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Development of an Electrochemical Bioassay Based on the Alkaline Phosphatase Activity of *Chlamydomonas reinhardtii* to Assess the Toxicity of Heavy Metals

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A novel electrochemical bioassay for the rapid and sensitive evaluation of heavy metals toxicity was developed using the alkaline phosphatase activity of *Chlamydomonas reinhardtii* (*C. reinhardtii*). The inhibition of alkaline phosphatase activity caused by heavy metals was measured by quantifying the oxidation of *p*-aminophenol (PAP), which was produced from the enzymatic reaction of a *p*-aminophenyl phosphate (PAPP) substrate. The quantification of PAP oxidation was performed via cyclic voltammetry in a micro-droplet on a screen-printed carbon electrode (SPCE). The heavy metals concentrations that produced a 50% inhibition of enzymatic activity (EC_{50}) for Cu, Pb, Cd, Zn, and Hg were 8.18, 0.10, 0.27, 1.99, and 0.07 μmol L^{-1}, respectively. The present method was compared with a micro-scaled algal growth inhibition test (μ-AGI) whereby the obtained values of EC_{50} were 36.2, 7.7, 8.5, 29.1, and 2.6 μmol L^{-1} for Cu, Pb, Cd, Zn, and Hg, respectively. By comparison with a conventional AGI test, the proposed method was much more sensitive and faster.

Keywords: electrochemical bioassay; *Chlamydomonas reinhardtii*; alkaline phosphatase activity; *p*-aminophenol; heavy metals; cyclic voltammetry

1. INTRODUCTION

Heavy metals are major environment pollutants due to the extreme toxicity, non-biodegradability and bioaccumulation properties that they exert in plants, animals and humans. Many of these metals are carcinogens that have been closely linked to several diseases such as Alzheimer's, Parkinson's, multiple sclerosis, osteoporosis, developmental disorders, and organ failures [1,2].

Therefore, the detection of heavy metals is very important, particularly in the sources of environmental water.

A wide variety of chemicals already exist in environmental water, and the mixture of heavy metals into these chemicals may exhibit complex toxicities. Bioassays are one of the most useful methods used to determine environmental and industrial wastewater toxicity. Many bioassays have been developed using algae [3-10], luminescence bacteria [11,12], plant tissues [13] and animal cells [14], especially algal bioassay which is one of the popular toxicity tests. Bioassays are characterized by high sensitivity, reproducibility and year-round growth [15]. Furthermore, algae are a primary producer of the ecosystem, and toxic impact measurements on algae help us to understand the impact on higher organisms by showing high sensitivity towards toxicants and by revealing any small changes in water quality [16,17]. Algal growth inhibition (AGI) toxicity tests standardized by OECD are frequently used to determine the presence of toxic chemicals in the environment [18]. On the other hand, bacterial growth inhibition tests could not be used in experiments with pure water because they compete when the nutrient medium is depleted. In such situations green algae could be used successfully for the toxic determination of heavy metals, pesticides and industrial wastes [19,20]. The disadvantages of AGI testing is the time that is required for algal growth monitoring, as well as the requirement of a large culture apparatus [21]. Therefore, the development of a more rapid and sensitive technique to determine toxicity is required.

Since the enzymatic activity is inhibited by the presence of heavy metals, the toxicity of these chemicals can be assessed by measuring the enzymatic activity [22-28]. Over the past three decades, several techniques using the various enzymes have been proposed: oxydases and dehydrogenases [22], and alkaline phosphatase [23-28]. In addition, some studies have been reported about utilization of the enzymatic activity inhibition of algae by heavy metals [29-35]. In these researches, they are using several enzymes in algae, such as alkaline phosphatase [29-31,35], acetylcholinesterase [31], and esterase [32-34]. The enzyme inhibition in microalgae is becoming a well-accepted indicator of environmental stress because of the rapid and sensitive endpoint [34]. Enzyme inhibition has been measured using both optical [23,24,29,32-35] and electrochemical methods [22,25,28,30,31]. In general, conventional optical detection is complicated, time-consuming, requires a large amount of equipment, and cannot be used to detect color samples.

