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Electro-enzymatic degradation of chlorpyrifos by immobilized hemoglobin

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ABSTRACT

Electro-enzymatic processes, which are enzyme catalysis combined with electrochemical reactions, have been used in the degradation of many environment pollutants. For some pollutants, the catalytic mechanisms of the electrochemical–enzyme reaction are still poorly understood. In this paper, the degradation of chlorpyrifos by a combination of immobilized hemoglobin and in situ generated hydrogen peroxide is reported for the first time. Hemoglobin was immobilized on graphite felts to catalyze the removal of chlorpyrifos in an electrochemical–enzyme system. Under the optimal conditions, more than 98% of the chlorpyrifos was degraded. Furthermore, the degradation products of chlorpyrifos were also studied and identified using liquid chromatography–mass spectrometry analysis. The results suggest a possible degradation mechanism for chlorpyrifos with low power and high efficiency, reveal the feasibility of hemoglobin as a substitute for some expensive natural enzymes, and demonstrate the application of an electro-enzymatic process in the treatment of organophosphorus compounds in wastewater.

1. Introduction

Organophosphorus (OP) pesticides, an alternative to organochlorine pesticides, have been widely used in both agricultural and residential environments [1]. However, their extensive and indiscriminate use may result in their ubiquitous presence in food and drinking water [2,3]. OP pesticides have strong reproductive toxicity, cytotoxicity, immunotoxicity, genotoxicity and inhibitory activity against cholinesterase [4,5]. They are a risk to human health as a consequence of bioaccumulation through the food chain.

Chlorpyrifos (CP, *o*,*o*-diethyl-*o*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is a broad spectrum OP insecticide widely used for pest control in agriculture and, to a lesser degree, for indoor applications [6]. Because the government of India has banned chlorinated hydrocarbon insecticides, such as aldrin, chlordane, and DDT, and recommended CP as an alternative due to its moderate toxicity to humans, the use of CP has increased [7]. However, excessive exposure to CP can produce typical symptoms of acute organophosphate poisoning. Poisoning from CP may affect the central nervous system, cardiovascular system and respiratory system [8,9], and CP acts as a skin and eye irritant [10]. Therefore, the detection and degradation of CP remaining in the environment has become a public concern.

Many scholars have reported the microbial degradation of CP. For instance, Mallick [11] reported the rapid degradation of CP by *Flavobacterium* sp. and *Arthrobacter* sp., which were isolated for the degradation of diazinon and methyl parathion, respectively. These species are able to degrade CP because the characteristic P–O–C linkage of CP is the same as in diazinon, parathion, methyl parathion and fenitrothion. Moreover, Zhang et al. has found that CP is degraded through ultrasound treatment [12]. With the increasing demands of environmental quality, many researchers are conducting further research to seek rapid, simple and economic treatments for the removal of CP from water.

There is a growing recognition that enzymes can be used in many remediation processes to target specific pollutants for treatment [13]. The treatment of organic compounds has been extensively explored with various enzymes, including peroxidases, tyrosinases and laccases [13,14]. Among these enzymes, horseradish peroxidase (HRP) is probably the most studied and, in many ways, can be considered the classical enzyme in the field [15]. However, peroxidase is a protein and shows limited stability, and frequent restandardization of the enzyme activity during storage is required [16]. Because of these issues, a considerable amount of attention has been focused on studies of stable and cheap mimetic enzymes.

Hemoglobin (Hb), a natural macromolecular protein, has a natural quaternary structure. It is a model protein for studies on structure–function relationships of proteins due to its high stability, commercial availability and well-documented structure information [17,18]. Hb is known to have some intrinsic peroxidase activity due to its close structural similarity to peroxidase [19], and this

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makes it possible for Hb to oxidize various organics in the presence of hydrogen peroxide (H_2O_2). Because of its high stability and low cost, Hb is a preferred material for wastewater treatment. In our previous work, we investigated and reported the degradation of bisphenol A in water catalyzed by Hb in an electrochemical reactor; the removal efficiency reached 50.7% under optimal operation conditions in 120 min [20]. This report demonstrated the utilization of Hb in the catalytic-degradation of an organic contaminant.

