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Article

Catalytic performance and stability of C–C bond hydrolase BphD immobilized onto single-wall carbon nanotubes

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ABSTRACT

Single-wall carbon nanotubes (SWCNTs) possess unique mechanical properties and extraordinary thermal conductivity, and were used as the matrix for immobilized biocatalysts. The C–C bond hydrolase BphD was immobilized on SWCNTs by physical adsorption and covalent bonding. The relative activity, stability, and reusability of the immobilized enzyme were investigated. BphD immobilized by physical adsorption retained 52.5% of the activity of free BphD, and BphD thermal stability and denaturant resistance were also improved. Covalently bound BphD exhibited the activity nearly the same as that of free BphD (99.7% of initial activity), but its stability showed no significant improvement over that of free BphD.

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1. Introduction

Single-wall carbon nanotubes (SWCNTs) are one-dimensional nanomaterials widely used in etching, photoelectrocatalysis, biosensors, and high-resolution imaging, because of their unique mechanical, electrical, and chemical properties [1]. Enzymes including horse radish peroxidase (HRP) and lipase have recently been immobilized on SWCNTs, demonstrating SWCNTs' potential as an immobilization matrix for biocatalysts [2–5]. Asuri et al. [6] adsorbed soybean peroxidase and α -chymotrypsin on SWCNTs, with enzyme loadings reaching 600 mg/g. They also covalently immobilized HRP on SWCNTs, with the loading amount reaching 1.3 mg/g and HRP retaining 40% of its initial activity. Pedrosa et al. [7] covalently immobilized parathion hydrolase on SWCNTs and multi-wall carbon nanotubes (MWNTs), obtaining biosensors for monitoring the presence of parathion. Immobilized parathion hy-

drolase retained 75% of its initial activity, even after storage for several months. While such SWCNT-immobilized enzymes retained high loading amounts and activities, some issues require further attention. First, the enzyme types immobilized on SWCNTs have been relatively limited, and the immobilization of other enzymes should be investigated. Second, the detailed effect of physical adsorption and covalent bonding on loading amounts and relative activities remains unclear. Last, the SWCNT binding site and enzyme binding mechanism remain unclear.

C–C bond hydrolases are involved in the *meta*-cleavage degradation pathway of aromatic compounds. They catalyze the C–C bond hydrolysis of *meta*-cleavage products. This step requires high energy and is the rate-limiting process of the overall degradation [8]. Researchers have recently demonstrated the unique catalytic promiscuity of C–C bond hydrolases, i.e. catalyzing different reactions using the same active site.

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For example, the C–C bond hydrolase MhpC can catalyze the hydrolysis of C–C, C–O bond, S–O and even the reverse synthesis of C–C bonds, so has potential biosynthesis applications [9]. We cloned the genes encoding the C–C bond hydrolase BphD from the strain *Dyella ginsengisoli* LA-4 and expressed in recombinant *E. coli* [10]. In this study, we immobilized BphD on SWCNTs, and compared the catalytic performance and stability of enzymes immobilized by physical adsorption and covalent bonding. A molecular simulation was used to account for differences in residual activity.

2. Experimental

2.1. Materials

The strain was recombinant *E. coli* BL21(DE3) containing the gene *bphD*, which encoded the C–C bond hydrolase BphD.

Lysogeny broth (LB) medium was used, which contained NaCl 10.0 g/L, tryptone 10.0 g/L and yeast extract 5.0 g/L. The pH was adjusted to 7.0.

SWCNTs (95+%) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). The SWCNT length was 5–15 μm , and their diameter was < 2 nm. 2-(*N*-Morpholino)-ethanesulfonic acid (MES), *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) were purchased from Sinopharm (Shanghai, China). Kanamycin sulfate, isopropyl β -D-1-thiogalactopyranoside (IPTG), and *p*-nitrophenyl benzoate were purchased from J&K Chemical (Beijing, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Culture of bacteria

Bacterial liquid (200 μl) was transferred into 25 ml of LB medium containing kanamycin sulfate (30 mg/L), and incubated at 30 °C and 150 r/min for 12 h. 2 ml of incubated bacterial liquid was transferred into 100 ml of LB medium containing kanamycin sulfate (30 mg/L), and incubated at 37 °C and 150 r/min until the optical density values reached 0.4–0.6. IPTG (1 mmol/L) was then added for 3 h to induce the expression of BphD. The broth was centrifuged at 4 °C and 10000 r/min for 5 min. The precipitate was washed twice with sodium phosphate buffer (PBS) and stored at –80 °C in a freezer until use.

2.3. Modification of SWCNTs

Step 1: Carboxylation. To functionalize with carboxyl groups, SWCNTs (0.1 g) were suspended in 400 ml of 3:1 v/v H_2SO_4 : HNO_3 , and sonicated at 30 °C for 5 h. The suspension was diluted with MilliQ water and filtered through a 0.22 μm polycarbonate membrane. The nanotube film was resuspended into MilliQ water and sonicated again to remove any residual acid. The suspension was centrifuged and the precipitate dried at 50 °C under vacuum for 8 h. The solid obtained was characterized by Fourier transform infrared spectroscopy (FTIR).