The aim of this study was to develop a new electrochemical bioassay for the evaluation of heavy metals toxicity using alkaline phosphatase induced by *C. reinhardtii.* A schematic diagram of the proposed assay procedure and the enzymatic/electrochemical reaction are illustrated in Figure 1. *p*-Aminophenyl phosphate (PAPP) was used as a substrate for the voltammetric determination of the alkaline phosphatase activity. The induced alkaline phosphatase converts the PAPP into *p*-aminophenol (PAP), which can then be oxidized to *p*-quinone imine (PQI) via reversible electrochemical reaction [36]. A sensitive electrochemical determination for the enzymatic activity of *C. reinhardtii* can be performed from the oxidation current of PAP by means of cyclic voltammetry in 50 μL droplets on a screen-printed carbon electrode (SPCE). This electrochemical measurement offers some advantages compared with conventional optical detection: the elimination of bulky equipment, disposability, on-site operation, and an ability to detect samples containing colored components or suspended solids. Another major advantage of this electrochemical technique is the lessened dilution

of the product of the enzymatic reaction, which results in rapid detection and high sensitivity. The toxicity test proposed in this study was carried out with Cu, Pb, Cd, Zn, and Hg. The results obtained from the proposed method were compared with a micro-scaled AGI test based on UV/vis spectrophotometric detection and the results from other published articles.

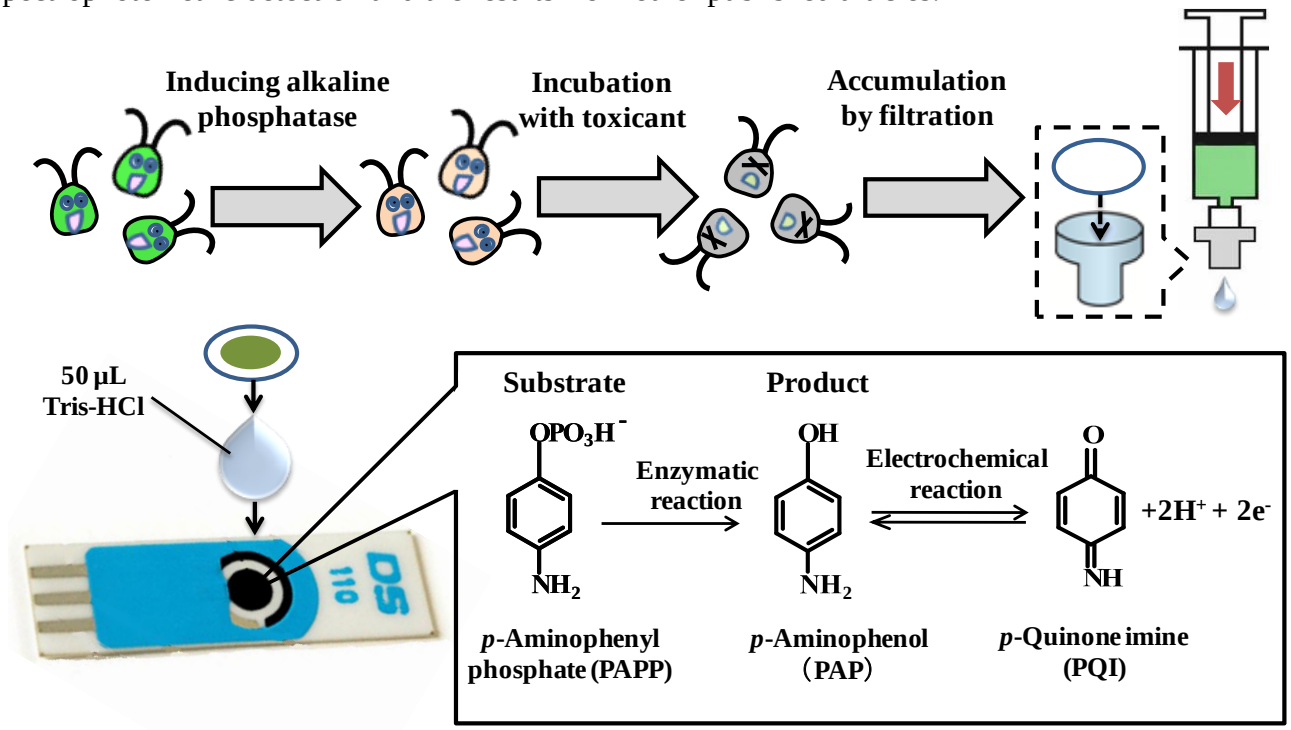

Figure 1. Schematic diagram of the proposed electrochemical bioassay based on the alkaline phosphatase activity of *C. reinhardtii*.

2. MATERIALS AND METHODS

2.1. Apparatus and Reagents

The cyclic voltammetry (CV) was performed with an Epsilon electrochemical workstation (Bioanalytical, systems, USA). The SPCE used was a solvent-resistant three-electrode system printed onto a PVC pad with a size of 3.4×1.0×0.05 cm (DRP-110, DropSens). The working (diameter is 4 mm) and counter electrodes were carbon and the reference electrode was Ag/AgCl. The boxed connector (DSC, DropSens) operated as an interface between the SPCE and the potentiostat.