H_2O_2 is a necessity for the enzymatic oxidation reaction [21]. Due to its instability, H_2O_2 is difficult to store and transport. To overcome these drawbacks, Lee [22,23] used an electrochemical reaction to produce H_2O_2. In the presence of an applied voltage, the consumption of dissolved oxygen leads to the formation of H_2O_2 or H_2O at the cathode according to the following two types of O_2 reduction: (i) $O_2 + 2H^+ + 2e^- \leftrightarrow H_2O_2$ and (ii) $O_2 + 4H^+ + 4e^- \leftrightarrow 2H_2O$ [24]. Accordingly, the electrogeneration of H_2O_2 is an attractive approach because it does not require additional chemicals and electricity is readily available [24]. Currently, some types of electrochemical reactors have been constructed to degrade a variety of phenolic compounds [25], and this technology has been proven to be feasible and effective. Nevertheless, few investigations have reported the use of an electrochemical–enzyme reactor in the removal of an OP insecticide.

In this study, Hb covalently immobilized on graphite felt was used as the cathode in an electrochemical reactor. The removal efficacies of CP through absorption, biochemical and electrochemical processes were compared with those obtained in the electro-enzymatic process with electrogenerated H_2O_2. The energy consumptions of the electrochemical and electro-enzymatic treatments were also compared. The degradation products were identified using LC/MS analysis, and a possible breakdown pathway of chlorpyrifos is proposed.

2. Experimental

2.1. Materials

Hemoglobin (Hb) from bovine blood (MW, 64,500) was obtained from Beijing Biodee Biotechnology Company Ltd. (China). Graphite felt was purchased from Hunan Jiuhua Carbon Company Ltd. (China). All other chemicals were obtained from Shanghai Jingchun Reagent Company (China), were of analytical reagent grade and were used as received without further purification.

2.2. Quantitative analysis

The concentration of electrogenerated H_2O_2 was determined by spectrophotometric analysis using copper (II) and 2,9-dimethyl-1,10-phenanthroline (DMP) at 454 nm in a UV-5301 visible spectrophotometer (Shimadzu, Japan) [26]. The concentrations of CP in the reaction mixtures were measured using high performance liquid chromatography (HPLC, Agilent 1100, USA) equipped with a UV–vis detector set at 300 nm. Analysis was performed on an Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm). The flow rate of the mobile phase, which consisted of methanol and water (90:10, v/v), was 0.8 mL/min. Liquid chromatography–mass spectrometry (LC/MS Agilent Q-TOF 6510, USA) analysis was performed to verify and identify the CP degradation products. Detection was achieved with a UV–vis detector set at 300 nm. Analysis was performed on a C18 column (150 mm × 2.1 mm, 3.5 μm). The mobile phase was methanol–0.1% formic acid (v/v = 90/10), and the flow rate of the mobile phase was 0.8 mL/min. The mass spectrometer was operated in positive ion mode in the m/z 50–800 range.

2.3. Preparation and characterization of enzyme electrode

To increase the stability of Hb, it was immobilized on graphite felt via a previously reported method [23]. As shown in Scheme 1, the graphite felt was cut into 2 cm × 5 cm pieces, which were oxidized at 500 °C for 8 h in air. The carboxylic acid groups formed on the graphite surface were treated by immersion of the oxidized graphite felt in 1 M hydrazine aqueous solution for 3 days, and excess hydrazine was removed by washing with Milli-Q water.

The carbohydrate side chains of Hb were first partially oxidized by adding 0.1 mL sodium periodate (0.1 M) to a 1 mL solution (0.05 M acetate buffer–0.1 M sodium chloride (pH 5)) of Hb (2 mg). The partial oxidation of the carbohydrate residues on Hb proceeded at room temperature for 30 min with the solution covered by aluminum foil to prevent exposure to light. The partially oxidized Hb was added to pretreated graphite felt cubes in 0.05 M acetate buffer–0.1 M sodium chloride (pH 5). The mixture (covered with aluminum foil) was gently tumbled in a shaker (200 rpm) at room temperature for 30 min and then stored at 4 °C overnight. The Hb-loaded graphite felt pieces were washed thoroughly with 0.2 M phosphate buffer (pH 5.5) prior to use.