Step 2: Carboxyl group activation. The acidified SWCNTs

were dispersed in a MES buffer, and an equal volume of 400 mmol/L NHS was added. The suspension was sonicated for 30 min. The addition of 20 mmol/L EDC initiated the coupling of NHS to the carboxylic groups, and the mixture was stirred at 150 r/min for 1 h. Excess NHS and EDC were separated through a 0.22 μm polycarbonate membrane. The precipitate was dried at 80 °C under vacuum for 8 h and named CM-SWCNT.

2.4. Purification of BphD

BphD was purified based on the affinity of the His-tag for nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography. *E. coli* cells harvested above were resuspended in 200 mmol/L PBS and lysed by sonication for 40 min. After centrifugation at 22000 r/min for 20 min, the supernatant was separated and used as the crude extract for further purification. Crude extracts were bound to the resin in the equilibrating buffer (100 mmol/L phosphate buffer containing 0.5 mol/L NaCl and 20 mmol/L imidazole, pH 7.4), and the target His-tagged BphD was eluted by the buffer containing 325 mmol/L imidazole. Fractions were collected, dialyzed and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Immobilization of BphD

The optimal immobilization pH was tested in a 3 ml reaction volume. The concentration of BphD was 30 $\mu\text{g}/\text{ml}$ and the SWCNT mass was 0.5 mg. The activity of immobilized BphD was monitored by absorption at 405 nm using a UV-visible spectrophotometer. The pH values were set to 6, 7, 8, 9, and 10 using different buffers. Tubes were incubated at 30 °C and 150 r/min. After ensuring the optimal pH was achieved, SWCNTs and CM-SWCNTs were each used to immobilize BphD. The concentrations of BphD were set as 15, 30, 60, and 90 $\mu\text{g}/\text{ml}$. SWCNTs or CM-SWCNTs (1 mg/ml), protein and a corresponding volume of PBS (pH 7.0) were mixed and incubated at 30 °C and 150 r/min. The relative activity and loading amount were monitored by absorption of 405 and 595 nm, respectively [11]. Sample (60 μl) was removed after a specific interval to determine the residual protein amount using the Bradford method [12].

2.6. Characterization of immobilized BphD

The reaction volume for characterizing immobilized BphD was 2 ml, which contained 40 μl of immobilized BphD, 40 μl of substrate and 1920 μl of PBS. One unit of enzymatic activity was defined as the quantity of enzyme required to produce 1 $\mu\text{mol}/\text{L}$ of *p*-nitrophenol.

Effect of temperature on the activity of immobilized BphD. Immobilized and free BphD were each incubated at 30, 40, 50, 60, and 65 °C for 15 min. The activity was measured when the solution was cooled to room temperature.

Thermostability of immobilized BphD. Immobilized and free BphD were each incubated at 60 °C, and the activity deter-

mined every 5 min. The initial activity was normalized as 100%, and the change in relative activity was recorded.

Effect of denaturing agent on the activity of immobilized BphD. Immobilized and free BphD were each incubated in guanidine hydrochloride (GdmCl) for 15 min, and the activity of the enzyme was then recorded. The concentrations of GdmCl used were 0, 0.2, 0.4, 0.8, and 1.6 mmol/L.

Reusability of immobilized BphD. After one cycle of use, immobilized BphD was collected by centrifuging at 12000 r/min for 5 min, and resuspended in the same volume of PBS. The activity of the resuspended immobilized enzyme was determined, and the change in relative activity was recorded.

2.7. Homology modeling, docking and prediction of the covalent binding site

The three-dimensional structure of BphD was modeled using the online server SWISS-MODEL program (<http://swissmodel.expasy.org>). The BphD protein sequence was set to “Automated Mode” [13]. Molecular docking of a SWCNT and BphD was performed using Patchdock and Fire-dock [14]. The covalent binding site was predicted using the method provided by Pamela et al. [15]. The pK_a values for residues containing amino groups, i.e. for the *N*-terminal residue, Lys and Tyr, were calculated using the online server PROPKA. The *LIG Re* value of each amino group was then calculated using:

$$LIG Re = 10^{(pH-pK_a)}$$

When *LIG Re* was < 0.1, the amino group was considered to have low activity. When *LIG Re* was 0.1–1 and > 1, it had moderate and high activity, respectively.

3. Results and discussion

3.1. Modification and characterization of SWCNTs

To covalently attach protein molecules, the SWCNT surface was functionalized with carboxyl groups that were active to NHS. The modification is shown in Fig. 1.

FT-IR spectra of pristine, carboxylated and acylated SWCNTs are shown in Fig. 2. The characteristic C=O stretching vibration at $\sim 1720\text{ cm}^{-1}$ indicating carboxyl group modification [16]. The band at 1645 cm^{-1} in the NHS/EDC modified SWCNT sample was due to the amide C=O stretching vibration.

Fig. 2. FT-IR spectra of pristine SWCNTs (1), $\text{H}_2\text{SO}_4/\text{HNO}_3$ oxidized SWCNTs (2), and NHS/EDC modified SWCNTs (3).

3.2. Optimal pH for immobilization

The main force determining the interaction between BphD and SWCNTs was electrostatic. The protein and SWCNT charges were both affected by pH, so it was important to determine the optimal pH for immobilization. Relative activities and loading amounts of immobilized BphD were compared. Figure 3 shows the effect of pH on the relative activities of free and immobilized BphD. BphD-SWCNT, BphD-CM-SWCNT, and BphD all had similar optimal pH values at around 7.0. BphD-SWCNT exhibited a slightly higher relative activity than the other two samples at pH 6.0, while BphD-CM-SWCNT had a slightly higher relative activity at pH >7.5. SWCNT and CM-SWCNT could each

Fig. 3. Effect of pH on the relative activities of free and immobilized BphD.

