3. A 0.10 M acetate buffer pH 3 was prepared by dissolving 3.402 g of $CH_3COONa{\cdot}3H_2O$ in de-ionized water and small amount of 99.8 % CH_3COOH, then adjusting to the volume of 250 mL with de-ionized water.

A spectrophotometric measurement was performed using visible spectrophotometer (Spectronic 15, Thermo Scientific, USA) and a spectrofluorophotometer (RF-5301PC, Shimadzu, Japan). A low pressure rotary evaporator (Model R-114, Buchi, Switzerland) was employed to eliminate the solvent. Centrifuge (EBA 20, Hettich, Germany), vertex mixer (Genie2, Scientific Industries, USA), centrifuge tube conical screw cap (Pyrex, Mexico), a pipetman P20-P1000 (Pipetman, France) micropipette and membrane filter, 0.45 μm, (Whatman International Ltd., UK) were used. A magnetic stirrer (SP 46920-26, Barusteal Thermolyne, Iowa, USA) and pH meter (Model 251, Denver Instrument, USA).

Solvent extraction of garlic: Fresh garlic clove was purchased from a local market in Khon Kaen, Thailand. The sample was stored cool (4 °C) in a plastic box before use. About 1.5 g of fresh garlic sample was extracted with 10 mL of 20 % (v/v) EtOH in de-ionized water under magnetic stirring for 1 h. The garlic extract was centrifuged at 3000 rpm for 20 min and the supernatant was filtered with Whatman No.1 filter paper and made up to 25 mL with the solvent used.

Solid-phase extraction of allicin: The garlic extract (5 mL) was added into a C-18 SPE cartridge which was conditioned with 5 mL methanol and 5 mL de-ionize water. Allicin was adsorbed onto the column while other water soluble compounds were removed by washing the cartridge with 10 mL de-ionized water. The main fraction of allicin extract was eluted with 5 mL methanol. The eluate solution was filtered through a 0.45 μm nylon filter membrane prior to preliminary identification and characterization by HPLC and FTIR, respectively, since none of allicin standard was used. The antioxidant acti-vity of the allicin extract was evaluated. Then, the allicin extract was subjected to form complex with some heavy metal ions.

Study of complex formation of allicin extract with Cu^{2+}, Co^{2+} and Pb^{2+}

Effect of the mixing ratio of the metal ion with allicin extract: The effect of sample amount on the complex formation between allicin and metal ion was determined by varying volumes of the allicin extract from 0.125 mL to 10 mL. Under suitable conditions, the complex formation was monitored by adding 5 mL of 0.5 mg/L metal ions into the allicin extract soluble in acetate buffer pH 3. The final volume was adjusted to be 15 mL with the buffer solution. The reaction mixture was kept at room temperature for 0.5 h. The fluorescence spectra of allicin extract and the metal-allicin complex were recorded.

Effect of pH on the metal-allicin complex: Effect of pH on the fluorescence spectra of metal-allicin complex was investigated. Five mL of metal ions solution (5 mg/L) was added into 2 mL of the allicin extract soluble in acetate buffer solutions (pH 2-6). The other conditions were the same as stated earlier. The fluorescence spectra of allicin extract and the metal-allicin complex were comparatively determined.

Effect of reaction time on the metal-allicin complex: The complex formation of Cu-allicin was monitored at 15, 30, 45, 60, 90 and 120 min. The optimum conditions were consisted of 5 mL of 0.5 mg/L Cu^{2+} solution and 2 mL of the allicin extract soluble in acetate buffer pH 3. The reaction mixture was kept at room temperature for 15-120 min prior to recording the fluorescence spectra of both allicin extract as a blank and the metal-allicin complex.

Effect of concentration of HNO_3 on the metal-allicin complex: The complex formation between allicin and metal ion in different acid media was determined. The complex solution was prepared by adding 5 mL of 0.5 mg/L metal ion solution of 0.01, 0.10, 1.0 and 2.0 % HNO_3 into 2 mL of the allicin extract soluble in acetate buffer pH 3. The volume was adjusted to be 15 mL with buffer solutions. The reaction mixture was kept at room temperature for 30 min. The fluorescence spectra of the allicin extract and the metal-allicin complex was determined. The fluorescence spectra for the mixed solutions of Cu-, Co- and Pb-allicin complexes were measured at 385, 380 and 400 nm, respectively.