Cyclic voltammetry was performed with a CHI832A electrochemical workstation (Shanghai Chenhua Co., China) to confirm the immobilization effect of the enzyme. Cyclic voltammetry was obtained from the pure graphite felt and Hb-modified graphite felt in 0.1 M phosphate buffer solution (PBS, pH 7.0). No redox peaks were observed with the pure graphite felt within the potential window (curve a in Fig. 1S). However, a cathodic peak was obtained from the Hb-coated graphite felt (curve b in Fig. 1S), which illustrated that Hb was covalently immobilized on the graphite felt (see supporting information, Fig. 1S).

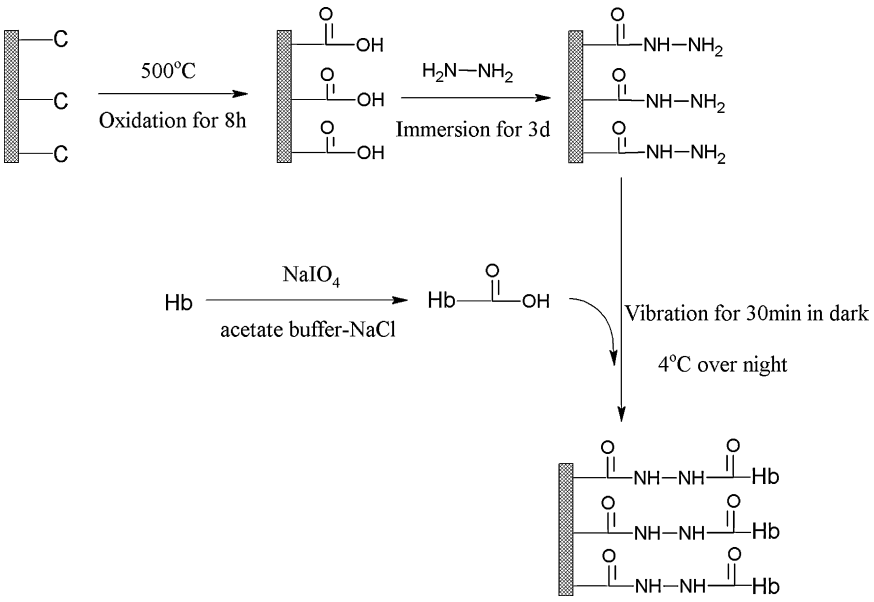
2.4. Electrochemical–enzyme reactor design

The electro-enzymatic oxidation of CP in the aqueous phase was studied in a membraneless electrochemical–enzyme reactor. The reactor had an effective volume of 35 cm^3, and stainless steel (2 cm × 5 cm) and graphite felt immobilized with the Hb (2 cm × 5 cm) was used as the anode and cathode, respectively. When a voltage was applied to the electrodes, the ions moved in the electric field according to their charges. With the two-electron reduction of oxygen [23] and protons generated from water dissociation, H_2O_2 generated at the cathode is continuously supplied to the electrolyte, preventing its decomposition at the anode. The Hb-immobilized electrode catalyzes the degradation of CP using in situ electrogenerated H_2O_2.

2.5. Electrogeneration of H_2O_2 and electrodegradation of CP

During H_2O_2 generation experiments, the electrolyte was a 0.1 M PBS (pH 7.0). The solution was continuously oxygenated at a rate of 25 mL/min for saturation of dissolved oxygen.

In the CP removal experiments, the reaction solution was PBS (0.1 M, pH 7.0) that contained 100 mg/L CP. The electro-enzymatic degradation of CP was studied at a current density of 25 A/m^2, and the experiments were conducted under potentiostatic conditions using a WLS power supply (Sangli, Nanjing). The working conditions of electrochemical degradation were the same as those of the electro-enzymatic method, except for the absence of enzyme. Biochemical degradation of CP with externally supplied H_2O_2 was performed on the Hb-immobilized electrode in the absence of a supplied voltage. Moreover, the adsorption degradation of CP was investigated under the same conditions as those of the biochemical degradation, except for the H_2O_2 was supplied externally.



Scheme 1. Immobilization of hemoglobin on the oxidized graphite felt by hydrazine linkage.

