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(82) CONTINUOUS TREATMENT OF ENDOCRINE DISRUPTING CHEMICALS BY AQUATIC PLANTS AND BIOLOGICAL FENTON REACTION

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In this paper, we report the treatment results of trace phenolic endocrine disrupting chemicals (EDCs) by aquatic plants. EDCs used in this study were bisphenol-A, 2,4-dichlorophenol, 4-*tert*-octylphenol, pentachlorophenol, and nonylphenol. Referring to reported detection levels in aquatic environments and municipal wastewater, the feed concentration of each EDC was set at 100 μg/L. From continuous experiments, it was found that every EDC except pentachlorophenol was efficiently removed by different aquatic plants through the following reaction catalyzed by peroxidases (POs): EDCs + H_2O_2 → Products + H_2O. Histochemical localization of POs show that they are located in every part of root cells, while highly concentrated zones were observed in epidermis and vascular tissues. In addition, it was also found that H_2O_2 concentrations and POs in aquatic plants were almost kept constants during the treatment of EDCs. The endogenous H_2O_2 concentrations were in the range of 0.1 to 0.6 mmol/kg-FW (fresh weight). Pentachlorophenol was not removed in continuous treatment. However, by the addition of Fe^{2+}, pentachlorophenol was quickly removed by different aquatic plants with the consumptions of endogenous H_2O_2, demonstrating the occurrence of a biological Fenton reaction. These results indicated that aquatic plants have a superior performance for removing trace EDCs as well as refractory pollutants.

Key Words *: Endocrine disrupting chemicals, peroxidase, hydrogen peroxide, biological Fenton reaction, constructed wetland, phytoremediation.*

1. INTRODUCTION

Endocrine disrupting chemicals (EDCs) have been defined as exogenous substances that cause health effects in an intact organism, or its progeny, consequent to changes in endocrine function[1]. These compounds may seriously alter some vital activities, such as reproduction, with dangerous and sometimes unpredictable consequences. Especially, the estrogenic effects of 17β-estradiol, nonylphenol (NP), 4-*tert*-octylphenol (4-*t*-OP), and bisphenol A (BPA) have been identified for aquatic life forms such as fish at very low concentrations, i.e. 0.001 to 0.1 μg/l[2]. These threshold concentration levels are much smaller than those in municipal sewage, one of the major pollution sources, which contains NP, 4-t-OP and BPA in the range of 0.01 to 100 μg/l[1),3)]. EDCs may enter fresh and sea water due to agricultural practices and by application, discharge and disposal of urban and industrial effluents, sludges, and other wastes[4].

The discharges of EDCs in the environment should be reduced because of identified endocrine disrupting effect; and therefore, effective methods for the removal of these EDCs from wastewater, surface water and contaminated sites are required[1].

Several methods proposed for treating phenolic waste, including adsorption, and different types of advanced oxidation process (AOPs) have been tried [5]. All of these methods, although certainly feasible and useful, suffer from certain serious drawbacks, such as high cost, incomplete removal, formation of hazardous byproducts[5]. In addition, economical feasibility should be considered if the processes were applied to wastewater treatment[6]. For these reasons, attention has been devoted to the development of alternative and/or complementary

processes for the removal of EDCs in surface water, wastewater or treated wastewater[1].

The process using aquatic plants or phyto remediation technology is proposed to be a cost-effective alternative for the treatment of contaminated water[7]. Phytodegradation, a kind of phytoremediation, is the breakdown of contaminants taken up by plants through metabolic processes within the plant, or the breakdown of contaminants external to the plant through the effect of compounds produced by the plants[8].

On the removal of EDCs, recent studies demonstrated the effectiveness of plant and/or related plant substances including enzymes. EDCs such as BPA, 4-*t*-OP, and NP could be removed efficiently by enzymes such as peroxidases (POs)[9), 10), 11)], laccase (LAC)[12), 13)], polyphenol oxidase (PPO)[14)], glutathione S-transferase (GST)[15)], plant cell or tissues containing enzymes[16), 17), 18)], and aquatic plants[19)]. In addition, Caza et al.[9)] and Imai et al.[18)] confirmed that the treatment of EDCs by plant enzymes or plant was effective to eliminate estrogenic activity.

This study was conducted to evaluate precise removal performance of trace EDCs by different types of aquatic plants under continuous conditions. In addition, the role and activities of enzymes such as POs, PPO, GST, and LAC extracted from aquatic plants were analyzed. Furthermore, the occurrence of a biological Fenton reaction by different aquatic plants was studied for the treatment of refractory compounds including pentachlorophenol (PCP).

2. MATERIAL AND METHODS

2.1 Plants and experimental design

Experiments were conducted using different aquatic plants, including floating plants: Amazon frogbit (*Limnobium laevigatum*), Giant Duckweed (*Spirodela polyrhiza*), Duckweed (*Lemma gibba*), and Crystalwort (*Riccia fluitans*); and submerged plant: Hornwort (*Ceratophyllum demersum*) and Willow Moss (*Fontinalis antipyretica*), respectively.

Continuous experimental apparatus used in this study is illustrated in Fig. 1, where each plant was cultivated in glass vessel after washing several times with distilled water. All vessels were maintained under intermittent illumination with light and dark conditions at 16h and 8h, respectively. Illumination at light condition was controlled at 3000 lux provided by white fluorescent lamps.