The standard solutions of Cu, Pb, Cd, Zn, and Hg were prepared daily by the appropriate dilution of standard stock solutions (1,000 mg L^{-1}). Tris-HCl buffer solution (pH 8.4) was prepared by mixing 0.1 M Tris, 0.1 M hydrochloric acid, and 0.01 mM $MgCl_2$. The substrate was 4-Aminophenyl phosphate monosodium salt (PAPP), and the substrate solution was prepared daily. All the solutions were prepared with Milli-Q water (resistivity ≥18.2 Ω cm^{-1}). For Sagar and Granick medium (SG1), all of the reagents were analytical grade and were manufactured by Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Test algae

In this study, a wild-type strain of *C. reinhardtii* (C-239: UTEX-90, mt+) was used. The stock cultures of *C. reinhardtii* were stored at room temperature on agar. Subsamples were transferred into flasks containing 100 mL of standard Sagar and Granick 1 (SG1) medium. *C. reinhardtii* was incubated for 72 hours in SG1 medium and then transferred into 100 mL of m-SG1 medium (without K_2HPO_4 and KH_2PO_4) at an initial cell density of 1×10^4 cells mL^{-1} and further incubated for another 72 hours to induce alkaline phosphatase. Previous studies on batch cultures indicated that the alkaline phosphatase activity of *Chlorella vulgaris* is usually negatively correlated with the phosphate level [35]. The cell suspension was incubated with aeration and maintained at a temperature of 24 ± 2 °C under fluorescent lamps (light intensities: 64 μmol m^{-2} s^{-1}) (FL20SS-D/18, Mitsubishi, Tokyo, Japan).

2.3. Procedure of electrochemical measurement

Two mL of *C. reinhardtii* suspension was filtered using glass microfiber filters GF/F (Whatman™) that were 5 mm in diameter with a pore size of 0.7 μm. The filter was then placed on the SPCE surface and 50 μL of 1 mM PAPP solution was added. After incubation for 5 min at room temperature, the cyclic voltammogram was recorded without deaeration at a potential range between -300 mV and 300 with a 20 mV s^{-1} scan rate.

2.4. Procedure for the enzyme inhibition test

For the determination of enzyme inhibition from heavy metals, *C. reinhardtii*-induced alkaline phosphatase was exposed with metal solutions at concentrations ranging from 0.001 to 10 ppm. After 5 hours incubation without aeration, the enzyme inhibition percentages were calculated from the peak current of PAP oxidation in the cyclic voltammograms and measured using the same procedure described above.

2.5. Procedure for the μ-AGI test

The μ-AGI tests were performed according to our previous report [37]. The initial cell density of *C. reinhardtii* established for the test was 1×10^4 cells mL^{-1}. The number of algae cells was counted using a hemocytometer (Fuchs-Rosenthal, Sunlead Glass Co., Tokyo, Japan). We prepared a 2-fold dilution series of heavy metals from 100 mg L^{-1} in the wells of a 96-well round-bottom polystyrene micro-plate (Falcon, Franklin Lakes, NJ, USA). In each of the wells, 100 μL of the metal solution, 50 μL of five-fold concentrated SG1 medium, 50 μL of sterilized Milli-Q water and 50 μL of 5×10^4 cells mL^{-1} *C. reinhardtii* suspension were mixed. The absorbance for *C. reinhardtii* was measured using a spectrophotometric micro-plate reader (Model 550, Bio-Rad, Hercules, CA, USA) at 655 nm ($Abs._{655}$) at 0 hour and after incubation at 24 ± 2 °C under fluorescent lamps for 96 hours. The relative growth amounts (RGA) were obtained using the following formula.

$$\text{RGA}(\%) = \frac{\{\text{Abs.}_{655}(96\text{hr.}) - \text{Abs.}_{655}(0\text{hr.})\}}{\{\text{Control Abs.}_{655}(96\text{hr.}) - \text{Control Abs.}_{655}(0\text{hr.})\}} \times 100$$

The values of the half-maximal effective concentration (EC_{50}) were calculated using the program EcoTox-Statics Version 2.6 d [38] that is distributed by the Japan Society for Environmental Toxicology.

3. RESULTS AND DISCUSSION

3.1. Electrochemical behavior of PAP and PAPP in a micro-droplet on a SPCE

In this assay, the presence of PAPP should not influence the electrochemical reaction of PAP, because PAPP molecules as an enzymatic substrate exist at a much higher concentration compared with PAP as a product of enzymatic reaction. The electrochemical behaviors of PAPP and PAP in 50 µL of a 0.1 M Tris-HCl buffer solution were investigated. The cyclic voltammograms of 1 mM PAPP and PAP on the SPCE are shown in Figure 2. Almost no current response was observed from the PAPP solution. On the other hand, the cyclic voltammogram of PAP had well-defined peaks for the oxidation of PAP to PQI and the reduction of PQI to PAP at potential of 80 mV and -5 mV, respectively. The difference between oxidation and reduction peak (ΔE_p) was 85 mV. Previous report also obtained the difference between the reduction and oxidation peaks (ΔE_p) of 89 mV with graphene-SPCE and considered as good reversibility [39]. Therefore, it seems that the electrochemical response of PAP which is produced by the enzymatic reaction could be measured in a solution that includes PAPP.