Evaluation of antioxidant activity by DPPH radical scavenging assay: Stock standard solution of 1000 mg/L of BHT was prepared by dissolving 0.02 g BHT with methanol and then adjusted volume to 20 mL. The BHT standard was applied for the calibration curve ranging from 2-50 mg/L. Each concentration was prepared in triplicates. A DPPH solution was prepared from 1,000 μM of DPPH, which dissolved 0.0086 g of DPPH with methanol then adjusted volume to 100 mL. The DPPH solution was diluted to 50, 100, 200, 250, 300, 400 and 500 μM by methanol and was used for DPPH antioxidant assay. The DPPH solution giving the absorbance in the range of 0.192-0.761 ABFS was selected.

The free radical scavenging activity was determined by using DPPH assay. Briefly, 2 mL of each extract was mixed with 3 mL of 300 μM DPPH solution. After that the solution was kept in the dark at ambient temperature for about 45 min and the absorbance of the mixture was read at 517 nm. The control solution was prepared from 3 mL of the DPPH solution, 2 mL of methanol and the absorbance was measured at the

same wavelength. The percentage of an inhibition was calculated according to following equation:

$$\% \text{ inhibition} = \frac{[Abs_{(t=0)} - Abs_{(t=45)}]}{Abs_{(t=0)}} \times 100$$

where $Abs_{(t=0)}$ is the absorbance of the control reaction (containing all reagents except the test extract) at 0 min. $Abs_{(t=45)}$ is the absorbance of the test extract at 45 min. The DPPH scavenging percentage of each sample was compared with the BHT calibration curve, which plotted the inhibition percentage against all BHT concentration. The antioxidant activity was expressed as µg BHT/g dry weight. Data are reported as means ± standard deviation (SD) for three replicates.

Evaluation of antioxidant activity by 1,10-phenanthroline assay: Stock standard solution of Fe^{2+} 2000 µmol/L was prepared by dissolving 0.0556 g of ferrous sulfate heptahydrates ($FeSO_4.7H_2O$) with methanol and then adjusted final volume to 5 mL. The Fe^{2+} solution was used for a calibration curve ranging from 0.1-1.0 µmol/L. 1,10-Phenanthroline solution (0.5 %, w/v) was prepared by dissolving 0.5 g of 1,10-phenanthroline with methanol and then adjusted final volume to 100 mL.

The ferric-ion-based total antioxidant activity assay was used for determination of antioxidant capacity of the phenolic extracts from the garlic extract. The antioxidant activities were determined by coloration of a methanolic solution of 1,10-phenanthroline with slightly modified method. Briefly, 2 mL of solution of the allicin extracts, 1 mL of 0.2 % $FeCl_3$ and 1 mL of 0.5 % 1,10-phenanthroline and made up to volume with solvent. The obtained solution was mixed and left at room temperature in a dark. After 20 min, the absorbance of an orange-red solution was measured at 510 nm. The calibration curve of Fe^{2+} complex was prepared in the range of 0.1-1.0 µmol/L. The absorbance that affected by the garlic products was compared with Fe^{2+} calibration curve. The antioxidant activity was expressed as µmol Fe^{2+}/g DW. Data are reported as means ± standard deviation (SD) for three replicates.

Effect of Cu^{2+}, Co^{2+} and Pb^{2+} on the antioxidant activity of the allicin extract: The effect of some heavy metal ions on the antioxidant activity of the organosulfurs in the garlic extract (as an allicin extract) was studied by using a visible spectrophotometer. 3 mL of 300 µM DPPH solution was used for the sample extract. Briefly, 1 mL of the garlic extract was mixed with a certain concentration of metal ions (5 mL). Blank and control solutions were also carried out. The absorbance of the reaction solution was recorded at 517 nm. The control solution was prepared from 2 mL of DPPH and 2 mL acetate buffer pH 2 and measured the absorbance at the same wavelength. In the same way, the antioxidant activity of the metal-allicin complex was also determined by Phen assay.