EDCs used in this study were BPA, NP, 4-t-OP, 2,4-dichlorophenol (2,4-DCP) and pentachloro phenol (PCP) purchased from WAKO Chemicals. Referring to reported detection levels in wastewater and aquatic environments, the feed concentration of each EDC was set at 100 μg/L. The stock solutions of each EDC were prepared using acetone and were mixed and dissolved in diluted Hoagland solution. The flow rate of the solution was set at 1.92 L/day using a peristaltic pump. Every experiment and analysis was conducted at a room temperature (around 25 degree Celcius). The effective volume of glass vessel was 8L.

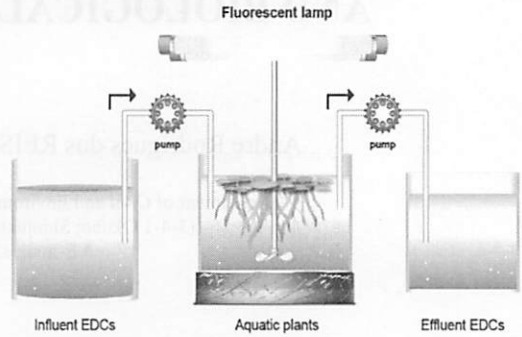

Fig.1 Scheme of experimental apparatus.

In continuous experiment each aquatic plant was set at 5 g-FW/L. The feed solution of reactors was prepared every 5 days, while the fresh biomass of aquatic plants were measured and the excess over than 5 g/L of biomass was withdrawn. For every experiment, blank test using no aquatic plant was conducted as a reference. After the continuous treatment, plants in vessels were taken out and the amount of EDCs accumulated in plant tissues were analyzed. All experiments were carried out under the same conditions.

2.2 Endogenous H_2O_2 assay

H_2O_2 concentrations in aquatic plants were measured according to former studies[20)]. Aquatic plants samples (0.3 g fresh weight) were ground with a mortar and pestle in liquid nitrogen and fine powdered material obtained was mixed with 1 mL of 0.2 M perchloric acid. The mixture was centrifuged at 12000 ×g and 4 °C for 15 min. Afterwards, the H_2O_2 concentration was measured using horseradish peroxidase in a spectrophotometer at 590 nm. A standard calibration curve was prepared with known concentration of H_2O_2 using the same method.

2.3 *In vitro* treatment by extracted enzyme

To know primal enzymes in the EDC treatments, different groups of crude enzymes (i.e. POs, LAC, PPO, and GST) were extracted from homogenized aquatic plants and *in vitro* batch treatments of phenolic EDCs were conducted.

The following extraction steps were carried out at 4 °C unless stated otherwise. Aquatic plant tissues were homogenized (buffer volume (ml):fresh weight

(g)=1:1) in a mortar with a pestle in a buffer for each enzyme. For PO fractions; soluble PO (SPO), ionically cell wall-bound PO (IPO) and covalently cell wall-bound PO (CPO) were extracted at pH 6.0 in 50 mM Tris maleate; 0.2 M $CaCl_2$ and 40 mM Tris maleate buffer, respectively[21]. The PPO fration was extracted at pH 6.0 in 0.1 M sodium phosphate[21]; while LAC and GST were extracted at pH 7.5 in 0.1 M potassium phosphate[12), 22].

The homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant was used for the measurement of enzyme activities as well as *in vitro* treatment of EDCs. To simulate the degradation of phenolic EDCs by enzymes in plant cell, each crude enzyme fraction was extracted. To avoid any interferences, *in vitro* treatments of phenolic EDCs was performed with a buffer for each enzyme. The reaction was performed in a capped Erlenmeyer flask, the initial concentration of each phenolic compound (BPA, 2,4-DCP, NP, 4-t-OP, and PCP) was set at 100 μg/L, and variable levels of each crude enzyme was added in 40 mM Tris maleate (pH 6.0) and 0.15 mM H_2O_2 for PO reactions; 0.1 M sodium phosphate (pH 6.0) for PPO reactions and 0.1 M potassium phosphate (pH 7.5) for LAC and GST reactions, respectively. The final volume was completed to 100 ml and after 1, 3 and 12 hours the enzymatic reactions at 25 °C were stopped, and the concentration of EDCs was measured. A control experiment without crude enzyme was conducted as reference.

2.4 Measurement of enzyme activities

Activities of POs, PPO, LAC, and GST were measured after the extractions of enzymes from plants according to former studies[21), 12), 22].

Activities of PO fractions, SPO, IPO, and CPO were measured according to the increase in absorbance at 470nm using 168mM guaiacol as an electron donor in the presence of 40 mM tris-maleate buffer and 30 mM H_2O_2[21]. The increase in absorbance was converted to the number of unit of PO, based on an extinction coefficient of 26.6 mM^{-1} cm^{-1} [31]. One unit of enzyme activity (U) represents the amount of enzyme catalyzing the corresponding reaction of 1 μmoL of substrate per 1 min. On the other hand, PPO activity was measured according to the increase in absorbance at 410 nm using 0.2 M 4-methyl catechol as an electron donor, and was converted to the number of unit for PPO based on extinction coefficient 1.42 mM^{-1} cm^{-1} [21]. The PO and PPO activities were expressed as per unit of fresh weight of biomass or per unit of protein measured according to the Bradford Protein Assay using BSA as standard[23].

The activity of LAC was assayed according Toyama et al.[12] with minor modifications. The reaction mixture was prepared by adding 2.0 ml of enzyme extract with 0.84 ml of 0.1 mM 2,2-azinobis (3-methyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 3.16 ml of phosphate buffer; and the increase in 420 nm was measured in triplicate. One unit of LAC activity (U) represents the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS per 1 min.