3. Results and discussion

3.1. Electrogeneration of H_2O_2

The enzyme activity is activated by H_2O_2, and consequently, the efficiency of the electro-enzymatic process is dependent on the generation of H_2O_2 [27]. To determine the optimal conditions, the effects of current density, reaction time, and concentration of buffer solution on the generation of H_2O_2 were examined. Fig. 1 presents the concentration of continuously electrogenerated H_2O_2 within the membraneless electrochemical–enzyme system as a function of current density in the range of 5–40 A/m^2. As shown in Fig. 1, the energy consumption increased with current density because the Ohmic relation governs in an electrochemical system [25]. The highest concentration of electrogenerated H_2O_2 was observed at 25 A/m^2. In addition, at this current density, the energy consumption was relatively low. According to the report of Lee et al. [23], a higher potential is needed to reduce O_2 to H_2O. That is, a voltage at a higher current density than 25 A/m^2 may exceed the electrode potential to electrochemically form H_2O_2 [27]. Therefore, in further experiments, the current density was fixed at 25 A/m^2 for the electrogeneration of H_2O_2.

In addition, the effect of reaction time on the electrogeneration of H_2O_2 was investigated. As shown in Fig. 2, the concentration of H_2O_2 increased with increasing reaction time due to its accumulation, while the lowest energy consumption was observed at 90 min or longer. From the point of view of economy, a shorter reaction time is advantageous for practical applications. Based on these results, a 90 min reaction time was selected for further experiments.

The effect of the buffer solution (electrolyte) concentration was also investigated in the range of 0–0.2 M (pH 7.0) at 25 A/m^2. As shown in Fig. 3, the highest H_2O_2 concentration was observed at 0.01 M of PBS, but in this region, the energy consumption was relatively high. Considering H_2O_2 generation and energy consumption, a reasonable concentration of PBS was determined to be 0.1 M.

3.2. Effects of pH and temperature on the enzymatic degradation of chlorpyrifos

The effects of pH and temperature on the degradation efficiency were examined in the pH range from 5 to 9 at 10, 20, 30 or 40 °C for 90 min. From Fig. 4, it was found that the removal efficiencies of CP initially increased and then decreased with pH at any temperature. The maximum degradation efficiencies were obtained at pH

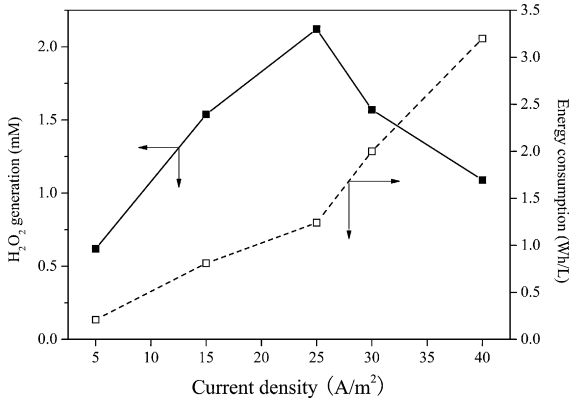


Fig. 1. The effect of current density on the generation of H_2O_2 (■) and the energy consumption (□). [PBS] = 0.1 M, reaction time = 90 min, pH 7 and temperature = 25 °C.

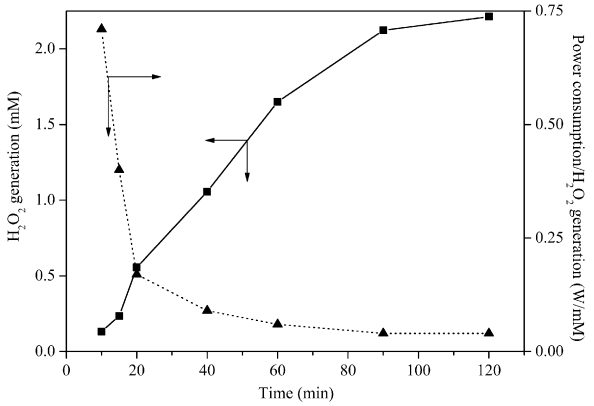


Fig. 2. The effect of reaction time on the generation of H_2O_2 (■) and the power consumption (▲). Current density = 25 A/m^2, [PBS] = 0.1 M, pH 7 and temperature = 25 °C.

