Enzyme Immobilization Using Single-Walled Carbon Nanotubes

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***Abstract*—**The *cphA-I* gene extracted from *Arthrobacter chorophenolicus* A6 was cloned, overexpressed, and purified to obtain hydroxyquinol 1,2-dioxygenase (cphA-I) enzyme cleaving aromatic hydrocarbons. The cphA-I enzyme was immobilized onto single-walled carbon nanotubes (SWCNTs) either by physical adsorption and covalent bond. SWCNTs was found to be an effective enzyme support owing to their high surface area for enzyme interaction, small size, good dispersion characteristics in solution, low mass transfer resistance, and effective functionalization at their surface. The Michaelis-Menten model parameters for the free and immobilized enzymes were obtained to examine their activities for the catechol and 4-chlorocatechol as substrates. The results showed that cphA-I enzyme retained a relatively high percentage of specific activity for substrates tested and that the physically adsorbed enzymes exhibited better catalytic efficiency than the covalently immobilized enzymes. This study supported an idea that the enzyme immobilization onto SWCNTs can provide a new enzyme immobilization technique.

***Keywords*—**catechol, 4-chlorocatechol, cphA-I, enzyme immobilization, single-walled carbon nanotubes

I. Introduction

CATECHOL and 4-chlorocatechol are intermediates produced in the microbial decomposition of various aromatic compounds and therefore, its level in waters is strictly regulated in many countries [1]. Biodegradation is an effective treatment way for those aromatic contaminants and it basically uses microorganisms based on their enzymatic activities. *Arthrobacter chorophenolicus* A6 is a gram-positive actinobacterium that can effectively decompose the 4-chlorophenol, catechol, and related aromatic compounds [2]. It was found that *cphA-I* gene extracted from *Arthrobacter chorophenolicus* A6 can be cloned, overexpressed, and purified and the hydroxyquinol 1,2-dioxygenase (cphA-I) enzyme can can cleave catechol and 4-chlorocatechol [2]. However, there are several disadvantages associated with biodegradation of pollutants, for example, slow induction of enzymes specific to pollutant degradation, slow growth of degrading microbes, difficulty in control and maintenance of environment for the microbial growth [3]. In spite of fast and highly effective decomposition of organic contaminants when enzymes are used, their direct application in the environmental treatment processes has been quite limited due to loss of enzyme activity and low operating stability. Enzyme immobilization is one of solutions for enzyme activity preservation. The potential of enzyme immobilization for practical application depends on the properties of carriers and immobilization techniques. Single-walled carbon nanotubes (SWCNTs) have been used as carrier because of their properties such as high surface area, low mass transfer resistance and easily functionalization on their surface [4]. Therefore, in this study, the cphA-I enzyme was immobilized onto (SWCNTs) to preserve its activity via physical adsorption and covalent bonding. The activity of the immobilized enzyme was quantitatively examined.

II. Materials and Methods

A. Materials

SWCNTs (diameter and length = 1-2 nm and 10 µm, respectively) were purchased from Carbon Nano-Material Technology Company (Gyeongbuk, Korea). Quick Start Bradford solution was purchased from Bio-Rad (Hercules, CA, USA). All other chemicals and reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received.

B. Cloning, Overexpression, and Purification of Enzyme

The *cphA-I* gene was extracted from *Arthrobacter chorophenolicus* A6 and it was cloned, overexpressed, and purified to obtain the hydroxyquinol 1,2-dioxygenase (cphA-I) enzymes. The procedure for cloning, overexpression, and purification of cphA-I enzyme was described in our previous study [5].

C. Physical Adsorption of Enzyme onto SWCNTs

Enzyme was immobilized onto SWCNTs by physical adsorption. 2 mg of SWCNTs was sonicated in 1.5 ml of

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dimethylformamide (DMF) for 30 min to obtain a uniformly dispersed solution. Then, the mixture was washed with 50 mM phosphate buffer (pH 7.0). This was achieved through repeated sonication. Four cycles of washings were performed to allow unfunctionalized SWCNTs dispersed more in the aqueous solution. SWCNTs were then added to the solution of cphA-I enzyme in the same buffer and the mixture was stirred on a rotator for 2 h at 25°C. After incubation, the SWCNTs were centrifuged at 8,000 × *g* and the supernatant was discarded. Immobilized cphA-I enzyme was washed with fresh buffer to remove unbound enzymes until no enzyme was detected from the washing solution.