Fig. 1. Covalent modification process of SWCNT.

be fully loaded with the added protein molecules. The following experiments were performed at pH 7.0.

3.3. Adsorption kinetics of BphD

The adsorption kinetics of BphD on the surface of SWCNTs and CM-SWCNTs were determined at different protein concentrations. The curves displayed in Fig. 4 show the relationship between adsorption percentage and time. Figure 4(a) shows that when the protein concentration was low (15 and 30 $\mu\text{g/ml}$), adsorption reached equilibrium within 2 h, and the adsorption percentage was nearly 100%. At higher concentrations (60 and 90 $\mu\text{g/ml}$), loading equilibrium was reached within 3 h, but the adsorption percentage only reached 60%. The adsorption kinetics of BphD on CM-SWCNT were similar to that on SWCNT, but the time required to reach adsorption equilibrium was typically much longer (for a constant protein concentration). This result could be explained by the interaction between BphD and CM-SWCNT being more specific.

According to calculations based on adsorption in Fig. 4, maximum loadings were obtained at a protein concentration of 90 $\mu\text{g/ml}$. Therefore, the following experiments were all performed at this concentration. Under these conditions, the loading amounts of SWCNT and CM-SWCNT were 255 and 518 mg/g, respectively. BphD-SWCNT retained 52.5% of its initial activity, while BphD-CM-SWCNT retained 99.7% of its initial activity. The loading amount and relative activity of BphD-CM-SWCNT were both higher than those of BphD-SWCNT, which may have been due to covalent bonding retaining the BphD conformation of its free form. The result where CM-SWCNTs exhibited a larger loading amount than pristine SWCNT was consistent with Asuri et al. [6].

3.4. SEM observations of morphology of immobilized BphD

The surface morphology of immobilized BphD was characterized by scanning electron microscopy (SEM). Figures 5(a)

Fig. 4. Adsorption of BphD on SWCNT (a) and CM-SWCNT (b).

and 5(c) show the morphology of SWCNTs and CM-SWCNTs, respectively. SWCNTs and CM-SWCNTs both existed as clusters, but the size of the CM-SWCNT clusters was more variable. Figures 5(b) and 5(d) show the morphology of BphD-SWCNT and BphD-CM-SWCNT, respectively. Amorphous particles appeared among nanotubes and could have been crystalline phosphate.

3.5. TEM observations of morphology of immobilized BphD

Protein molecules cannot be observed using SEM, so immobilized enzymes were characterized using transmission electron microscopy (TEM). The morphologies of SWCNTs and CM-SWCNTs were not significantly different, as shown in Figs. 6(a) and 6(b), respectively. Figures 6(c) and 6(d) show the morphologies of BphD-SWCNT and BphD-CM-SWCNT, respectively. These both appeared to show colloidal materials covering the nanotubes, which may have been protein molecules.

Fig. 5. SEM images of SWCNT (a), BphD-SWCNT (b), CM-SWCNT (c), and BphD-CM-SWCNT (d).

Fig. 6. TEM images of SWCNT (a), CM-SWCNT (b), BphD-SWCNT (c), and BphD-CM-SWCNT (d).

Fig. 7. Effect of temperature on the activity of free BphD and BphD-CNT.

More colloidal material was apparent in the TEM image of BphD-CM-SWCNT, consistent with the higher loading amount observed earlier.

3.6. Characterization of immobilized BphD

BphD-SWCNT and BphD-CM-SWCNT were each characterized, and the effects of temperature, pH, and denaturant on enzyme activity were investigated. The thermal stability and reusability of immobilized BphD were also determined.

3.6.1. Effect of temperature on BphD activity

Figure 7 shows the effect of temperature (30, 40, 50, 60, and 65 °C) on the relative activities of the enzymes. The highest relative activity of free BphD and BphD-SWCNT were both observed at 50 °C, while BphD-CM-SWCNT exhibited its highest activity at 40 °C. Basha et al. [17] immobilized an invertase enzyme from thermophilic fungus using phenyl-sepharose, and found the optimal temperature for immobilized invertase was lowered by 10 °C. This was due to a conformation change of the enzyme during immobilization, which enhanced the product inhibition at higher temperatures.

3.6.2. Effect of pH on BphD activity

The effect of pH on the activity of BphD immobilized at pH 7.0 was investigated. Figure 8 shows that the relative activity changed with pH, and that the optimal pH for both BphD-SWCNT and BphD-CM-SWCNT was 7.0. At lower pH, the relative activity of immobilized BphD was significantly higher than that of free BphD. At pH 6.0, 62% of BphD activity was retained for BphD-SWCNT, while only 25% was retained for free BphD. This indicated that the tolerance for changing pH could be enhanced through immobilization and the change in microenvironment.

3.6.3. Thermostability of immobilized BphD

The thermostability of immobilized BphD was investigated at 60 °C, and the result is shown in Fig. 9. After incubation at 60 °C for 40 min, both immobilized and free BphD lost at least part of their activity. Free BphD lost all activity within 25 min, while BphD-SWCNT exhibited improved thermostability compared

Fig. 8. Relative activity of free and immobilized BphD at different pH values.

with free BphD. The activity of BphD-CM-SWCNT decreased more quickly than that of free BphD, the reason for which may be similar to the decreased optimal temperature.