GST was assayed by using 1-chloro-2,4-dinitrobenzene (CDNB), referring to Habig et al.[23] The reaction mixture was prepared by mixing 900 μl of 0.1 M potassium phosphate (pH 6.5), 25μl of 0.4 mM CDNB, 50 μl of 1 mM glutathione-SH (GSH) and 25 μl of the enzyme extract in a final volume of 1 ml. The reaction was started by adding CDNB. The enzyme activity was measured by monitoring the increase in absorbance at 340 nm, and was converted to the number of unit for GST, based on an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

2.5 Histochemical localization of POs

The localization of PO in roots of *Limnobium laevigatum* was conducted by placing samples in 40 mM tris maleate buffer containing 84 mM guaiacol and 3 mM H_2O_2. Transverse sections of the roots were obtained using a freehand microtome. The enzymatic reaction for staining sample was carried out in 1 min, where PO activity in plant cell structures was manifested as a brown color formation. No staining of the samples using tris maleate buffer without H_2O_2 were also conducted and were compared with stained samples. After these treatments with and without guaiacol/H_2O_2, the sections or samples were washed with distilled water and observations were made using an epifluorescent microscope (Olympus BX60) at an accelerating voltage of 50 kV.

2.6 Biological Fenton treatment

In the continuous experiments, PCP was not removed by aquatic plants, while endogenous H_2O_2 levels were kept in the range of 0.1 to 0.6 mM in aquatic plants used in this study. Based on these results, the possibility of PCP treatment by biological Fenton reaction was studied experimentally.

Experiments were carried out using *Spirodela polyrhiza*, *Ceratophyllum demersum* and *Riccia fluitans*. The fresh biomass of aquatic plants was set at 10 g/L and the PCP concentration at 100 μg/L. A blank experiment with no aquatic plant was set as reference. Ferrous sulfate as source of Fe^{2+} was added in vessels and time course changes of PCP and endogenous H_2O_2 concentrations of different aquatic plants were measured.

2.7 GC/MS measurements

In the chemical analysis of EDCs in solutions, 100 ml of water samples were extracted two times with 10 ml of dichloromethane 5000 (Wako Pure Chemical Industries, Ltd.), after adding 5 g of NaCl, 0.2 ml of 1M HCl solution, and 5 μl of surrogate. The surrogate was spiked to confirm the analytical recoveries and precision. After that, the extracted samples were transferred to erlenmeyer capped flasks, and 3g of sodium sulfate was added and kept for over 12h. Thereafter, trimethylsilyl (TMS) derivatives were obtained and were measured using GC/MS (Shimadzu QP5050A). Procedures used in the analysis were referred to tentative investigation manual[24]. Details of the analysis were shown in our former studies[1),2)].

Measurements of EDCs in aquatic plants were also conducted, where the extractions of EDCs were carried out after the following pretreatments. First, aquatic plants were washed with distilled water and submerged in liquid nitrogen. The samples were ground in methanol with a mortar and pestle. After that, EDCs were extracted with dichloromethane and measurements were made in the same manner for EDCs in water samples.

3. RESULTS AND DISCUSSION

3.1 Continuous treatments

Fig. 2 (A)-(E) shows experimental results of continuous treatment of phenolic EDCs. Effluent concentrations of most EDCs declined sharply during the first 10 to 30 days and thereafter gradually increased and approached to steady-state values. This might indicate that adsorption or sorption of EDCs to plant tissues occurred initially, and thereafter the EDCs such as 2,4 DCP, 4-t-OP, BPA, and NP were removed biologically. These results demonstrate that treatments by aquatic plants were very effective for continuous treatments. However, no removal of PCP was observed. In blank experiments, every EDCs was kept constant at almost feed concentration of 100 μg/L.

In the continuous treatments, HRT was set at 3 days, which is corresponding to or less than those used in stabilization ponds or constructed wetlands[25]. Therefore, it is considered that cultivations of aquatic plants would enhance the treatment performance of most EDCs in ponds or constructed wetlands.

After 110 days of operation in continuous experiment, aquatic plants were taken out and accumulated EDCs in plant tissues were analyzed. However, the amounts of accumulated EDCs were around a few % or less of the EDCs removed during the continuous treatments. Referring to former