D. Covalent Immobilization of Enzyme onto SWCNTs

32 mg of SWCNTs sample was added in 8 mL of concentrated H_2SO_4:HNO_3 (3:1, v/v) and sonicated in an ultrasonic bath for 4 h. Distilled water was added to sample (16 mL) and centrifuged at 4°C and 11,000 × *g* for 20 min. The supernatant was separated out and rinsed four times with fresh distilled water (pH~7). This SWCNT-COOH was freeze-dried and stored at room temperature. SWCNT-COOH (1 mg) was added in 1 mL of 2- (*N*-morpholino) ethanesulfonic acid (MES) buffer (50 mM, pH 7.0), followed by addition of 1 mL of 400 mM NHS in MES buffer. The mixture was sonicated in an ultrasonic bath for 30 min. The 20 mM of EDC was added to initiate coupling of NHS to the carboxylic groups on SWCNT-COOH. This mixture was mixed on a rotating shaker 30 min at 65 rpm, 25°C. The suspension was filtered through a Nylon 66 membrane (pore size 0.2 μm) and rinsed thoroughly with MES buffer (50 mM, pH 7.0) to remove the excess of NHS, EDC and by-product urea. The nanotube film was re-dispersed to cphA-I enzyme and sonicated by for 1 min. The mixture was mixed on a rotating shaker 2 h at 65 rpm, 25°C. The CNT–enzyme conjugate was then filtered through a Nylon 66 membrane (pore size 0.2 μm) and washed three times with phosphate buffer and once with 1% Tween-20 to remove unbound cphA-I enzyme. The CNT–enzyme conjugates were then re-dispersed in fresh buffer.

E. Determination of Enzyme Loading onto SWCNTs

Protein concentration was measured by a spectrophotometer (UV/VIS Spectrophotometer, Optizen POP, Deajeon, Korea, wavelength of 595 nm) using Quick Start Bradford solution. The amount of enzyme loaded was determined by measuring the difference in concentrations of enzymes in solution before and after the immobilization onto SWCNTs.

F. Enzyme Activity Test

The activity of cphA-I enzyme was measured using catechol and 4-chlorocatechol as a substrate. Free and immobilized enzymes were mixed with substrate in the presence of H_2O_2 (molar ratio of 1:2) at room temperature. The initial reaction rates were measured at various initial concentrations of substrate using a high-pressure liquid chromatography (HPLC, Agilent Technologies, Palo Alto, USA). HPLC was performed on an Eclipse XDB C18 column (5 µm) at a mobile phase composed of acetonitrile and water (3:7) and a flow rate of 1.0 ml/min. One unit of activity was defined as the amount of enzyme required to catalyze the oxidation of 1 µmol substrate in 1 min. The Michaelis-Menten parameters were determined by measuring the initial rates of reaction with substrate (0.1 – 20 mM) at room temperature and the model parameters were achieved by the best fitting of the experiment results to model equation. The Michaelis–Menten model was employed to describe the kinetic behavior:

$$v = \frac{v_{\max} C_S}{K_M + C_S} \tag{1}$$

where v is the rate of substrate utilization, v_{max} is the maximum rate of substrate utilization, K_M is the half-substrate constant indicating enzyme–substrate affinity, and C_S is the initial substrate concentration. The Lineweaver–Burk plots were used to determine kinetic parameters by linear regression.

III. Results and Discussion

A. Enzyme Immobilization Yield

Enzymatic loading values and immobilization yields in the case of physical adsorption (Table I) were much higher than those in the case of covalent bonding. The enzyme loading values using physical adsorption were similar to those reported in previous studies that used soybean peroxidase and alpha-chymotrysin [6] and amyloglucosidase [7]. For enzyme immobilization using covalent bonding, the reported enzyme loading for horseradish peroxidase and Subtilisin Carlsberg were as high as 1.3 mg-enzyme/mg-SWCNT) [8]. Moreover, the previous study demonstrated that enzyme immobilization yields on carbon-based nanomaterials using both non-covalent and covalent bonding were increased by increasing enzyme dosage [9].