3.6.4. Effect of denaturant on BphD activity

GdmCl is a commonly used protein denaturant, and can denature protein molecules by capturing bound water necessary for function. Figure 10 shows the effect of different concentrations of GdmCl on the relative activity of BphD. BphD-SWCNT exhibited an improved tolerance to GdmCl compared with the other two samples. When the GdmCl concentration reached 1.6 mmol/L, BphD-SWCNT still retained 33% of its initial activity. BphD-CM-SWCNT did not exhibit any improvement to denaturant compared with free BphD.

3.6.5. Reusability of immobilized BphD

The reusability of BphD-SWCNT and BphD-CM-SWCNT was tested over 10 cycles. Figure 11 shows that BphD-SWCNT retained 90% of its initial activity after five cycles, while BphD-CM-SWCNT lost all activity. The stability of a covalently bound enzyme is usually greater than that of a physically adsorbed enzyme. However, in the current situation the reusability of BphD-CM-SWCNT was worse than BphD-SWCNT. This may have resulted from few ionized amino groups being present in BphD at pH 7.0. According to the calculation method

Fig. 9. Thermostability of free and immobilized BphD at 60 °C.

Fig. 10. Effect of denaturant concentration on the activity of free and immobilized BphD.

provide by Torres-Salas et al. [15], the pK_a values of the *N*-terminal residues, Lys and Tyr in BphD were estimated. The *N*-terminal residue possessed moderate activity ($0.1 < \text{LIG Re} < 1$), while those of the Lys and Tyr residues possessed low activities. This could explain the lower reusability observed for BphD-CM-SWCNT.

3.7. Homology modeling and molecular docking

Because of limited available experimental techniques, it was not possible to directly observe the binding site of the enzyme and SWCNT, so the binding site was modeled through molecular docking. The three-dimensional structure of BphD was first constructed by SWISS-MODEL, using the crystal structure of BphD from *Burkholderia xenovorans* LB400 as a template (sequence identity was 88.73%). The BphD model is shown in Fig. 12, and suggested that BphD existed as a homotetramer. The model quality was evaluated by the PROCHECK program in the online server UCLA-DOE (<http://www.doe-mbi.ucla.edu/>). The Ramachandran plot showed a reasonable conformation of the main chain. 90.7% of the residue dihedral angles φ and ψ were located in favor of the allowed region. Therefore, the model was suitable for further docking studies [18].

The binding mode of BphD and SWCNT was generated by Patchdock and Firedock. All possible binding sites of SWCNT were far removed from the active site of BphD (Fig. 13(a)). The

Fig. 11. Reusability of immobilized BphD.

Fig. 12. Three-dimensional structure of BphD generated from homology modeling.

decreased catalytic efficiency of BphD-SWCNT could not have resulted from the insertion of the SWCNT into the BphD active site. When an inhibitor is bound at the interface of adjacent subunits, it can decrease catalytic efficiency through disrupting the interaction between subunits. We identified the interaction force between subunits. Taking subunits B and C as an example, two salt bridges, Glu717-Lys438 and Lys720-Glu435, were important for the interaction of subunits. Figure 13(b) shows the interaction site of SWCNT and BphD. When the size of the SWCNT was 0.5 nm, it could insert into the subunit interface and disrupt the salt bridges, decreasing the catalytic efficiency of BphD-SWCNT. Zuo et al. [19] investigated the toxic effect of

Fig. 13. Interaction between BphD and SWCNT, showing an overview (a) and enlargement of the interaction site (b).

Graphical Abstract

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Catalytic performance and stability of C–C bond hydrolase BphD immobilized onto single-wall carbon nanotubes

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The C–C bond hydrolase BphD was immobilized on single-wall carbon nanotubes by physical adsorption and covalent bonding. The former imparted higher stability while the latter imparted higher activity on the immobilized enzyme.

SWCNTs on the WW domain of the human Yes-associated protein using molecular dynamics. They suggested that SWCNTs could disrupt and block the active sites, thus reducing the possibility of direct binding of the ligand. The active site of BphD was buried in a hydrophobic core not easily accessed by the SWCNT. The binding site of CM-SWCNT could not be predicted through molecule docking, because the covalent interaction was much stronger than intermolecular interactions (i.e. hydrogen bonding, salt bridges and Van der Waals forces). The most probable binding site was the *N*-terminal residue of BphD, but because this residue had only moderate activity, the BphD conformation may not have changed during covalent bonding. This could explain why CM-SWCNT-BphD had a comparable catalytic efficiency to that of free BphD.

4. Conclusions

BphD immobilized by covalent modification (BphD-CM-SWCNT) had a higher activity than that immobilized by physical absorption (BphD-SWCNT). BphD-CM-SWCNT retained 99.7% of the activity of free BphD, compared with 52.5% retained by BphD-SWCNT. Meanwhile, BphD-SWCNT exhibited improved stability to thermal treatment, pH changes and denaturant exposure, while BphD-CM-SWCNT did not exhibit any significant improvement. Molecular docking suggested that the decreased catalytic efficiency of SWCNT-BphD resulted from the disruption of salt bridges between adjacent subunits. Therefore, single-wall carbon nanotubes could be used for immobilizing C–C bond hydrolase, thus provide an alternative matrix for immobilization.