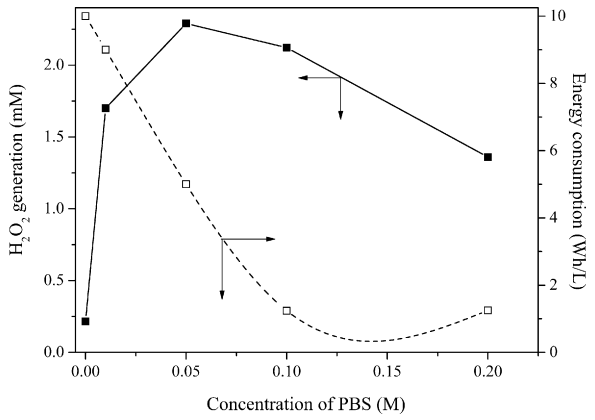


Fig. 3. The effect of concentration of buffer solution on the generation of H_2O_2 (■) and the energy consumption (□). Current density = 25 A/m^2, reaction time = 90 min, pH 7 and temperature = 25 °C.

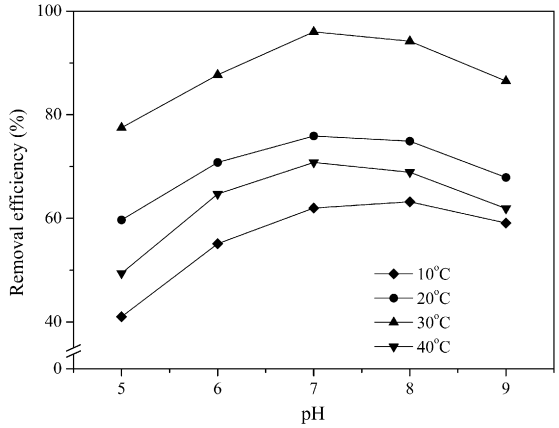


Fig. 4. The effects of pH and temperature on the removal efficiency of CP using the electro-enzymatic method. Current density = 25 A/m^2, reaction time = 90 min and [CP] = 100 mg/L in PBS (0.1 M).

7.0 and 30 °C. Under these conditions, a reducing substrate such as CP was degraded, and the degradation efficiency was highly dependent on the enzyme activity. It has been reported that variations in the pH of the medium affect the ionic form of the active site of the enzyme and change its activity and three-dimensional structure [28].

The rate of enzyme-catalyzed reactions increases with temperature up to a certain limit, but above a certain temperature, it decreases due to denaturation [28]. In addition, an increase in temperature also leads to a lower concentration of dissolved oxygen and the self-decomposition of H_2O_2 [29]. As shown in Fig. 4, the removal efficiency increased for the immobilized enzymes with increasing temperature up to 30 °C. Therefore, the optimal pH and

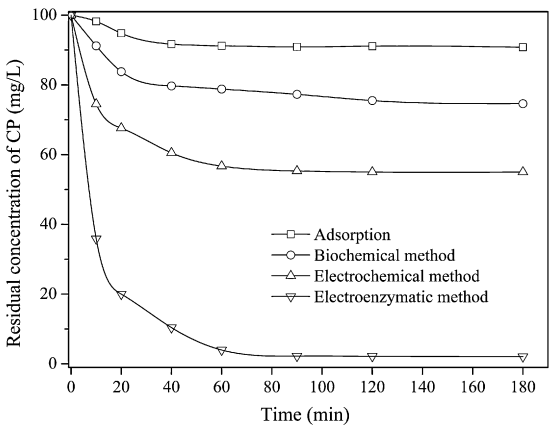


Fig. 5. Removal of CP through the electro-enzymatic (▽), electrochemical (△), biochemical (○) and adsorption method (□)). The electro-enzymatic method was performed in the presence of Hb immobilized on graphite felt under 25 A/m^2. The electrochemical method was performed under 25 A/m^2. The biochemical method was performed in the presence of immobilized Hb and externally supplied H_2O_2. The adsorption method was performed only in the presence of Hb immobilized on graphite felt. [CP] = 100 mg/L in PBS (0.1 M, pH 7), temperature = 30 °C and reaction time = 180 min.

temperature for the catalytic removal of CP were pH 7 and 30 °C, respectively.