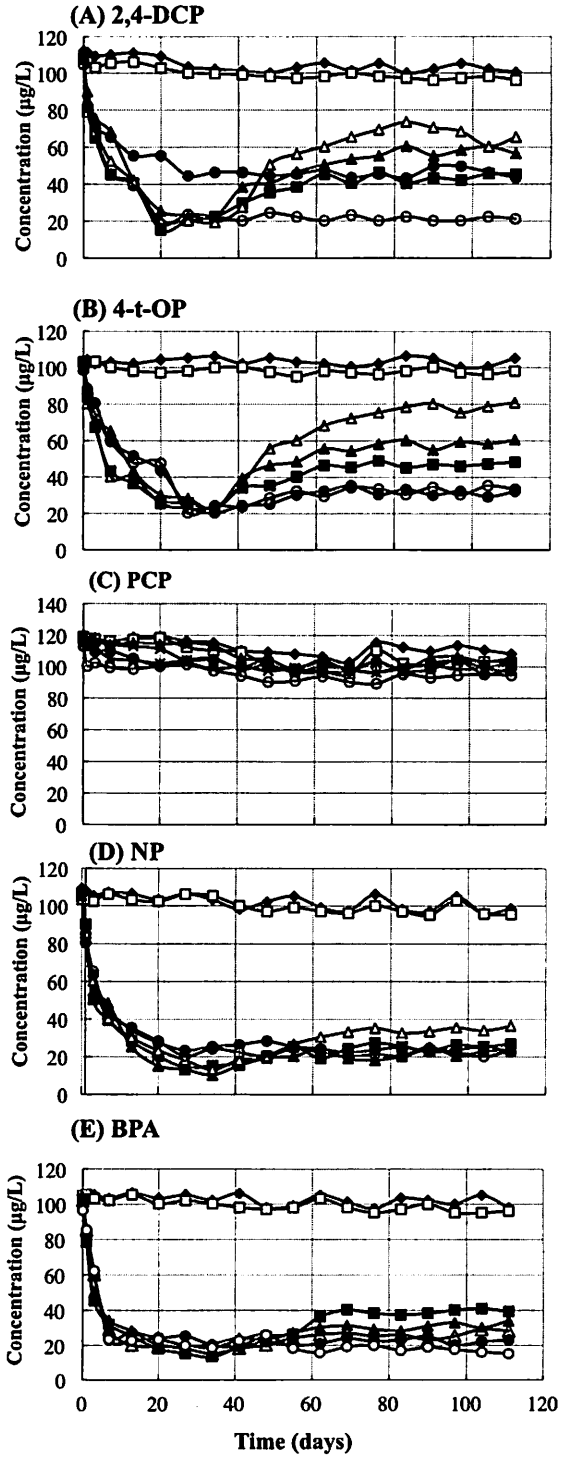

Fig. 2 Time course changes of (A) 2,4-DCP, (B) 4-t-OP, (C) PCP, (D) NP and (E) BPA in continuous treatment, where, ◆ Influent, □ Blank (with no aquatic plants); ■ *Fontinalis antipyretica*; Δ *Limnobium laevigatum*; ▲ *Ceratophyllum demersum*; ● *Spirodela polyrhiza*, O *Lemma aoukikusa*.

studies such as Caza et al.[9], Schröder[8], Xuan et al.[10], Reis et al.[34] and Imai et al.[18], this result suggests that EDCs were removed by plants through biological enzymatic reactions in a long term continuous treatment over 100 days.

3.2 Primal Enzyme in EDCs Treatments

3.2.1 Enzyme activities of aquatic plants

Activities of POs, which are composed of SPO, IPO and CPO fractions, PPO, LAC, and GST as well as endogenous H_2O_2 concentrations of aquatic plants used in this study are analyzed before the continuous treatments and are summarized in Table 1. Activities of POs were in the range of 0.69 to 103.52 (U/g-FW) or 0.17 to 25 (U/mg-protein), depending on species of aquatic plants. Similar activities for POs were reported in the literature. For example, Pandolfini et al.[26] reported the activities of SPO, IPO and CPO of *Triticum aestivum L.* were in the range of 0.2 to 3.49 (U/mg-protein). As shown in Table 1, relatively larger activities of POs were observed for *Ceratophyllum demersum* and *Riccia fluitans*, while lower activities for *Spirodela polyrhiza.*

PPO activities were in the range of 1.94 to 4.93 (U/g-FW), or 0.48 to 4.45 (U/g-protein), depending on species of aquatic plants. *Limnobium laevigatum* and *Spirodela polyrhiza* had relatively higher activities. Observed activities were almost equal to 0.35 to 2.85 (U/mg-protein) reported by Ghanati et al.[21]. Activities of LAC in this study were in the range of 0.39 (U/kg-FW) for *Ceratophyllum demersum* to 4.84 (U/kg-FW) for *Riccia fluitans.*

Comparing with POs, LAC activities were relatively low. Similar results were reported for LAC in aquatic plants by Toyama et al.[12]. Furthermore, the LAC activities are more than 5 orders of magnitude smaller than 1 to 5 U/g-mycelium solution of a fungus, *Trametes versicolor* reported by Mougin et al.[27], indicating LAC activities in plants are very low comparing with that in fungi.

Activities of GST were in the range of 0.5 to 35 (U/g-FW) or 45 to 71 (U/mg-protein). GST catalyzes the detoxification of number of hydrophobic compounds through the conjugation of glutathione to the compounds[28]. In this study, *Ceratophyllum demersum* showed relatively high GST activities around 1 to 30 (U/g-FW).

Endogenous H_2O_2 concentrations in aquatic plants were in the range of 99.1 (μmol/kg-FW) for *Ceratophyllum demersum* to 611.3 (μmol/kg-FW) *Limnobium laevigatum.* In addition, there was a tendency that aquatic plants possessing higher POs activity had lower endogenous H_2O_2 concentration as shown by *Ceratophyllum demersum* (Table 1). This is because one of the key functions of POs is to protect plant cells through the reduction of H_2O_2 generated by photosynthetic reactions and respiration[29].

3.2.2 *In vitro* assays for extracted enzymes

In order to evaluate actual performances of enzymes, *in vitro* enzyme assays for EDCs removals were conducted using SPO, IPO, CPO, PPO, GST and LAC extracted from aquatic plants. As shown in 2.3, every enzyme was extracted into 1:1 (ml buffer/g-FW) of aquatic plant; and different amounts of the extracted solution was finally solved into 100 mL of EDCs solution. That is, in case of

Table 1. Enzyme activities and H_2O_2 concentration of aquatic plants.