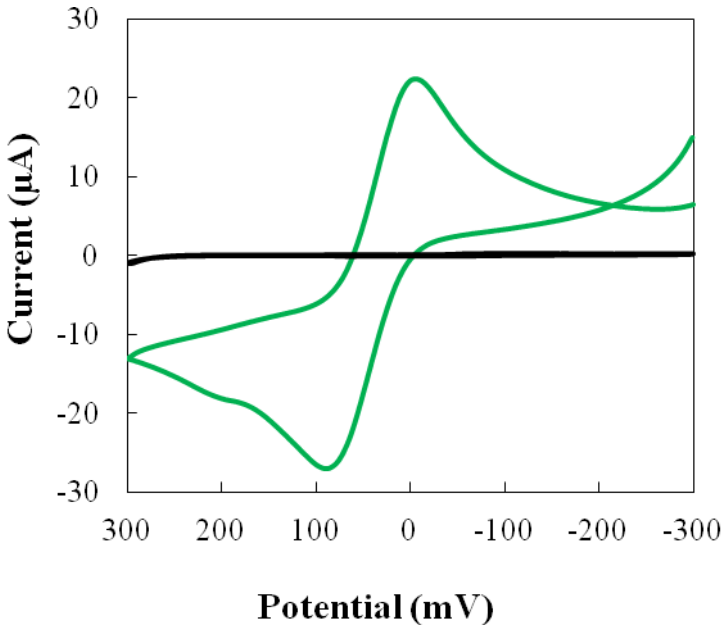

Figure 2. The cyclic voltammograms of 1 mM PAPP (black line) and PAP (green line) measured in 50 µL of Tris-HCl (pH 8.4) on a SPCE at a scan rate of 20 mV s^{-1}.

3.2. Alkaline phosphatase activity of C. reinhardtii

The alkaline phosphatase activities of *C. reinhardtii* after incubation in both SG1 and m-SG1 medium for 72 hours was compared via cyclic voltammetry, and the results are shown in Figure 3. The measurements were carried out using a filter that had accumulated 2 mL of the *C. reinhardtii* suspension. Prior to measurements, the filter was incubated in 50 µL of a Tris-HCl (pH 8.4) droplet including 1 mM PAPP on the SPCE for 5 min. The *C. reinhardtii* growing in the m-SG1 medium showed significant response according to the electrochemical reaction of PAP as the product of an enzymatic reaction. This result clearly indicates that alkaline phosphatase was induced successfully by cultivation into the m-SG1 medium. In this study, phosphatase starvation method has been used. Some other researchers also induced the phosphatase activity of algae by cultivating in low levels of nutrient concentrations [40,41]. Kruskopf and Plessis obtained about 4 time higher alkaline phosphatase activity in two chlorophyceaes after treatment with limited concentrations of phosphate level [40].

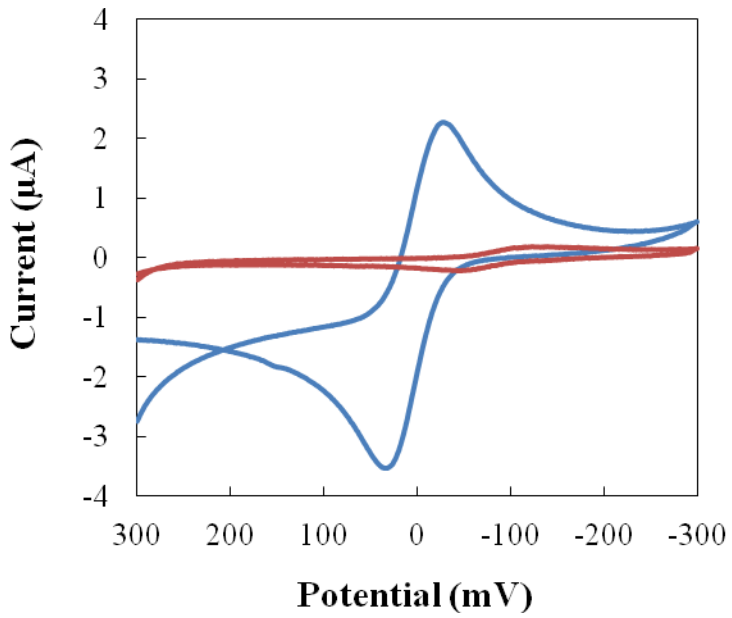

Figure 3. The cyclic voltammograms of *C. reinhardtii* incubated in SG1 (red line) and m-SG1 (blue line) medium. The measurements were performed in 50 µL of Tris-HCl (pH 8.4) including 1 mM PAPP on a SPCE at a scan rate of 20 mV s^{-1} after incubation for 5 min.