TABLE I
Enzyme Immobilization Yield

Immobilized Enzyme	Enzyme Loading (µg-enzyme/mg-SWCNT)	Immobilization Yield (%)
Physical adsorption	523	31
Covalent immobilization	360	21

B. Kinetic Comparison

The Michaelis-Menten kinetics of the reactions catalyzed by free and immobilized cphA-I enzyme were examined using catechol and 4-chlorocatechol as substrates. The model parameters were determined graphically (Fig. 1 (a) and (b)) and summarized in Table II. For catechol substrate, the enzyme immobilized by physical adsorption retained a maximum reaction velocity (V_{max}) of 3.20 mM/min with a substrate affinity constant (K_m) of 0.67 mM. These kinetic parameters corresponded to a relative substrate utilization activity of 30%. The catalytic efficiency (V_{max}/K_m) was 89% of that for the free enzyme. For the enzyme immobilized by covalent bonding, the V_{max} values (1.49 mM/min) was smaller than that of the enzyme immobilized by physical adsorption. The relative

substrate utilization activity was 14%. The V_{max}/K_m values retained 11% of that for the free enzyme. For 4-chlorocatechol substrate, the enzyme immobilized by physical adsorption retained a maximum reaction velocity (V_{max}) of 2.62 mM/min with a substrate affinity constant (K_m) of 0.54 mM. These kinetic parameters corresponded to a relative substrate utilization activity of 35%. The catalytic efficiency (V_{max}/K_m) of physical adsorption sample retained 62% of that of the free enzyme. For the enzyme immobilized by covalent bonding, the V_{max} values (2.06 mM/min) was smaller than that of the enzyme immobilized by physical adsorption. The relative substrate utilization activity was 28%. The V_{max}/K_m values retained 9% of that for the free enzyme. Typically, the differences in K_m for free and immobilized enzymes are due to their diffusional limitations, steric effects, and ionic strength. The change in affinity of enzyme to substrate probably resulted from structural changes in the enzyme introduced by the immobilization procedure or by lower accessibility of the substrate to the active site of immobilized enzyme.

TABLE II
MICHAELIS-MENTEN MODEL PARAMETERS

Enzyme	V_{max} (mM/min)	K_m (mM)	V_{max}/K_m (min^{-1})
Catechol			
Free enzyme	10.76	2.03	5.31
Physical adsorption	3.20	0.67	4.75
Covalent immobilization	1.49	2.55	0.58
4-Chlorocatechol			
Free enzyme	7.45	0.95	7.81
Physical adsorption	2.62	0.54	4.83
Covalent immobilization	2.06	2.88	0.72

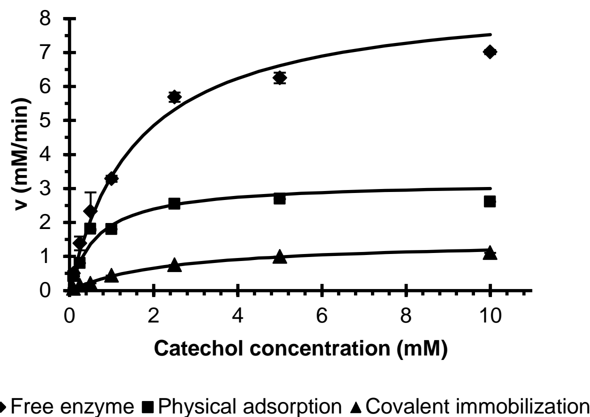

Fig. 1(a) Michaelis-Menten plot for catechol as substrate. Error bars denote standard deviations (n = 3).

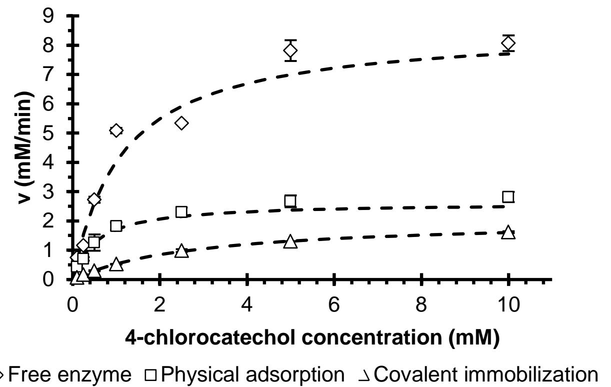

Fig. 1(b) Michaelis-Menten plot for 4-chlorocatechol as substrate. Error bars denote standard deviations (n = 3).

IV. CONCLUSIONS

The cphA-I enzyme interacted with SWCNTs, depending on the enzyme immobilization methods. The cphA-I enzyme retained high activity when it was physically adsorbed onto or covalently bound to SWCNTs. These results demonstrated that the potential interaction of enzyme and CNT surface in each enzyme is unique, depending on its properties and substrates. Therefore, more investigation needs to be performed, including, different types of enzymes and chemical functionalization based on our understanding of environmental engineering field.

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