Plant species	SPO		IPO		CPO	
	U/g-FW	U/mg-protein	U/g-FW	U/mg-protein	U/g-FW	U/mg-protein
Ceratophyllum demersum	103.52	11.98	27.49	13.22	35.24	24.47
Fontinalis antipyretica	14.20	1.20	6.17	4.28	2.96	0.22
Limnobium laevigatum	13.91	0.50	7.10	0.28	4.34	0.27
Lemma aoukikusa	22.55	5.34	11.27	2.74	1.50	1.95
Spirodela polyrhiza	5.63	2.12	5.26	1.16	0.57	0.95
Riccia fluitans	26.71	0.17	9.51	0.34	3.80	0.29

Plant species	PPO		GST		LAC		H_2O_2
	U/g-FW	U/mg-protein	U/g-FW	U/mg-protein	U/kg-FW	U/mg-protein	μmol/kg-FW
Ceratophyllum demersum	1.94	2.23	35.27	60.76	0.39	0.126	99.1
Fontinalis antipyretica	3.28	0.64	0.55	69.38	0.64	0.006	105.4
Limnobium laevigatum	3.41	1.27	0.50	55.64	1.12	0.012	611.3
Lemma aoukikusa	4.05	4.45	3.23	62.43	2.45	0.104	240.5
Spirodela polyrhiza	4.93	1.21	2.12	45.65	3.01	0.113	350.9
Riccia fluitans	2.05	0.48	1.17	70.40	4.84	0.062	149.4

* Abbreviations of enzymes are as follows:
SPO: Soluble Peroxidase, IPO: Ionically Cell Wall-Bound Peroxidase, CPO: Covalently Cell Wall-Bound Peroxidase, PPO: Polyphenol oxidase, GST: Glutathione S-transferase, LAC: Laccase, H_2O_2: Hydrogen peroxide

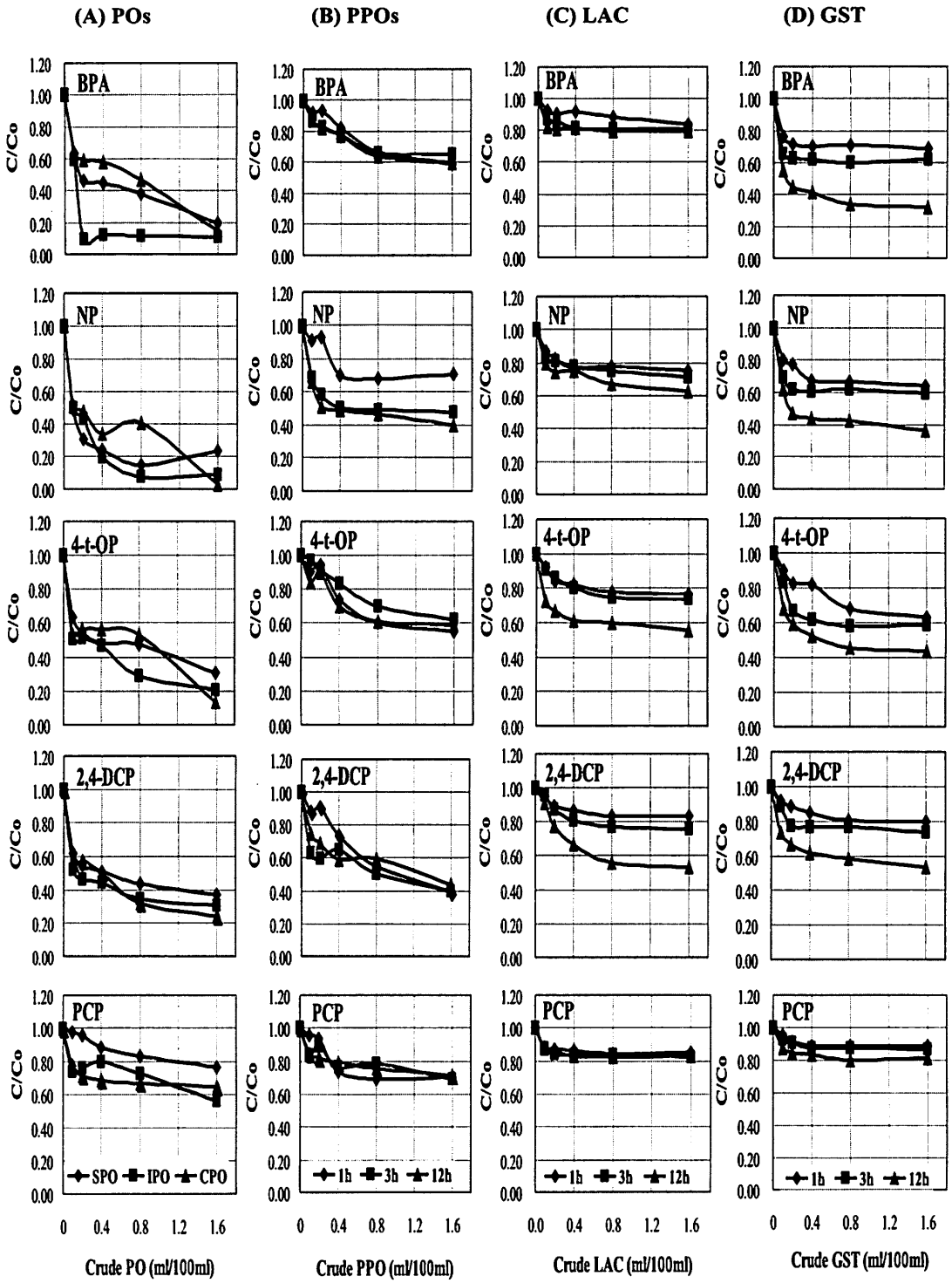

Fig. 3 EDCs removal by enzymes extracted from *Ceratophyllum demersum*, where (A) Peroxidases (SPO – Soluble Peroxidase, IPO – Ionically Cell Wall-Bound Peroxidase, CPO – Covalently Cell Wall-Bound Peroxidase), (B) Polyphenol oxidase, (C) Laccase, and (D) Glutathione S-transferase, respectively.

solving 1 ml of crude enzymes into 100 ml of buffered EDCs solution, enzyme concentration is corresponding to an aquatic plant ratio of 10 g-FW/L. Furthermore, for the use of crude enzymes it was assumed no significant interaction among the enzymes existed.