3.3. Energy consumption

Table 1 shows the energy consumption during the electro-enzymatic and electrochemical treatments. In the electro-enzymatic treatment of CP, a significant power reduction was observed when compared with the electrochemical oxidation at 25 A/m^2. The results showed that the electrochemical degradation required more energy than the electro-enzymatic method. The electrochemical reaction gave a mole ratio of 36.34 at 1.5 h but led to a CP reduction of only 52.7%. The electro-enzymatic reaction, however, had a mole ratio of 19.09 but led to CP reduction of nearly 100%. It is evident that the electro-enzymatic process significantly improved the efficiency of electrolysis.

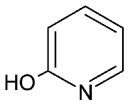
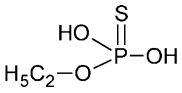
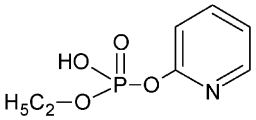
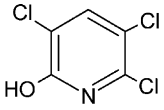
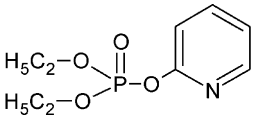
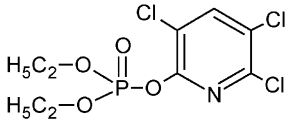
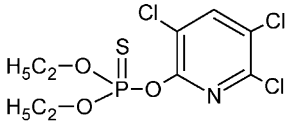
3.4. Comparison of the different treatment technologies

To confirm the effectiveness of the electro-enzymatic degradation, the electro-enzymatic, electrochemical, biochemical and adsorption methods were compared. Fig. 5 presents the degradation of CP using the different procedures. To investigate the effect of adsorption, a control experiment was conducted without an electric current. The results indicated that the removal of CP was partly affected by its adsorption to the graphite felt. It was found that approximately 9.2% of CP was adsorbed.

Table 1
Relationship between CP degradation and energy consumption during the course of the CP degradation reactions at 25 A/m^2 carried out with either the electrochemical or electroenzymatic method.

Time (min)	Electroenzymatic method			Electrochemical method		
	CP degraded (mg/L)	Energy consumed ($\times10^{-5}$ kWh)	Energy consumed/CP degraded ($\times10^{-7}$)	CP degraded (mg/L)	Energy consumed ($\times10^{-5}$ kWh)	Energy consumed/CP degraded ($\times10^{-7}$)
5	35.9	1.01	2.81	18.8	1.04	5.53
10	64.2	2.05	3.19	25.5	2.13	8.35
20	80.0	4.12	5.15	32.4	4.30	13.27
40	89.6	8.29	9.25	45.5	8.57	18.84
60	96.0	12.45	12.97	50.3	12.79	25.43
90	98.7	18.67	18.92	52.7	19.10	36.24

Table 2
Main chlorpyrifos intermediates identified by LC/MS.

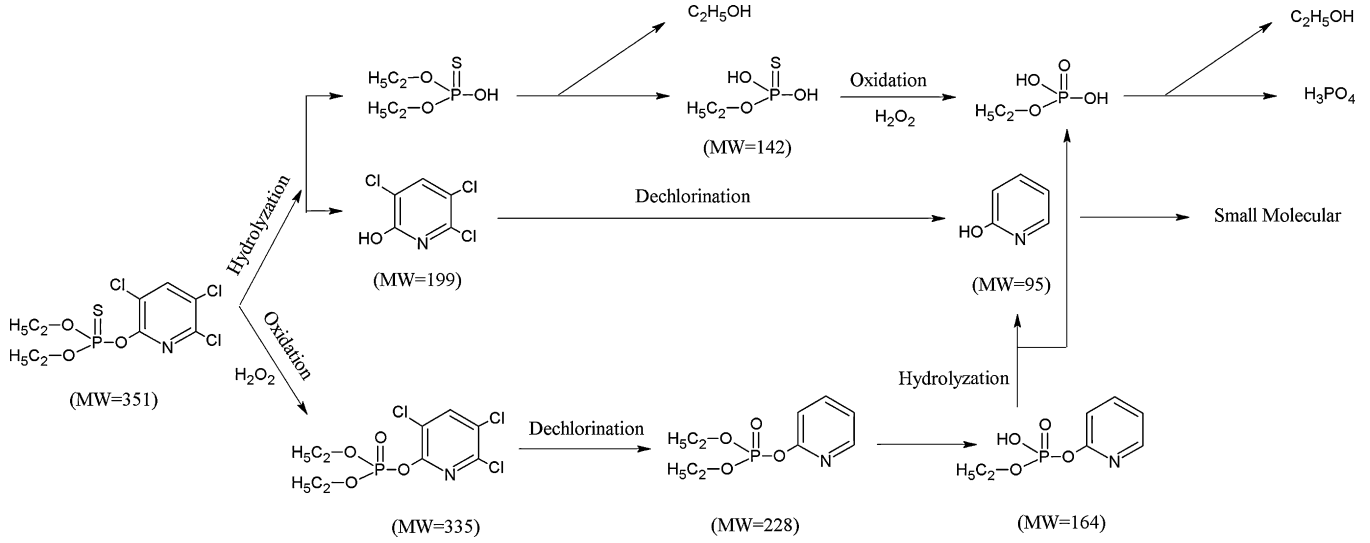
Peak no.	Retention time (min)	*m*/*z*	Molecular weight	Molecular structure	Chemical names
1	0.841	96	95		2-Hydroxypyridine
2	1.593	143	142		Ethyl thiophosphoric acid
3	1.843	165	164		O-ethyl-O-(2-pyridyl)-phosphate
4	2.103	200	199		3,5,6-Trichloro-2-pyridinol
5	5.570	229	228		O,O-diethyl-O-(2-pyridyl)-phosphate
6	16.363	336	335		Chlorpyrifos oxon
7	16.909	352	351		Chlorpyrifos