RESULTS AND DISCUSSION

The preliminary identification of allicin in the garlic extract was carried out by using HPLC. Characterization of the main component of allicin eluted from C-18 SPE was done by FTIR. The competitive complexation of allicin extract with some metal ions was monitored using spectrofluorophotometry. The effect of some heavy metal ions on the antioxidant activity of the allicin extract was investigated.

Complex formation of allicin extract with Cu^{2+}, Co^{2+} and Pb^{2+}: The competitive complexation of allicin extract with some metal ions was measured using a spectrofluorophotometer. In order to investigate the optimum conditions for metal-allicin complex, the allicin extracted from the fresh garlic clove was chosen. The study was carried out under various pH at 25 °C. The parameters affecting the metal-allicin complex were investigated in details including metal ion/sample ratio, pH value, reaction time and concentration of HNO_3.

Effect of the mixed ratio of metal ion/allicin extract: Various ratios of metal ion and allicin were experimentally tried. Practically, a volume (mL) of the allicin extract was varied with fixed concentration of the metal ion in the ratios of 1:50, 1:100, 1:200, 1:400, 1:800, 1:1000, 1:2000, 1:3000 and 1:4000. For example, the fluorescence spectra of the Cu-allicin complex solutions were shown in Fig. 1, indicating the maximum emission wavelength at 334 nm after excited at 238 nm. The fluorescence intensity of the Cu-allicin complex increased with the ratios between 1:50 and 1:800 and decreased at 1:1000 down to 1:4000 as shown in Fig. 2. Differences in the relative fluorescence intensity at which the ratio of 1:800 would attribute to the Cu-allicin complex formation. The results of both Co^{2+} and Pb^{2+} which used for the metal-allicin complexes were also found in similar manner (data not shown). From the results, the metal ion and allicin ratios of 1:800, 1:400 and 1:400 for Cu^{2+}, Co^{2+} and Pb^{2+} were chosen to monitor their complex formation.

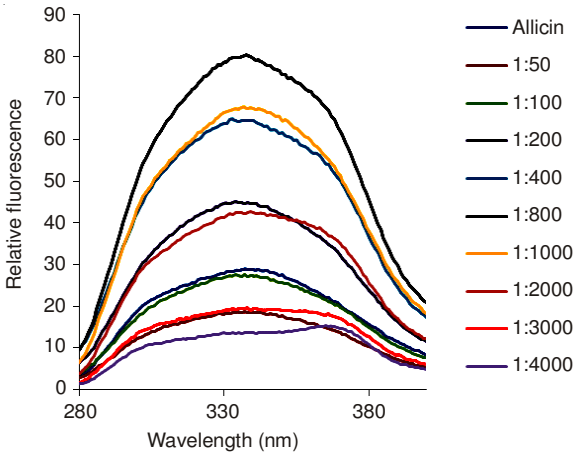

Fig. 1. Relative fluorescence spectra of various ratios of Cu-allicin complex; Cu^{2+} 0.5 mg/L in 0.01 % nitric acid, acetate buffer solution pH 2 and 30 min reaction time; Ex/Em = 238/334 nm

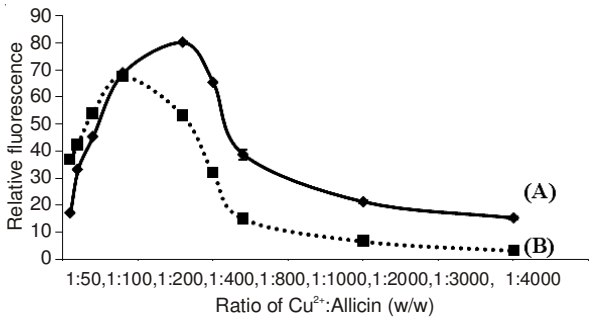

Fig. 2. Relative fluorescence intensity (Ex/Em: 238/334 nm) of Cu-allicin complex: (A) allicin extract alone, (B) Cu^{2+} 0.5 mg/L in 0.01 % nitric acid plus allicin extract

Effect of reaction time on the metal ion-allicin complex: The effect of reaction/incubation time was also studied as shown in Fig. 3. The relative fluorescence intensity of the metal-allicin complex was the highest at 15 min incubation time and it decreased at longer time, probably due to the stability of the metal-allicin complex occurred. The other metal ions (Co^{2+} and Pb^{2+}) for their allicin complexes were investigated in the same manner, indicating that 0.5 h reaction time was chosen.