The alkaline phosphatase activity for a single cell of *C. reinhardtii* was calculated using the following formula:

$$(i_p^a)=0.4463nF[\mathrm{PAP}]D^{\frac{1}{2}}\left(\frac{nFv}{RT}\right)^{\frac{1}{2}}$$

Where *n* is the number of electrons, *D* is the diffusion coefficient of PAP in solution, and *v* is the scan rate. As shown in Figure 3, the oxidation peak current was 3.72 µA. Using this value, the molar concentration of PAP that was produced by *C. reinhardtii* after 5 min incubation was calculated to be

0.143 mM. Therefore, the alkaline phosphatase activity of *C. reinhardtii* was 7.15×10^{-10} Unit/cell because 2 mL of 1×10^{6} cells mL^{-1} *C. reinhardtii* solution was filtered and used for this measurement.

3.3. Optimization of incubation time for the enzymatic reaction

The incubation time in the substrate solution was optimized as follows. First, two mL of the *C. reinhardtii* solution was filtered and then the filter was placed on the SPCE for a reaction with 50 µL of 1 mM PAPP solution at times ranging from 0 to 15 min. The results obtained from this investigation are shown in Figure 4. As time increased, the current value also increased for up to 10 min of reaction time, after which it became a constant value. The optimum time was more than 10 min. However, as time increased, the solution started to evaporate since the volume of the reaction solution was only 50 µL. Therefore, we investigated the impact of evaporation and found that at 10 min of reaction time about 12% of the solution had evaporated, which could have influenced the electrochemical measurement (data is not shown). For further experimental purposes, an optimal reaction time of five min was selected for the enzymatic reaction, because the evaporation rate was only 5.8%. This incubation time is much shorter than the similar algal assays based on the enzyme activity inhibition using spectroscopic detection which was optimized 90 min as incubation time with a substrate [35]. This is due to the high sensitivity of this electrochemical detection strategy using a micro-droplet and algae accumulated through filtration.

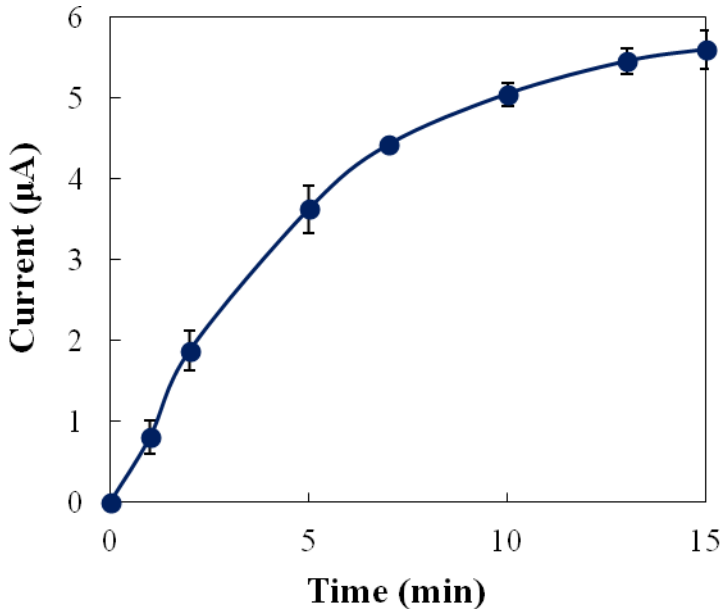

Figure 4. Optimization of the incubation time to determine the enzyme activity. The current values were obtained from the cyclic voltammograms measured in 50 µL of Tris-HCl (pH 8.4) including 1 mM PAPP on the SPCE at a scan rate of 20 mV s^{-1}.

3.4. Optimization of the C. reinhardtii solution volume for accumulation by filtration

The volume of *C. reinhardtii* solution for the filtration procedure was investigated. The *C. reinhardtii* suspension after inducing alkaline phosphatase was filtered at volumes ranging from 0 to

2.5 mL, and their current response was measured to obtain the optimum algal solution volume. The results in Figure 5 show that by increasing the filtration volume, the current value was increased to 2 mL until reaching a constant value based on the alkaline phosphatase activity. This could have been caused by the 2.5 mL algal solution containing too much *C. reinhardtii* on the surface of the filter, which formed a thick layer of algae and prevented the transfer of the substrate reaction with the enzyme on the surface of the filter. Taking this result and the filtration time into consideration, the volume of *C. reinhardtii* solution for filtration was set at 2 mL.