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单壁碳纳米管对C–C键水解酶BphD的固定化性能

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摘要: 采用修饰与未修饰单壁碳纳米管固定C–C键水解酶BphD, 并对固定化酶的相对活性、稳定性、重复使用性进行了考察. 结果表明, 未修饰单壁碳纳米管固定的BphD相对活性为游离态的52.5%, 其热稳定性和在变性剂中的稳定性均有所提高, 且重复使用10次仍可保持初始活力的90%. 修饰单壁碳纳米管固定的BphD相对活性可达99.7%, 但其稳定性没有明显提高. 同源模建及分

子对接分析结果显示,未修饰的单壁碳纳米管对BphD亚基之间的联系可能存在干扰作用,从而对其活性产生影响。

关键词: C–C键水解酶; 单壁碳纳米管; 固定化; 分子模拟; 物理吸附; 共价结合

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1. 前言

作为新兴的一维纳米材料,单壁碳纳米管(SWCNT)具有独特的力学、电学和化学性能,因而广泛应用于纳米刻蚀、纳米光电催化、纳米传感以及高解析度成像等领域^[1]. 近年来,研究者陆续将辣根过氧化物酶、脂肪酶等成功地固定在SWCNT上,表明SWCNT在生物催化剂固定领域同样具有应用价值^[2–5]. Asuri等^[6]首先采用物理吸附法在SWCNT上固定豌豆过氧化物酶和糜蛋白酶,负载量均可达到600 mg/g,但固定化后酶二级结构的改变导致其活性的保留程度存在很大差异. 随后,他们又利用共价修饰法将辣根过氧化物酶固定在SWCNT上,负载量为1.3 mg/g,并能保留40%左右的初始活性. Pedrosa等^[7]采用共价修饰法将对硫磷水解酶分别固定在单壁和多壁碳纳米管上,并制备用于检测对硫磷的生物传感器. 结果表明,经过共价修饰的固定化对硫磷水解酶存放7个月后,仅损失25%的活性. 尽管利用SWCNT固定酶研究可以获得较高的负载量和保留活性,但仍然存在一些亟待解决的问题: (1)利用SWCNT固定化的酶种类较少,应探索SWCNT作为更多类型酶固定化载体的可行性; (2)酶分子与SWCNT通过物理吸附或共价作用结合对于负载量和保留活性的具体影响; (3)SWCNT与酶具体结合位点及结合机制尚不明确.

C–C键水解酶来源于芳香化合物间位开环降解途径,催化儿茶酚类物质间位开环产物中高能C–C键的水解反应,是整个芳香化合物间位开环降解途径中的限速步骤^[8]. 近年来的研究表明, C–C键水解酶具有独特的催化混杂性,即利用同一活性位点能催化多种反应,如C–C键、C–O键、S–O键的水解以及C–C键的可逆合成,因而,具有潜在的生物催化应用前景^[9]. 本课题组从菌株*Dyella ginsengisoli* LA-4中克隆得到了一段编码C–C键水解酶的基因**bphD**,并在大肠杆菌中成功地异源表达^[10]. 本文利用SWCNT对C–C键水解酶BphD进行固定化,比较直接物理吸附和共价修饰后得到的固定化酶的活性和稳定性,并利用分子模拟手段说明两者活性保留的差异.

2. 实验部分

2.1. 材料

本文所用菌株为含有携带编码C–C键水解酶BphD基因**bphD**的重组大肠杆菌BL21 (DE3). 培养基为Lysogeny broth (LB)培养基(g/L): NaCl 10.0, 蛋白胨 10.0, 酵母浸粉5.0, pH = 7.0. SWCNT购自深圳市纳米港有限公司,其长度为5~15 μm ,直径小于2 nm,纯度>95%; 2-(N-吗啉基)乙磺酸(MES)、N-羟基丁二酰亚胺(NHS)、1-(3-二甲基氨丙基)-3-乙基碳二胺(EDC)购自国药集团化学试剂有限公司; 卡那霉素、异丙基硫代半乳糖苷(IPTG)、对硝基苯基苯甲酸酯等购自百灵威公司,其余试剂均为国产分析纯.

2.2. 菌株的培养

将200 μL 菌液转接至含25 ml LB培养基的锥形瓶(50 ml)中,加入30 mg/L的卡那霉素,置于摇床中培养12 h作为种液(30 $^{\circ}\text{C}$, 150 r/min). 取2 ml种液转接至含100 ml LB培养基的锥形瓶(250 ml)中,并加入30 mg/L的卡那霉素,在37 $^{\circ}\text{C}$, 150 r/min的条件下培养至 $\text{OD}_{600} = 0.4\sim 0.6$,加入1 mmol/L IPTG诱导3 h后,在4 $^{\circ}\text{C}$, 10000 r/min条件下离心5 min收集菌体,并用磷酸盐缓冲溶液清洗两次,放置于–80 $^{\circ}\text{C}$ 冰箱中保存备用.