H_2O_2 concentration was set at 0.15 mM in the treatment of EDCs, because *in vivo* concentrations were in the range of 0.1 to 0.6 mM/kg-FW as show in Table 1. This concentration is nearly equal to those of *Ceratophyllum demersum* and *Riccia fluitans*, but is very low compared to *Limnobium laevigatum* (about 0.6 mM), as shown in Table 1.

Figure 3 (A)-(D) shows the results of enzymes assays, where decreases of EDCs concentratios were shown for different enzymes (POs, PPO, LAC, and GST) and different enzymes concentrations which were represented by the volume ratio of extracted enzyme solution (ml) to the 100 ml of final solution.

Reaction times for POs and other enzymes were 3 and 1 to 12 h, respectively. As shown in the Figure 3(A), most EDCs except PCP were degraded within 3h by every PO. Comparing to these results, the activities of other enzymes in removing EDCs were relatively low or longer reaction times were required to reduce EDCs. Therefore, in removing EDCs except PCP, POs are the primal enzymes which catalyze the following reaction[9];

$$\text{EDCs} + H_2O_2 \xrightarrow{\text{Peroxidases}} \text{Products} + H_2O \qquad (1)$$

From the results in Figures 2 and 3, we expected that removal performances of EDCs in constructed wetland or phytoremediation processes can be enhanced if aquatic plants which possessing larger amounts of POs and higher concentration of endogenous H_2O_2 are selected and cultivated.

To determine an optimum condition of the EDC removals by POs, batch treatments of EDCs by SPO, IPO and CPO were conducted under different pH conditions. Crude PO concentration was prepared as 0.6ml/100ml EDCs solution, while H_2O_2 concentration and reaction time were set at 0.15 mM and 3 h. Figure 4 (A)-(E) shows the removals of EDCs as a function of pH values. It is clearly observed that except PCP, POs had catalytic abilities over a pH range of 4 to 8. This result may suggest aquatic plants possessing weak acidic conditions in plant cells are useful in the treatment. Regarding PCP, slight removals by POs at pH above 8.0 were observed. The reason for this result is not known, and a further study will be needed to explain this phenomena.

3.2.3 Histochemical localization of POs

In the histochemical localization using guaiacol and H_2O_2, it is possible to visualize the location of POs in plant cells. The results of *Limnobium laeviga tum* were shown in Figure 5 (A)-(D), indicating that

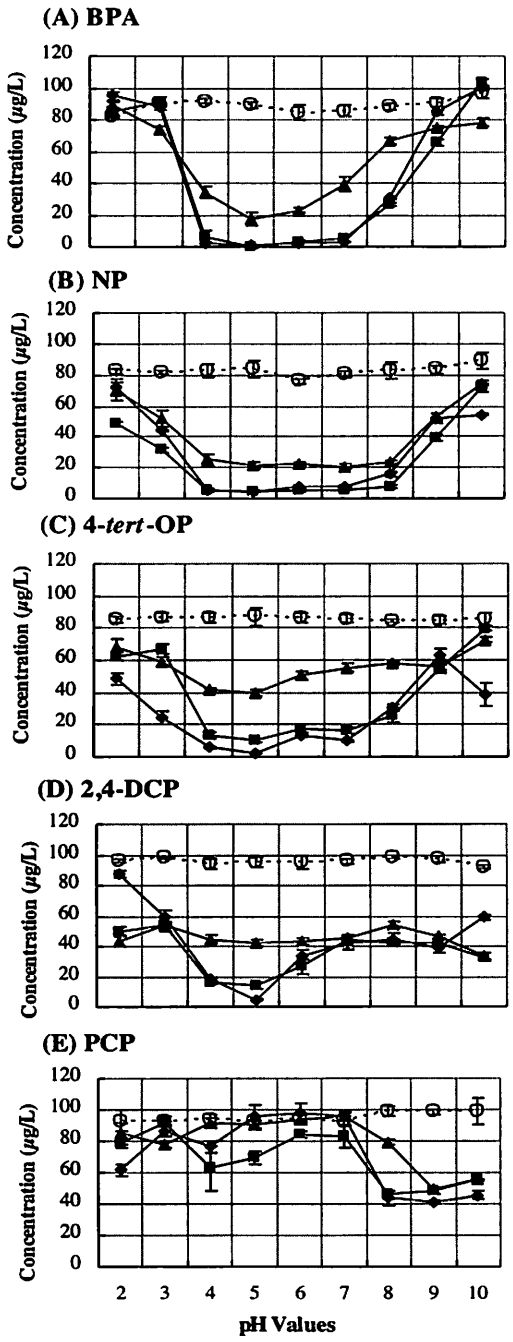

Fig. 4 pH optimization for EDCs degradation by peroxidase fractions extracted from *Ceratophyllum demersum*, where, ○ control: without peroxidase and H_2O_2; ◆ soluble peroxidase fraction; ■ ionically cell wall-bound peroxidase fraction and ▲ covalently cell wall-bound peroxidase fraction.

almost every part of the root cells possesses POs. Figure 5 (B) shows the POs on root epidermis. In transverse sections of the roots, highly concentrated zones of POs were observed in epidermis and vascular tissues including phloem and xylem (Figure 5D). Similar results regarding localization of POs in epidermis and vascular tissues for *Vicia faba* roots were reported by Jensen[32]. In addition to Figures 5(A)-(D), its was found that abaxial side of the leaf of *Limnobium laevigatum* also possesses relatively concentrated zones of POs (data not shown).