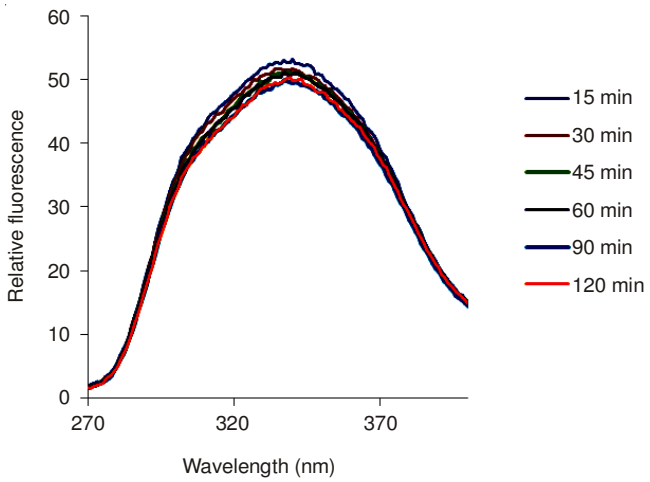

Fig. 3. Relative fluorescence spectra of the Cu-allicin complex after incubating between 15-120 min: Cu^{2+} 0.5 mg/L in 0.01 % nitric acid and allicin extract, acetate buffer pH 2; Ex/Em = 238/334 nm

Effect of pH on the metal-allicin complex: The effect of pH on the metal-allicin complex was investigated. The most important variable parameter for the complex formation is the pH of the solution. It was found that the metal-allicin complex is strongly dependent on pH value of the reaction mixture. As shown in Fig. 4, at pH 2, the relative fluorescence intensity of metal ions binding with the ligand was higher than other pH values, when the pH value increased, which is absolutely because of the competitive binding with the hydronium ion. The relative fluorescence intensity decreased again at pH > 2 because of the above reasons. The other metal ions (Co^{2+} and Pb^{2+}) for the metal-allicin complexes were investigated in the same manner. From the results, pH 2 of metal-allicin complexes of Cu^{2+}, Co^{2+} and Pb^{2+} was chosen for further study.

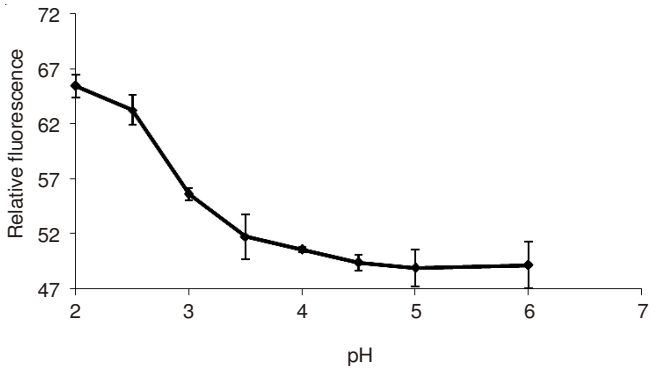

Fig. 4. Relative fluorescence intensity of the Cu-allicin complex at pH 2-6; Cu^{2+} 0.5 mg/L in 0.01 % (v/v) nitric acid, 30 min reaction time; Ex/Em = 238/334 nm

Effect of concentration of HNO_3 on the metal-allicin complex: The effect of concentration of HNO_3 on metal-allicin complex was also investigated. In this study, the complex formation between metal ion and allicin extract is strongly dependent on the concentration of HNO_3. Fig. 5 showed that the relative fluorescence intensity of the reaction mixture in 0.01 % HNO_3 decreased when increasing of the concentration of HNO_3. The other metal ions (Co^{2+} and Pb^{2+}) for their metal-allicin complexes were investigated in the same manner, indicating that 0.01 % HNO_3 was used for the metal-allicin complex formation of Cu^{2+}, Co^{2+} and Pb^{2+}.