2.3. 碳纳米管的修饰

第一步修饰(羧基化): 称0.1 g SWCNT溶于400 ml浓硫酸和浓硝酸的混合物(V:V=3:1)中,在30 $^{\circ}\text{C}$ 超声5 h. 冷却至室温后小心将其倒入去离子水中,混合均匀进行抽滤,将滤饼放入去离子水中超声振荡. 将振荡后的混合液离心(22000 r/min, 20 min),收集沉淀于50 $^{\circ}\text{C}$ 真空干燥8 h. 干燥后的固体采用红外光谱(FT-IR)仪表征特征官能团.

第二步修饰(羧基活化): 将上述SWCNT加入MES缓冲溶液(50 mmol/L, pH = 6.2)中,加入等体积的NHS 400 mmol/L. 超声30 min后加入终浓度为20 mmol/L的EDC (固体),反应混合液置于摇床中振荡(30 $^{\circ}\text{C}$, 150 r/min, 1 h). 利用0.22 μm 膜过滤洗涤除去多余的NHS和EDC. 得到的SWCNT在超纯水中超声2 min后悬起,离心之后置

于真空干燥箱中80 °C下干燥8 h后, 室温下储存备用. 修饰后的SWCNT命名为CM-SWCNT.

2.4. BphD的纯化

从-80 °C冰箱中取出菌体, 用磷酸盐缓冲溶液悬浮, 得到的悬浮液利用超声破碎仪破碎40 min, 得到的样品离心20 min (22000 r/min, 4 °C), 上清液即为BphD的粗酶液. 纯化采用Ni柱亲和层析法, 65%咪唑梯度(325 mmol/L)下洗脱得到的组分即为BphD纯酶液, 蛋白纯度采用聚丙烯酰胺凝胶电泳确定.

2.5. BphD/碳纳米管的固定化

实验中采用3 ml反应体系进行固定化最优pH摸索, 其中BphD浓度为30 µg/ml, 碳纳米管含量为0.5 mg. 缓冲溶液pH值分别为6, 7, 8, 9和10. 将反应体系放入摇床中振荡(150 r/min, 30 °C)使其混合均匀, 利用紫外-可见分光光度法在405 nm下测定固定化前后反应产物的吸光度值变化. 实验中将不同浓度的BphD分别用SWCNT和CM-SWCNT进行固定化. 两组反应体系中BphD浓度分别为15, 30, 60和90 µg/ml, 在50 ml锥形瓶中加入初始浓度为1 mg/ml的碳纳米管材料, 一定量BphD及相应体积pH=7.0的磷酸盐缓冲溶液, 最终反应体系为6 ml. 将反应体系放入摇床中振荡(150 r/min, 30 °C). 每隔一定时间取出60 µl样品, 离心(12000 r/min, 2 min)后测定上清液中蛋白的残余量, 利用紫外分光光度计法对BphD固定化过程进行吸附动力学的考察, 并计算不同蛋白浓度下固定化酶的相对活性及负载量^[11]. 蛋白质定量采用Bradford法^[12].

2.6. 固定化BphD特性考察实验

本实验反应体系包括40 µl固定化酶, 40 µl底物, 1920 µl磷酸盐缓冲溶液(pH=7.0). 单位酶活的定义为每分钟生成1 µmol/L对硝基苯酚所需的酶量.

为考察温度对固定化酶活性的影响, 分别将固定化酶和游离酶置于恒温水浴锅中, 温度分别设定为30, 40, 50, 60和65 °C. 15 min后取出, 待其冷却至室温后进行活性的测量.

对于固定化酶热稳定性考察, 分别取10组固定化酶和游离酶置于60 °C恒温水浴锅中, 每隔5 min取样, 待其冷却至室温进行活性的测量. 以初始的活性为100%, 计算活性随时间的变化趋势.

对于变性剂对固定化酶活性的影响, 分别取40 µl固定化酶和游离酶于1920 µl不同浓度盐酸胍溶液中放置15 min后, 进行活性的测量. 盐酸胍溶液浓度分别为0, 0.2, 0.4, 0.8和1.6 mmol/L.

对于固定化酶重复使用性的考察, 将固定化酶离心(12000 r/min, 5 min), 弃去上清液, 用同样体积的磷酸缓冲溶液(pH=7.0)将固定化酶悬起, 测量每次悬浮后固定化酶的活性. 重复上述过程多次. 以初始活性为100%, 计算活性随时间的变化趋势.

2.7. 同源建模、分子对接及共价结合位点预测

利用在线服务器对蛋白质进行三维结构的构建. 通过<http://swissmodel.expasy.org>访问SWISS-MODEL服务器, 在自动模式(Automated Mode)中输入通过NCBI数据库中得到的菌株*Dyella ginsengisoli* LA-4中C-C键水解酶BphD的蛋白质序列, 构建C-C键水解酶BphD的三级结构模型^[13]. 碳纳米管与BphD的分子对接利用Patchdock以及Firedock进行^[14]. 针对共价结合, 采用Pamela等^[15]的方法进行计算, 首先利用在线程序PROPKA计算具有氨基的残基(N-端残基, Lys和Tyr)的pKa值, 再利用下式计算LIGRe值:

$$LIGRe = 10^{(pH - pKa)}$$

当LIGRe < 0.1时, 该氨基被认为是低活性的; 当0.1 < LIGRe < 1时, 该氨基被认为是中等活性的; 当LIGRe > 1时, 该氨基被认为是高活性的.