Figure 6 shows the POs activities and endogenous H_2O_2 concentrations of aquatic plants in continuous experiments shown in Fig. 2. *Ceratophyllum demersum* and *Lemma aoukikusa* possessed relatively higher activities of SPO and IPO. Furthermore, the fractions of POs (SPO, IPO, and CPO) were kept constants during the continuous experiments. In addition, H_2O_2 concentrations were also kept constants around 550, 400, 300, 200 and 130 μmol/kg-FW in *Fontinalis antipyretica*, *Limnobium laevigatum*, *Lemma aoukikusa*, *Spirodela polyrhiza*, and *Ceratophyllum demersum*, respectively.

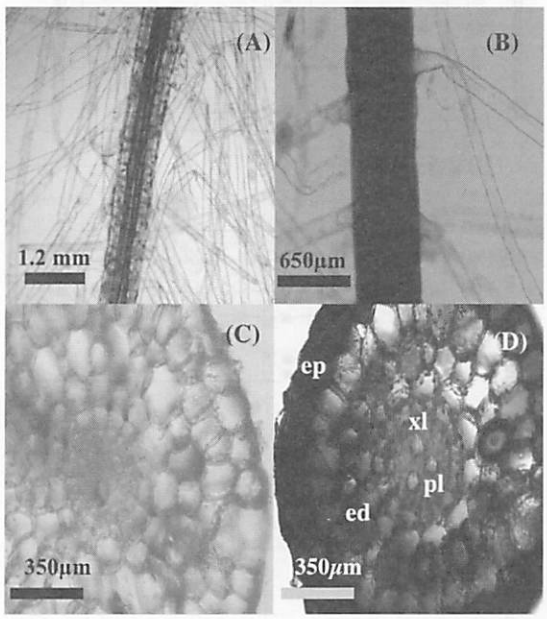

Fig. 5 Epifluorescent micrographs showing localization of POs in root with and without guaiacol/H_2O_2 treatment of *Limnobium laevigatum*, where, (A) roots without treatment (40x); (B) roots with treatment (100x); (C) transverse section of root without treatment (200x); (D) transverse section of root with treatment (200x). Abbreviations are as follows: ep - epidermis; ed – endodermis; ph – phloem, and xl – xylem.

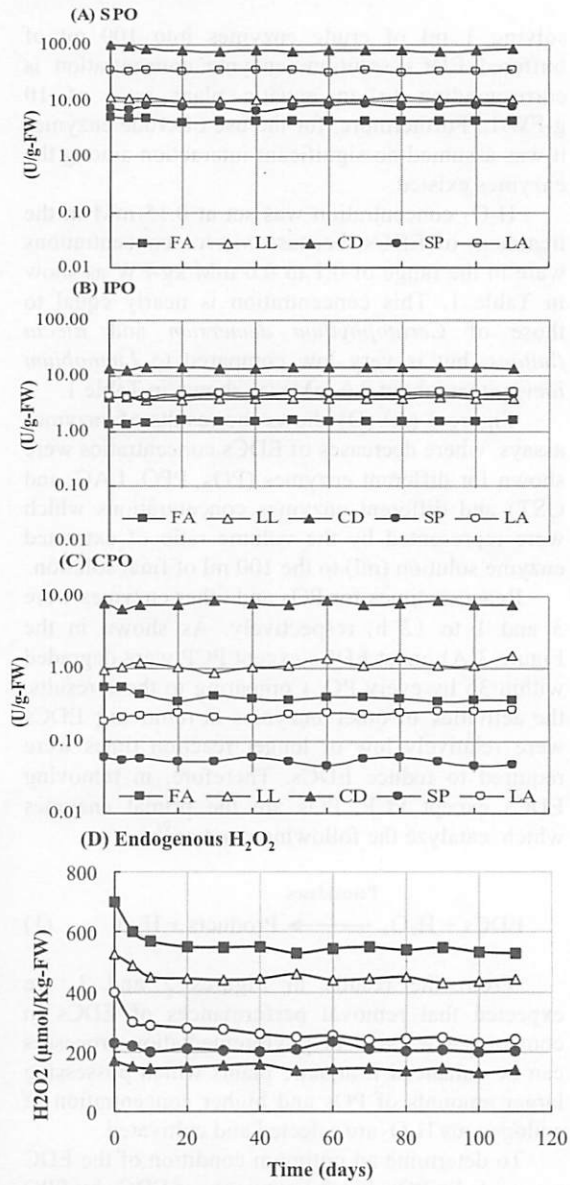

Fig. 6 Peroxidase activity (A) SPO – Soluble peroxidase; (B) IPO – Ionically cell wall-bound peroxidase; (C) CPO – Covalently cell wall-bound peroxidase, and (D) H_2O_2 concentrations in aquatic plants, where, ■ *Fontinalis antipyretica*; Δ *Limnobium laevigatum*; ▲ *Ceratophyllum demersum*; ● *Spirodela polyrhiza*; ○ *Lemma aoukikusa*, respectively.