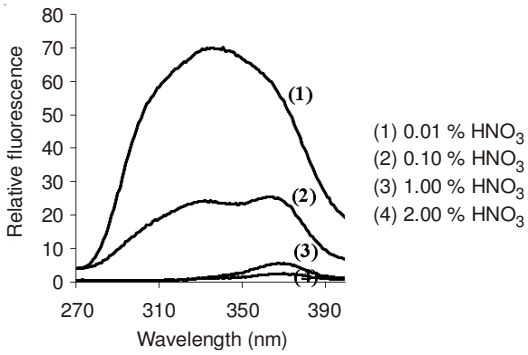

Fig. 5. Relative fluorescence spectra of the Cu-allicin complex in 0.01-2 %(v/v) HNO_3, Ex/Em = 238/334 nm

Effect of Cu^{2+}, Co^{2+} and Pb^{2+} on the antioxidant activity of the allicin extract: The effect of some heavy metals (Cu^{2+}, Co^{2+} and Pb^{2+}) on their antioxidant activity was investigated based on the calibration curves from both DPPH assay (Fig. 6) and 1,10-phenanthroline assay (Fig. 7).

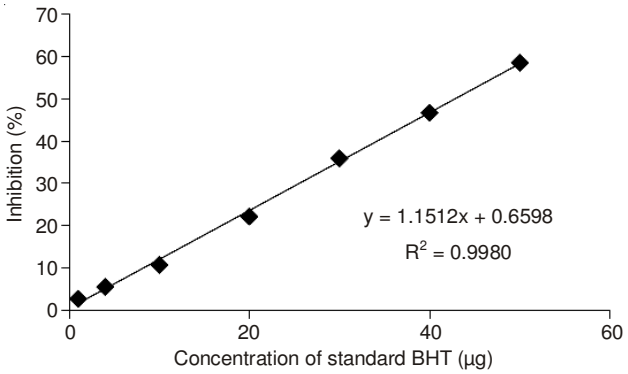

Fig. 6. Calibration curve for an inhibition (%) of standard BHT

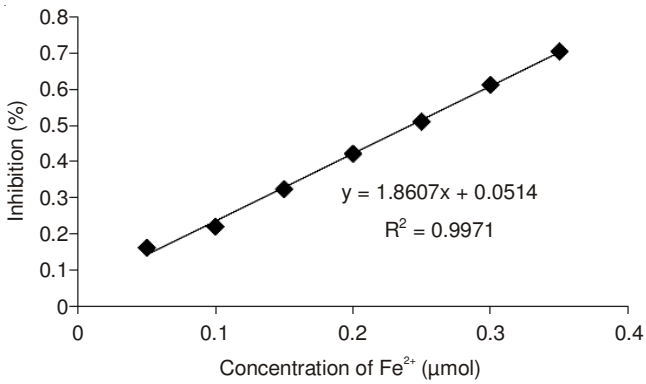

Fig. 7. Calibration curve of standard solution of Fe^{2+}

From the results (Table-1 and Fig. 8), it was found that the antioxidant activity of the allicin extract (about 550 µg BHT) decreased in the presence of heavy metal ions (about 400 µg BHT). This is probably due to the complex formation between allicin, the main component in the garlic extract and the metal ions under optimum conditions (about 300 µg BHT). The trend of an antioxidant activity of the complex using Phen assay was similar to that of the complex determined by DPPH radical scavenging assay. In addition, it was noted that the antioxidant activities of these metal ions determined by both methods were not significantly different under their optimum conditions of the metal ion-allicin extract used. Thus, the antioxidant activity of the Cu-allicin complex was found in the same trend with other metal-allicin complexes.