3. 结果与讨论

3.1. 碳纳米管的修饰及表征结果

为了实现蛋白质分子在碳纳米管上的共价结合, 需要对碳纳米管进行修饰, 在其表面添加经过NHS活化的羧基, 修饰过程原理如图1所示.

图2为修饰前后碳纳米管的FT-IR谱. 由图可见, 经混酸处理的碳纳米管在1720 cm⁻¹附近均有一个比较强的峰, 归属于羧基的C=O伸缩振动峰, 表明经过混酸修饰后的碳纳米管表面含有羧基^[16]. 经NHS/EDC处理的碳纳米管在1645 cm⁻¹左右有一个比较强的峰, 表明材料表面的羧基得到活化, 对应的C=O伸缩振动峰受新形成的N-O键的影响而出现红移.

3.2. BphD固定化最优pH值

BphD与SWCNT结合时, 主要作用力为静电作用. 蛋白质所带电荷受pH值影响, 因此pH值是二者结合过程中的重要影响因素. 本文考察了不同pH条件下固定化酶的负载量及相对活性, 其中相对活性随pH值的变化趋势见图3. 可以看出, 两种固定化酶的最适pH值与游离酶类似, 均在pH=7时具有最高的比活力. BphD-SWCNT在pH值较低(pH=6)时具有较高的相对活性; 而BphD-CM-SWCNT在pH=7.5~8.0时, 比游离酶

具有更高的相对活性. 当 $\text{pH} = 7$ 时, BphD在两种碳纳米管上都能达到100%的吸附率. 因此, 下文选取 $\text{pH} = 7$ 的缓冲溶液来进行固定化.

3.3. BphD固定化过程的吸附动力学

对不同浓度的BphD与碳纳米管的固定化过程进行了吸附动力学考察, 测定了蛋白质吸附百分比随时间的变化曲线, 如图4所示. 由图4(a)可见, 对于BphD-SWCNT复合体系, 当蛋白浓度较低(15或30 $\mu\text{g/ml}$)时, 吸附2 h后可以达到平衡, 吸附率几乎为100%; 而蛋白浓度较高(60或90 $\mu\text{g/ml}$)时, 3 h后吸附可以达到平衡, 吸附率为60%左右. 由图4(b)可见, BphD-CM-SWCNT复合体系所呈现的吸附趋势与BphD-SWCNT的基本相同, 但吸附达平衡所需时间均有所增加. 这可能是因为共价修饰后的SWCNT与蛋白质分子是特异性的结合.

通过计算吸附达到平衡时不同蛋白浓度下SWCNT和CM-SWCNT的负载量可知, 当蛋白浓度为90 $\mu\text{g/ml}$ 时, 负载量达最大值. 因此, 以下实验蛋白浓度均为90 $\mu\text{g/ml}$. 此时BphD-SWCNT复合体系负载量为255 mg/g , 相对活性为游离酶的52.5%, 而BphD-CM-SWCNT复合体系分别为518 mg/g 和99.7%. 可见BphD与CM-SWCNT结合时, 负载量及相对活性均高于BphD-SWCNT. 这可能是因为BphD与CM-SWCNT属于特异性结合, 使得活性得以较高度度的保留. 而共价修饰可提高固定化酶负载量的实验结果也与Asuri等^[6]的研究结果类似.

3.4. 固定化BphD扫描电子显微镜(SEM)结果

利用SEM对碳纳米管及碳纳米管-BphD复合物的表面形貌进行了表征, 结果示于图5. 可以看出, 修饰前后的碳纳米管均以团簇形式存在, 但CM-SWCNT的长短更不均一; 而在修饰前后的碳纳米管-BphD复合物中, 除了碳纳米管外, 还有部分无定形的固定颗粒, 可能为磷酸盐的结晶体.

3.5. 固定化BphD透射电子显微镜表征结果

为了进一步观察碳纳米管上蛋白质分子的分布情况, 利用透射电镜对单壁碳纳米管-蛋白质复合物的形貌进行了表征. 图6(a)和(b)分别为修饰前后的SWNT, 其形貌并未观察到明显的变化. 比较图6(c)和(d)可以发现, 当BphD与CM-SWCNT结合时, 表面的蛋白质覆盖的更为致密, 这也与CM-SWCNT上BphD的负载量更多的实验结果相吻合.

3.6. 固定化BphD的基本特性

考察了BphD与碳纳米管两种复合物的基本特性,

包括温度、 pH 值及变性剂盐酸胍对其活性的影响、固定化酶的热稳定性和重复利用性.

3.6.1. 温度的影响

图7为温度对固定化酶相对活性的影响. 由图可知, 50 $^{\circ}\text{C}$ 时游离BphD和BphD-SWCNT复合物的相对活性均达最高; 而BphD-CM-SWCNT复合物在40 $^{\circ}\text{C}$ 时最高. Basha等^[17]利用苯基琼脂糖固定了来源于真菌的转移酶, 发现固定化后的转移酶催化反应的最适温度比游离酶的低10 $^{\circ}\text{C}$, 其原因可能是固定化后酶的构象发生改变, 使得温度较高时对产物的抑制作用增加, 从而导致固定化酶最适温度下降.