That is, POs and endogenous H_2O_2 concentrations were kept constants in all aquatic plants, indicating that H_2O_2 and POs are being produced stably in plants themselves. Moreover, these results suggest that phenolic compounds such as BPA, NP and 4-t-OP were oxidized by H_2O_2, and the removal rates were affected by H_2O_2

concentration and PO activity, because it was considered that POs were primal enzymes in aquatic plants as shown in Fig. 3. A further study will be needed to analyze kinetic relationships among these parameters and overall removal rates of EDCs.

We observed the growth of aquatic plants, however, as described in material and methods, every 5 days, the plants were measured and the excess over than 5g-FW/L was withdrawn. That is, total enzyme activities or H_2O_2 concentration was kept constant in each treatment system.

In continuous experiments, PCP was not removed by aquatic plants themselves. PCP was used as a pesticide and its persistency and toxicity were reported in the literature[30]. It is supposed that enzymatic reaction in aquatic plants is not effective in oxidizing or decomposing PCP, and therefore, more powerful oxidant or oxidation reaction may be required to treat PCP.

3.3 Biological-Fenton treatment

Based on experimental results in continuous treatments, we tried to use endogenous H_2O_2 for destroying PCP through the generation of OH radical according to the following Fenton reaction[33]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \qquad (2)$$

Figure 7 shows experimental results, where PCP was treated by a *Spirodela polyrhiza*, *Ceratophyllum demersum*, and *Riccia fluitans* in the presence and absence of Fe^{2+}. In presence of Fe^{2+}, the total iron concentration in liquid decreased with time. It was confirmed that endogenous H_2O_2 in aquatic plants decreased in parallel with iron and PCP removal, as well as chloride formation in water (data not shown). These results indicated that PCP was decomposed through a biological Fenton reaction, and H_2O_2 in aquatic plants was a key endogenous substance in treatments of refractory toxic pollutants.

As shown in Fig. 7, PCP was quickly removed within 2 days in the presence of Fe^{2+}, while endogenous H_2O_2 concentration also decreased. The decrease of H_2O_2 was larger at higher Fe^{2+} concentration (data not shown). In the absence of Fe^{2+}, no significant decreases or sligh decreases of PCP and H_2O_2 were observed. Referring to Songa and Huang[30], slight decreases of PCP were considered attributable to the adsorption of PCP by aquatic plants.

When Fe^{2+} concentrations were higher than 10 mM, most plants changed their green colour and the PCP removals tend to decrease. Therefore, it was though that Fe^{2+} concentration around 2.8 mM or less was suitable for the PCP treatment. Furthermore, these results strongly suggested that the Fenton

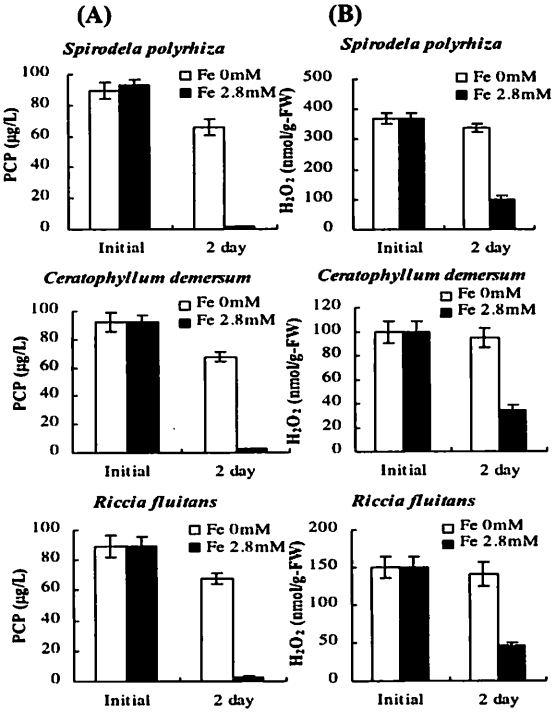

Fig. 7 Batch treatment of PCP by *Ceratophyllum demersum*, *Spirodela polyrhiza* and *Riccia fluitans*, where concentration changes of (A) PCP at bulk liquid and (B) endogenous H_2O_2 were compared in the presence and absence of Fe^{2+}.

reaction proceeded in plant cells and decomposed PCP regardless of the species of aquatic plants. To our knowledge, this result is the first experimental finding on the occurrence of a biological Fenton reaction. Further studies will be needed to evaluate more precise mechanisms, kinetics, optimum conditions, and the applicability to constructed wetland or phytoremediation process.

CONCLUSIONS

Continuous treatments of trace phenolic EDCs were conducted using different aquatic plants. Experimental results demonstrated that EDCs, except PCP, were removed stably and effectively by aquatic plants through Reaction (1) catalyzed by peroxidases (POs). In relation to EDCs removals, it was found that endogenous H_2O_2 and 3 different fractions of POs were kept constants during continuous treatments. POs were located in almost every part of root cells, while highly concentration zones of POs were observed in epidermis and vascular tissues.

Moreover, in the presence of Fe^{2+}, PCP was quickly removed with the consumption of endogenous H_2O_2, indicating the occurrence of a biological Fenton reaction. These results

demonstrated the effectiveness of aquatic plants for the treatments of trace EDCs as well as refractory and toxic compounds in water and wastewater.

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