TABLE-1
ANTIOXIDANT ACTIVITY OF THE METAL-ALLICIN EXTRACT COMPLEX

Complex	DPPH assay (µg BHT)	Phen assay (µmol Fe^{2+})
Cu^{2+}-allicin	298.97 ± 0.01	2.09 ± 0.03
Co^{2+}-allicin	298.97 ± 0.02	1.87 ± 0.02
Pb^{2+}-allicin	300.87 ± 0.02	1.81 ± 0.03

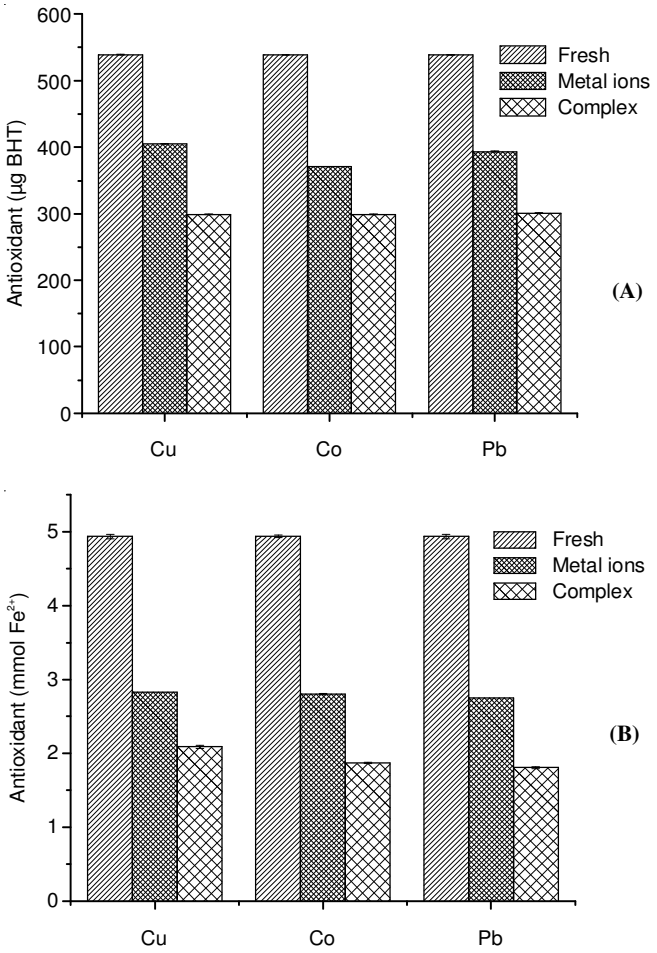

Fig. 8. Antioxidant activities of the metal-allicin extract comparing with those of allicin extracted from fresh garlic clove or metal ions themselves by (A) DPPH assay and (B) Phen assay

The antioxidant activities of the metal ions exerted in some extents since these metals are essential elements and important building components in biological systems in humans[11]. The metal-allicin complex gave low antioxidant activity because the metal ions can form complex with allicin, so that their free radical disappeared, the antioxidant activity decreased.

Conclusion

The competitive complexation of the allicin extract with some metal ions was monitored using spectrofluorophotometry. The mixed ratios of metal ion-allicin extract of 1:800, 1:400 and 1:400 were chosen for Cu^{2+}, Co^{2+} and Pb^{2+} complex formation, respectively. The reaction/incubation time for the complex formation in 0.01 % HNO_3, acetate buffer pH 2 was about 0.5 h. The fluorescence spectra of the Cu-, Co- and Pb-allicin complexes exhibited a maximum wavelength at 334, 335 and 313 nm, respectively. In addition, it was found that the antioxidant activity of the garlic extract decreased in the present of heavy metal ions, probably due to the complex formation between allicin and/or other organosulfurs and the metal ions under the optimum conditions. The antioxidant activity of the metal ion itself showed somewhat extents, since some metals are essential elements and important building components in biological systems. However, in this study, the metal-allicin complex gave lower antioxidant activity when compared with those of either allicin extract or metal ions, since these transition metal ions could be chelated by the organosulfurs, resulting in loss of its antioxidant activity.

ACKNOWLEDGEMENTS

Research financial support from the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand and the Hitachi Scholarship Foundation, Tokyo, Japan is gratefully acknowledged.

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