The H_2O_2 supply strategy (in situ generation or externally supplied) had a significant effect on the rate and on overall degradation efficiency. In the biochemical treatment, the reaction rate was high during the initial 40 min period. However, the overall removal efficiency was much lower than that of the electro-enzymatic method due to the depletion of active H_2O_2 [23].

Only with the H_2O_2 generated in situ was the highest removal efficiency in the electrochemical treatment achieved, which was 52.7%. This finding illustrates that the electrochemical method could remove part of the CP through radical reactions or/and H_2O_2 oxidation, but that the degradation was not satisfactory. When Hb was added to the electrochemical reactor, CP was rapidly degraded by the immobilized Hb and the electrogenerated H_2O_2. It was clear that Hb played an important role in the electro-enzymatic oxidation of CP.

3.5. Degradation pathway of chlorpyrifos

To identify the breakdown species of CP during the electro-enzymatic process, the samples were analyzed by LC/MS. The *m*/*z*



Scheme 2. Possible degradation pathways of CP during the electro-enzymatic oxidation.

values for each peak correspond to $[M+1]^+$ ions in positive ion mode. Table 2 lists the main fragments obtained for the intermediate products.

In most cases described to date, there are two main degradation reactions for CP, hydrolysis and oxidation [30]. As shown in Scheme 2, CP was first degraded by hydrolysis to produce diethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP). Next, the DETP was further hydrolyzed and oxidized to form alcohols and H_3PO_4. The TCP was then dechlorinated on the pyridine ring by reacting with the immobilized Hb and in situ generated H_2O_2. The CP was also oxidized to form chlorpyrifos-oxon (CPO). Subsequently, the CPO was dechlorinated and hydrolyzed to produce the O,O-diethyl-O-(2-pyridyl)-phosphate and 2-hydroxypyridine, respectively. All of these intermediates will eventually decompose to generate small molecules such as alcohols, H_3PO_4, CO_2 and H_2O.

4. Conclusion

This study focused on the degradation of chlorpyrifos using a membraneless electrochemical reactor with immobilized hemoglobin. The electrochemical reactor had a stainless steel anode and graphite felt covalently immobilized with hemoglobin as a cathode. Compared with other methods, the electro-enzymatic process provided better removal efficacy in a shorter period of time. From the point of view of economy, the electro-enzymatic treatment is an effective method for the removal of chlorpyrifos with low energy consumption. Moreover, the LC/MS data illustrated that CP was degraded by hydrolysis and oxidation to form various small molecules such as alcohols, H_3PO_4, CO_2 and H_2O. The results clearly demonstrate that the electro-enzymatic process with both Hb and H_2O_2 generated in situ is a notable approach in degrading organopollutants through enzyme catalysis and electrochemical reactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.01.080.

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