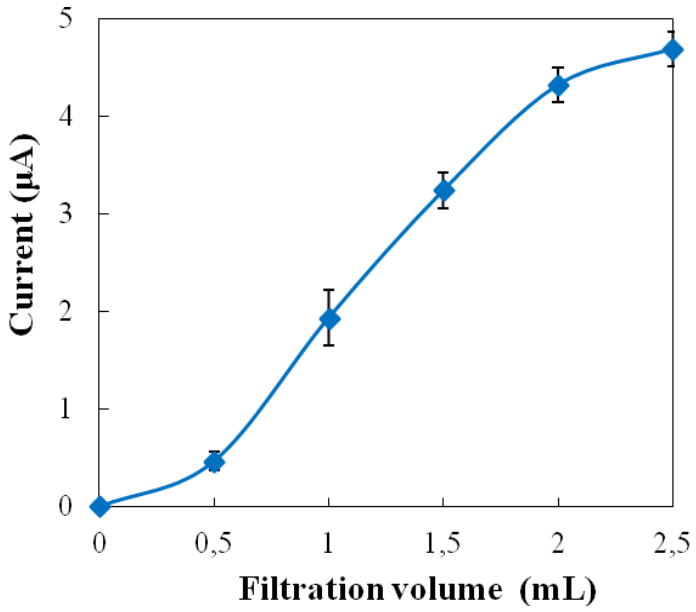

Figure 5. The optimization of the *C. reinhardtii* solution for filtration. The current values were obtained from the cyclic voltammograms measured in 50 μL of Tris-HCl (pH 8.4) including 1 mM PAPP on the SPCE at a scan rate of 20 mV s^{-1} after incubation for 5 min.

3.5. Optimization for exposure time with toxicant

To clarify the optimum exposure time, *C. reinhardtii* solution was exposed with different concentrations of Cu solution for up to 24 hours, and the relative phosphatase activity was calculated. In order to carry out the experiment, Cu was added to the *C. reinhardtii* suspension that had already cultured in m-SG1 for 72 hours for Cu final concentrations of 0, 1, 10, and 100 mg L^{-1}. As shown in Figure 6, the activity of alkaline phosphatase induced by *C. reinhardtii* was inhibited by the addition of Cu, and the degree of inhibition corresponded to the concentration of Cu. When the concentration of Cu was 1 mg L^{-1}, the relative phosphatase activity reached a minimum level after 5 hours of exposure, and then the relative phosphatase activity rebounded slightly. Here, it could be assumed that the concentration of Cu was insufficient, and that the *C. reinhardtii* restored it gradually. In the case of a Cu concentration of approximately 10 mg L^{-1}, the relative phosphatase activity dropped sharply during 5 hours before stabilizing at a constant value. For a Cu concentration of 100 mg L^{-1}, the relative phosphatase activity dropped sharply to almost zero within 5 hours. Based on these results, 5 hours was chosen as the optimum exposure time with a toxicant which is much lower than the conventional

AGI test requires 72-96 hours [18]. The algal bioassay conducted by Simpson *et al.* for obtaining the effect of declining toxicant concentrations with *Phaeodactylum tricornutum* have measured the decline in growth rate in a 72 hours exposure [42]. Durrieu *et al.* investigated the effects of heavy metals on alkaline phosphatase activity of intact *Chlorella vulgaris* with dialysis system [35]. In their study, the algae were exposed with metals for 24 hours and then screening experiments were carried out.