3.6.2. pH 值的影响

图8是两种固定化酶($\text{pH} = 7$ 时制得)在不同 pH 值时的相对活性. 可以看出, BphD-SWCNT和BphD-CM-SWCNT仍在 $\text{pH} = 7$ 时活性达最高; 但经过固定化后的BphD在较低 pH 值下的活性保留明显要高于游离的BphD. 其中当 $\text{pH} = 6$ 时, BphD-SWCNT具有62%的相对活性, 远远高于游离酶的25%. 这表明通过固定化后酶对 pH 的耐受能力得到强化, 可能与酶所处的微环境有关.

3.6.3. 酶的热稳定性

图9为游离酶和固定化酶的热稳定性. 由图可知, 在60 $^{\circ}\text{C}$ 恒温孵化40 min后, 酶的相对活性均有所下降. 25 min后游离BphD的活性完全丧失; BphD-SWCNT复合物的热稳定性较游离酶有所提高; 但BphD-CM-SWCNT复合物热稳定性不如游离酶. 这可能与BphD-CM-SWCNT最适温度下降的原因类似.

3.6.4. 变性剂的影响

盐酸胍是一种常用的蛋白质变性剂, 通过夺取蛋白质分子中的结合水使得蛋白质变性. 图10为盐酸胍浓度对游离BphD以及两种固定化BphD相对活性的影响. 由图可知, 在不同浓度盐酸胍下BphD-SWCNT复合物的相对活性较游离酶均有所提高; 当盐酸胍浓度高达1.6 mmol/L 时, BphD-SWCNT复合物仍能保持33%的初始活性, 而BphD-CM-SWCNT复合物与游离酶类似, 几乎完全丧失.

3.6.5. 酶的重复使用性

图11为两种固定化酶分别重复使用10次的相对活性. 由图可知, BphD-SWCNT复合物重复使用10次后, 其相对活性仍保持在90%以上; 而BphD-CM-SWCNT复合物重复使用5次后, 其活性完全丧失, 固定化酶效果不理想. 通常来说, 通过共价修饰的酶一般具有比游离酶

更高的稳定性,但是利用修饰后碳纳米管固定的BphD稳定性降低.这可能是由于pH = 7时BphD可供与活化羧基结合的游离氨基较少有关.根据Torres-Salas等^[15]的方法,我们计算了BphD的N-端残基、Lys和Tyr的pKa值,发现只有N-端残基所带的氨基在pH = 7时具有中等活性($0.1 < LIG Re < 1$),主链上Lys和Tyr的氨基均活性较低.这可以解释BphD-CM-SWCNT重复使用性不如BphD-SWCNT的原因.

3.7. 同源建模与分子对接

通过现有的实验手段,我们无法直接观察到酶与纳米材料的结合位点.因此,通过引入分子模拟手段,可以对结合位点进行合理的预测.首先,通过SWISS-MODEL构建了菌株LA-4中BphD的三级结构模型,采用的模板蛋白为*Burkholderia xenovorans* LB400中的BphD,两者的序列一致性达88.73%,可以用于同源建模,构建结果如图12所示,表明BphD可能为同源四聚体.采用UCLA-DOE服务器(<http://www.doe-mbi.ucla.edu/>)的PROCHECK对模型进行质量检测,PROCHECK中的Ramachandran Plot结果显示,模建结构蛋白的主链结构合理:主链二面角 ϕ , ψ 落于中心区域和可接受区域的残基占90.7%,可应用于进一步的分子对接^[18].

利用Patchdock以及Firedock获得的对接结果表明,BphD/碳纳米管复合物中可能的结合模式都是远离活性位点的.因此,BphD-SWCNT复合物活性降低可能不是由于SWCNT进入活性位点内部所致,如图13(a)所示.而当抑制剂结合在亚基界面,可以通过干扰亚基间的相互作用而抑制酶的活性.因此,我们对亚基之间的相互

作用残基进行了识别,以亚基B, C为例,两者之间通过两对盐桥进行结合(Glu717-Lys438, Lys720-Glu435).如图13(b)所示,当SWCNT的直径为0.5 nm时,它可以深入到两个亚基的界面处,破坏这两对盐桥的作用,从而导致BphD-SWCNT活性降低. Zuo等^[19]利用分子动力学模拟研究了碳纳米管对双色氨酸功能域的毒性效应,得到可能的作用机制是碳纳米管进入活性口袋,并阻止正常的配体与双色氨酸功能域的结合.在本文中,由于BphD的活性位点深埋在疏水内核之中,因而不大可能通过直接作用于活性位点而使得酶活性降低.需要说明的是,CM-SWCNT与BphD的结合位点并不能通过对接进行预测,因为共价作用要远远强于分子对接所考虑的分子间相互作用力.正如上文所述,CM-SWCNT与BphD最有可能的结合位点是BphD的N-末端游离氨基.由于两者之间的结合力并不是特别强,因此共价结合对于BphD本身构象的影响不大,从而保留了大部分的活性.

4. 结论

修饰单壁碳纳米管固定化的BphD能更好地保持游离酶的活力,其相对活性可达99.7%;而未修饰单壁碳纳米管固定化的BphD仅能保留52.5%的相对活性,但其稳定性较游离酶有所提高,而修饰单壁碳纳米管固定化的变化不大.分子对接结果表明,未修饰单壁碳纳米管固定化的BphD活性的降低可能与单壁碳纳米管破坏了BphD亚基间相互作用有关.由此可见,单壁碳纳米管可用作生物催化剂的载体,为酶固定化载体的选择提供新的思路.