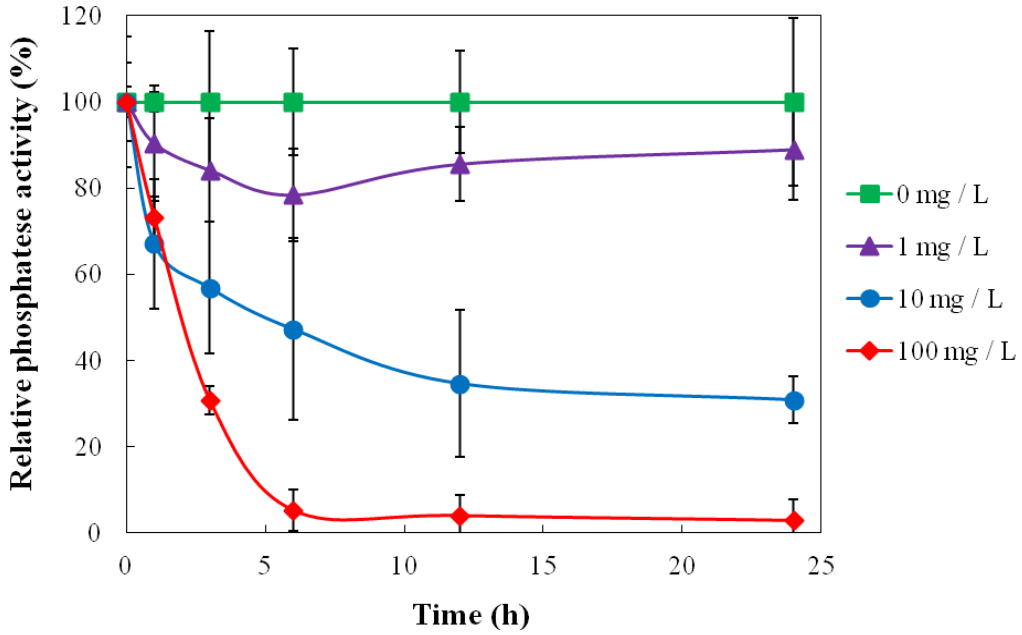

Figure 6. The relationship between the relative alkaline phosphatase activity of *C. reinhardtii* and the exposure time with Cu. The relative phosphatase activities were calculated using the current values obtained from the cyclic voltammograms measured in 50 µL of Tris-HCl (pH 8.4) including 1 mM PAPP on a SPCE at a scan rate of 20 mV s^{-1} after incubation for 5 min.

3.6. Toxicity test for heavy metals

A total of five heavy metals, Cu, Pb, Cd, Zn, and Hg, were tested by this proposed electrochemical bioassay, and the results are shown in Figure 7. The EC_{50} for Cu, Pb, Cd, Zn, and Hg were calculated as 8.18, 0.10, 0.27, 1.99, and 0.07 µmol L^{-1}, respectively, and the toxicity order was Hg>Pb>Cd>Zn>Cu. On the other hand, the EC_{50} values evaluated from the µ-AGI test using *C. reinhardtii* were 36.2, 29.1, 8.5, 7.7, and 2.6 µmol L^{-1} for Cu, Zn, Cd, Pb, and Hg respectively, and these were comparatively higher than the values obtained from the enzyme inhibition test. The summary of the comparative analysis of EC_{50} values obtained from this proposed method, the µ-AGI test and the reference method [35] are presented in Table 1. The reference method is based on the inhibition of alkaline phosphatase of *Chlorella vulgaris* using *p*-nitrophenyl phosphate as the substrate for spectrophotometric detection. Comparison of the EC_{50} values obtained from all the above methods clearly indicated that the enzyme inhibition test proposed in this study shows the most sensitive response to heavy metals, particularly for Hg, Pb and Cd. Moreover, the double logarithmic plots of the EC_{50} values appeared to show good linearity, as shown in Figure 8. The present assay and the

reference method both measured the inhibition of alkaline phosphatase activity with two different species of algae. The lower EC_{50} values in our study, however, established that the sensitivity of *C. reinhardtii* towards heavy metals was higher by comparison with *C. vulgaris.* Previous studies based on algal toxicity bioassay also found similar results for these two species [43-45]. Thus, the present electrochemical bioassay demonstrated significant advantages over other existing methods in terms of sensitivity and measurement duration.

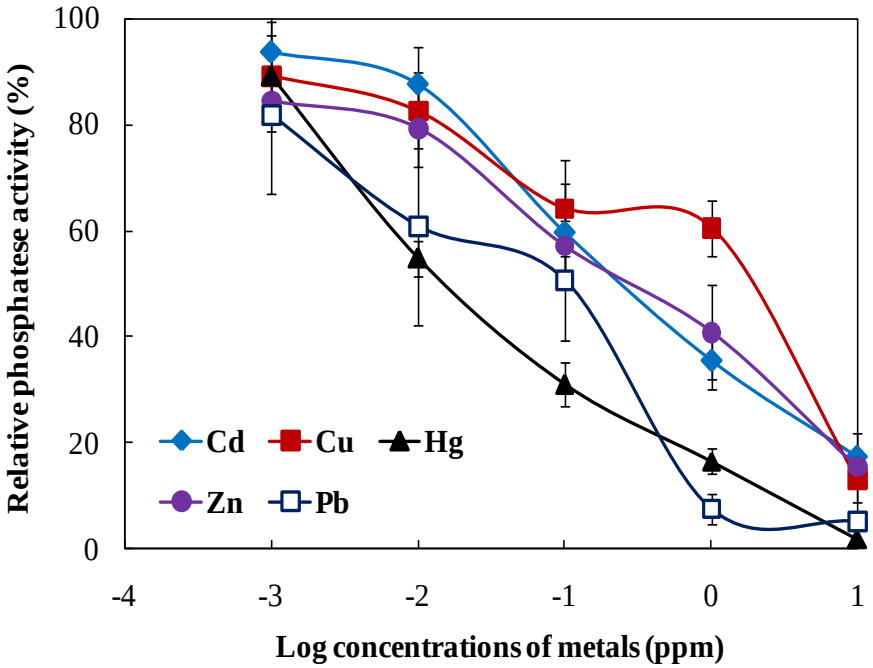

Figure 7. The dose response curves for Cu, Pb, Cd, Zn, and Hg obtained from the proposed electrochemical bioassay based on the inhibition of alkaline phosphatase activity of *C. reinhardtii*. The relative phosphatase activities were calculated from the current values obtained from the cyclic voltammograms measured in 50 µL of Tris-HCl (pH 8.4) including 1 mM PAPP on the SPCE at a scan rate of 20 mV s^{-1} after incubation for 5 min.

Table 1. The comparative analysis of the EC_{50} values obtained from various techniques

Detection method	EC_{50} (µM)				
	Cu	Zn	Cd	Pb	Hg
Present method	8.18	1.99	0.27	0.10	0.07
µ-AGI test	36.2	29.1	8.5	7.7	2.6
Reference method	7.40	6.73	1.51	1.11	0.947

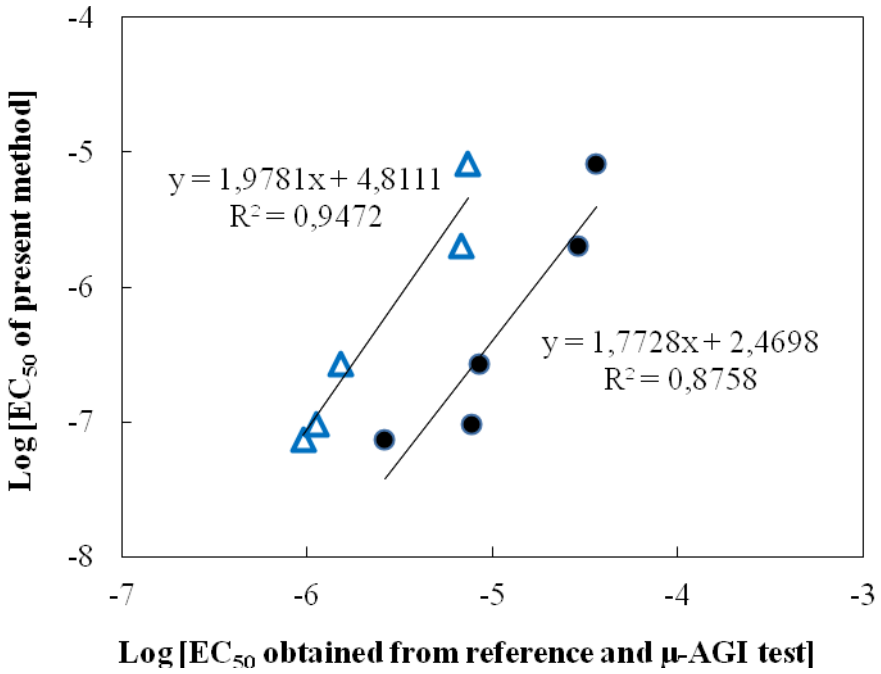

Figure 8. Relationships among the EC_{50} values obtained from the proposed electrochemical bioassay, the µ-AGI test, and the reference method [35]. The blue triangle (Δ) represents the reference method and the black circle (●) is for the µ-AGI test.

4. CONCLUSIONS

The algal bioassay based on the inhibition of alkaline phosphatase activity with electrochemical detection in a micro-droplet on a screen-printed carbon electrode was demonstrated to evaluate the toxicity of heavy metals. The alkaline phosphatase *C. reinhardtii* was used to convert *p*-aminophenyl phosphate (PAPP) to *p*-aminophenol (PAP). Voltammetric detection was used to measure the oxidation current of PAP. In order to promote high enzymatic activity, alkaline phosphatase was successfully induced by cultivating *C. reinhardtii* in a modified-SG1 (m-SG1) medium that was without phosphate, and the algae activated by this procedure was accumulated through filtration. This enzymatic reaction can be conducted in a 50 µL micro-droplet, which ultimately reduced the reaction time with the substrate and enabled the proposed technique to be carried out using lesser chemicals. Comparisons with a conventional AGI test and enzyme inhibition based on spectrophotometric detection indicated that the sensitivities were much better than those of two other conventional techniques. Furthermore, the present assay can be used for detecting samples of colored and suspended solids due to the electrochemical detection feature. The demonstration of *C. reinhardtii* with an electrochemical technique presents new possibilities as a much more effective tool for aquatic toxicity monitoring in terms of cost, speed and sensitivity.

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